Effects of Diethyl Ether on Membrane Lipid Ordering and on Rotational Dynamics of the Anion Exchange Protein in Intact Human Erythrocytes: Correlations with Anion Exchange Function†

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ABSTRACT: The roles of lipid ordering and protein dynamics on the function of the anion exchange protein (band 3) in *intact* human erythrocytes have been investigated. The effects of diethyl ether on the ordering of membrane lipids and on the rotational dynamics of band 3 were measured by EPR and saturation-transfer EPR spectroscopies, respectively, and correlated with the anion exchange function of band 3. With increasing concentration, diethyl ether monotonically decreased the ordering of membrane lipids near the polar head-group region, as reported by the lipid-soluble spin probe 5-doxylstearic acid, but produced comparatively little change in the ordering of lipids in the hydrophobic midzone, as reported by 16-doxylstearic acid. The rotational mobility of band 3, as reported by the affinity spin-label bis(sulfo-*N*-succinimidyl) doxyl-2-spiro-5′-azelate [Anjaneyulu et al. (1989) *Biochemistry 28*, 6583–6590], also increased monotonically with increasing ether concentration. This increase in rotational mobility was not due to a demonstrable change in its state of oligomerization, since band 3 was readily cross-linked by bis(sulfo-*N*-succinimidyl) suberate to covalent dimers in the presence or absence of ether. At concentrations up to 2 vol % ether, hemolysis of erythrocytes was negligible, and the spectroscopic changes observed were completely reversed following its removal. K_m, V_{max}, and $E_{act.}$ for sulfate uptake into chloride-loaded erythrocytes were not significantly affected by addition of ether. These data indicate that the rate-limiting step in sulfate–chloride exchange is not affected by decreased ordering of membrane lipids, suggesting that structural rearrangements of band 3 which may be involved in anion translocation probably do not involve regions of the protein which are in contact with the lipid head-group regions of the bilayer.

The anion exchange protein (band 3) in human erythrocytes facilitates the physiologically important exchange of Cl^- for HCO_3^- across the lipid bilayer [reviewed in Jennings (1989)]. Additionally, other small anions including sulfate (Ku et al., 1979) can traverse the bilayer via band 3. The catalytic mechanism of anion exchange has been extensively studied over the past decade [for reviews, see Cabantchik et al. (1978), Fröhlich and Gunn (1986), Passow (1986), and Jennings (1989)]. Considerable experimental evidence from exchange kinetics measurements suggests that translocation involves a ping-pong mechanism of an anion binding site on band 3 which alternates between inward- and outward-facing conformations with a low probability for the protein to alternate between those two conformations in the absence of a bound, exchangeable anion. Interestingly, one study has suggested that a remarkably large volume change may be associated with sulfate exchange (Canfield & Macey, 1984).

Elucidation of structure–function relationships of band 3 remains an important objective, due both to the importance of this protein in the erythrocyte and also to its use as a model for other transmembrane proteins. Recent advances including determination of the complete amino acid sequence of human erythrocyte band 3 (Tanner et al., 1988; Lux et al., 1989) and expression of site-directed mutants in oocytes (Garcia & Lodish, 1989) have opened new avenues for determination of structural features of the protein itself which are relevant to its function as an anion exchanger. However, it is also of interest to understand how the membrane lipids surrounding the protein affect its anion exchange function. Such information could provide important insights into the nature of physical rearrangements of band 3 structure which are involved in anion translocation across the bilayer.

Band 3 is an intrinsic transmembrane protein. Therefore, the physical state of membrane lipids surrounding the protein could potentially contribute to its function, particularly if the band 3 volume changes associated with anion translocation involve the transmembrane segments of the protein. Spectroscopic methods including electron paramagnetic resonance (EPR)[1] using lipid spin-label probes have provided a convenient means of assessing the ordering of membrane lipids in cellular systems [reviewed in Robinson et al. (1985)]. Additionally, recent advances in spectroscopic techniques and in the development of affinity probes have enabled studies of band 3 rotational dynamics by ST-EPR spectroscopy (Beth et al.,

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[1] Abbreviations: EPR, electron paramagnetic resonance; V_1, first harmonic, in-phase absorption EPR signal; ST-EPR, saturation-transfer EPR; V_2', second harmonic, out-of-phase absorption EPR signal; 5PBS7.4, 5 mM sodium phosphate/0.15 M NaCl buffer, pH 7.4; 106P7.4, 106 mM sodium phosphate buffer, pH 7.4; BSSDA, bis(sulfo-*N*-succinimidyl) doxyl-2-spiro-5′-azelate; doxyl, 4,4-dimethyloxazolidine-*N*-oxyl; 5-SASL, 5-doxylstearic acid; 16-SASL, 16-doxylstearic acid; *S*, order parameter; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BS^3, bis(sulfo-*N*-succinimidyl) suberate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; v_0, initial rate of sulfate uptake into chloride-loaded erythrocytes; $E_{act.}$, apparent activation energy for sulfate uptake into chloride-loaded erythrocytes.

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1986; Anjaneyulu et al., 1989) and by transient optical anisotropy approaches (Cherry et al., 1976; Nigg & Cherry, 1979), thereby enabling direct assessment of how changes in membrane order are experienced by band 3.

In the present work, we have evaluated the effects of diethyl ether on the ordering of erythrocyte membrane lipids by EPR spectroscopy using 5- and 16-SASL as probes. Having established concentrations of ether which lead to measurable changes in the order of membrane lipids without causing significant hemolysis or changes in cell shape, we have examined how these changes in bulk membrane lipid order affect band 3 by comparing the rotational diffusion of the protein in control and ether-treated cells. Finally, we have carried out functional characterization of band 3 in parallel studies by measuring kinetic and thermodynamic parameters of sulfate–chloride exchange in control and ether-treated erythrocytes. We conclude that decreasing the order of membrane lipids in the polar head-group region of the bilayer with diethyl ether leads to a significant increase in the rotational motion of band 3 with no apparent change in its oligomeric state and with no measurable alteration of functional parameters of sulfate–chloride exchange. Portions of this work have been published in the form of an abstract (Cobb et al., 1990).

Experimental Procedures

General Procedures. All experiments were carried out on *intact* erythrocytes isolated from freshly drawn venous blood which was collected in heparinized vacutainer tubes and immediately placed on ice. Erythrocytes were washed free of plasma and other cells by three suspension and centrifugation cycles in 5PBS7.4. Packed erythrocytes obtained from the final wash were further prepared for individual experiments as described below. All isolation steps and preparative procedures were carried out at 0–4 °C unless otherwise noted.

BSSDA (Anjaneyulu et al., 1989) and BS^3 (Staros, 1982) were prepared as described previously. 5-SASL and 16-SASL were purchased from Aldrich Chemical Co.

Labeling of Erythrocytes with 5-SASL or 16-SASL. Stock solutions of 5-SASL and 16-SASL (Figure 1) were prepared in ethanol at 2 mM concentration. Twenty microliters of 5-SASL or 16-SASL was then transferred to a glass tube, and the ethanol was evaporated under a stream of nitrogen. One milliliter of packed erythrocytes in 5PBS7.4 was added to the dried spin probe and incubated for 10 min with hand-warming. This procedure resulted in essentially complete incorporation of spin probe into erythrocyte membranes at a final concentration of 40 nmol/mL of suspension. EPR spectra were recorded on the packed erythrocytes and analyzed for determination of the probe order parameter as described below.

Labeling of Erythrocytes with BSSDA. Packed erythrocytes in 5PBS7.4 were added to 20 volumes of 106P7.4 and respun to pellet the cells. After gentle resuspension, the packed cells were diluted with an equal volume of 106P7.4 containing freshly dissolved 100 μM BSSDA (Figure 1; 50 μM final concentration), and the sample was incubated at room temperature for 15 min. The reaction was quenched by adding 40 volumes of 5PBS7.4 containing 0.5% (w/v) bovine serum albumin and incubating for an additional 30 min at room temperature. The labeled erythrocytes were pelleted by centrifugation and washed twice in 40 volumes of 5PBS7.4 by centrifugation and resuspension. Packed cells from the final wash were used to record EPR and ST-EPR spectra as described below.

Addition of Diethyl Ether to Erythrocytes. Diethyl ether and a Hamilton syringe were placed in an ice bath and allowed to come to thermal equilibrium. Packed erythrocytes (500 μL) labeled with 5-SASL, 16-SASL, or BSSDA were transferred to a 1.5-mL polypropylene microfuge tube and placed on ice. Ether was drawn into the cooled Hamilton syringe and slowly injected directly into the erythrocyte suspension below the surface at the selected volume percent. The microfuge tube was then tightly capped and the sample inverted several times to allow thorough mixing. Samples were transferred via a cold glass pipet into the EPR sample cell which was then tightly capped to minimize loss of ether by evaporation during the time of data acquisition.

EPR and ST-EPR Measurements. X-band EPR and ST-EPR spectra were recorded on a Bruker ESP-300 spectrometer equipped with an ER-4103 TM_{110} cavity and an ER-4111VT variable-temperature unit. Sample temperature was maintained during data acquisition by blowing precooled nitrogen into the cavity through the front optical port and was continuously measured with a digital thermometer by placing the temperature probe into the top of the cell in contact with the sample. Samples were contained in a WG-813 (Wilmad Glass Co.) aqueous flat cell for recording all spectra. V_1 EPR spectra were recorded at 100-kHz field modulation of 1.0-G amplitude (peak-to-peak) using a microwave observer power of 10 mW which corresponded to 0.07 G in the rotating frame (peroxylamine disulfonate calibrated; Beth et al., 1983). V_2' ST-EPR spectra were recorded at 50-kHz field modulation (100-kHz detection) of 5.0-G amplitude (peak-to-peak) using a microwave observer power of 79 mW which corresponded to 0.2 G in the rotating frame. The phase setting for detecting the 90° out-of-phase signal was determined by the self-null method using a microwave observer power of 1 mW (Thomas et al., 1976). Spectra were accumulated with the ESP-300 data system by signal averaging and were 100-G scans consisting of 1024 data points. A point-by-point spectral subtraction routine with linear base-line correction developed for this system was employed for data analyses.

Analysis of EPR and ST-EPR Spectra. V_1 EPR spectra obtained from 5-SASL- and 16-SASL-labeled erythrocytes were analyzed for determination of the nitroxide order parameter (S) as described by Griffith and Jost (1976) using the relationship:

$$S = (A_{max} - A_{min})/[2A_{zz} - \tfrac{1}{2}(2A_{xx} + 2A_{yy})]$$

The splittings A_{max} and A_{min} were measured from experimental spectra recorded on triplicate samples. Values for the magnetic tensor elements A_{zz}, A_{xx}, and A_{yy} were taken from Gaffney (1976) for 5-doxylpalmitate bound to lyophilized bovine serum albumin. S values were calculated without polarity or nuclear manifold overlap corrections (Gaffney, 1976).

V_2' ST-EPR spectra obtained from BSSDA-labeled erythrocytes were analyzed by measuring the motionally sensitive ratio parameters L''/L and C'/C (Thomas et al., 1976). Over the range of line shapes observed in the present studies, these two ratio parameters increase approximately linearly with increasing correlation time for isotropic rotational motion (Thomas et al., 1976) and with increasing correlation time for uniaxial anisotropic rotational motion for the case where the nitroxide z axis is orthogonal to the uniaxial diffusion axis (Beth et al., unpublished computations). Therefore, data from the ST-EPR measurements are presented as the measured ratio parameters versus diethyl ether concentration and are discussed in the text by using the approximation that their values are proportional to the rotational mobility of band 3.

BS^3 Cross-Linking of Control and Ether-Treated Erythrocytes. Two 0.5-mL aliquots of packed erythrocytes in 5PBS7.4 were transferred to 1.5-mL polypropylene microfuge tubes on ice. One percent (v/v) diethyl ether was added to

one of the tubes, the tube was capped, and the sample was inverted several times to allow mixing. An equal volume of freshly prepared 10 mM BS^3 in 5PBS7.4 was rapidly added to each sample, the samples were mixed and then tightly capped, and the BS^3 reaction was allowed to proceed at room temperature for 15 min. The reaction was quenched by addition of 40 volumes of 5PBS7.4 containing 0.5% (w/v) bovine serum albumin followed by incubation for 30 min at room temperature. The erythrocytes were then washed 2 times with 40 volumes of 5PBS7.4. Membranes were prepared by lysing labeled cells in 40 volumes of 5 mM sodium phosphate buffer, pH 8.0, with three subsequent washes to remove hemoglobin and other cytosolic components as described previously (Anjaneyulu et al., 1989). Efficacy of band 3 dimer cross-linking was assessed by SDS–PAGE separation of solubilized ghost membrane proteins (Fairbanks et al., 1971).

Measurement of Sulfate–Chloride Exchange. Packed erythrocytes from freshly drawn whole blood were washed once in 27 mM glycylglycine/139.5 mM NaCl, pH 7.4. The packed cells were washed 2 additional times with 40 volumes of uptake buffer consisting of 271 mM sucrose/27 mM glycylglycine/1 mM KCl/1.4 mM $MgCl_2$/1.4 mM $CaCl_2$, pH 7.4. Following the final wash, the packed erythrocytes were incubated on ice for 1 h prior to the start of the assay. The hematocrit was then adjusted to 20% with the same buffer, and all solutions used in the uptake experiments were preequilibrated to the desired temperature in a refrigerating circulator. Uptake experiments were initiated by quickly mixing a 0.35-mL aliquot of the 20% Hct cell suspension in a 1.5-mL polypropylene microfuge tube with 0.35 mL of uptake buffer containing the desired concentration of total sulfate which included $^{35}SO_4^{2-}$ (New England Nuclear) at a known tracer specific activity and a variable concentration of sucrose in order to maintain isoosmolarity. Uptake was terminated at selected time points by rapid transfer of 0.2 mL of cell suspension into 5.0 mL of ice-cold glycylglycine/NaCl buffer containing 200 μM DIDS. The cells were immediately pelleted by centrifugation at 5 °C and then washed once with 5 mL of the DIDS-containing buffer. The packed erythrocytes were lysed with 0.5 mL of ice-cold water followed by addition of 0.5 mL of 1 M perchloric acid to precipitate hemoglobin and other intracellular proteins. Following centrifugation of these samples, the quantity of SO_4^{2-} transported into the cells was determined from the specific activity by counting $^{35}SO_4^{2-}$ in 0.7 mL of the supernatant in 2.0 mL of ScintiVerse II (Fischer) in a liquid scintillation counter (Beckman LS3100). Initial rates of uptake (v_0's) were calculated by linear regression analysis of cellular radioactivity versus time and are expressed as micromoles of intracellular SO_4^{2-} per milliliter of packed cells per minute. Kinetic parameters, V_{max} and K_m, were determined from Haynes-Woolf plots of SO_4^{2-} concentration/v_0 versus SO_4^{2-} concentration. Apparent activation energies ($E_{act.}$'s) were determined from plots of ln v_0 versus T^{-1} by linear regression analyses. v_0's were determined as described above at 10 mM sulfate concentration at each temperature.

Uptake measurements on diethyl ether treated cells were carried out by using the same protocols described for control cells. Ether was added slowly below the surface of the cell suspension to the desired volume percent from a cold Hamilton syringe; the sample was tightly capped and mixed, and then allowed to equilibrate at the desired temperature for 5 min in the refrigerating circulator. Uptake experiments were initiated by addition of SO_4^{2-}, the tubes were again capped and then incubated until uptake was stopped by rapid transfer of 0.2 mL of cell suspension into uptake buffer containing 200 μM DIDS as described above. Samples were prepared for determining intracellular SO_4^{2-} content, and the resulting data were analyzed by using the same approaches described for control cells.

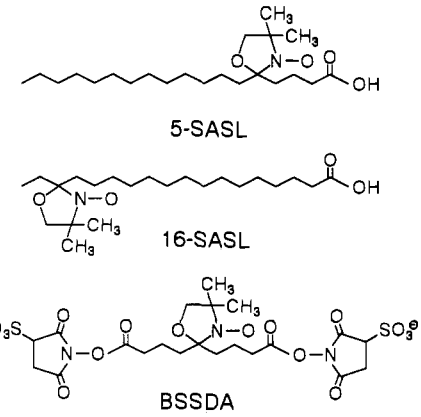

FIGURE 1: Chemical structures of the spin-labels employed. The two stearic acid spin probes, 5-SASL and 16-SASL, were employed to monitor the ordering of lipids in the polar head-group and the hydrophobic midzone regions of the erythrocyte membrane, respectively. The membrane-impermeant bifunctional spin-label, BSSDA, was employed to affinity-label band 3 in *intact* erythrocytes, thereby providing a probe of band 3 rotational motion.

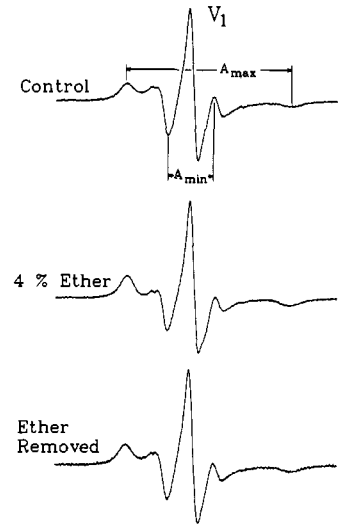

FIGURE 2: Effects of ether on V_1 EPR spectra of 5-SASL-labeled erythrocytes. *Intact* erythrocytes were labeled with 5-SASL (40 nmol/mL of packed cells) as described under Experimental Procedures. The upper spectrum was obtained from control erythrocytes. The middle spectrum was obtained from erythrocytes treated with 4 vol % diethyl ether. The lower spectrum was obtained from erythrocytes treated with 4 vol % ether followed by two washes to remove the ether. All spectra were recorded at 20 °C. Spectral structurings which were employed to measure the splittings A_{max} and A_{min} are defined in the upper spectrum.

Efficacy of SO_4^{2-} uptake inhibition by DIDS was determined under the same assay conditions. Erythrocytes were diluted to 20% Hct in uptake buffer, and DIDS was added to a final concentration of 50 μM, followed by a 5-min incubation at 20 °C. Uptake experiments were initiated by addition of SO_4^{2-}, and exactly the same assay procedures were followed as with the control and ether-treated cells.

Results

Effects of Diethyl Ether on the Order Parameter of 5-SASL- and 16-SASL-Labeled Erythrocytes. Addition of diethyl ether to *intact* erythrocytes labeled with 5-SASL (Figure 1) resulted in progressive changes in the EPR spectrum

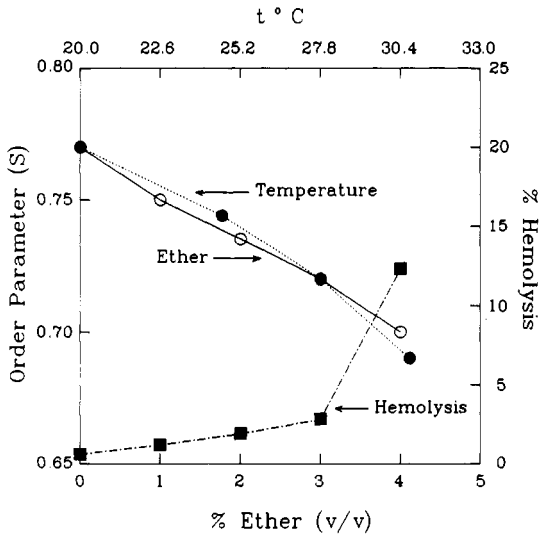

FIGURE 3: *S* versus ether concentration and temperature for 5-SASL-labeled erythrocytes. The order parameter (*S*) for the 5-SASL probe was determined from the two upper EPR spectra in Figure 2 plus from additional spectra recorded at 1, 2, and 3 vol % ether (spectral data not shown). *S* values determined from the spectra are plotted as open circles and connected with solid lines. Also plotted as closed circles connected with dotted lines are *S* values determined from EPR spectra obtained from control erythrocytes as a function of temperature (spectral data not shown). The closed squares connected by dot–dash lines are the percent of total cells hemolyzed by addition of ether at the indicated concentrations. The percent hemolysis was determined as the ratio of A_{415} from the supernatant of ether-treated cells to the A_{415} of supernatant following hemolysis of an equal volume of control cells in water.

indicative of "fluidization" or disordering of the membrane (Figure 2). The observed spectral changes were completely reversed after removal of added ether by repeatedly washing the cells (Figure 2, lower). The spectra changes produced by ether treatment are quantitated in terms of the order parameter (*S*) for the 5-SASL probe in Figure 3 where it is apparent that *S* decreases approximately linearly with increasing ether concentration from 0 to 4% by volume. Also shown in Figure 3 is the change in *S* resulting from increasing the sample temperature of 5-SASL-labeled control erythrocytes. Comparison of these data indicates that addition of 4 vol % ether decreased *S* by approximately the same amount as a 10 °C increase in sample temperature. However, addition of ether at levels higher than 3 vol % resulted in significant hemolysis as shown. Therefore, subsequent studies on band 3 rotational dynamics and anion exchange function presented below were carried out at ether concentrations of 2 vol % or lower. At these lower concentrations, hemolysis was negligible (Figure 3), and the erythrocytes maintained their biconcave disk shape when viewed under a light microscope (data not shown).

With the 16-SASL probe, addition of diethyl ether resulted in only very subtle changes in the EPR spectrum (Figure 4). These data are quantitated in terms of *S* for the 16-SASL probe in Figure 5. Also shown is the change in *S* resulting from increasing the sample temperature of 16-SASL-labeled control cells. Clearly, ether produces less of an effect on the ordering of membrane lipids in the hydrophobic midzone of the bilayer than it does near the polar head-group region. This statement is supported by comparing the ether/temperature data obtained with 5-SASL (Figure 3) with those obtained with 16-SASL (Figure 5). With 16-SASL, 4 vol % ether produced much less change in *S* than did a 10 °C change in sample temperature.

Effect of Diethyl Ether on the Rotational Motion of Band 3. In previous work, it has been shown that BSSDA affinity

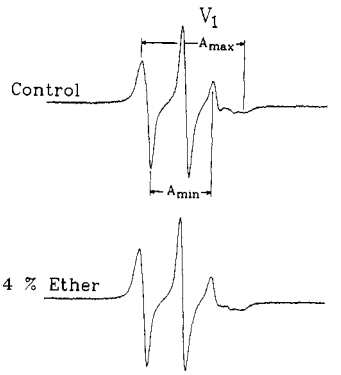

FIGURE 4: Effects of ether on V_1 EPR spectra of 16-SASL-labeled erythrocytes. *Intact* erythrocytes were labeled with 16-SASL (40 nmol/mL of packed cells) as described under Experimental Procedures. The upper spectrum was obtained from control erythrocytes. The lower spectrum was obtained from erythrocytes treated with 4 vol % diethyl ether. Both spectra were recorded at 20 °C. Spectral structurings which were employed to measure the splittings A_{max} and A_{min} are defined as the upper spectrum.

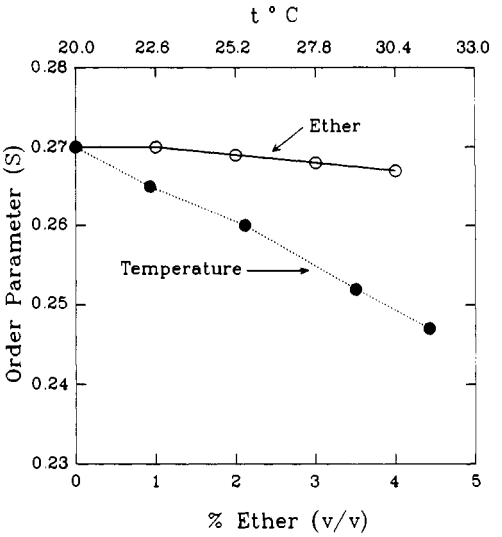

FIGURE 5: *S* versus ether concentration and temperature for 16-SASL-labeled erythrocytes. The order parameter (*S*) for the 16-SASL probe was determined from the two EPR spectra shown in Figure 4 plus from additional spectra recorded at 1, 2, and 3 vol % ether (spectral data not shown). *S* values determined from the spectra are plotted as open circles connected with solid lines. Also plotted as closed circles connected with dashed lines are *S* values determined from EPR spectra obtained of control erythrocytes as a function of temperature (spectral data not shown).

spin-labels band 3 in *intact* erythrocytes and that the rotational mobility of the spin-labeled protein can be inferred from the ST-EPR spectrum (Anjaneyulu et al., 1989). As shown in Figure 6, changes in the V_2' ST-EPR spectrum of BSSDA-labeled band 3 following addition of 2 vol % ether indicated a significant increase in the rotational mobility of the protein (Thomas et al., 1976). The observed spectral changes were completely reversed by removal of added either by repeated washing of cells (Figure 6, lower). Changes in the motionally sensitive ratio parameters, L''/L and C'/C, are quantitated in Figure 7 for the two ether concentrations shown in Figure 6 plus the same parameters from additional ST-EPR measurements made at 0.5 and 1 vol% ether (spectral data not shown). Since the ratio parameters increase approximately linearly with increasing rotational correlation time over the range of line shapes observed in this study (see Experimental Procedures), the data presented in Figure 7 suggest that the rotational mobility of band 3 increases rather abruptly between control cells and those treated with 0.5 vol % ether followed

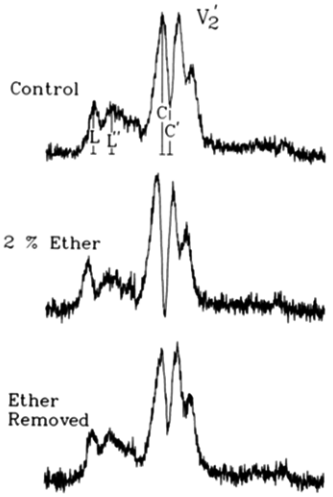

FIGURE 6: Effects of ether on V_2' ST-EPR spectra of BSSDA-labeled erythrocytes. The upper spectrum was obtained from control erythrocytes labeled with 50 μM BSSDA as described under Experimental Procedures. The middle spectrum was obtained from BSSDA-labeled erythrocytes treated with 2 vol % ether. The lower spectrum was obtained from erythrocytes treated with 2 vol % ether followed by two washes to remove the ether. In the upper display, field positions where spectral amplitudes were measured for computation of motionally sensitive ratio parameters are defined. All spectra were recorded at 20 °C, and each had the contribution from BSSDA-labeled membrane lipid removed by spectral subtraction (Anjaneyulu et al., 1989).

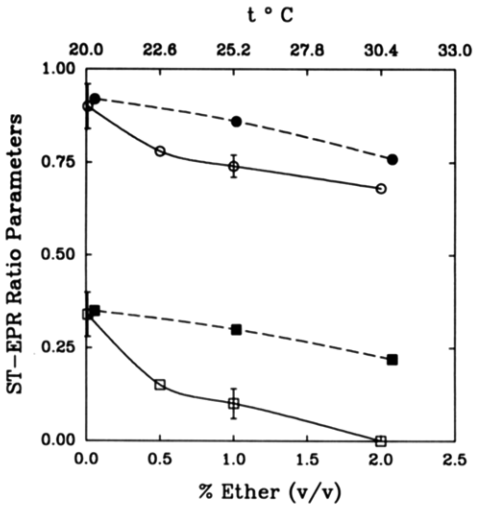

FIGURE 7: Ratio parameters from ST-EPR spectra versus ether concentration and versus temperature. Ratios of spectral amplitudes at field positions L'' and L and at field positions C' and C were measured from the experimental spectra shown in Figure 6 plus from additional spectra recorded on erythrocytes treated with 0.5 and 1 vol % ether (Thomas et al., 1976; spectral data not shown). Values for the ratio parameter L''/L are plotted as open circles. Values for C'/C are plotted as open squares. Data points from control and 1 vol % ether-treated erythrocytes are plotted as the average value ± SD (n = 5). Data points from 0.5 to 2 vol % ether-treated samples are plotted as the average value (n = 2). ST-EPR spectra from the 0% control and ether treated samples were recorded at 20 °C. Values for L''/L from control erythrocytes at 20.3, 25.3, and 30.8 °C are plotted as closed circles. Values for C'/C from control erythrocytes at the same temperatures are plotted as closed squares. The small contribution from BSSDA-labeled membrane lipid was digitally subtracted from all spectra before measurement of ratio parameters (Anjaneyulu et al., 1989).

by a more subtle, linear increase between 0.5 and 2 vol % ether.

Effect of Diethyl Ether on the Oligomeric State of Band 3. Previous work has shown that band 3 subunits in *intact* erythrocytes can be nearly quantitatively cross-linked to covalent dimers with the membrane-impermeant bifunctional reagent BS^3 (Staros, 1982; Staros & Kakkad, 1983). We have addressed the question of whether diethyl ether disrupts the band 3 dimer by comparing the BS^3 cross-linking efficiency in control and 1 vol % ether-treated erythrocytes. As shown in Figure 8, 1 vol % ether did not detectably alter the yield of band 3 dimers produced by BS^3 treatment.

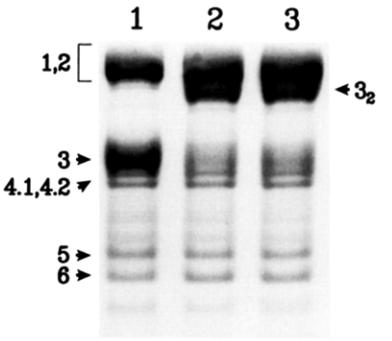

FIGURE 8: SDS–PAGE of erythrocyte membrane proteins. Ghost membranes were prepared from erythrocytes as described under Experimental Procedures. Membrane proteins were solubilized by addition of 1% (w/v) SDS and the proteins separated by electrophoresis (Fairbanks et al., 1971). Lane 1 is from control, untreated erythrocytes. Lane 2 is from *intact* erythrocytes treated with 5 mM BS^3. Lane 3 is from *intact* erythrocytes treated with 5 mM BS^3 in the presence of 1 vol % ether. Bands are identified to the left of lane 1. The band labeled 3_2 is band 3 dimer produced by cross-linking of subunits with BS^3 (Staros & Kakkad, 1983).

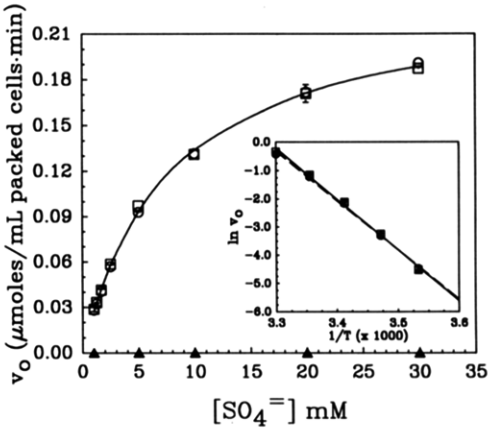

FIGURE 9: Initial rate of sulfate uptake versus sulfate concentration and apparent activation energy of sulfate–chloride exchange. Sulfate uptake into chloride-loaded erythrocytes was measured as described under Experimental Procedures. The open squares are from control, untreated erythrocytes. The open circles are from erythrocytes treated with 1 vol % ether. The closed triangles are from control erythrocytes pretreated with 50 μM DIDS before addition of sulfate. Points are average values ± SD (n = 4). Initial rates of uptake were determined at 20 °C. The solid line through the points was computed from the Michaelis–Menten equation using a K_m of 7.7 mM and a V_{max} of 0.24 μmol (mL of packed cells)$^{-1}$ min^{-1} which were determined from a Hanes–Woolf plot of the pooled control and 1 vol % ether data (plot not shown). *Inset*: The initial rate of sulfate uptake (v_0) into chloride-loaded erythrocytes was determined at the indicated temperatures as described under Experimental Procedures. These data are plotted as ln v_0 versus the reciprocal of absolute temperature. The open squares are from data obtained on control, untreated erythrocytes. The open circles are from erythrocytes treated with 1 vol % ether. The solid straight line was computed by linear regression of the data points from control erythrocytes (r = 0.998). The dashed straight line was computed by linear regression of the data points from 1 vol % ether-treated erythrocytes (r = 0.998). The points at each temperature are the mean of three determinations (±SD). The computed $E_{act.}$'s were 35.4 and 34.6 kcal/mol for control and 1 vol % ether-treated erythrocytes, respectively.

Effects of Diethyl Ether on Kinetic and Thermodynamic Parameters of Sulfate–Chloride Exchange. The uptake of

sulfate into chloride-loaded erythrocytes (sulfate–chloride exchange) exhibits conventional saturation behavior as shown in Figure 9. Hanes–Woolf replots of these data were linear ($r > 0.99$). Linear regression analysis of the pooled data from control and 1 vol % ether-treated erythrocytes yielded values for K_m and V_{max} of 7.7 mM and 0.24 μmol of sulfate (mL of packed cells)$^{-1}$ min^{-1}, respectively. The K_m value reported above may be slightly overestimated because of the presence of 6.6 mM Cl^- in the assay, but there was no difference between control and ether-treated cells. In both control and ether-treated cells, sulfate uptake was effectively (>98%) blocked at all concentrations of sulfate used by addition of 50 μM DIDS, indicating that it was band 3 mediated exchange which was being measured (control data shown in Figure 9).

The $E_{act.}$ for sulfate–chloride exchange was determined in control erythrocytes and in erythrocytes pretreated with 1 vol % ether as shown in Figure 9, *inset*. Plots of ln v_0 versus T^{-1} were linear over the range of temperature from 0 to 30 °C. Linear regression analyses of the data yielded $E_{act.}$'s of 35.4 and 34.6 kcal/mol for control and 1 vol % ether-treated cells, respectively.

Discussion

A primary goal of the studies presented here was to gain insight into how the physical state of membrane lipids contributes to the functional properties of anion translocation via band 3. Such information can be obtained, in theory, by altering the physical state of the bilayer and then measuring how this alteration affects functional properties of anion exchange. Temperature is the most obvious experimental parameter which can be easily manipulated to alter the ordering of membrane lipids. However, temperature will also have a direct effect upon the protein, making assessment of the relative contributions to altered function by lipid and protein difficult. An alternate approach, which we have pursued, is to select an agent which disorders lipids at constant temperature without causing significant perturbations to band 3. Previous studies of functional properties of sarcoplasmic reticulum Ca^{2+}-ATPase (Salama & Scarpa, 1980; Bigelow & Thomas, 1987) suggested that diethyl ether would be an appropriate lipid-disordering agent which might have minimal direct effects on membrane proteins.

Numerous studies have shown that diethyl ether reversibly incorporates into the erythrocyte membrane, producing a number of measurable effects including disordering of lipids [e.g., see Vanderkooi et al. (1977)] and expansion of the membrane [e.g., see Bull et al. (1982)]. In the present work, we observed significant disordering of lipids by ether in the polar head-group region of the bilayer, as reported by the 5-SASL probe (Figures 2 and 3), with apparently less pronounced effects in the hydrophobic midzone, as reported by the 16-SASL probe (Figures 4 and 5). It is important to recognize that the spin-labeled stearic acid probes used in these studies partition into the erythrocyte membrane rather nonspecifically and, thus, the order parameters determined reflect a weighted "average" over all environments accessible to the probes. Therefore, it is important to determine if the bulk membrane disordering effect of ether reported by the stearic acid spin probes reflects the disordering of lipids surrounding band 3 since it is these lipids which would potentially have the greatest effect on anion exchange function.

Direct assessment of the effects of ether on the EPR signal from 5-SASL or 16-SASL probes in the annulus of "boundary lipid" (Jost et al., 1973) surrounding band 3 in *intact* erythrocytes is not practical due to the relatively high lipid/protein ratio. However, it is possible to observe changes in the rotational mobility of band 3 produced by ether from the ST-EPR spectrum (Figure 6). If we assume that disordering of the lipids in the vicinity of band 3 reduces their "effective viscosity", then the rotational mobility of band 3 would be a reasonable indicator of the ordering of these lipids. It should be emphasized that the rotational mobility of band 3 is not a direct indicator of the physical state of its layer of boundary lipid. However, it does provide a measurable parameter for estimating the magnitude of the effect of ether on band 3 which can be quantitatively compared with other perturbations such as temperature.

The data presented in Figures 6 and 7 show that the rotational mobility of band 3 increases monotonically with increasing ether concentration. These data strongly suggest that the bulk membrane disordering effect of ether reported by the 5-SASL probe is also produced to an appreciable extent in the lipids surrounding band 3. In fact, the data in Figure 7 suggest that at low concentrations, ether may preferentially affect these lipids since motional enhancement was greatest between control and 0.5 vol % ether-treated samples. In comparison, previous studies have demonstrated selective mobilization of lipids by ether at the lipid–protein interface in sarcoplasmic reticulum vesicles containing Ca^{2+}-ATPase (Bigelow & Thomas, 1987).

It is important to understand what the changes in ST-EPR line shapes and associated spectral ratio parameters produced by ether (Figures 6 and 7) reflect, both in terms of the magnitude of the change in the rotational mobility of band 3 and also in terms of the potential alterations of the membrane at the molecular level which lead to this change in rotational mobility. First, ether produced substantial changes in the V_2' ST-EPR line shape in the extrema regions of the spectrum (L'' parameter, Figure 7, and H'' parameter, data not parameterized) as well as in the central region of the spectrum (C' parameter, Figure 7). Since the orientation of the nitroxyl moiety of BSSDA relative to the transmembrane axis of band 3 is unknown, it is presently not feasible to determine true rotational correlation times for the protein by computer modeling of experimental spectra [reviewed in Beth and Robinson (1989)]. However, it is instructive to compare the magnitude of spectral changes produced by ether with data obtained from BSSDA-labeled erythrocytes as a function of temperature (Figure 7). The change in L''/L between control and 1 vol % ether-treated cells corresponded to the change in this ratio parameter observed by increasing temperature from 20 to approximately 31 °C. The change in C'/C in the same samples corresponded to an even larger change in temperature. Clearly, both of the ratio parameters indicate that 1 vol % ether alters the apparent rotational mobility of band 3 by an amount exceeding that produced by a 10 °C increase in temperature. By comparison, S for the 5-SASL probe decreased between control and 1 vol % ether-treated cells by an amount equal to the change in this parameter produced by increasing the sample temperature from 20 to approximately 24 °C (Figure 3), again suggesting that ether may produce larger effects at, or near, the band 3–lipid interface than in the bulk lipid of the bilayer.

Next, the spectral data obtained permit some useful conclusions to be drawn concerning the effects of ether on the tertiary structure of band 3. Diethyl ether readily partitions into the membrane bilayer due to its high solubility in nonpolar environments. Therefore, it is reasonable to believe that it might also partition into hydrophobic regions of proteins including band 3. A priori estimates of the effects of such partitioning on the structure and function of band 3 are difficult to make. However, if ether produced substantial al-

terations in the tertiary structure of band 3 subunits through local or global unfolding of the protein, it is not unreasonable to expect that the V_1 EPR spectrum of BSSDA-labeled band 3 might reflect these alterations. Specifically, if ether caused a "loosening" of its structure by increasing the amplitudes of high-frequency internal motional modes of the protein in the vicinity of the spin-label, then measurable changes in EPR spectral widths should be observed (Mason & Freed, 1974). It is possible that the introduction of an intrasubunit cross-link by reaction with BSSDA could stabilize the structure of band 3, reducing ether-induced perturbations; however, we did not observe *any* change in the V_1 EPR spectrum of BSSDA-labeled band 3 between control cells and 2 vol % ether-treated cells (data not shown). This result indicates that the structure and motional modes of BSSDA-labeled band 3 in the region of the BSSDA reaction site, which overlaps the DIDS (Anjaneyulu et al., 1989) and the eosinyl-5-maleimide (Cobb & Beth, 1990) reaction sites, are largely unaffected by addition of ether.

Finally, by the same arguments presented in the previous paragraph, ether could potentially alter the oligomeric state of band 3 in the membrane. Previous studies have provided convincing evidence that band 3 exists primarily as noncovalent dimers in *intact* erythrocytes (Steck, 1972; Staros et al., 1981; Staros, 1982; Staros & Kakkad, 1983; Jennings & Nicknish, 1985; Cuppoletti et al., 1985). Band 3, when solubilized in Triton X-100, has been shown to sediment as a noncovalent dimer (Yu & Steck, 1975; Clarke, 1975) which can be made covalent by Cu^{2+}–*o*-phenanthroline–catalyzed oxidation of endogeneous thiols. One group, however, has presented evidence of a detectable monomer–dimer–tetramer equilibrium for band 3 solubilized in other nonionic detergents (Pappert & Schubert, 1983).

If ether caused concentration-dependent dissociation of band 3 dimers in the membranes of *intact* erythrocytes and the dissociated subunits had a smaller cross-sectional area in the plane of the bilayer, then one would expect to observe an increase in the rotational mobility of band 3 upon addition of ether (Saffman & Delbruck, 1975). However, as shown in Figure 8, addition of 1 vol % ether to *intact* erythrocytes did not alter the efficiency of BS^3 cross-linking of band 3 subunits, indicating that on the time scale of the cross-linking experiment, ether had no detectable effect on the association of band 3 subunits in the membrane.

It has been suggested, though not universally accepted, that band 3 dimers may associate to form higher order structures including tetramers in the erythrocyte membrane [reviewed in Jennings (1984)]; however, no conclusive assay for such structures, e.g., the cross-linking of band 3 subunits into covalent tetramers in *intact* erythrocytes, has yet appeared,[2] making it difficult to address the question of what effect ether has on such hypothesized structures. Therefore, we have interpreted the rotational mobility data on band 3 in these studies in terms of the dimer. Additionally, protein–protein interactions between band 3 and glycophorin A have been suggested in previous studies (Pinto da Silva & Nicolson, 1974; Nigg et al., 1980). It remains a possibility that ether could disrupt interactions between hand 3 and glycophorin A, leading to an increase in the rotational mobility of band 3 which is independent of the ordering of membrane lipids. However, the majority of the mass of glycophorin A is extracellular and, hence, not subject to impediment of rotational mobility by the viscous drag of the lipid bilayer. This fact, taken with estimates of only one glycophorin A (Gahmberg et al., 1979) per band 3 subunit (Fairbanks et al., 1971) in the membrane, suggests that even if ether does disrupt this interaction, it would not produce the magnitude of change in rotational mobility observed in these studies (Figures 6 and 7).

Nigg and Cherry (1980) and Clague et al. (1989) observed an increase in the rotational mobility of a constrained population of band 3 upon proteolytic removal of its cytoplasmic domain in ghost membranes. This observation has been interpreted as suggesting that the rotational diffusion of a subpopulation of band 3 may be controlled by interactions with proteins of the membrane skeleton. Therefore, it is important to consider the possibility that the increase in the rotational mobility of band 3 observed in the presence of ether in these studies may be due to disruptions of interactions between its cytoplasmic domain and the membrane skeleton or in the organization of the membrane skeleton itself. On the basis of numerous studies of erythrocytes from patients with a variety of hemolytic anemias, reasonable indicators of such molecular disruptions in *intact* erythrocytes are the shape and fragility of individual cells [reviewed in Palek and Lux (1983)]. As shown in Figure 3, concentrations of ether up to 2 vol % caused almost no hemolysis above control cells. Also, at 1 and 2 vol % ether, the cells appeared as biconcave disks when viewed under a light microscope (data not shown). These observations suggest that major alterations of interactions between band 3 and the membrane skeleton or disruption of the membrane skeleton itself did not result from addition of ether at the concentrations used in this study. However, we cannot exclude ether having some effect on the equilibrium of binding of the cytoplasmic domain of band 3 to the membrane skeleton or on other binding equilibria between components of the membrane skeleton.

Collectively, the results discussed in the preceding paragraphs strongly suggest that the increase in rotational mobility of band 3 observed in the presence of ether was largely the result of decreased ordering of membrane lipids in the vicinity of the transmembrane domain of the protein. From a mechanistic standpoint, it is of interest to determine if kinetic and thermodynamic parameters of anion exchange reflect this alteration in membrane lipids.

The data presented in Figure 9 demonstrate that the K_m and the V_{max} for sulfate uptake into chloride-loaded erythrocytes were not significantly altered by 1 vol % ether treatment. Similarly, the apparent $E_{act.}$ for sulfate–chloride exchange was approximately 35 kcal/mol in both control and 1 vol % ether-treated erythrocytes (Figure 9, *inset*). However, the data in Figure 9, *inset*, demonstrate that the v_o for sulfate–chloride exchange is very sensitive to temperature as reported previously for sulfate–sulfate exchange [reviewed in Passow (1986)]. If the ordering of lipids surrounding band 3 contributed in any substantial way to the large energy of activation for sulfate–chloride exchange, then disordering of these lipids by diethyl ether would be expected to lower the energy of activation with an accompanying increase in V_{max}. The studies presented clearly demonstrate that this was not observed. Thus, the effects of temperature on kinetic parameters of anion exchange are likely due to direct effects on internal dynamics of the protein and/or its substrate binding affinity and not due to its accompanying effects on the ordering of the head-group regions of membrane lipids.

Extrapolations of these results to mechanistic aspects of the anion translocation event are of interest. It has been reported

[2] Subsequent to the submission of this paper, the authors became aware of a paper in press (Salhany et al., 1990) in which a method is described for detecting the noncovalent association of BS^3-cross-linked pairs of band 3 subunits.

that band 3 mediated sulfate exchange exhibits a remarkably high activation volume of 150 cm^3/mol (Canfield & Macey, 1984), suggesting comparatively large conformational changes in the protein during the transport cycle. If these conformational changes involve domains of the protein in contact with lipid, then altering the ordering of these lipids would be expected to contribute to the functional parameters of anion exchange. By analogy, enzymatic activation of Ca^{2+}-ATPase by diethyl ether has been hypothesized to arise from selective mobilization of lipids surrounding the enzyme, thereby facilitating protein motions which are required for calcium transport (Bigelow & Thomas, 1987). Our finding of substantial disordering of membrane lipids in the polar head-group regions of the bilayer by ether without substantial alterations of kinetic (K_m and V_{max}) or thermodynamic ($E_{act.}$) parameters of anion exchange suggests that conformational changes in band 3 which are necessary for anion translocation are not likely to involve motions of the protein in contact with these regions of the bilayer.

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References

Anjaneyulu, P. S. R., Beth, A. H., Cobb, C. E., Juliao, S. F., Sweetman, B. J., & Staros, J. V. (1989) *Biochemistry 28*, 6583–6590.

Beth, A. H., & Robinson, B. H. (1989) *Biol. Magn. Reson.* 179–253.

Beth, A. H., Balasubramanian, K., Robinson, B. H., Dalton, L. R. Venkataramu, S. D., & Park, J. H. (1983) *J. Phys. Chem. 87*, 359–367.

Beth, A. H., Conturo, T. E., Venkataramu, S. D., & Staros, J. V. (1986) *Biochemistry 25*, 3824–3832.

Bigelow, D. J., & Thomas, D. D. (1987) *J. Biol. Chem. 262*, 13449–13456.

Bull, M. H., Brailsford, J. D., & Bull, B. S. (1982) *Anesthesiology 57*, 399–403.

Cabantchik, Z. I., Knauf, P. A., & Rothstein, A. (1978) *Biochim. Biophys. Acta 515*, 234–302.

Canfield, V. A., & Macey, R. I. (1984) *Biochim. Biophys. Acta 778*, 379–384.

Cherry, R. J., Bürkli, A., Busslinger, M., Schneider, G., & Parish, G. R. (1976) *Nature (London) 263*, 389–393.

Clague M. J., Harrison, J. P., & Cherry, R. J. (1989) *Biochim. Biophys. Acta 981*, 43–50.

Clarke, S. (1975) *J. Biol. Chem. 250*, 5459–5469.

Cobb, C. E., & Beth, A. H. (1990) *Biochemistry 29*, 8283–8290.

Cobb, C. E., Juliao, S. F., Staros, J. V., & Beth, A. H. (1990) *Biophys. J. 57*, 95a.

Cuppoletti, J., Goldinger, J., Kang, B., Jo, I., Berenski, C., & Jung, C. Y. (1985) *J. Biol. Chem. 260*, 15714–15717.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.

Fröhlich, O., & Gunn, R. B. (1986) *Biochim. Biophys. Acta 864*, 169–194.

Gaffney, B. J. (1976) in *Spin Labeling: Theory and Applications* (Berliner, L. J., Ed.) pp 567–571, Academic Press, New York.

Gahmberg, C. G., Jokinen, M., & Andersson, L. C. (1979) *J. Biol. Chem. 254*, 7442–7448.

Garcia, A. M., & Lodish, H. F. (1989) *J. Biol. Chem. 264*, 19607–19613.

Griffith, O. H., & Jost, P. C. (1976) in *Spin Labeling: Theory and Applications* (Berliner, L. J., Ed.) pp 453–523, Academic Press, New York.

Jennings, M. L. (1984) *J. Membr. Biol. 80*, 105–117.

Jennings, M. L. (1989) *Annu. Rev. Biophys. Biophys. Chem. 18*, 397–430. c

Jennings, M. L., & Nicknish J. S. (1985) *J. Biol. Chem. 260*, 5472–5479.

Jennings, M. L., Monaghan, R., Douglas, S. M., & Nicknish, *J. S.* (1985) *J. Gen. Physiol. 86*, 653–669.

Jost, P. C., Griffith, O. H., Capaldi, R. A., & Vanderkooi, G. (1973) *Proc. Natl. Acad Sci. U.S.A. 70*, 480–484.

Ku, C. P., Jennings, M. L., & Passow, H. (1979) *Biochim. Biophys. Acta 553*, 132–144.

Lux, S. E., John, K. M., Kopito, R. R., & Lodish, H. F. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9089–9093.

Mason, R. P., & Freed, J. H. (1974) *J. Phys. Chem. 78*, 1321–1323.

Nigg, E. A., & Cherry, R. J. (1979) *Biochemistry 18*, 3457–3465.

Nigg, E. A., & Cherry, R. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4702–4706.

Nigg, E. A., Bron, C., Girardet, M., & Cherry, R. J. (1980) *Biochemistry 19*, 1887–1893.

Palek, J., & Lux, S. E. (1983) *Semin. Hematol. 20*, 189–224.

Pappert, G., & Schubert, D. (1983) *Biochim. Biophys. Acta 730*, 32–40.

Passow, H. (1986) *Rev. Physiol. Biochem. Pharmacol. 103*, 61–203.

Pinto da Silva, P., & Nicolson, G. L. (1974) *Biochim. Biophys. Acta 363*, 311–319.

Robinson, B. H., Thomann, H., Beth, A. H., Fajer, P., & Dalton, L. R. (1985) in *EPR and Advanced EPR Studies* of *Biological Systems* (Dalton, L. R., Ed.) pp 257–293, CRC Press, Boca Raton, FL.

Saffman, P. G., & Delbruck, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3111–3113.

Salama, G., & Scarpa, A. (1980) *J. Biol. Chem. 255*, 6525–6528.

Salhany, J. M., Sloan, R. L., & Cordes, K. A. (1990) *J. Biol. Chem.* (in press).

Staros, J. V. (1982) *Biochemistry 21*, 3950–3955.

Staros, J. V., & Kakkad, B. P. (1983) *J. Membr. Biol. 74*, 247–254.

Staros, J. V., Morgan, D. G., & Appling, D. R. (1981) *J. Biol. Chem. 256*, 5890–5893.

Steck, T. L. (1972) *J. Biol. Biol. 66*, 295–305.

Tanner, M. J. A., Martin, P. G., & High, S. (1988) *Biochem. J. 256*, 703–712.

Thomas, D. D., Dalton, L. R., & Hyde, J. S. (1976) *J. Chem. Phys. 65*, 3006–3024.

Vanderkooi, J. M., Landsberg, R., Selick, H., & McDonald, G. G. (1977) *Biochim. Biophys. Acta 464*, 1–16.

Yu, J., & Steck, T. L. (1975) *J. Biol. Chem. 250*, 9176–9184.