

# Synthesis and Membrane Interactions of Spin-Label Bifunctional Reagents<sup>†</sup>

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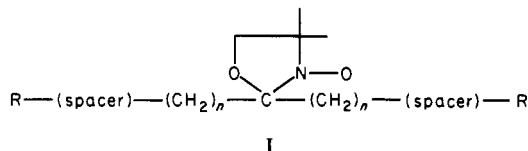
**ABSTRACT:** The design of chemical reagents that interact with biological membranes requires knowledge of the orientation and location of the reagents with respect to the membrane surface and of the partition coefficients of the reagents between membrane and water. These properties are explored here for a class of molecules having the following properties: (1) they have long, flexible chains which are, in some cases, long enough to span the membrane; (2) there is a polar, or functional, group at each end of the chain; (3) the structures are symmetrical about a center atom which is part of a paramagnetic, spin-label ring. Electron paramagnetic resonance (EPR) spectra were used to determine whether molecules with the above features take up an orientation in a lipid membrane such that the polar or functional groups on each end of the molecule are on the same side of a membrane or are on opposite sides, indicating a membrane-spanning orientation. The only case of a membrane-spanning orientation found was for a dicarboxylic acid of total chain length equal to twice that of the fatty acyl chains of the lipid. When amide linkages are substituted for two methylenes in the structure that spans the membrane, a different orientation is observed. All of the molecules extended by amide linkages take up an orientation in a membrane in which both polar ends of the molecule are on the same side of the membrane, giving EPR signals in oriented membranes similar to those of bifunctional molecules that are too short to span the membrane. The EPR spectra do not provide unambiguous proof of whether the flexible molecules with both functional groups on one side of a membrane are extended on the surface or are bent and intercalated into the membrane hydrophobic interior, so two other tests of these possibilities

In the presence of a membrane, an amphiphilic molecule in aqueous solution partitions between the membrane and water. This sort of process is involved in membrane binding of amphiphilic drugs (Seeman, 1972), of fluorescent and paramagnetic probes (Radda, 1975; Berliner, 1976, 1979), of chemical reagents directed to membrane components (Peters & Richards, 1977), and, probably, also of nascent polypeptide chains (Inouye et al., 1982), among others. Our own interest in binding of amphiphilic molecules to membranes is based on a desire to design protein modification reagents with specificity for particular cellular components. We have synthesized a series of long, flexible bifunctional molecules. In the following paper (Willingham & Gaffney, 1983) we will show that variations in these structures result in specificities

were made. First, partition coefficients (membrane/water) were determined for three dicarboxylic acid spin-label chains of 15-, 19-, and 23-carbon lengths, and virtually *no* contribution per methylene to the free energy of binding was found. In contrast, the contribution per methylene in binding of a series of spin-label, monofunctional fatty acids was similar to that for the series of saturated, unlabeled fatty acids. Second, no change in partition coefficient at the phase transition of dimyristoyllecithin was found for the 15-carbon diacid. These results suggest that the dicarboxylic acid spin-labels may seek an interfacial region between lipid and water. Olive oil/water and octanol/water partition coefficients tend to support this conclusion. It has recently been suggested that the binding of amphiphiles is very different for lipid model membranes compared to biological membranes with proteins [Conrad, M. J., & Singer, S. J. (1981) *Biochemistry* 20, 808-818]. Accordingly, we compared the partition coefficients of one of the dicarboxylic acid spin-labels and of two monofunctional spin-label fatty acids in membranes of red cell extracted lipids and in red cell ghosts. The largest difference that we observe in comparing ghosts and pure erythrocyte lipid membranes is a less than 2-fold decrease in the partition coefficient of the diacid spin-label in ghosts compared to their lipids. Partition coefficients for fatty acid spin-labels are virtually identical in ghosts and ghost lipids. Thus, the large effects of membrane proteins observed by Conrad and Singer for other amphiphiles and another measurement technique are not observed for the molecules for which we measured partition coefficients by EPR techniques.

for different regions of the red blood cell. Here, the effects of structure on the orientation of a molecule in a lipid membrane and on the partition coefficients,  $K^1$  of these molecules between membrane and water are examined. Specifically, we ask, if a molecule is long enough to go across a membrane, what orientation does it adopt when it partitions into a membrane?

The molecules which are the subject of this report have the general structure I. They contain a paramagnetic, or spin-



label, group in the middle of a symmetrical, bifunctional molecule. The geometry of the 3-oxoazolidine spin-label ring is such that the direction of the  $\pi$  orbital with the unpaired

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<sup>1</sup> Abbreviations:  $K$ , partition coefficient; EPR, electron paramagnetic resonance; TLC, thin-layer chromatography; IR, infrared; DCC, dicyclohexylcarbodiimide; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy; DML, dimyristoyllecithin; BHT, butylated hydroxytoluene; Tempol, 2,2,6,6-tetramethyl-4-hydroxypiperidinyl-1-oxy; RBCL, red blood cell lipids; RBCG, red blood cell ghosts; NHS, *N*-hydroxysuccinimide ester; SR, rabbit muscle sarcoplasmic reticulum.

electron is parallel to the extended, long molecular axis. As has been demonstrated numerous times before, an oxazolidine nitroxide so oriented on a rod-shaped molecule is a convenient indicator of the orientation of the molecular axes with respect to the plane of a phospholipid membrane (Seelig, 1970; Hubbell & McConnell, 1971; Gaffney-McFarland & McConnell, 1971; Libertini et al., 1974; Seelig et al., 1972; Gaffney & McConnell, 1974). This is so because the magnitude of the nitrogen nuclear hyperfine interaction with the unpaired electron depends on the angle between the direction of the  $z$  axis, or  $\pi$  orbital, in the molecule and the direction of the external magnetic field. When the magnetic field is perfectly aligned parallel to the  $\pi$  orbital, the hyperfine splitting in the electron paramagnetic resonance (EPR) spectrum is about 30 G; when it lies along the plane perpendicular to the  $\pi$  orbital, the splitting is about 6 G. For membrane or liquid crystal samples the orientation of a spin-label relative to a sample axis can be determined if it is possible to achieve macroscopic ordering of the membrane or liquid crystal with respect to the magnetic field direction in an EPR spectrometer. Seelig et al. (1972) used this approach to determine the orientation of long-chain, dicarboxylic acid spin-labels in a smectic liquid crystal. The molecules studied by these authors have the general structure I, but without the spacer, and with  $R = \text{COOH}$ . When the number of methylene groups,  $n$ , was approximately equal to the number of methylenes in the amphiphiles from which the liquid crystal was composed, the preferred orientation was the transmembrane one, that is, with the two ends of the molecule on opposite sides of a bilayer plane in the liquid crystal. Shorter molecules had an orientation such that both carboxyl groups were on one side of a bilayer. These observations were the starting point in our work with molecules having spacers and other  $R$  groups, including protein-modifying groups. We have reported elsewhere (Tse-Tang et al., 1980) on the orientation in a membrane of a series of bifunctional amphiphiles having a different nitroxide geometry.

The presence of a nitroxide group also renders EPR spectra sensitive to partitioning of amphiphiles between membrane and water. There are two effects that allow the EPR spectra of the amphiphiles in these two regions to be distinguished. First, the amphiphiles move more slowly, and anisotropically, in membranes than they do in water, and consequently the anisotropic hyperfine interactions are less averaged in a randomly oriented membrane sample than they are for the amphiphile tumbling more rapidly in water. The result is a spectrum broadened to varying degrees. A second effect of membranes on the EPR spectra is a polarity effect: the hyperfine splittings are smaller for nitroxides in media of low polarity than they are for the molecules in a polar medium such as water. This is an effect of smaller magnitude than motional effects and is most evident in cases where the nitroxide is small and hydrophobic enough that it tumbles rapidly in the fluid hydrocarbon interior of a lipid bilayer (Hubbell & McConnell, 1968). Analysis of EPR spectra of spin-label amphiphiles in terms of the partition coefficients of these molecules between membranes and water has been useful for measuring the temperature dependence of membrane fluidity (Shimshick & McConnell, 1973) and for evaluating membrane surface charge (Gaffney & Mich, 1976; Castle & Hubbell, 1976; Cafiso & Hubbell, 1981). There are several advantages in using EPR spectra to measure partition coefficients. As discussed above, spectra of membrane-bound and "free" (in water) labels are distinguishable. In addition, a third type of spectrum would be observed should the nitroxide amphiphile

form micelles. And finally, the area under a magnetic resonance absorption curve, recorded under nonsaturating conditions, is a linear function of the number of molecules giving rise to the spectrum so that effects of a "quantum-yield type" do not have to be considered, as they do in fluorescence spectroscopy, to extract quantitative information. Our results on partition coefficients for the spin-label amphiphiles of general structure I have been particularly revealing in model lipid membranes which undergo a gel-liquid-crystalline phase transition. There is little change in the partition coefficient, when  $R = \text{COO}^-$  in structure I, at the phase transition of dimyristoyllecithin. This fact, combined with data on the orientation of molecules in oriented lipid bilayers, suggests that molecules of structure I have a preference for the membrane-water interfacial region.

Much of the now conventional wisdom on partitioning of drugs and anesthetics between membranes and water has recently been called into question as a result of experiments using a new technique called "hygroscopic desorption" (Conrad & Singer, 1979, 1981). Using this technique, Conrad and Singer find enormous (several orders of magnitude) differences in partition coefficients for chlorpromazine, methochlorpromazine, 2,4-dinitrophenol, and 1-decanol partitioning into lipid model membranes compared to partitioning into biological membranes which contain proteins. They suggest that while these amphiphiles do partition into model lipid membranes, they are excluded almost completely from biological membranes such as red blood and lymphoma cell membranes and from sarcoplasmic reticulum vesicles. The positive evidence for binding to biological membranes obtained from centrifugation studies (Seeman, 1972) they attribute to the external association of micelles of the amphiphiles, or comicelles of these molecules with extracted membrane components, with surfaces of cell membranes. Using EPR techniques, we have obtained data on partitioning of several spin-label amphiphiles between water and model membranes or red cell ghosts. We report here that these measurements reveal very little difference in partition coefficient for the spin-labels in membranes with and without proteins.

#### Materials and Methods





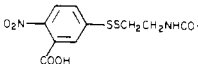
**Materials.** Dilauroyl- and dimyristoyllecithins were purchased from Calbiochem or from Sigma. Thin-layer chromatography employed prepared plates provided by Analtech (silica gel GF, 250  $\mu\text{m}$ ). Inulin ( $^3\text{H}$ , 2.2 Ci/mmol) was from Amersham. Unlabeled inulin was from Sigma. *N*-Hydroxysuccinimide (Aldrich) was recrystallized from ethanol before use. Human blood was obtained from the Baltimore American Red Cross and was used within 1 week of the time it was drawn.

**Syntheses of Reagents.** The structures, compound numbers, and microanalytical data for the final products of the syntheses are given in Table I.

(1) *Preparations of Keto Diacids.* Zantour et al. (1972) reported the synthesis of 8-oxo-1,15-pentadecanedioic acid. The oxopentadecane-, oxononadecane-, and oxotricosanedioic acids used as precursors to reagents II, III, and IV were prepared essentially by the Zantour et al. procedure. Details of our preparation of the pentadecaneketodioate are given here. Only yield and analytical data for the other two derivatives are given.

Ethyl 7-(chloroformyl)heptanoate was prepared in 94% yield from ethyl hydrogen suberate by using thionyl chloride (Jones, 1947). This acid chloride (30.0 g, 0.136 mol) was dissolved in 300 mL of diethyl ether (Mallinckrodt, freshly opened can) in a three-necked flask equipped for vigorous mechanical

Table I: Symmetrical, Paramagnetic Amphiphiles Synthesized

compound no.	$n^a$	$R^a$	spacer <sup>a</sup>	total <sup>b</sup> length (atoms)	calculated			found		
					C	H	N	C	H	N
Diacids										
II	6	COOH	none	15	61.26	9.20	3.76	61.44	9.19	3.93
III	8	COOH	none	19	64.50	9.90	3.30	64.71	9.71	3.18
IV	10	COOH	none	23	insufficient quantity for analysis					
V	6	COOH	-CONH(CH <sub>2</sub> ) <sub>5</sub> -	29	62.13	9.43	6.69	62.18	9.43	7.02
VI	6	COOH	-CONH(CH <sub>2</sub> ) <sub>3</sub> -	25	analyzed as NHS ester XI					
VII	6	COOH	-CONH(CH <sub>2</sub> ) <sub>5</sub> -CONH(CH <sub>2</sub> ) <sub>3</sub> -	39	60.91	9.18	9.11 <sup>c</sup>	59.74	8.81	8.35
Bis( <i>N</i> -hydroxysuccinimide esters)										
VIII	6		none	15+	57.23	7.12	7.42	57.52	7.31	7.26
IX	8		none	19+	59.79	7.77	6.75	60.11	7.57	6.50
X	6		-CONH(CH <sub>2</sub> ) <sub>5</sub> -	29+	59.07	7.88	8.83	59.37	8.08	8.68
XI	6		-CONH(CH <sub>2</sub> ) <sub>3</sub> -	25+	57.05	7.39	9.51	56.88	7.50	9.30
5-Dithio-2-nitrobenzoic Acid Derivative										
XII	6		none	23+	50.09	5.91	7.90 <sup>c</sup>	48.82	5.96	7.69

<sup>a</sup> With reference to the general structure I. <sup>b</sup> A plus sign (+) after the total length in atoms means that the R group adds a small increment to the total length of the extended molecule. <sup>c</sup> The analyses of compounds VII and XII indicate the presence of some impurities. In the case of VII, low values were found for all atoms analyzed. Compound VII was prepared in very small quantity and could not be filtered repeatedly to remove traces of silica gel from the final TLC purification. The analysis is consistent with some silica gel remaining. In the case of XII, the low value for carbon may be due to slight oxidation of the reagent. When XII was cleaved by base, the amount of dithio-nitrobenzoate anion detected by visible spectroscopy was two per molecule within the limits of detection.

stirring. The contents of the flask were kept under an atmosphere of nitrogen throughout the reaction. The stirred acid chloride solution was cooled to -15 °C in an ice-methanol bath, and triethylamine (Baker, 13.75 g, 0.136 mol; freshly distilled from potassium hydroxide) in 25 mL of diethyl ether was added to it from a dropping funnel during 10 min. After the addition, the mixture was heated with a 40 °C water bath for 5 min and then stirred vigorously at room temperature for 3 h. Triethylamine hydrochloride was removed by filtering through a glass-sintered funnel, ether was removed from the filtrate, and the residue was heated under reflux for 4 h with aqueous potassium hydroxide (200 mL of a 2 N solution in 20% water in ethanol). Ethanol was removed by evaporation, 40 mL of water was added, and two 20-mL extracts with ether were taken. The aqueous solution at 0 °C was acidified with concentrated hydrochloric acid to pH 2-3. The precipitated keto diacid was collected by filtration and was recrystallized once from water to give 11.3 g (58% yield) of 8-oxo-1,15-pentadecanedioic acid (mp 113-115 °C). A second recrystallization gave an analytically pure sample melting at 114-115 °C [Zantour et al. (1972) report 113-114 °C].

Melting points of 10-oxo-1,19-nonadecanedioic acid and 12-oxo-1,23-tricosanedioic acid were 118-120 and 119-120 °C, respectively, and these compounds were prepared in 41 and 18% yields, respectively. Reported melting points are 117-119.5 (Blomquist et al., 1952) and 120.5-121.5 °C (Zantour et al., 1972), respectively.

(2) *3-Oxy-4,4-dimethylloxazolidine Derivatives of Keto Diacids*. Several of these spin-labeled derivatives of keto

dicarboxylic acids have been reported by Seelig et al. (1972), who used the method of Keana et al. (1967) to introduce the 3-oxoxazolidine group. Using essentially these procedures but with varied chromatography conditions, we prepared the spin-labeled derivatives of the diethyl esters of 15- and 19-carbon keto diacids in yields of 59% and 51%, respectively. The derivative of the 23-carbon diacid was prepared similarly. The diester labels were purified by column chromatography on silica gel (Mallinckrodt CC-4; 500 g of silica gel/material from 10 g of keto diester; 5% methanol in benzene as solvent). Diesters were converted to diacids by basic hydrolysis (Hubbell & McConnell, 1971). Diacids were purified by chromatography on silica gel (ICN-Woelm, 100-200 mesh, 2% methanol increasing to 3%, in chloroform as solvent). Microanalytical data for spin-labeled diacids are given in Table I.

(3) *Preparations of Bis(*N*-hydroxysuccinimide esters) of Spin-Label Dicarboxylic Acids*. The 3-oxy-4,4-dimethyl-oxazolidine derivative of 8-oxo-1,15-pentadecanedioic acid (0.424 g, 1.14 mmol) was dissolved in 10 mL of distilled acetonitrile. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDAC; Bio-Rad; 4.8 g, 2.5 mmol) was dissolved in 5 mL of acetonitrile, and this solution was added to the solution of spin-label diacid and 0.288 g (2.5 mmol) of *N*-hydroxysuccinimide. The mixture was stirred at room temperature for 20 h, and the acetonitrile was removed under reduced pressure. The remaining yellow oil was dissolved in chloroform, and this solution was extracted with three 20-mL portions of saturated, aqueous sodium chloride. After drying over sodium sulfate, the chloroform solution was evaporated

to give 0.7 g of an oil which was purified by silica gel chromatography (60 g; ICN; 1% methanol in benzene as solvent) to give 0.33 g of slightly impure (TLC) 3-oxy-2,2-bis[6-((*N*-succinimidylloxy)carbonyl)hexanyl]-4,4-dimethyloxazolidine (compound II, Table I). Two further passes through silica gel columns, using a gradient increasing from 2 to 5% acetone in benzene as eluent, gave an analytically pure oil (IR in  $\text{CHCl}_3$ , succinimidyl ester bands at 1815, 1780, and 1730  $\text{cm}^{-1}$ ). Analytical data are reported in Table I for this and the corresponding derivative of the  $\text{C}_{19}$ -keto diacid.

(4) *Preparations of Spin-Labeled Bis(N-hydroxysuccinimide esters) with Spacer Arms.* The chains of the bifunctional spin-label molecules were lengthened by reaction of shorter bis(*N*-hydroxysuccinimide esters) with  $\omega$ -aminocarboxylic acids. A representative procedure follows for preparation of X, the 3-oxy-4,4-dimethyloxazolidine derivative of bis[5-((*N,N'*-succinimidylloxy)carbonyl)pentanyl]-8-oxo-1,15-pentadecanediamide.

6-Aminocaproic acid (2.83 g, 21.6 mmol; Aldrich) in 20 mL of 0.1 M sodium borate buffer, pH 8.7, and 10 mL of acetone was stirred vigorously while the bis(*N*-hydroxysuccinimide ester) VIII, 3-oxy-2,2-bis[5-((*N*-succinimidylloxy)carbonyl)hexanyl]-4,4-dimethyloxazolidine (0.244 g, 0.43 mmol), dissolved in 10 mL of acetone was added over 30 min. After it was stirred at room temperature for 20 h, the solution was cooled in an ice bath, acidified with cold, concentrated HCl to pH 1, and extracted 3 times with ice-cold ethyl acetate. The ethyl acetate solutions were combined and washed 3 times with cold, saturated salt solution and were dried over anhydrous sodium sulfate. Filtration and evaporation gave a yellow oil which was purified on TLC (chloroform-methanol-acetic acid, 92:8:0.1 v/v/v) to give 0.2413 g of product (0.34 mmol; 83% yield) moving on TLC as above with  $R_f$  0.44 and IR bands at 1650 (amide) and 1710 (carboxylic acid)  $\text{cm}^{-1}$ . This diacid was converted to the bis(*N*-hydroxysuccinimide ester) in an acetonitrile solution (35 mg of diacid in 5 mL of dry solvent) with dicyclohexylcarbodiimide (DCC; Aldrich) as the coupling agent. The procedures were the same as those for preparation of bis(*N*-hydroxysuccinimide ester) VIII except that the solvent for silica gel column chromatography was 2% methanol in chloroform increasing to 4% methanol. The product was obtained after two silica gel column purifications in 71% yield. It moved on TLC with  $R_f$  0.51 (chloroform-methanol, 95:5); IR bands were at 1650 (amide) and 1815, 1780, and 1730 (succinimidyl ester)  $\text{cm}^{-1}$ .

When a similar compound (XII) was prepared by extending VIII with 3-aminopropionic acid, the diacid was obtained in 68% yield ( $R_f$  0.53; chloroform-methanol-acetic acid, 90:8:0.1). The bis(*N*-hydroxysuccinimide ester) was subsequently obtained in 60% yield (TLC; chloroform-methanol, 95:5;  $R_f$  0.45). Both compounds exhibited the expected IR bands.

The diacid with two spacer arms, VII, was prepared from X in 40% yield by the above procedures except that the reaction was carried out under nitrogen and at 50 °C for 22 h. The product was purified by preparative TLC (chloroform-methanol-acetic acid, 92:8:0.1;  $R_f$  0.61) and had prominent IR bands at 1650 and 1720  $\text{cm}^{-1}$ .

(5) *Preparation of Bifunctional Disulfide Exchange Reagent: 3-Oxy-2,2-bis[(((2-((3-carboxy-4-nitrophenyl)dithio)ethyl)amino)carbonyl)hexanyl]-4,4-dimethyloxazolidine (XII).* A solution of cysteamine (82 mg, 0.72 mmol; Aldrich) in 2 mL of 0.2 M ammonium bicarbonate (pH 7.0) was added by drops to a stirred solution of 5,5'-dithiobis(2-nitrobenzoic acid) (570 mg, 1.43 mmol; Aldrich) dissolved in 5 mL of 0.2

M ammonium bicarbonate, pH 7.2. The solution turned deep red-orange instantly. A precipitate formed while the solution was stirred at room temperature, and after 1 h, it was collected and washed with 0.2 M ammonium bicarbonate and with distilled water to give a yellow powder identified as 5-[(2-aminoethyl)dithio]-2-nitrobenzoic acid (173 mg, 0.63 mmol): 88% yield; mp 219–220 °C dec. Anal. Calcd for  $\text{C}_9\text{H}_{10}\text{N}_2\text{O}_4\text{S}_2$ : C, 39.40; H, 3.67; N, 10.21. Found: C, 39.27; H, 3.71; N, 10.17.

5-[(2-Aminoethyl)dithio]-2-nitrobenzoic acid (60 mg, 0.22 mmol) in 4 mL of 0.1 M aqueous sodium phosphate (pH 7.5) was stirred under nitrogen while VIII (20 mg, 0.035 mmol) in 500  $\mu\text{L}$  of dimethylformamide was added in several aliquots over 15 min at room temperature. After continued stirring for 4 h, the mixture was filtered, the pH of the filtrate was adjusted to 6.5 with 0.1 N hydrochloric acid, and the solution was evaporated under vacuum at no more than 35 °C to 1 mL. The concentrated solution was applied to a Sephadex G-15 column (Pharmacia; 2  $\times$  45 cm) prepared in 0.01 M sodium phosphate (pH 6.5) and eluted with the same buffer. Four-milliliter fractions were collected, and 10 fractions at the top of the third peak showing absorbance at 323 nm were combined, cooled on ice, and acidified to pH 1.0 with cold, concentrated hydrochloric acid. The acidic solution was extracted with three 100-mL portions of cold ethyl acetate, and these combined solutions were washed twice with 25 mL of saturated salt solution, dried over sodium sulfate, filtered, and evaporated to give 19 mg of XII (0.021 mmol): 60% yield; TLC  $R_f$  0.51 (chloroform-methanol-acetic acid, 80:20:0.1 v/v/v);  $\lambda_{\text{max}}^{\text{water}}$  323 nm; IR 1710 (acid), 1660 (amide), and 1515 (aromatic nitro)  $\text{cm}^{-1}$ .

(6) *Spin-Label Fatty Acids (XIII).* Standard procedures were used to prepare the spin-labeled fatty acids (Hubbell & McConnell, 1971). Anal. Calcd for XIIIa: C, 65.82; H, 10.43; N, 4.27. Found: C, 65.84; H, 10.30; N, 4.29. Anal. Calcd for XIIIb: C, 67.38; H, 10.74; N, 3.93. Found: C, 67.19; H, 10.86; N, 3.82.

*Membrane Samples.* Dispersions of lipids in buffer (liposomes) were prepared from either synthetic dimyristoyllecithin or extracted red blood cell lipids by evaporating 1–5 mg of a chloroform solution of the lipid as a thin film in a 10-mL flask. Traces of solvent were removed by placing the lipid sample under vacuum (1 mm) for 1 h. Lipid was dispersed in buffer by alternatively heating to 37 °C and agitating at room temperature with a Vortex mixer over a period of 1 h. Erythrocyte lipids were extracted by the procedure of Korten et al. (1980) and stored under nitrogen with 1 ppm of BHT at –20 °C in chloroform (5 mg/mL). Lipids were suspended in 20 mM sodium phosphate and 150 mM sodium chloride at pH 8.0 or 6.0.

Lipid samples for oriented bilayers were prepared by mixing nitroxide amphiphile and dilauroyllecithin or egg yolk lecithin (mole ratio 1/100) in a chloroform solution, evaporating the solvent under 1-mm vacuum to leave a thin film of lipid, and hydrating the lipid overnight in water vapor in a closed container at room temperature. Hydrated lipids were scraped from the flask with a spatula and pressed between two quartz plates for EPR measurements.

Erythrocyte ghosts were prepared according to the method of Dodge et al. (1963) by using 5 mM sodium phosphate, pH 8.0, as the buffer for lysis and the subsequent three washes. The determination of membrane weight in a sample was based on phosphate analysis (Rouser et al., 1970) of a sample which had been dialyzed 12 h against isotonic saline to remove buffer phosphate. In calculating the weight of lipid, the average

phospholipid molecular weight was taken as 765, and a cholesterol/phospholipid weight ratio of 0.42 was used (Rose & Oklander, 1965). A typical value of total lipid per milliliter for normal ghosts was 4.1 mg/mL. It was necessary to concentrate ghosts further than by the Dodge et al. (1963) procedure for partition coefficient measurements. This was done by centrifugation at 40000g for 15 min at 4 °C to give 12.8 mg of total lipid/mL or by dehydration through dialysis membrane with dry Sephadex G-50 (overnight, 4 °C) to give 15.8 mg of total lipid/mL.

For estimation of the actual volume of ghost membranes from which solutes of intermediate molecular weight are excluded, the volume excluded to radioactive [<sup>3</sup>H]inulin was determined. A measured volume of ghosts, or an equal volume of buffer for the controls, was mixed with inulin dissolved in buffer, the mixture was centrifuged, and the radioactivity in the supernatant was counted. A sample containing 5.7 mg of ghost lipid (phospholipid and cholesterol) gave an inulin excluded volume of 0.021 mL. By use of a phospholipid to protein weight ratio of 0.40 and a density of red cell ghosts of 1.15 g/mL (Steck, 1974), 5.7 mg of ghost lipid corresponds to 0.0123 mL. Thus, the inulin excluded volume exceeds that based on membrane protein and lipid weights by a factor of about 1.7.

**EPR Spectra.** In general, EPR spectra were recorded with the following instrument settings: modulation amplitude 0.5 G; microwave power nominal 10 mW; scan time 8 min; time constant 0.5 s; scan range 100 or 200 G. The spectra were recorded on a Varian E-9 spectrometer. Temperature control was obtained with a stream of nitrogen by using the Varian variable temperature controller and a sample holder [R. D. Kornberg (1973) design presented in Gaffney & McNamee (1974)] which held the sample in the center of the temperature control Dewar. Temperatures were measured with a copper-constantan thermocouple placed just above the measuring region of the sample. The actual microwave powers at the sample were higher than the instrument setting by about a factor of 2 when the quartz insert was used. For samples that settled during measurement, the EPR cavity was mounted horizontally.

**Determination of Partition Coefficients.** EPR spectra were used to determine partition coefficients of <sup>14</sup>N amphiphiles between water and membrane. When EPR spectra are recorded under nonsaturating conditions, the area under the absorption curve is linearly proportional to the number of molecules in the sample. For nitroxide amphiphiles in aqueous membrane suspensions, there are two overlapping spectra: a broad one from label in membrane and a sharper, three-line spectrum from label in water. If line widths remain constant, peak heights can be used as an indicator of the relative amounts of label in the two environments. We have used changes in the height of the amphiphile water signal to measure, by difference, how much label is bound to membrane. However, because EPR signal intensities from sample to sample are determined by several factors in addition to concentration, an external standard of [<sup>15</sup>N]nitroxide was used for quantitation. [<sup>15</sup>N]Nitroxides have a two-line spectrum in water (see Figure 3). The <sup>15</sup>N standard was 2,2,6,6-tetramethyl-4-hydroxy[1-<sup>15</sup>N]piperidinyl-1-oxy [prepared by the method of Gaffney et al. (1981)], and it was contained as an aqueous sample in sealed, fine capillaries. One of these capillaries was inserted into the sample tube containing the <sup>14</sup>N amphiphile and membrane suspension, and the relative intensities of <sup>15</sup>N-labeled standard and <sup>14</sup>N amphiphile in the aqueous phases were determined. The absolute concentration

of the <sup>14</sup>N signal was obtained with reference to a calibration sample. For each of the <sup>15</sup>N reference capillaries, the calibration sample was obtained by preparing samples of <sup>14</sup>N amphiphile in buffer at known concentrations but with no membrane present.

Samples for determination of distribution coefficients were prepared by evaporating an aliquot of a benzene solution of the amphiphile in a test tube, adding a known volume of membrane suspension and/or buffer, and incubating the mixture for 30 min at room temperature. Each solution was transferred to three sample tubes consisting of a Corning 50- $\mu$ L glass pipet sealed at one end. An <sup>15</sup>N standard capillary was inserted into the middle of each sample tube for measurement and the <sup>14</sup>N/<sup>15</sup>N ratio of signal intensities was determined in triplicate. Reported values of partition coefficients are in units of moles of nitroxide per 1000 g of dry lipid divided by moles of nitroxide per 1000 g of aqueous buffer.

## Results

**Orientation of Bifunctional Spin-Labels in Membranes.** Whether the orientation of a bifunctional reagent in a membrane is perpendicular or parallel to the surface of a membrane may influence the course of a chemical cross-linking reaction. We have therefore examined the orientations of II-VIII and X-XII in oriented lipid bilayers.

When phosphatidylcholines are exposed to water vapor, they take up a limited amount of water for form a smectic phase that is easily oriented by shear. This sort of sample has been used to investigate the orientation of nitroxide phospholipids with respect to the normal to a phospholipid bilayer plane (Gaffney & McConnell, 1974). Figure 1a shows an EPR spectrum of IV in oriented membranes composed of hydrated dilauroyllecithin. The magnetic field (*H*) is perpendicular to the plane of the membrane bilayers for the spectrum shown in Figure 1a. Figure 1b shows the spectrum when the sample is rotated by 90° so that the field is perpendicular to the membrane normal (*N*). This amphiphile has a number of methylene groups on each side of the nitroxide equal to the number (10) of methylenes of the fatty acids of the lipid. The spectrum reveals that the predominant orientation of the amphiphile must be the transmembrane one because the molecular *z* axis is clearly approximately parallel to the membrane normal and to the direction along which the magnetic field is applied for the spectrum shown in Figure 1a. Because the amphiphile is symmetrical, it is very unlikely that it assumes a conformation other than the extended one which can give an orientation in which the molecular *z* axis is parallel to the membrane normal. The spectrum resulting from the transmembrane orientation is identified by arrows at the position of each of the three hyperfine components in Figure 1a. A minor component of the spectrum has features consistent with a conformation of the molecule such that both carboxyl groups of a single molecule are on the same side of the membrane. Parts c and d Figure 1 show computer-simulated EPR spectra for a molecule with the transmembrane orientation to support the assignment of spectra in Figure 1a. The results shown in Figure 1a are similar to those of the study of Seelig et al. (1972) except that the line widths are somewhat wider in our phospholipid bilayers compared to those reported for the liquid crystal sample. In both cases, the amphiphiles take up the transmembrane orientation if they are long enough to do so and if their chains are composed of *only* methylene groups.

In contrast, all of the other amphiphiles we have examined (II, III, V-VIII, X, and XII) take up an orientation with the *z*-molecular axis parallel to the surface of the lipid bilayer. Two general molecular conformations can give this result:

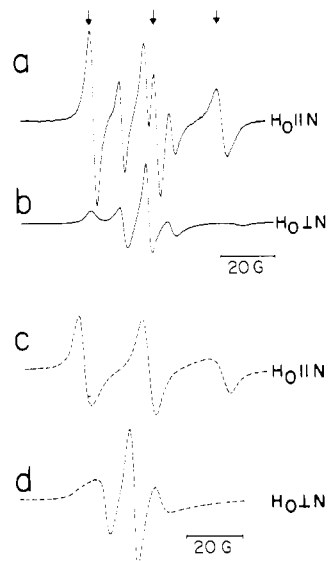


FIGURE 1: Experimental paramagnetic resonance spectra resulting from oriented bilayers of dilauroyllecithin containing one diacid spin-label, IV, for 100 lipids are shown in (a) and (b). The magnetic field is applied parallel to the membrane normal in (a) and perpendicular to it in (b) ( $H$  designates magnetic field;  $N$  designates the membrane normal). The arrows in (a) are at the positions of the spectral features which arise from the fraction of molecules that are in the transmembrane orientation. Spectrum b also contains contributions from molecules in two orientations in the same macroscopically aligned sample from which spectrum a was obtained. Although spectrum b accidentally resembles a spectrum from an isotropic distribution of membranes, it should not be confused with one. The relative contributions of molecules in the two orientations is about 4 to 1 in favor of the transmembrane orientation, based on the peak heights and line widths shown in (a). Computer-simulated spectra are shown in (c) and (d) for the spectra which would arise from the transmembrane conformation with the magnetic field applied parallel (c) and perpendicular (d) to the membrane normal. The spectra in (c) and (d) are taken from Gaffney & McConnell (1974), Figure 9a, for  $\theta = 0^\circ$ . A wider distribution of label orientations is assumed in the simulated spectra (c and d) than is apparently observed in the experimental spectra (a and b).

either the molecule may be bent in half and intercalated into the membrane or it can lie in the surface at the membrane/water interface. Only very small differences due to  $g$ -factor anisotropy and motional freedom should distinguish these molecular arrangements. Thus, if the extended chain of the amphiphile is too short to span the membrane or if a short chain is extended by *amide linkages* until it has a number of atoms equal to or greater than twice the number of carbons in the fatty acid chains of the membrane lipid, the molecule does *not* adopt a predominant transmembrane orientation. Figure 2 shows representative spectra for molecules which are *not* in the transmembrane orientation. The spectrum of bis-( $N$ -hydroxysuccinimide ester) VIII does show a second, minor component (indicated by arrows) that probably results from a small fraction of this molecule in the transmembrane conformation. An even smaller, minor-component signal is evident in the spectra of two of the longer molecules studied: VII and XII. In these cases, we cannot determine whether this additional component is from molecules with the transmembrane orientation or from disordered and essentially isotropic regions of the bilayers.

**Bifunctional Spin-Labels Partition between Membranes and Water.** As discussed above, there are two possible interpretations of the data for oriented bilayers. The EPR spectra could arise from molecules extended parallel to the surface of the lipid bilayer or bent with both ends on a single side of the membrane and the nitroxide group toward the center of

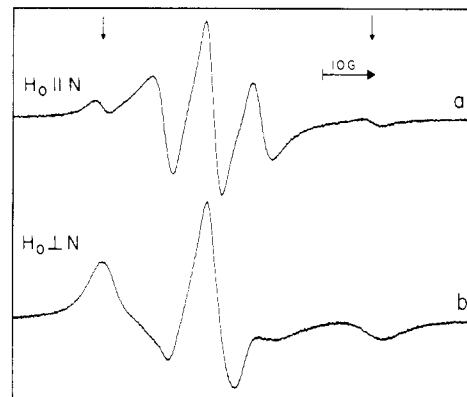


FIGURE 2: EPR spectra are shown for the bis( $N$ -hydroxysuccinimide ester), VIII, in oriented bilayers of dilauroyllecithin. In (a) the magnetic field is parallel to the bilayer normal, and in (b) it is perpendicular. The arrows in (a) indicate the positions of spectral components from molecules that may have the transmembrane orientation.

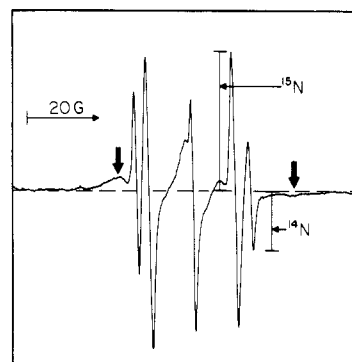


FIGURE 3: This EPR spectrum is representative of the spectra used to determine partition coefficients. The spectrum has three components; those from  $^{14}\text{N}$  amphiphile in membrane [the broad outer (heavy arrows) and central features of the spectrum], from  $^{14}\text{N}$  amphiphile in water (marked on the spectrum as  $^{14}\text{N}$ ) and from  $^{15}\text{N}$ -labeled standard in water (marked as  $^{15}\text{N}$ ). For this spectrum, the  $^{14}\text{N}$  amphiphile is the dicarboxylic acid, II. The standard in all measurements was [ $^{15}\text{N}$ ]Tempol in aqueous solution sealed in a fine capillary which was inserted into the wider capillary containing the membrane sample. The membrane in this case was a 30% by weight dispersion of dimyristoyllecithin in PBS, pH 6.0 at  $22^\circ\text{C}$ . The base line is shown as a dashed line. Spectral amplitudes were measured above or below the base line as shown.

the bilayer. These two arrangements of the molecules should have quite different binding isotherms for lipid membranes in water. We therefore examined the partition coefficients between membrane and water for the series of dicarboxylic acid nitroxides (II–IV) of varied chain length.

Figure 3 shows a representative EPR spectrum used in determining partition coefficients. The portions of the  $^{15}\text{N}$ -labeled standard and  $^{14}\text{N}$  nitroxide amphiphile signal amplitudes which are used for quantitative measurement of binding are indicated. These portions of the spectrum were chosen because they are relatively free of overlap with other spectral components. The linearity of the response of the  $^{14}\text{N}/^{15}\text{N}$  amplitude ratio to absolute concentration of  $^{14}\text{N}$  nitroxide in water was measured in order to determine that overlap does not interfere with measurements. By use of  $^{15}\text{N}$ -labeled standard solutions of several concentrations, the response of the ratio of signal amplitudes,  $^{14}\text{N}/^{15}\text{N}$ , is found to be a linear function of  $^{14}\text{N}$  concentration for the range from  $10^{-6}$  to  $10^{-4}$  M nitroxide as long as the standard capillary is chosen so that the signal amplitude ratio ranges from 0.15 to 10. The ratio is, of course, expected to be linear, given the right choice of  $^{15}\text{N}$ -labeled standard, below  $10^{-6}$  M, but poor signal-to-noise

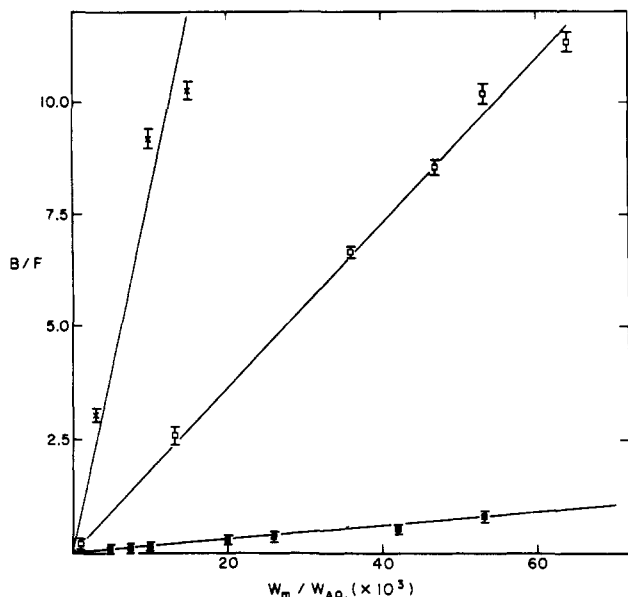


FIGURE 4: Plot of data from EPR spectra that was used to determine the partition coefficients for the diacid label II and the *N*-hydroxysuccinimide ester VIII of this diacid. The increase in partition coefficient that results when the pH of the aqueous phase is dropped to the point that the carboxyls of the dicarboxylic acid II are less ionized is demonstrated by plots that compare data for aqueous phases at two pHs. The samples for the data shown all contain dimyristoyllecithin, labels, and aqueous phase pHs as follows: (X) di-NHS label VIII, pH 6.0; (□) diacid label II, pH 6.0; (■) diacid label II, pH 8.0. *B/F* means moles of label bound to membrane per mole of label in the aqueous phase;  $W_M/W_{AQ}$  signifies dry weight of dimyristoyllecithin per weight of aqueous phase, assuming that the density of aqueous buffer at the temperature of measurement (22 °C) is 1. Conversion of EPR signal amplitudes to concentration units is described under Materials and Methods. The scale of the plot shown does not allow all of the data points from which partition coefficients were determined to be shown. Additional data points were obtained at the following *B/F* ( $W_M/W_{aq} \times 10^3$ ) values: for VIII (X), 41.5 (71), 31.1 (53), 25.6 (42), and 13.6 (20); for II, at pH 8 (■), 1.7 (111). Error bars represent the range of values for *B/F* obtained in three EPR measurements in separate tubes for a membrane sample at the given concentration. Spin-label concentrations were  $(2-5) \times 10^{-5}$  M.

at lower concentrations makes this a practical lower concentration limit. The upper concentration limit is that at which EPR spectra begin to be broadened by intermolecular spin exchange. The lower limit for detection of the EPR signal also sets a lower limit to the concentration of lipid for which a partition coefficient can be measured by this technique. If the partition coefficient were such that 10% of the label partitioned into membrane and it is desired to have no more than one label per 100 lipids, a  $10^{-5}$  M lipid suspension could be used if the partition coefficient were about  $10^4$ .

Figure 3 also contains a third component in addition to the sharp signals from the  $^{14}\text{N}$  amphiphile and  $^{15}\text{N}$ -labeled standard in aqueous phases. The third component is the broad signal from  $^{14}\text{N}$  amphiphile bound to membranes. In principle, partition coefficients could be determined from the relative amplitudes of the double integral of the broad and sharp  $^{14}\text{N}$  amphiphile signals. In practice, however, this approach would be limited to higher concentrations of membranes than those used in most of the experiments reported here.

If a true partition coefficient governs the equilibrium of the spin-label amphiphile, the ratio of bound to free label concentrations should be a linear function of the membrane concentration with a slope equal to the partition coefficient. This is indeed the case, as shown in Figures 4 and 5, for experiments with amphiphiles in varying concentrations of dimyristoyllecithin (DML). Figure 4 compares the binding

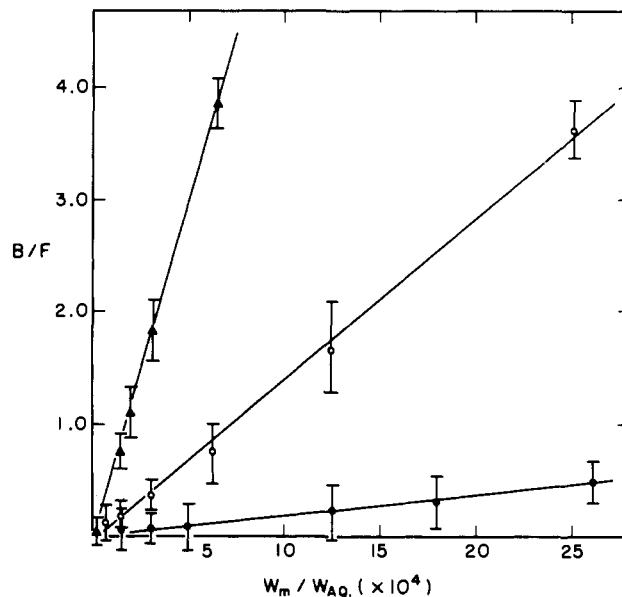
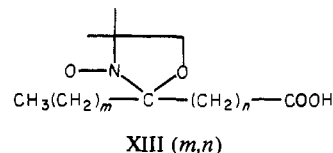


FIGURE 5: Contribution of additional methylene groups to the partition coefficients of fatty acid spin-labels. The plot is similar to that shown in Figure 4. Data are for XIIIa (3,8 FA) (●), XIIIb (5,8 FA) (○), and XIIIc (7,8 FA) (▲). Aqueous phase was at pH 8, and the temperature was 37 °C. Additional points for XIIIc which are off the scale of the figure are at 11.5 (20) and 6.8 (10) for *B/F* ( $W_M/W_{aq} \times 10^3$ ). Spin-label concentrations were  $(2-5) \times 10^{-5}$  M.

of II at pHs 6 and 8 with that of the *N*-hydroxysuccinimide ester of II. Binding data for monofunctional spin-label fatty acids, XIII (*m,n*) with *n* = 8 and *m* = 3, 5, or 7, are shown



in Figure 5. The results are self-consistent: the partition coefficient of the diacid II increases 8 times when the pH is dropped from above to nearer the expected aqueous carboxyl group  $\text{p}K_a$  (pH 8 to pH 6), and the NHS ester of the diacid has a partition coefficient 25 times that of the diacid at the higher pH. In both cases, loss of negative charge on the amphiphile increases binding to the membrane considerably. At the higher pH where the carboxyl group is partially ionized, the monofunctional fatty acid XIIIa binds to membranes much more strongly than the dicarboxylic acid with the same number of methylenes (counting the methyl group of the fatty acid with the methylenes for this comparison).

So that the contributions of charge could be distinguished from effects due to hydrophobic groups on binding of the spin-label amphiphiles to membranes, analogues extended by pairs of methylenes were compared in the binding assay. The results are given in Table II. For the fatty acid molecules, there is a substantial increase in binding as the number of methylene groups is increased. In contrast, when pairs of methylenes are added on both sides of the nitroxide in the dicarboxylic acids, there is virtually no increase in binding. This certainly suggests that the dicarboxylic acids bind to the lipid membranes in a way that does not involve much intercalation into the hydrocarbon interior of the bilayers.

The partition coefficients of diacids III and II are shown in Table II for two lipid systems, DML and lipids extracted from red blood cells. The lower partition coefficients in liposomes from erythrocyte lipids may reflect the influences of two effects. There may be a charge repulsion effect because

Table II: Partition Coefficients ( $K$ )<sup>a</sup> between Buffer and Lipid Dispersions

compound no.	diacids or diester <sup>b</sup>	pH	T (°C)	$K_{DML}$	$K_{RBCL}$
IV	di-C <sub>10</sub>	8	22	21.8	
III	di-C <sub>8</sub>	8	22	18.1	4.7
III	di-C <sub>8</sub>	8	37		6.3
II	di-C <sub>6</sub>	8	22	15.1	3.9
II	di-C <sub>6</sub>	8	37	22.2	4.4
II	di-C <sub>6</sub>	6	22	181.2	
cross-linking reagents					
VIII	di-C <sub>6</sub> -NHS	6	22	557.3	
XII	di-C <sub>6,2</sub> -TNB	8	22	55.1	
XII	di-C <sub>6,2</sub> -TNB	8	37	64.4	
<i>m,n</i> fatty acids					
XIIIa	3,8 FA	8	37	179	
XIIIb	5,8 FA	8	37	1449	279.2
XIIIc	7,8 FA	8	37	5926	

<sup>a</sup> The partition coefficient is defined as the ratio of the fraction of spin-label molecules bound to the membrane to the fraction of labels in the aqueous phase for a mixture containing equal weights of lipid and buffered water. Values of partition coefficients given are good to at least  $\pm 10\%$ ; most are  $\pm 2\%$ . <sup>b</sup> The abbreviations for molecules II-IV, VIII, and XII-XIII can be discerned from Table I: for instance, di-C<sub>10</sub> means ten methylenes on either side of the nitroxide in the diacid, IV. For the cross-linking reagents, NHS is *N*-hydroxysuccinimide ester and TNB is dithionitrobenzoate. Molecules XIIIa-c are fatty acids of general structure XIII (*m,n*) (see text) and have *m* methylenes between the terminal methyl group and the nitroxide and *n* methylenes from nitroxide to carboxyl group.

both the diacids and some of the phospholipid components are negatively charged. Alternatively, the presence of cholesterol in erythrocyte lipids makes a more rigid lipid phase and thus may diminish binding.

Table II also gives data for the partition coefficients of a dithionitrobenzoate, spin-label cross-linking reagent. This aromatic diacid binds a little more strongly than the aliphatic diacids do. In oriented bilayers, it was found to have the same orientation with respect to the membrane plane as the aliphatic diacids.

*Some Amphiphiles Prefer the Membrane/Water Interface to the Membrane Interior.* Table II also shows binding data taken for several bifunctional amphiphiles at 22 and 37 °C. There is little dependence of the partition coefficient on temperature. So that this could be tested further, the partitioning of II between DML and buffer was measured over the temperature range from 15 to 37 °C. For comparison, the partitioning of Tempo, the spin-label which has been widely used in measurements of the thermal behavior of lipids (Hubbell & McConnell, 1968; Shimshick & McConnell, 1973), was measured in DML over the same temperature range. Figure 6 shows the results. As expected, the partition coefficient of Tempo changes by a factor of 9 at the phase transition of DML (Shimshick & McConnell, 1973). In contrast, the partition coefficient of label II is virtually unresponsive to the phase transition of DML, even though at least 15% of the label in the sample is bound to the membrane under the conditions used for the experiment reported in Figure 6. Again, this is a result that is consistent with the amphiphile binding at the surface of the membrane. Label II was also unresponsive to the phase transition in a membrane suspension at pH 6.0.

As a final test that the majority of the bifunctional spin-labels binds preferentially at the lipid/water interface, partition coefficients between olive oil and water and between octanol and water were compared for the diacid, II, and the fatty acid,

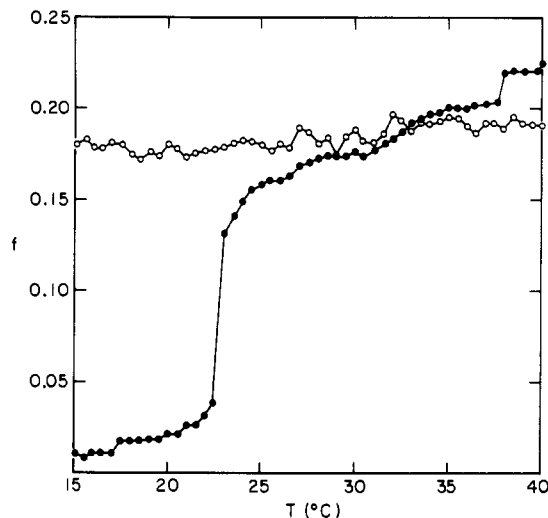


FIGURE 6: Fraction of spin-label in dimyristoyllecithin as a function of temperature is shown for two spin-label molecules: (O) the diacid II (C<sub>6</sub> diacid) and (●) Tempo. Clearly, only Tempo partitioning responds to the phase transition of DML. The concentration of DML in buffer was 1% by weight. Therefore, the fractions shown correspond to *B/F* ratios (for Tempo) of label at 20 °C of 0.0218 for *f* = 0.0213 and at 25 °C of 0.188 for *f* = 0.158. The partition coefficient of Tempo changes from 2.16 to 18.61 below and above the phase transition, respectively. Spin-label concentrations were  $2.5 \times 10^{-5}$  M for both spin-labels.

Table III: Comparison of Partition Coefficients for Several Nonpolar Phases<sup>a</sup>

amphiphile	pH	partition coefficient		
		$K_{DML}$	$K_{olive\ oil}$	$K_{octanol}$
II (di-C <sub>6</sub> )	8	15	0.006	ND
II (di-C <sub>6</sub> )	6	181	0.09	4
XIIIa (3,8 FA)	8	ND	2	24
XIIIb (5,8 FA)	8	620	24	277

<sup>a</sup> All partition coefficients are for distribution between the non-polar phase indicated and phosphate-buffered saline at the pH given. Measurements were made at 22 °C. Errors in measurement of partition coefficients are no greater than 10%. ND, not determined.

XIIIa. Data are shown in Table III. Partition coefficients for the monocarboxylic acid spin-label vary in predictable ways based on numerous literature values of partition coefficients for amphiphiles: the partition coefficient is 1-2 orders of magnitude smaller for olive oil/water than for membrane/water. In comparison, the partition coefficient for the diacid is more than 3 orders of magnitude higher in membranes than it is in bulk olive oil, again consistent with the label preferring the interfacial region between water and a nonpolar medium.

## Discussion

The aim of the experiments described here is to understand how the structures of hydrophobic, bifunctional molecules affect the way that these reagents bind to membranes. Although several molecules which are long enough to span a membrane have been studied, only in one case is the transmembrane orientation the preferred one. That is when the reagent is an oxazolidine nitroxide substituted with two, identical  $\omega$ -carboxyl polymethylene chains and when the number of methylenes on each side of the nitroxide ring is equal to or greater than the number of methylenes in the fatty acids of the lipid from which the membrane is prepared. Because these molecules with 20 or more methylenes are so insoluble in water that it would be difficult to introduce them



or derivatives of them into a biological membrane, we have also prepared analogues, of varied length, in which two methylene groups are replaced by an amide linkage. The amide group indeed gives a reagent with enhanced water solubility. However, all of the molecules extended by amide linkages take up a conformation in a membrane in which both polar ends of the molecule are on the same side of the membrane, yielding EPR signals in oriented membranes similar to those of bifunctional molecules which are of too short a length to span the bilayer. As we will discuss below, some of these molecules probably lie on the surface of the membrane and apparently do not intercalate into the hydrocarbon region even though they are sufficiently flexible that they could bend in half and insert their less polar portion into the membrane interior if this arrangement were the preferred one. This information about the orientation of long, flexible chains in a membrane adds to what is already known about the geometry of other chromophores in a membrane. The orientation of a number of fluorescent probes in lecithin and lecithin/cholesterol membranes has been determined (Badley et al., 1973; Andrich & Vanderkooi, 1976). Depending on the structure, these aromatic chromophores can either lie on the surface of the membrane or intercalate so that the molecular long axis is preferentially aligned with the bilayer normal. For instance, 2,2'-(vinylenedi-*p*-phenylene)bis(benzoxazole), which has length approximately equal that of 18 extended methylenes, has its long axis parallel the membrane normal and is located in the hydrocarbon interior of the membrane. Variations of this structure might be good candidates for a membrane-spanning reagent. Another generality about the orientations of molecules in membranes is that long hydrocarbon tails will anchor certain chromophores in defined orientations at the membrane surface. This is the case with diindocarbocyanine dyes, anchored by two alkyl chains (Waggoner, 1979), and chlorophyll *a*, anchored by its phytol chain (Steinmann et al., 1972; Lee, 1975, 1976). The orientations of local anesthetics and flavins at the lipid/water interfaces of model lamellar membranes have also been studied by polarized optical absorption (Johansson & Lindblom, 1980, 1981).

One of the most striking results of our study is the complete insensitivity of the partition coefficient of a spin-label dicarboxylic acid to the phase transition of a synthetic phospholipid membrane (Figure 6), even though this amphiphile clearly does associate with the lipid membrane. Several other experiments support the interpretation of this result that the amphiphiles I, in general, seek an interfacial region between membrane and water. (1) The disposition of the nitroxide chromophore in oriented lipid membranes as discussed above suggests an orientation of the long molecular axis that may be parallel to the surface of the membrane. (2) The partition coefficients of molecules, I, are hardly affected by varying the number of hydrophobic methylene groups whereas the partition coefficients of the fatty acids, XIII, depend strongly on the number of methylenes in the chain. (3) Bulk phase (olive oil/water or octanol/water) partition coefficients for the dicarboxylic acid labels, compared to those for membrane/water, are unusually low (membrane suspensions have a much larger surface to volume ratio than bulk phases). In contrast, for the fatty acid spin-labels, the respective partition coefficients are similar to those that have been reported for other monofunctional amphiphiles (Roth & Seeman, 1972). An alternative explanation of the orientation and binding results is that the bifunctional spin-labels seek a region of intermediate polarity. The fact that the ratio of  $K_{\text{octanol}}$  to  $K_{\text{olive oil}}$  is about 4 times higher for the dicarboxylic acid spin-labels compared

to the monocarboxylic acid labels (Table III) would favor this idea. Diamond & Katz (1974; Katz & Diamond, 1974a-c) used a similar argument in discussing the partitioning of solutes such as urea, ethyl acetate, and low molecular weight alcohols. Recently, Sturtevant has shown that while ethanol, ethyl acetate, and 1-butanol form well-defined solutions in lipid membranes, there is no evidence that urea dissolved in the hydrocarbon region of DML membranes (Sturtevant, 1982). It should also be noted that insensitivity of a partition coefficient to a lipid phase transition (Figure 6) does not in itself imply a surface orientation: it simply means that the free energy of transfer of the solute between solid and fluid lipid phases is zero. A near zero free energy of transfer may also explain the surprisingly small change in partition coefficient for *cis*-parinaric acid at a phase transition (Sklar et al., 1977). This probe clearly partitions into the hydrocarbon region of a membrane.

The increasing partition coefficients with increasing chain length of spin-label fatty acids (Table II) are in qualitative agreement with other experimental evidence that the spin-label fatty acids intercalate into the hydrocarbon region of a lipid bilayer. For instance, spin-label order parameters, when corrected for the differing time scales of this and other magnetic resonance techniques (Gaffney & McConnell, 1974), are quite similar to those obtained by deuterium NMR (Seelig & Seelig, 1977). The free energy of transfer of aliphatic molecules with chains of varying length from water to an organic solvent has been shown, for a number of amphiphiles, to be a regular function of the chain length with a contribution of about +800 cal/mol of methylenes (Tanford, 1973), with interaction of the amphiphile with water molecules being a dominant contribution to this result. For transfer of amphiphiles from water to micelles, the incremental free energy per methylene is slightly smaller. On the basis of the partition coefficients of the spin-label fatty acids listed in Table II, the difference in free energy of transfer from water to DML bilayers at 37 °C is 655 cal/single methylene in comparing XIIIa with XIIIb (with 14- and 16-carbon chain lengths, respectively) and 430 cal/methylene when XIIIb and XIIIc (16 and 18 carbons) are compared. While these numbers are suggestive of a similarity for spin-label and normal fatty acids in thermodynamics of transfer between water and micellelike structures (i.e., lipid bilayers), there are a number of reasons that the present spin-label data are only crude approximations to acceptable thermodynamic quantities. The partitioning of fatty acids poses special problems because of the state of ionization of the carboxyl group and the existence of dimers in both aqueous and organic phases (Smith & Tanford, 1973). To the extent that spin-label fatty acids behave like their aliphatic counterparts, it is likely that there is an appreciable fraction of dimers in the aqueous phase at the concentrations which must be used for spin-label measurements ( $10^{-5}$ – $10^{-4}$  M). There is also evidence from the response of the partitioning of fatty acid spin-labels to membrane surface charge that the labels may not be fully ionized in the lipid bilayer at pH 8.0 and physiological ionic strength (Gaffney & Mich, 1976). In fact, NMR measurements show that the  $pK_a$ s of fatty acids are about 7.3 in lecithin membranes and considerably higher in negatively charged membranes (Ptak et al., 1980). Also, although it has been pointed out that the bulk properties of bilayers composed of phospholipids in which one chain is spin-labeled are similar to those in which one chain has a double bond or methyl group at the same position (Chen et al., 1982), the contributions of the "branching" effect of the nitroxide group to the interactions of a fatty acid with water

Table IV: Comparison of Partition Coefficients for Amphiphiles in Lipid Model Membranes and Red Cell Ghosts<sup>a</sup>

amphiphile	pH	partition coefficient		
		$K_{DML}$	$K_{RBCL}$	$K_{RBCG}$ <sup>b</sup>
II (di-C <sub>6</sub> )	6	234 <sup>c</sup>	9	5
XIIIb (5,8 FA)	6	ND	495	678
	8	1449	279	303

<sup>a</sup> Measurements were made at 37 °C. Lipids were suspended in buffer as indicated under Materials and Methods. Ghosts were dialyzed for 18 h against this buffer prior to experiments to determine partition coefficients. Values of  $K$  for ghosts are good to at least  $\pm 20\%$ . Others are at least  $\pm 10\%$ . ND, not determined. <sup>b</sup> These values of  $K$  are based on weight of lipid for comparison with model membranes. The weight of lipid in red cell ghosts was estimated as described under Materials and Methods. A value of  $K$  based on total membrane weight (lipid and protein) can be obtained by multiplying these numbers by 0.4 (Steck, 1974). A value of  $K$  based on inulin excluded volume is obtained by multiplying the values shown by 0.23. <sup>c</sup> This value is less well determined than the others because it is based on a measurement at a single lipid concentration. This measurement was part of a study of the temperature dependence of partitioning (data similar to that that shown in Figure 6 except that the pH was 6.0). The value of  $K_{DML}$  at 22 °C was well determined by using data shown in Figure 4. This 22 °C value was multiplied by fractional change determined from the temperature dependence plot to get the value of  $K_{DML}$  at 37 °C.

have not been determined. It is of interest that Swanson et al. (1980) found the membrane binding contribution per methylene of octapeptins, substituted with fatty acids of varied lengths, to be of the order of 100–200 cal/mol of methylene. From this they concluded that the conformation of octapeptin in water is such that the peptide portion partially protects the fatty acid from normal interactions with solvent water. One additional comparison can be made between data in this report on partitioning of fatty acid spin-labels and published data for unlabeled fatty acids. The spin-label XIIIb is a 16-carbon fatty acid bearing an oxazolidine ring in place of a methylene in a normal fatty acid. The partition coefficient of XIIIb (Table IV) between water and red cell ghosts at pH 8.0 and 37 °C is similar to that of tridecanoic acid into ghosts at the same temperature but at pH 7.4 (Sallee, 1978). At pH 8.0, the latter partition coefficient should be slightly lower (Smith & Tanford, 1973). To the extent that the experiments are comparable, the data suggest that the oxazolidine nitroxide group lowers the partition coefficient from that of the parent fatty acid by an amount equal to the loss of about three methylenes. It will be of interest in future experiments to determine the contributions to binding of the methylene groups between the nitroxide and the carboxyl group.

Conrad & Singer (1979, 1981) report differences as large as 4 orders of magnitude in partition coefficients for several amphiphiles when results with membranes containing only lipids are compared with those composed of both lipids and proteins. These authors suggest that the binding of amphiphiles, of which many spin-labels are an example, is generally quite different for membranes with and without proteins. These effects are attributed to forces called a "large internal pressure" provided by membrane proteins. We found the suggestion of these authors that spin-labels may also be subject to the large internal pressure to be apparently at variance with previous studies of partitioning of spin-labels in both model and biological membranes. For instance, earlier attempts to quantitate the fraction of fluid lipid in a membrane compared the lipid/water distribution of Tempo in membranes of egg yolk lecithin with those from rabbit muscle sarcoplasmic reticulum (SR) (Robinson et al., 1972; McConnell et al., 1972).

When equal weights of lipid in the two membranes were compared for Tempo binding, the sarcoplasmic reticulum membrane bound 84% as much of the amphiphile as did egg lecithin. Thus, there seems to be only a slight effect of the proteins in the SR on a molecule that dissolves in the center of the lipid bilayer. In the present report, we ask whether the presence of membrane proteins might have a much different effect on the partition coefficients of molecules that seek a membrane/water interface from the effects of amphiphiles that intercalate into the hydrophobic regions of a membrane. Accordingly, we have compared partition coefficients of monofunctional and bifunctional spin-label carboxylic acids in membranes of dimyristoyllecithin, lipids extracted from red blood cells, and red cell ghosts. These data are presented in Table IV. Partition coefficients for both types of amphiphile are lower for red cell lipids than they are for DML. This difference could result from the "condensing effect" of cholesterol on the red blood cell lipids and/or from charge repulsion by the net negative surface charge of red cell lipids. Given the insensitivity of partitioning of the bifunctional carboxylic acids to the phase transition of DML (Figure 6), and thus to changes in the area per molecule at the surface, the surface charge argument offers the more plausible explanation for the diminished partition coefficients in red cell lipids compared to DML. In contrast, a comparison of partition coefficients for red cell *ghosts* with those for red cell *lipids* shows very little difference in binding of either the bifunctional amphiphiles that appear to lie on the membrane surface or the monofunctional ones that intercalate. These data are in sharp contrast with the trends of hygroscopic desorption measurements.

In summary, then, we find less effect of membrane proteins on partition coefficients than we do of lipid composition. Although we have not made measurements with any of the same molecules that have been the subjects of other studies (because the other molecules are not paramagnetic), it seems reasonable to compare our results with other binding measurements. Our data rely primarily on signals from label in water and are thus more similar to centrifugation techniques (Roth & Seeman, 1972) than to hygroscopic desorption (Conrad & Singer, 1979, 1981). The partition coefficient from centrifugation of the monofunctional alcohol decanol is only lower by a factor of 4 in red cell ghosts than it is in octanol. Our comparison of partition coefficients for a monofunctional fatty acid spin-label in the same systems shows a difference of the same magnitude. (Note, our data in Tables III and IV are for temperatures of 22 and 37 °C, respectively, and thus are not strictly comparable, but the temperature coefficient of  $K$  in RBCG is small.) In contrast, the hygroscopic desorption technique (Conrad & Singer, 1979, 1981) gives a partition coefficient for decanol in erythrocyte ghosts that is 0.04 compared to the value of 1226 obtained by centrifugation (Roth & Seeman, 1972). The partition coefficients from hygroscopic desorption for decanol in protein-free lipid membranes are 990–1130, close to the values from centrifugation for decanol in the protein-containing ghosts. Conrad and Singer suggest, as an explanation of their differences between membranes with and without protein, that the amphiphile may be present in three phases: the membrane, water, and micelles. According to this argument, micelles would remain with the membrane in centrifugation experiments and with the water in hygroscopic desorption. We are able to inquire about the possibility of one type of micelle in our EPR spectra by examining the line shapes. If a micelle containing  $\sim 20\%$  or more of the spin-label were present, it would contribute a very broad,

featureless line rising in the low-field region, passing through zero in the middle and dropping below the base line in the high-field region. In Figure 3, two features are apparent in the center of the spectrum. The wider one is from membrane-bound label. In contrast, a micelle should give little absorption in this region. We have examined spectra like those in Figure 3 for several membrane concentrations and several of our labels and find no evidence for the presence of substantial amounts of the label in micellar form. Thus, we do not believe the labels form pure micelles by themselves, but we cannot rule out the presence of some comicelles formed from low concentrations of the label and lipids extruded from red cell ghosts. The fact that we observe the same partition coefficient for the fatty acid label XIIIb at concentrations of lipid in red cell ghosts varying from 0.5 to 2 mg/mL and for the diacid in ghost samples that have 4–15 mg of total lipid/mL argues against gross artifacts resulting from micelle formation. All of our partition coefficient data are taken at label and membrane concentrations where the value of  $K$  is independent of either. Conrad and Singer have pointed out that the apparent  $K$ 's from centrifugation for chlorpromazine, and particularly for methochlorpromazine, are strongly dependent on membrane concentration in the region for which their data were obtained. The  $K$ 's are thus not true partition coefficients, and micelle formation could be involved. Whether there are similar nonlinearities in  $K$  vs. concentration for their decanol measurements is not made clear. However, if comicelles are to be taken as a plausible explanation for differences in hygroscopic desorption measurements and those of other techniques, a further experiment by the former technique is suggested. If all of the label in comicelles passes through the desorption filter with the aqueous phase, then a defined and measurable amount of red cell ghost lipid should also be lost.

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#### References

- Andrich, M. P., & Vanderkooi, J. M. (1976) *Biochemistry* 15, 1257–1261.
- Badley, R. A., Martin, W. G., & Schneider, H. (1973) *Biochemistry* 12, 268–275.
- Berliner, L. J. (1976) *Spin Labeling Theory and Applications*, Vol. I, Academic Press, New York.
- Berliner, L. J. (1979) *Spin Labeling Theory and Applications*, Vol. II, Academic Press, New York.
- Blomquist, A. T., Johnson, J. R., Diuguid, L. T., Shillington, J. K., & Spencer, R. D. (1952) *J. Am. Chem. Soc.* 74, 4203–4204.
- Cafiso, D. S., & Hubbell, W. L. (1981) *Annu. Rev. Biophys. Bioeng.* 10, 217–244.
- Castle, J. D., & Hubbell, W. L. (1976) *Biochemistry* 15, 4818–4831.
- Chen, S.-C., Sturtevant, J. M., Conklin, K., & Gaffney, B. J. (1982) *Biochemistry* 21, 5096–5101.
- Conrad, M. J., & Singer, S. J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76, 5202–5206.
- Conrad, M. J., & Singer, S. J. (1981) *Biochemistry* 20, 808–818.
- Diamond, J. M., & Katz, Y. (1974) *J. Membr. Biol.* 17, 121–154.
- Dodge, J. T., Mitchell, C., & Hanahan, D. J. (1963) *Arch. Biochem. Biophys.* 100, 119–130.
- Gaffney, B. J., & McConnell, H. M. (1974) *J. Magn. Reson.* 16, 1–28.
- Gaffney, B. J., & McNamee, C. (1974) *Methods Enzymol.* 32B, 161–198.
- Gaffney, B. J., & Mich, R. J. (1976) *J. Am. Chem. Soc.* 98, 3044–3045.
- Gaffney, B. J., Elbrecht, C. H., & Scibilia, J. P. A. (1981) *J. Magn. Reson.* 44, 436–446.
- Gaffney-McFarland, B. J., & McConnell, H. M. (1971) *Proc. Natl. Acad. Sci. U.S.A.* 68, 1274–1278.
- Hubbell, W. L., & McConnell, H. M. (1968) *Proc. Natl. Acad. Sci. U.S.A.* 61, 12–16.
- Hubbell, W. L. & McConnell, H. M. (1971) *J. Am. Chem. Soc.* 93, 314–326.
- Inouye, S., Soberon, X., Francheschini, T., Nakamura, K., Itakura, K., & Inouye, M. (1982) *Proc. Natl. Acad. Sci. U.S.A.* 79, 3438–3441.
- Johansson, L. B. A., & Lindblom, G. (1980) *Q. Rev. Biophys.* 13, 63–118.
- Johansson, L. B. A., & Lindblom, G. (1981) *Biophys. J.* 36, 735–741.
- Jones, R. G. (1947) *J. Am. Chem. Soc.* 69, 2350–2354.
- Katz, Y., & Diamond, J. M. (1974a) *J. Membr. Biol.* 17, 69–86.
- Katz, Y., & Diamond, J. M. (1974b) *J. Membr. Biol.* 17, 87–100.
- Katz, Y., & Diamond, J. M. (1974c) *J. Membr. Biol.* 17, 101–120.
- Keana, J. F. W., Keana, S. B., & Beetham, D. (1967) *J. Am. Chem. Soc.* 89, 3055–3056.
- Korten, K., Sommer, T. J., & Miller, K. W. (1980) *Biochim. Biophys. Acta* 599, 271–279.
- Lee, A. G. (1975) *Biochemistry* 14, 4397–4402.
- Lee, A. G. (1976) *Biochemistry* 15, 2448–2454.
- Libertini, L. J., Burke, C. A., Jost, P. C., & Griffith, O. H. (1974) *J. Magn. Reson.* 15, 460–476.
- McConnell, H. M., Wright, K. L., & McFarland, B. J. (1972) *Biochem. Biophys. Res. Commun.* 47, 273–281.
- Peters, K., & Richards, F. M. (1977) *Annu. Rev. Biochem.* 46, 523–551.
- Ptak, M., Egret-Charlier, M., Sanson, A., & Bouloussa, O. (1980) *Biochim. Biophys. Acta* 600, 387–397.
- Radda, G. K. (1975) *Methods Membr. Biol.* 4, 97–188.
- Robinson, J. D., Birdsall, N. J. M., Lee, A. G., & Metcalfe, J. G. (1972) *Biochemistry* 11, 2903–2909.
- Rose, H. G., & Oklander, M. (1965) *J. Lipid Res.* 6, 428–431.
- Roth, S., & Seeman, P. (1972) *Biochim. Biophys. Acta* 255, 207–219.
- Rouser, G., Fleischer, S., & Yamamoto, A. (1970) *Lipids* 5, 494–496.
- Sallee, V. L. (1978) *J. Membr. Biol.* 43, 187–201.
- Seelig, A., & Seelig, J. (1977) *Biochemistry* 16, 45–50.
- Seelig, J. (1970) *J. Am. Chem. Soc.* 92, 3881–3887.
- Seelig, J., Limacher, H., & Bader, P. (1972) *J. Am. Chem. Soc.* 94, 6364–6371.

- Seeman, P. (1972) *Pharmacol. Rev.* 24, 583-655.
- Shimshick, E. J., & McConnell, H. M. (1973) *Biochemistry* 12, 2351-2360.
- Sklar, L. A., Hudson, B. S., & Simoni, R. D. (1977) *Biochemistry* 16, 819-823.
- Smith, R., & Tanford, C. (1973) *Proc. Natl. Acad. Sci. U.S.A.* 70, 289-293.
- Steck, T. L. (1974) *J. Cell Biol.* 62, 1-19.
- Steinemann, A., Stark, G., & Läuger, P. (1972) *J. Membr. Biol.* 9, 177-194.
- Sturtevant, J. M. (1982) *Proc. Natl. Acad. Sci. U.S.A.* 79, 3963-3967.
- Swanson, P. E., Paddy, M. R., Dahlquist, F. W., & Storm, D. R. (1980) *Biochemistry* 19, 3307-3314.
- Tanford, C. (1973) *The Hydrophobic Effect*, Wiley, New York.
- Tse-Tang, M. W., Gaffney, B. J., & Kelly, R. E. (1980) *Heterocycles* 15, 965-974.
- Waggoner, A. P. (1979) *Annu. Rev. Biophys. Bioeng.* 8, 47-68.
- Willingham, G. L., & Gaffney, B. J. (1983) *Biochemistry* (following paper in this issue).
- Zantour, H., Pousse, A., & Brini, M. (1972) *Bull. Soc. Chim. Fr.*, 4715-4722.

## Reactions of Spin-Label Cross-Linking Reagents with Red Blood Cell Proteins<sup>†</sup>

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**ABSTRACT:** The reactions of two spin-label cross-linking reagents with components of red blood cells have been studied. A bifunctional bis(*N*-hydroxysuccinimide ester) reagent passes readily through the membrane of intact red cells and reacts almost exclusively with hemoglobin. The rotational motion of hemoglobin inside of red cells may be studied by electron paramagnetic resonance (EPR) by using this spin-labeling method. A second cross-linking molecule, a negatively charged, disulfide-exchange reagent, reacts both with membrane components and with hemoglobin when intact red cells are labeled. Upon reaction with membranes stripped of components other than band 3, this reagent readily produces dimers and higher oligomers of this protein. The EPR spectrum of band 3 labeled in this way shows that most of the spin-label molecules are highly immobilized on the protein. When the same reagent reacts with intact red cells, the cross-linked membrane products include components identified as band 3 dimers and a high molecular weight product consisting primarily of bands 1 and 2 but also containing lesser amounts of most of the other red cell membrane components and a slight amount of band 3. The reagent was made radioactive to demonstrate that the spin-label disulfide exchange reagent is indeed involved in formation of cross-linked products. Comparison of Coomassie Blue staining and radioactivity in the region where band 3 dimers are expected on a one-dimensional sodium dodecyl sulfate-polyacrylamide gel shows that there is approximately 0.5 cross-linking reagent per protein

in the region of band 3 dimers. Further proof that the spin-label reagent actually is involved in cross-linking some of the band 3 dimers is complicated by the possibilities of sulfhydryl oxidation and disulfide rearrangement. Chymotrypsin cleavage at the external, red cell face results in no significant cross-linked fragments on two-dimensional gels. However, chymotrypsin cleavage of red cell ghosts does reveal two peptides off the diagonal of a two-dimensional gel. The approximate molecular weights of these peptides are 13 000 and 8000. There are two possible explanations of the results of the chymotrypsin treatments. The more likely is that the 13 000- and 8000-dalton bands arise from an intramolecular cross-link or disulfide bond. The other explanation is that they do arise from an intermolecular cross-link, but this link is unstable during the chymotrypsin treatment of intact erythrocytes even though it is stable when cross-linked ghosts are treated with the protease. The 13 000- and 8000-dalton fragments may arise from disulfide formation rather than from cross-linking by the spin-label reagent because they are also observed when intact red cells are treated with the monofunctional reagents 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and [(2-aminoethyl)dithio]-2-nitrobenzoic acid. The results are consistent with a new disulfide bond being formed between an ~21 000-dalton, transmembrane portion of band 3 and a small fragment of the 35 000-dalton segment released by internal chymotrypsin cleavage of ghosts.

**T**he reactions of chemical reagents with components of the red blood cell have been used in studies of the geometry of erythrocyte membrane proteins, of the mobility of the band 3 protein in membranes, and of potential antisickling agents. Questions pertaining to the geometry of erythrocyte membranes have been addressed by using cross-linking reagents,

in particular ones that can be cleaved for positive identification of the components of the cross-linked products (Wang & Richards, 1974), by application of reagents to which the membrane is impermeable (Staros et al., 1975, 1981) and by chemical modification of specific proteolysis products (Steck, 1978; Rao & Reithmeier, 1979; Ramjeesingh et al., 1980). It has been possible to use optical techniques to study the lateral mobility of band 3 in intact erythrocytes (Fowler & Branton, 1977; Koppel & Sheetz, 1981) and the rotational motion of this integral membrane protein in ghosts (Nigg & Cherry, 1979) because several fluorescein and eosin derivatives are highly specific for band 3 among the protein components

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