Bis(sulfo-*N*-succinimidyl) [^{15}N,$^{2}H_{16}$]Doxyl-2-spiro-4′-pimelate, a Stable Isotope-Substituted, Membrane-Impermeant Bifunctional Spin Label for Studies of the Dynamics of Membrane Proteins: Application to the Anion-Exchange Channel in Intact Human Erythrocytes†

P. S. R. Anjaneyulu,‡ Albert H. Beth,*,§ Brian J. Sweetman,‖ Lee Anne Faulkner,‡ and James V. Staros*,‡

Department of Biochemistry, Department of Molecular Physiology and Biophysics, and Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Received March 21, 1988; Revised Manuscript Received May 6, 1988

ABSTRACT: We have synthesized and characterized an isotopically substituted homologue of the membrane-impermeant bifunctional spin label bis(sulfo-*N*-succinimidyl) doxyl-2-spiro-4′-pimelate (BSSDP) [Beth et al. (1986) *Biochemistry 25*, 3824–3832] in which the nitroxide N is substituted with ^{15}N and all of the protons in the doxylpimelate moiety are replaced by deuterons ([^{15}N,$^{2}H_{16}$]BSSDP). Like its normal isotope homologue, [^{15}N,$^{2}H_{16}$]BSSDP reacts with the anion-exchange channel in intact human erythrocytes at a site that spans the single extracytoplasmic chymotryptic cleavage site and that overlaps the stilbenedisulfonate site. The narrower line widths in the EPR spectrum of [^{15}N,$^{2}H_{16}$]BSSDP-labeled anion channels allow calculation of a *minimum* separation of 16 Å between spin labels bound at the functionally important stilbenedisulfonate sites on adjacent subunits of an anion channel dimer. The ^{15}N and ^{2}H isotopic substitutions also provide substantial improvement in signal to noise of motionally sensitive regions of the ST-EPR spectrum of [^{15}N,$^{2}H_{16}$]BSSDP-labeled anion channels in intact erythrocytes. [^{15}N,$^{2}H_{16}$]BSSDP-labeled anion channels in intact erythrocytes were cross-linked to covalent dimers in the extracytoplasmic domain with the membrane-impermeant cross-linking reagent bis(sulfo-*N*-succinimidyl) suberate [Staros (1982) *Biochemistry 21*, 3950–3955], and the saturation-transfer EPR spectrum of these cells was compared with that of cells treated with [^{15}N,$^{2}H_{16}$]BSSDP but not subsequently cross-linked. The spectra were essentially identical, supporting the hypothesis that anion channel subunits form stable dimers in the membranes of intact erythrocytes.

Recently, we introduced a new bifunctional spin-labeling reagent BSSDP[1] and applied this reagent to a study of the rotational dynamics of the anion-exchange channel in intact human erythrocytes (Beth et al., 1986). At low concentrations, BSSDP acts as an affinity label for the extracytoplasmic anion binding site of the channel with sufficient specificity to result in highly selective labeling of this protein when intact erythrocytes are treated with this reagent (Beth et al., 1986, 1987). Due to the bidentate covalent attachment of the doxyl-2-spiro-4′-pimelate moiety to the protein, dynamic measurements of the probe reflect the very slow dynamic state of the protein in the membrane.

When BSSDP-labeled erythrocytes are examined by linear EPR, the spectrum corresponding to the labeled anion channel is at the no-motion limit, indicating an effective rotational correlation time ($\tau_{r,eff}$) ≥ 1 μs. Therefore, it is necessary to use saturation-transfer EPR methods (Hyde & Dalton, 1972; Hyde & Thomas, 1973) to obtain estimates for $\tau_{r,eff}$ of spin-labeled anion channels. In our previous studies (Beth et al., 1986, 1987), first-order analyses of ST-EPR spectra were accomplished by comparison of motionally sensitive ratio parameters L''/L, C'/C, and H''/H with plots of these parameters from a model study in which the isotropic rotational correlation time τ_r of spin-labeled hemoglobin was adjusted by changing the glycerol/buffer proportions of the medium (Thomas et al., 1976). There are several difficulties with this approach for characterizing the dynamic state(s) of the anion channel in *intact* erythrocytes. First, the concentration of anion channel monomers, though substantial by membrane protein standards, is relatively low for conventional spin-labeling reagents, leading to rather poor signal to noise in the experimental ST-EPR spectrum. Next, in addition to affinity labeling of the anion channel, BSSDP also reacts with membrane lipids giving rise to a partially immobilized EPR and ST-EPR spectrum that overlaps the very slow motion signal from spin-labeled anion channels (Beth et al., 1986). Though it is possible to correct for this unwanted signal by digital subtraction procedures (Beth et al., 1986, 1987), it is difficult to determine the end point in the subtraction, particularly in the central portion of the ST-EPR spectrum from nitrogen-14 spin labels. This problem is further exacerbated in cases where there is poor signal to noise in the experimental data. Since it is the central region of the nitrogen-14 ST-EPR spectrum that provides the greatest signal to noise and, hence, the best opportunity for observing changes in the dynamic state of spin-labeled anion channels (Beth & Robinson, 1988), the lack of a precise marker for determining the end point in the subtraction procedure is a serious limitation for data analysis.

† Supported by Grants R01 DK31880, R01 HL34737, P01 CA43720, T32 GM07347, and S10 RR01688 from the National Institutes of Health.

‡ Department of Biochemistry.
§ Department of Molecular Physiology and Biophysics.
‖ Department of Pharmacology.

[1] Abbreviations: BSSDP, bis(sulfo-*N*-succinimidyl) doxyl-2-spiro-4′-pimelate; BS[3], bis(sulfo-*N*-succinimidyl) suberate; $NaDodSO_4$, sodium dodecyl sulfate; 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 ST-EPR signal; HPLC, high-performance liquid chromatography; EI, electron impact; FAB, fast atom bombardment; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; IR, infrared.

0006-2960/88/0427-6844$01.50/0 © 1988 American Chemical Society

It has previously been shown that isotopic replacement of protons on the nitroxide ring system with deuterons (Beth et al., 1980) leads to a lessening of inhomogeneous line broadening and, hence, increased signal to noise and resolution of the ST-EPR spectrum throughout the very slow motional time range. Moreover, isotopic replacement of the ^{14}N ($I = 1$) of the nitroxide moiety with ^{15}N ($I = {}^1/_2$) leads to a reduction in number of nuclear spin states from three to two, thereby providing an additional increase in resolution and a further increase in signal to noise without any loss in sensitivity to very slow rotational motion (Beth et al., 1981a,b). These combined isotopic substitutions offered the potential for overcoming many of the data analysis problems outlined in the previous paragraph, as well as the potential for quantitating the dynamic state(s) of anion channels in terms of an appropriate anisotropic rotational diffusion model using computer modeling approaches (Beth et al., 1983).

Here we describe the synthesis and characterization of an isotopically substituted homologue of our bifunctional spin-labeling reagent BSSDP, in which the nitroxide N is substituted with ^{15}N and all H atoms in the doxylpimelate moiety are substituted with ^{2}H, with a total isotopic substitution in the doxylpimelate moiety of 96%. We apply this new reagent to a study of the dynamics of the anion-exchange channel in intact human erythrocytes and to the effects on dynamics of chemically cross-linking anion channel subunits to dimers. The ^{15}N,^{2}H substitution in BSSDP is shown to provide the necessary resolution enhancement for reliable subtraction of the unwanted signal from spin-labeled lipids and significant improvements in signal to noise and resolution of motionally sensitive regions of the ST-EPR spectrum, enabling direct comparisons of the dynamic state of anion channels in their native oligomeric form with their dynamic state when cross-linked to covalent dimers in their extracytoplasmic domains.

Experimental Procedures

Melting points were determined on a Thomas–Hoover capillary melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer 727 spectrophotometer. Proton NMR spectra were obtained on an IBM/Bruker NR-300 spectrometer. Mass spectra (70 eV) were obtained on a VG-70/250 GC–MS instrument having extended geometry and equipped with a VG 11/250 data system and capability for fast atom bombardment (FAB). Deuterium composition was determined from the molecular ion cluster. Accurate mass measurements were made with the instrument set to a resolving power of 10000. Thin-layer chromatographic (TLC) analyses were carried out on E. Merck 60F-254 precoated silica gel plates with fluorescent indicator. Spots were visualized under ultraviolet light or by exposure to iodine vapor. All reagents and solvents were of ACS-certified grade or of a comparable or higher grade. Solvents were purified when necessary. Molecular sieves (3 Å) were activated at 140 °C prior to use.

Deuterium oxide (99.8% ^{2}H), methanol-*d* (99.5% ^{2}H), ethanol-*d* (99.5% ^{2}H), sulfuric acid-d_2 (99.5% ^{2}H, 98% solution in $^{2}H_2O$), deuterium chloride (99% ^{2}H, 30% solution in $^{2}H_2O$), sodium deuterioxide (99% ^{2}H, 30% solution in $^{2}H_2O$), *m*-chloroperbenzoic acid (80–85%), dicyclohexylcarbodiimide, and 4-ketopimelic acid were purchased from Aldrich Chemical Co. [^{15}N,$^{2}H_{11}$]-2-Amino-2-methyl-1-propanol (99% ^{15}N, 97.5% ^{2}H) was purchased from MSD Isotopes. *N*-Hydroxysulfosuccinimide was prepared from *N*-hydroxymaleimide as reported previously (Staros, 1982).

Erythrocytes were prepared from fresh herparinized blood and were treated with BSSDP (either normal isotope or isotopically substituted) as previously described (Beth et al., 1986), except as noted below.

4-Ketopimelic Acid-d_8 (**1**). The deuteriated diacid was prepared from its normal isotope homologue by base-catalyzed deuterium exchange (Atkinson et al., 1968). 4-Ketopimelic acid (20 g, 0.115 mol) was added slowly to a solution of deuterium oxide (90 mL) containing potassium hydroxide (37 g) and was refluxed for 5 days. The extent of deuteriation and the positions exchanged were monitored by NMR spectroscopy by following disappearance of triplets at 2.44 and 2.70 ppm, corresponding to the 2,6- and 3,5-methylene protons, respectively. Solvent was removed with a rotary evaporator, fresh deuterium oxide (90 mL) was added, and the solution was refluxed for $3^1/_2$ days. After a third exchange for another $3^1/_2$ days, the solution was slowly acidified with sulfuric acid-d_2 and filtered, and the filtrate was extracted with ethyl acetate. Combined ethyl acetate extracts were washed with saturated sodium chloride solution, and the ethyl acetate was removed by rotary evaporation to give 4-ketopimelic acid-d_8 (**1**)[2] (6.25 g), mp 139–141 °C. Mass spectrum *m/z* (rel intensity) 182.103 (2.8) (calcd for $C_7{}^1H_2{}^2H_8O_5$, 182.103), 164 (1.5), 137 (3.4), 105 (100), 77 (30). Mass spectral analysis also indicated isotopic composition to be as follows: d_8, 67.3%; d_7, 20.1%; d_6, 8.6%; d_5, 3.8%; d_4, 0.2%.

Diethyl 4-Ketopimelate-d_8 (**2**). 4-Ketopimelic acid-d_8 (**1**) (5.59 g, 30.7 mmol), ethanol-*d* (20 mL), benzene (40 mL), and concentrated sulfuric acid-d_2 (1 mL) were refluxed with stirring for 8 h, azeotropically removing deuterium oxide formed by using a Dean–Stark apparatus (Leonard & Goode, 1950). The solution was cooled, diluted with benzene (30 mL), washed with saturated solutions of sodium bicarbonate and sodium chloride, and concentrated on a rotary evaporator. The crude ester was distilled under vacuum to give diethyl 4-ketopimelate-d_8 (**2**) (6.5 g, 89%), bp 99–104 °C/0.2 mm; IR (thin film) 1730 cm^{-1} (ester, carbonyl); NMR ($CDCl_3$) δ 1.25 (t, 6 H, ester CH_3), 2.57 (s, 2,6-CH_2), 2.74 (s, 3,5-CH_2), 4.14 (q, 4 H, ester CH_2); mass spectrum *m/z* (rel intensity) 238.166 (0.1) (calcd for $C_{11}{}^1H_{10}{}^2H_8O_5$, 238.166), 193 (29.6), 165 (23.5), 133 (91.2), 105 (100). Isotopic composition was found to be as follows: d_8, 66.4%; d_7, 26.9%; d_6, 5.9%; d_5, 0.9%.

[^{15}N,$^{2}H_{16}$]*Doxyl-2-spiro-4'-pimelic Acid* (**4**). This compound was synthesized by modifications of procedures previously reported for the normal isotope synthesis (Beth et al., 1986). Diethyl 4-ketopimelate-d_8 (3.53 g, 14.8 mmol), [^{15}N,$^{2}H_{11}$]-2-amino-2-methyl-1-propanol (6 g, 59.3 mmol), *p*-toluenesulfonic acid (50 mg), and molecular sieves (2 g) in *o*-xylene (30 mL) were gently refluxed with stirring for 9 days. The dark orange solution was filtered, and the molecular sieves, which had been reduced to a powder, were washed with methanol-*d*. The filtrate was concentrated on a rotary evaporator, and unreacted [^{15}N,$^{2}H_{11}$]-2-amino-2-methyl-1-propanol was removed under vacuum (0.01 mm) between 45 and 50 °C to yield an orange semisolid (6.03 g). This was dissolved in methanol-*d* (75 mL), stored over anhydrous sodium carbonate at room temperature for 48 h, and filtered, and methanol-*d* was removed on a rotary evaporator at 50 °C.

The resulting orange semisolid was dissolved in diethyl ether (75 mL), and residual insoluble material (2.7 g) was filtered

[2] While 4-ketopimelic acid-d_8 (**1**) and [^{15}N,$^{2}H_{16}$]doxyl-2-spiro-4'-pimelic acid (**4**) were synthesized under conditions expected to yield deuteriated carboxylic acids, these compounds were analyzed for isotopic content under conditions in which the carboxylic acid deuterons would readily exchange with protons in the medium. We therefore report these compounds as protonated in the carboxylic acid groups and deuteriated in positions resistant to exchange in aqueous solution.

out. The orange solution was cooled with ice, and a cold solution of *m*-chloroperbenzoic acid (3.37 g, 15.6 mmol) in diethyl ether (30 mL) was added dropwise with stirring. The reaction mixture was stirred at 4 °C for 36 h, by which time the solution became increasingly greenish yellow. The solution was washed with a saturated solution of sodium bicarbonate and sodium chloride and was concentrated on a rotary evaporator to give a green oil (1.34 g). This was dissolved in methanol/10 mM ammonium formate, pH 3.5 (50/50 v/v, 5.6 mL), filtered, and purified by preparative reverse-phase HPLC. The HPLC system consisted of a Constametric III pump (Laboratory Data Control), an OPG/S ternary gradient controller (Autochrom), a Model 7125 manual injector with 1-mL sample loop (Rheodyne), a 2.14 by 25 cm Dynamax column (Rainin), and a Model 788 dual variable-wavelength detector (Micromeritics) set at 254 and 214 nm. The sample was purified by using a mobile phase of methanol/10 mM ammonium formate, pH 3.5 (50/50 v/v), isocratic for 30 min, and then a 60-min gradient to methanol/ammonium formate (55/45 v/v) with elution at 9.5 mL/min. Fractions having EPR signal were collected, and corresponding fractions from successive runs were combined. Methanol was removed on a rotary evaporator, and the product was lyophilized to yield a yellow semisolid (0.555 g).

Lyophilized material (0.555 g) was dissolved in methanol-*d* (20 mL), and a 20% sodium deuterioxide solution (4.6 mL) was added dropwise with stirring. After the solution was stirred for 48 h at room temperature, methanol was removed on a rotary evaporator, and the aqueous residue was acidified to pH 3 with chilled 1 N deuterium chloride. The solution was extracted with ethyl acetate, washed with a saturated sodium chloride solution, and concentrated on a rotary evaporator to give yellow liquid (0.304 g). This was dissolved for preparative HPLC in 4.5 mL of a mobile phase of methanol/ammonium formate, pH 3.5 (15/85 v/v), filtered, and purified on the above system, using a 1 × 25 cm reverse-phase Econosil 6231 column (Alltech) and elution at 3 mL/min. Fractions having EPR signal were collected, and corresponding fractions from each of the runs were combined. Methanol was removed on a rotary evaporator, and the product was lyophilized to yield a pale yellow hygroscopic solid (56.1 mg). This was dissolved in deuterium oxide (1 mL), acidified with chilled 1 N deuterium chloride to pH 3, extracted with ethyl acetate, and washed with a saturated sodium chloride solution. Ethyl acetate was removed on a rotary evaporator, and the sample was dried over phosphorus pentoxide in vacuo to give a pale yellow solid (45.1 mg).[2]

Bis(sulfo-N-succinimidyl) [^{15}N,$^{2}H_{16}$]Doxyl-2-spiro-4′-pimelate (**5**). [^{15}N,^{2}H$_{16}$]BSSDP was synthesized by the general method for the synthesis of bis(sulfosuccinimidyl) esters of dicarboxylic acids (Staros, 1982). Briefly, dicyclohexylcarbodiimide (74 mg, 0.3 mmol), *N*-hydroxysulfosuccinimide (66 mg, 0.30 mmol), and [^{15}N,^{2}H$_{16}$]doxyl-2-spiro-4′-pimelic acid ((**4**) (42 mg, 0.15 mmol) in *N*,*N*-dimethylformamide 0.8 mL) were stirred at room temperature overnight and then at 4 °C for 3 h. Dicyclohexylurea was filtered out and washed with *N*,*N*-dimethylformamide (0.8 mL). The product was precipitated from the filtrate by addition of ethyl acetate (100 mL), collected by filtration, and dried in vacuo to give a pale yellow solid, 39.2 mg (39%). Negative ion FAB–mass spectral analysis of the product yielded a major peak (*m*/*z* 653) corresponding to loss of Na^+ from the hydroxylamine of [^{15}N,^{2}H$_{16}$]Na$_2$BSSDP (calcd for $C_{19}{}^{1}H_7{}^{2}H_{16}O_{16}{}^{14}N_2{}^{15}NS_2Na_2$, 676). In addition, the fragmentation pattern was found to be consistent with that of normal isotope BSSDP (Beth et al., 1986) and related sulfosuccinimidyl esters examined by this technique (Anjaneyulu & Staros, 1987).

Treatment of Intact Erythrocytes with [^{15}N,$^{2}H_{16}$]BSSDP and BS^3. Erythrocytes were prepared from freshly drawn, heparinized blood as previously described (Staros & Richards, 1974). After three washes at 3 °C in phosphate-buffered saline (0.15 M NaCl, 14 mM sodium phosphate, pH 7.4), the cells were washed once in 106 mM sodium phosphate, pH 7.4, and resuspended at an approximately 50% hematocrit in the same buffer. An aliquot of cell suspension was added to a freshly prepared 1.0 mM stock solution of [^{15}N,^{2}H$_{16}$]BSSDP in the 106 mM sodium phosphate buffer, to give a final reagent concentration of 50 μM. After 15-min incubation at room temperature with gentle agitation, the sample was split and BS^3 was added to one of the samples from a 25 mM stock in the same buffer to a final concentration of 5.0 mM. After an additional 15-min incubation of both samples at room temperature with gentle agitation, the reaction with BS^3 was quenched by addition of ~10 volumes of cold phosphate-buffered saline containing 1% (w/v) bovine serum albumin. Cells were washed three times in this buffer, and samples were resuspended in a small volume of the same buffer for spectroscopic analysis. The remaining cells were washed twice in phosphate-buffered saline, lysed, and prepared for NaDodSO$_4$–polyacrylamide gel electrophoresis as previously described (Staros & Richards, 1974).

EPR and ST-EPR Measurements. EPR and ST-EPR spectra were recorded with a Varian E-112 spectrometer equipped with an E-238 high-volume aqueous cavity. Samples were contained in a WG-813 flat cell (Wilmad). Sample temperature was continuously monitored with a digital thermometer (Baily BAT12-R) by placing a temperature probe into the flat cell in contact with the sample but out of the active region of the cavity. Sample temperature was regulated during measurements with an E-257 variable temperature unit by passing precooled nitrogen into the cavity through the front optical port. This resulted in a 1-deg temperature gradient over the active dimensions of the sample. EPR signals (first-harmonic, in-phase absorption; V_1) were recorded at 100-kHz field modulation of 0.5-G amplitude at a microwave observer power of 10 mW. ST-EPR signals (second-harmonic, out-of-phase absorption; V'_2) were recorded at 50-kHz field modulation (100-kHz detection) of 5.0-G amplitude at a microwave observer power of 70 mW which corresponded to 0.2 G in the rotating frame [peroxylaminedisulfonate calibrated, Beth et al. (1983)]. Signals were recorded digitally by a PDP 11/73 microcomputer, which also drove the magnetic field sweep. Spectral subtraction and integration (trapezoidal rule) routines developed on this system were employed for data analysis.

Results and Discussion

Preparation and Characterization of [^{15}N,$^{2}H_{16}$]BSSDP. The scheme used for the synthesis of [^{15}N,^{2}H$_{16}$]BSSDP (**5**) is shown in Figure 1. 4-Ketopimelic acid was deuteriated under basic conditions, and the diacid (**1**) was esterified. The purity and identity of 4-ketopimelic acid-d_8 (**1**) and diethyl 4-ketopimelate-d_8 (**2**) were ascertained by analytical TLC, IR, NMR, and mass spectral fragmentation pattern,[2] by comparison with the corresponding normal isotope compounds. In agreement with the mass spectral analysis, NMR spectral analysis of diethyl 4-ketopimelate-d_8 (**2**) indicated deuterium isotopic purity to be 95.6 atom %, with a hydrogen content of 0.4% on the 2,6-methylenes and 4% on the 3,5-methylenes. HPLC analyses indicated that 4-ketopimelic acid-d_8 (**1**), di-

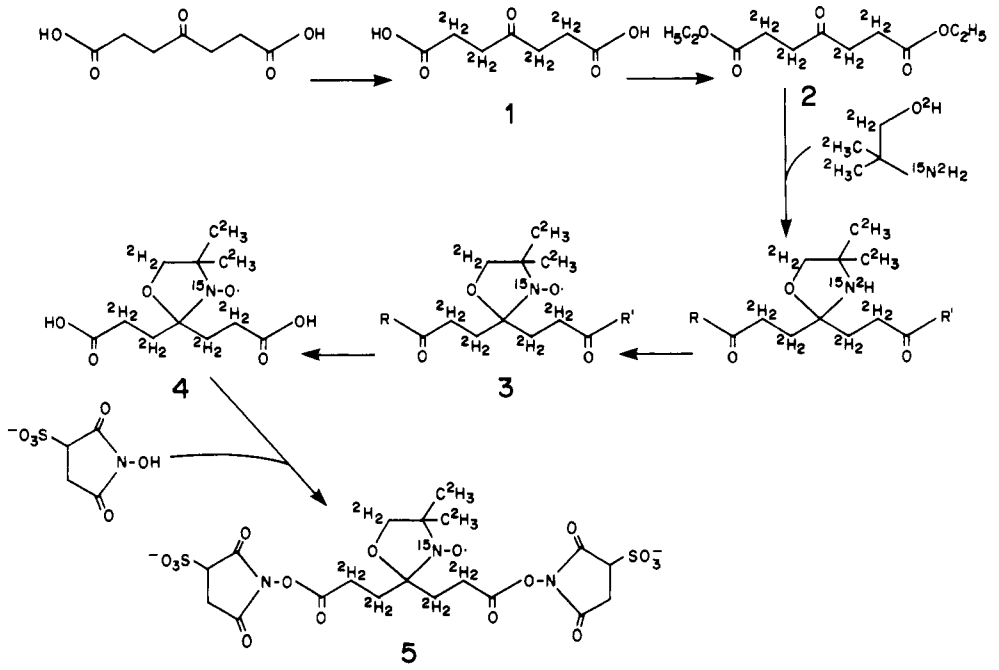

FIGURE 1: Scheme for synthesis of bis(sulfo-*N*-succinimidyl) [^{15}N,$^{2}H_{16}$]doxyl-2-spiro-4′-pimelate. 4-Ketopimelic acid was subjected to base-catalyzed deuterium exchange to give **1**, which was esterified with ethanol-*d* to give **2**. The doxyl ring was formed by condensation of [^{15}N,$^{2}H_{11}$]-2-amino-2-methyl-1-propanol with the keto function, followed by oxidation of the resulting oxazolidine with *m*-chloroperbenzoic acid to give **3**. The carboxylic acid groups were deprotected by saponification to yield **4**, which was converted to the title compound **5** by dicyclohexylcarbodiimide-mediated coupling of *N*-hydroxysulfosuccinimide to each of the carboxylate groups.

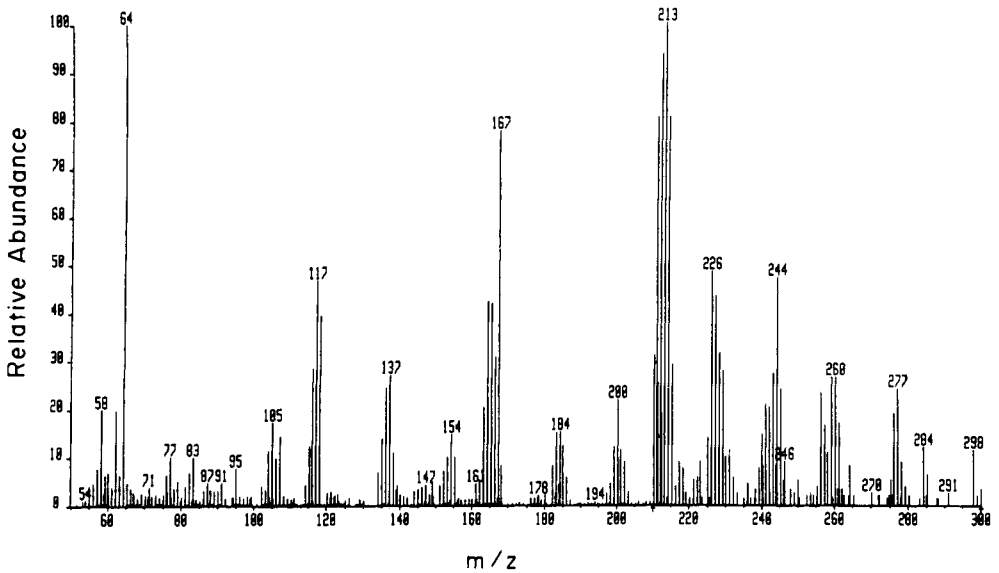

FIGURE 2: Mass spectrum of [^{15}N,$^{2}H_{16}$]doxyl-2-spiro-4′-pimelic acid. The parent (M^+) peak is seen at *m/z* 277 ($C_{11}{}^{1}H_2{}^{2}H_{16}{}^{15}NO_6$). The peaks at *m/z* 260, 244, and 226 correspond to the loss of OH, OH plus O, and OH plus O plus C^2H_3 from the parent ion. The base peak at *m/z* 213 corresponds to loss of $C_4{}^2H_8$ (from the doxyl ring), and the peak from $C_4{}^2H_8{}^+$ is seen at *m/z* 64. The peak at *m/z* 200 corresponds to loss of $C^2H_2C^2H_2COOH$, and the peaks at *m/z* 184 and 167 correspond to the loss of O and O plus OH, respectively, from the *m/z* 200 fragment. The isotopic substitution was calculated from the molecular ion cluster.

ethyl 4-ketopimelate-d_8 (**2**), and [^{15}N,$^{2}H_{16}$]doxyl-2-spiro-4′-pimelic acid (**4**) exhibited shorter retention times compared with their normal isotope counterparts. The EI mass spectrum of [^{15}N,$^{2}H_{16}$]doxyl-2-spiro-4′-pimelic acid (**4**) is shown in Figure 2. Loss of isobutene-d_8 accounted for the base peak. Other fragments were found to be consistent with the structure and with the fragmentation pattern of normal isotope doxyl-2-spiro-4′-pimelic acid. Further, positive ion FAB mass spectral analysis revealed the hydroxylamine as the molecular ion at 278, and a base peak was observed at 201 corresponding to loss of one chain adjacent to the hydroxylamine of the doxyl ring. The isotopic composition of **4** was found to be as follows: d_{16}, 50%; d_{15} 39.4%; d_{14}, 10.6%; the total ^2H isotopic purity was 96%. Extensive IR, NMR, and mass spectral analyses of the fractions obtained by HPLC purification from normal isotope as well as isotopically substituted reaction mixtures after oxidation with *m*-chloroperbenzoic acid indicated that three intermediates are formed: a dimethyl ester (**3a**), a methyl ester–amide (**3b**), and a structurally related amide (**3c**). All three intermediates yielded doxyl-2-spiro-4′-pimelic acid on saponification.

[^{15}N,$^{2}H_{16}$]Doxyl-2-spiro-4′-pimelic acid from the saponification of the pooled intermediates **3a–c** was converted to [^{15}N,$^{2}H_{16}$]BSSDP by a dicyclohexylcarbodiimide-mediated coupling of the diacid and *N*-hydroxysulfosuccinimide (Staros, 1982). The product was characterized by negative ion FAB–

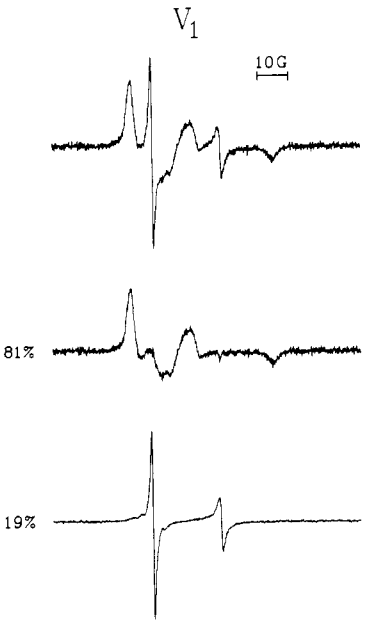

FIGURE 3: V_1 EPR spectrum of [^{15}N,$^{2}H_{16}$]BSSDP-labeled intact erythrocytes and its component spectra. The upper spectrum is from intact erythrocytes labeled with 50 μM [^{15}N,$^{2}H_{16}$]BSSDP as described under Experimental Procedures. This spectrum is composed of signals from BSSDP in two distinct motional environments, as illustrated in the lower two spectra. The bottom spectrum was obtained from intact erythrocytes labeled with 50 μM 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS) to block the anion channel reaction site for BSSDP (Beth et al., 1987) and then with 50 μM [^{15}N,$^{2}H_{16}$]BSSDP, resulting in labeling of membrane lipids. Subtraction of this partially immobilized lipid signal (bottom) from the composite spectrum (top) reveals the immobilized EPR spectrum from spin-labeled anion channels (middle). Double integration of the two component spectra, shown at the amplitude at which they appear in the composite, indicated that 81% of the spin label was in the immobilized environment due to reaction with the anion channel and that 19% was in the partially immobilized environment due to reaction with membrane lipids. Each spectrum was recorded at 20 °C.

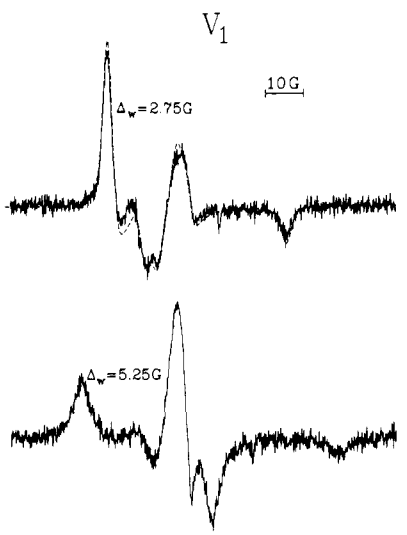

FIGURE 4: Comparison of V_1 EPR spectra from [^{15}N,$^{2}H_{16}$]BSSDP- and [^{14}N,^{1}H]BSSDP-labeled anion channels. The solid tracings are the experimental V_1 EPR spectra from [^{15}N,$^{2}H_{16}$]BSSDP- (upper) and [^{14}N,^{1}H]BSSDP- (lower) labeled anion channels in intact erythrocytes at 20 °C. In each display, the partially immobilized signal from spin-labeled lipids has been digitally subtracted to reveal the line shape from spin-labeled anion channels. The spectra were accumulated as described under Experimental Procedures by using modulation amplitudes of 0.5 and 1.0 G for the upper and lower displays, respectively. The superimposed dashed line in the upper display is the computer-generated V_1 spectrum, which was calculated by using the following parameters: g_{xx} = 2.0083; g_{yy} = 2.0059; g_{zz} = 2.0022;[4] A_{xx} = 9.25 G; A_{yy} = 8.0 G; A_{zz} = 46.0 G; T_{1e} = 15 μs; T_{2e} = 50 ns; H_m = 0.5 G (peak to peak); H_1 = 0.07 G (rotating frame); and a Gaussian postbroadening of 0.4 G.

mass spectral analysis. The isotopic purity estimated from the FAB–mass spectrum was consistent with the 96% isotopic purity found for **4**.[3] Linear EPR spectral analysis of an aqueous solution of [^{15}N,$^{2}H_{16}$]BSSDP reveals a line width for the $m_i = -1/2$ line of 0.45 G, as compared with a line width for the $m_i = 0$ line of 1.0 G for normal isotope BSSDP under the same conditions (data not shown).

EPR Spectrum from [^{15}N,$^{2}H_{16}$]BSSDP-Labeled Erythrocytes. When *intact* erythrocytes were labeled with 50 μM [^{15}N,$^{2}H_{16}$]BSSDP and subsequently washed to remove unreacted label, a composite EPR spectrum was observed (Figure 3, top) which was indicative of two distinct motional environments. The major component (Figure 3, middle), which comprised between 75 and 90% of the total integrated signal intensity in separate experiments, indicated very slow rotational motion ($\tau_r \geq 1$ μs) of the spin label. The minor component (Figure 3, bottom), which comprised the remaining erythrocyte-bound integrated signal intensity, indicated partial immobilization ($\tau_r \sim 1$ ns) of the spin label. Previous work has provided definition of the membrane reaction sites for the spin labels in these two motional environments. The very slow signal arises from spin labels that form an intramolecular cross-link which spans the unique, extracellular chymotryptic cleavage site on each subunit of the anion channel (Beth et al., 1986). This reaction site is the same, or overlapping with, the extracellular stilbenedisulfonate binding site, since pretreatment of erythrocytes with 4,4′-diisothiocyano-2,2′-stilbenedisulfonate (DIDS) blocks reaction of BSSDP with the anion channel (Beth et al., 1987). The partially immobilized signal arises from labeling of other membrane components, the large majority of which are extractable from ghost membranes with a 1:1 solution (v/v) of chloroform/methanol, suggesting a reaction product of the spin label with amino lipids in the membrane (Beth et al., 1986).

The EPR spectrum in Figure 3, middle, is characteristic of an immobilized and spatially isolated ^{15}N-nitroxide spin label. The resolution enhancements provided by the ^{15}N and ^{2}H isotopic substitutions allow determination of the principal elements of the nitrogen hyperfine (A) and electron–Zeeman (g) magnetic tensors from the slow-motion EPR signal as shown by the computer-simulated line shape (superimposed dashed line) in Figure 4, upper.[4] Characteristic spectral structurings are clearly resolved with the isotopically substituted BSSDP label which are absent when using conventional [^{14}N,^{1}H]BSSDP (Figure 4, lower). Since ST-EPR line shapes are strongly dependent on the values for these intrinsic magnetic parameters (Beth et al., 1981b, 1983), their accurate determination is crucial for extracting reliable information on the dynamic states of spin-labeled anion channels. Thus, the double isotopically substituted BSSDP label has provided a substantial advantage by allowing reliable determination of the A and g tensor values for spin-labeled anion channels which can be employed in subsequent studies aimed at extracting rotational correlation times from ST-EPR spectra by computer modeling approaches (Robinson & Dalton, 1980; Beth et al., 1981a,b; 1983).

[3] Isotopic purity was determined for intermediates **1**–**4** by EI–mass spectral analysis. The final product **5** could not be subjected to EI–mass spectral analysis because of its highly charged character.

[4] A value of 2.0022 was arbitrarily assigned for g_{zz} and the values for g_{xx} and g_{yy} were determined relative to this number.

The decreased line width resulting from deuterium substitution in BSSDP allows more rigorous assessment of the radial separation between the nitroxide moieties of labels bound to adjacent anion channel monomers of the anion channel dimer. Specifically, the width (at half-maximum amplitude) of the low-field EPR line from spin-labeled anion channels is 5.25 and 2.75 G for the conventional and [^{15}N,^{2}H$_{16}$]BSSDP labels, respectively (Figure 4). With this narrow line width, dipolar interactions between static, uniquely oriented spin labels should be observable in the EPR spectrum if the two labels are within 16 Å. Even if both nitroxide z axes were oriented at the magic angle (54.7°) with respect to the interelectron axis, a geometry of minimum relative sensitivity to dipole–dipole interactions in the extrema regions of the spectrum, resolved splittings of minor element spectral structurings are resolved in the center of the EPR spectrum at 16-Å separation with a 2.75-G line width (Beth et al., 1984). Our observation of no detectable interactions between [^{15}N,^{2}H$_{16}$]BSSDP-labeled anion channels thus suggests a minimum separation of 16 Å between the binding sites on adjacent monomers of the membrane oligomer.

The spatial separation of the BSSDP sites is of substantial interest since these sites are likely to be the same as, or to overlap, the functionally important stilbenedisulfonate binding sites (Beth et al., 1987). Though cooperativity has not been observable under some experimental conditions (Frölich, 1982; Rao et al., 1979), another study has reported cooperativity in binding of stilbenedisulfonates to the anion channel (Dix et al., 1979), suggesting that these sites may interact through steric or allosteric mechanisms. Our finding of a significant (>16-Å) separation between the BSSDP sites does not support steric mechanisms for cooperative ligand binding and, in addition, dictates that allosteric mechanisms be operative over considerable distances.

ST-EPR Spectrum from [^{15}N,^{2}H$_{16}$]BSSDP-Labeled Erythrocytes. ST-EPR spectroscopy has been employed to characterize the very slow rotational motions of spin-labeled anion channels in the erythrocyte membrane (Beth et al., 1986). As shown in Figure 5, top display, the V′$_2$ ST-EPR signal from spin-labeled erythrocytes is also composed of two distinct motional components. However, the nanosecond component from spin-labeled lipids can be digitally subtracted to reveal the line shape from spin-labeled anion channels as shown in Figure 5, second display. This spectrum may be compared with the corresponding normal isotope spectrum [see Beth et al. (1986), Figure 8, upper display]. The end point in this subtraction with [^{15}N,^{2}H$_{16}$]BSSDP can be determined much more precisely than with the normal isotope homologue by virtue of the narrower widths for lines from both motional components present. In general, a change of ±2% in the amplitude of the lipid signal relative to the visually observed optimum was found to result in a detectably worse subtraction in both the low- and high-field regions of the spectrum. By comparison, ±5% changes were required with normal isotope BSSDP, and even then, the most reliable place to judge the end point of the subtraction was in the $m_i = +1$ manifold of the ST-EPR spectrum. Following the subtraction, the motionally sensitive ratio parameters L''/L and H''/H (Beth et al., 1981a) indicate that the anion channel is highly hindered in its rotational motion, in agreement with previous work using the normal isotope spin label of BSSDP (Beth et al., 1986).

A number of intermolecular interactions may contribute to the very slow rotational motion of the anion channel in the membrane. First, the cytoplasmic domain of the anion channel is known to interact with ankyrin (Bennett, 1978) which in turn interacts with spectrin of the membrane skeleton (Bennett & Stenbuck, 1979a,b; Luna et al., 1979). Additional interactions that have been observed in ghost membrane preparations include binding of the cytoplasmic domain of the anion channel by protein 4.1 (Pasternack et al., 1985) and protein 4.2 (Korsgren & Cohen, 1986). Using a flash-induced transient optical technique, Nigg and Cherry (1980) provided evidence that proteolytic removal of the cytoplasmic domain of the anion channel led to increased rotational mobility of the transmembrane domain, suggesting that at least a subpopulation of anion channels are bound to large structures by their cytoplasmic domains. Further, the anion channel may also interact with other intrinsic membrane proteins including glycophorin (Pinto da Silva & Nicholson, 1974; Nigg et al., 1980).

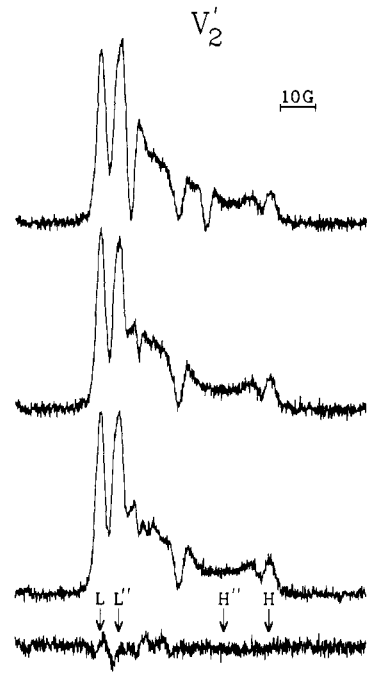

FIGURE 5: V′$_2$ ST-EPR spectra of [^{15}N,^{2}H$_{16}$]BSSDP-labeled anion channels in intact human erythrocytes. The top spectrum was obtained from intact erythrocytes labeled with 50 μM [^{15}N,^{2}H$_{16}$]BSSDP as described under Experimental Procedures. The second display shows the line shape from spin-labeled anion channels following digital subtraction of the V′$_2$ signal from spin-labeled lipids (spectrum not shown). The third display shows the line shape from spin-labeled anion channels following formation of covalent anion channel dimers with non-spin-labeled BS^3. The fourth display is the difference spectrum for the second minus the third displays. Field positions where the motionally sensitive spectral amplitudes L'' and H'' are measured from the V′$_2$ signal from ^{15}N spin labels are indicated (fourth display). All spectra were recorded on samples maintained at 20 °C.

In addition to its interactions with other proteins of the membrane and components of the cytoskeleton, the oligomeric state of the anion channel itself could significantly affect its rotational diffusion (Saffman & Delbrück, 1975). The oligomeric state of the anion channel has been the subject of many studies [reviewed in Jennings (1984)]. In physical studies of preparations of the anion channel purified in detergent solutions, stable dimers of anion channel subunits have been reported by most groups [e.g., Yu and Steck (1975), Clarke (1975), Reithmeier (1979), and Macara and Cantley (1983)], though there have been reports of evidence for dynamic monomer–dimer–tetramer interconversions (Dorst & Schubert, 1979; Pappert & Schubert, 1983). Chemical cross-linking of isolated membranes [e.g., Steck (1972), Wang and Richards (1974), and Reithmeier and Rao (1979)] or of intact erythrocytes with membrane-permeant (Wang &

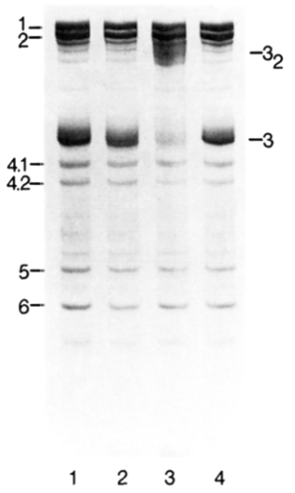

FIGURE 6: $NaDodSO_4$–polyacrylamide gel electrophoresis of $[^{15}N,^2H_{16}]$BSSDP-labeled anion channel subunits cross-linked to covalent dimers with BS^3. Samples of cells used for the experiments described in Figure 5 were hemolyzed, and membranes from these cells were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis. The resulting Coomassie blue stained gel is shown. Lanes 1 and 4 correspond to control membranes, prepared from untreated cells. Lane 2 corresponds to cells treated with 50 μM $[^{15}N,^2H_{16}]$BSSDP (Figure 5, second display). Lane 3 corresponds to cells treated sequentially with 50 μM $[^{15}N,^2H_{16}]$BSSDP and 1.0 mM BS^3. The numbers to the *right* of the gel lanes correspond to the migration positions of anion channel subunits (3) and covalent dimers of anion channel subunits (3_2). The numbers to the *left* of the gel lanes correspond to the positions of migration of other membrane-associated proteins [nomenclature modified from Fairbanks et al. (1971)].

Richards, 1975; Willingham & Gaffney, 1983) or with membrane-impermeant (Staros et al., 1981; Staros, 1982; Staros & Kakkad, 1983) reagents mostly results in the formation of covalent dimers of anion channel subunits, though in some instances tetramers have been observed; however, chemical cross-linking could not by itself be used to distinguish between stable dimers and a dynamic monomer–dimer equilibrium. Using optical techniques, Nigg and Cherry (1979) demonstrated that cross-linking of anion channel subunits by oxidation of endogenous thiols in the cytoplasmic domain does not alter their rotational diffusion, suggesting that in isolated membranes, at least, the predominant form of the channel is a stable, noncovalennt dimer.

Here we address the effects of chemical cross-linking on the rotational dynamics of anion channels in *intact* erythrocytes. Previous studies (Staros & Kakkad, 1983; Jennings & Nicknish, 1985) have led to the elucidation of two reaction products of the membrane-impermeant cross-linking reagent BS^3 with anion channels in intact erythrocytes. The first product formed, which is intrasubunit, spans the extracytoplasmic chymotryptic cleavage site, and is protected by stilbenedisulfonates, is presumably at the same site as that labeled by BSSDP at low concentrations. The second product, which forms at higher BS^3 concentrations, results in the formation of covalent dimers of anion channel subunits cross-linked in the extracytoplasmic domain.

Intact erythrocytes were first treated with 50 μM $[^{15}N,^2H_{16}]$BSSDP to form the intrasubunit labeled product (Figure 5, upper display; Figure 6, lane 2). The $[^{15}N,^2H_{16}]$BSSDP-labeled erythrocytes were then treated with 1 mM BS^3 to form covalent anion channel dimers (Figure 6, lane 3). The ST-EPR spectrum of the labeled, cross-linked anion channels in these cells indicated no detectable slowing of rotational motion as compared with the spectrum from $[^{15}N,^2H_{16}]$BSSDP-labeled cells that had not been subsequently cross-linked with BS^3 (Figure 5, third and fourth displays). This experiment provides the first direct evidence that, in the *intact* erythrocyte, the rotational diffusion of the anion channel is indistinguishable from that of a covalent dimer of anion channel subunits, an observation that supports the hypothesis that the native anion channel is a noncovalent dimer.

It is useful to consider the inherent sensitivity of the V'_2 ST-EPR signal to changes in motion of $[^{15}N,^2H_{16}]$BSSDP-labeled anion channels to establish confidence limits for these conclusions. Studies in which ST-EPR spectra were collected at various temperatures from *intact* erythrocytes or ghosts labeled with normal isotope BSSDP (Beth et al., 1986) or with $[^{15}N,^2H_{16}]$BSSDP (data not shown) have demonstrated a high sensitivity of the L''/L and H''/H ratio parameters to relatively modest changes in rotational motion. The less sensitive of these ratio parameters in the very slow motional range characteristic of the anion channel in the erythrocyte membrane is L''/L, and with this parameter, changes in τ_r of $\pm 15\%$ should be easily observable.

The remaining unknown in this analysis is the hydrodynamic size in the membrane of anion channel dimers relative to that of channel subunits. The correlation time for Brownian rotational diffusion of a cylindrical transmembrane protein about the axis normal to the membrane plane ($\tau_\parallel$) has been predicted to be proportional to the square of the cross-sectional radius of the protein in the plane of the membrane (Saffman & Delbrück, 1975). In the most favorable case, the cross-sectioned radius of the dimer would be twice that of the monomer, resulting in a 4-fold difference in $\tau_\parallel$. In the least favorable case, a subunit with an elongate cross-section could form a dimer with essentially the same cross-sectional radius as the monomer, resulting in essentially the same value for $\tau_\parallel$. While the actual case is likely to be something intermediate between these two extremes, additional structural studies are required before this can be determined.

The availability of $[^{15}N,^2H_{16}]$BSSDP with its high chemical reactivity, leading to bidentate labeling of the anion channel and thereby a tight motional coupling with it, in combination with its high sensitivity to small changes in rotational motion, should prove very useful in future investigations focused on the modulation of interactions of the anion channel with the cytoskeleton.

ACKNOWLEDGMENTS

We thank Dr. K. Balasubramanian for assistance with graphical display of spectral data, O. E. Morales and S. Juliao for excellent technical assistance, and S. Heaver for assistance in the preparation of the manuscript.

Registry No. **1**, 115420-11-6; **2**, 115420-12-7; **3**, 115436-78-7; **4**, 115420-13-8; **5**, 115420-14-9; 4-ketopimelic acid, 502-50-1; $[^{15}N,^2H_{11}]$-2-amino-2-methyl-1-propanol, 96681-27-5; 2-bis[2-(ethoxycarbonyl)ethyl]-4,4-dimethyloxazolidine, 115420-15-0; *N*-hydroxysulfosuccinimide, 82436-78-0.

REFERENCES

Anjaneyulu, P. S. R., & Staros, J. V. (1987) *Int. J. Pept. Protein Res. 30*, 117–124.

Atkinson, J. G., Csakvary, J. J., Morse, A. T., & Stuart, R. S. (1968) in *Proceedings of the Second International Conference on Methods of Preparing and Storing Labelled Compounds* (Sirchis, J., Ed.) pp 675–681, European Atomic Energy Community, Brussels.

Bennett, V. (1978) *J. Biol. Chem. 253*, 2292–2299.

Bennett, V., & Stenbuck, P. J. (1979a) *J. Biol. Chem. 254*, 2533–2541.

Bennett, V., & Stenbuck, P. J. (1979b) *Nature (London) 280*, 468–473.

Beth, A. H., & Robinson, B. H. (1988) in *Spin Labeling—Theory and Applications* (Berliner, L. J., & Reubin, J., Eds.) Vol. 3, Plenum Press, New York (in press).
Beth, A. H., Perkins, R. C., Venkataramu, S. D., Pearson, D. E., Dalton, L. R., Park, C. R., & Park, J. H. (1980) *Chem. Phys. Lett. 69*, 24–28.
Beth, A. H., Venkataramu, S. D., Balasubramanian, K., Dalton, L. R., Robinson, B. H., Pearson, D. E., Park, C. R., & Park, J. H. (1981a) *Proc. Natl. Acad. Sci. U.S.A. 78*, 967–971.
Beth, A. H., Balasubramanian, K., Wilder, R. T., Venkataramu, S. D., Robinson, B. H., Dalton, L. R., Pearson, D. E., & Park, J. H. (1981b) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4955–4959.
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., Robinson, B. H., Cobb, C. E., Dalton, L. R., Trommer, W. E., Birktoff, J. J., & Park, J. H. (1984) *J. Biol. Chem. 259*, 9717–9728.
Beth, A. H., Conturo, T. E., Venkataramu, S. D., & Staros, J. V. (1986) *Biochemistry 25*, 3824–3832.
Beth, A. H., Conturo, T. E., Abumrad, N. A., & Staros, J. V. (1987) in *Membrane Proteins: Proceedings of the Membrane Protein Symposium* (Goheen, S. C., Ed.) pp 371–382, Bio-Rad Laboratories, Richmond, CA.
Clarke, S. (1975) *J. Biol. Chem. 250*, 5459–5469.
Dix, J. A., Verkman, A. S., Solomon, A. K., & Cantley, L. C. (1979) *Nature (London) 282*, 520–522.
Dorst, H.-J., & Schubert, D. (1979) *Hoppe-Seyler's Z. Physiol. Chem. 360*, 1605–1618.
Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.
Frölich, O. (1982) *J. Membr. Biol. 65*, 111–123.
Hyde, J. S., & Dalton, L. R. (1972) *Chem. Phys. Lett. 16*, 568–572.
Hyde, J. S., & Thomas, D. D. (1973) *Ann. N.Y. Acad. Sci. 222*, 680–692.
Jennings, M. L. (1984) *J. Membr. Biol. 80*, 105–117.
Jennings, M. L., & Nicknish, J. S. (1985) *J. Biol. Chem. 260*, 5472–5479.
Korsgren, C., & Cohen, C. M. (1986) *J. Biol. Chem. 261*, 5536–5543.
Leonard, N. J., & Goode, W. E. (1950) *J. Am. Chem. Soc. 72*, 5404–5407.
Luna, E. J., Kidd, G. H., & Branton, D. (1979) *J. Biol. Chem. 254*, 2526–2532.
Macara, I. G., & Cantley, L. C. (1983) in *Cell Membranes: Methods and Review* (Elson, E., Frazier, W., & Glaser, L., Eds.) Vol. 1, pp 41–87, Plenum Press, New York.
Nigg, E., & Cherry, R. J. (1979) *Nature (London) 277*, 493–494.
Nigg, E., & Cherry, R. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4702–4706.
Pappert, G., & Schubert, D. (1983) *Biochim. Biophys. Acta 730*, 32–40.
Pasternack, G. R., Anderson, R. A., Leto, T. L., & Marchesi, V. T. (1985) *J. Biol. Chem. 260*, 3676–3683.
Pinto da Silva, P., & Nicholson, G. L. (1974) *Biochim. Biophys. Acta 363*, 311–319.
Rao, A., Martin, P. M., Reithmeier, R. A. F., & Cantley, L. C. (1979) *Biochemistry 18*, 4505–4516.
Reithmeier, R. A. F. (1979) *J. Biol. Chem. 254*, 3054–3060.
Reithmeier, R. A. F., & Rao, A. (1979) *J. Biol. Chem. 254*, 6151–6155.
Robinson, B. H., & Dalton, L. R. (1980) *J. Chem. Phys. 72*, 1312–1324.
Saffman, P. G., & Delbrück, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3111–3113.
Staros, J. V. (1982) *Biochemistry 21*, 3950–3955.
Staros, J. V., & Richards, F. M. (1974) *Biochemistry 13*, 2720–2726.
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. Mol. Biol. 66*, 295–305.
Thomas, D. D., Dalton, L. R., & Hyde, J. S. (1976) *J. Chem. Phys. 65*, 3006–3024.
Wang, K., & Richards, F. M. (1974) *J. Biol. Chem. 249*, 8005–8018.
Wang, K., & Richards, F. M. (1975) *J. Biol. Chem. 250*, 6622–6626.
Willingham, G. L., & Gaffney, B. J. (1983) *Biochemistry 22*, 892–898.
Yu, J., & Steck, T. L. (1975) *J. Biol. Chem. 250*, 9176–9184.