Lipid–Protein Interactions in Reconstituted Membranes Containing Acetylcholine Receptor†

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ABSTRACT: Functional membranes containing purified *Torpedo californica* acetylcholine receptor and dioleoylphosphatidylcholine (DOPC) were prepared by a cholate dialysis procedure with lipid to protein ratios of 100–400 to 1 (mol/mol). Spin-labeled lipids were incorporated into the reconstituted membranes and into native membranes prepared from *Torpedo* electroplax, and electron paramagnetic resonance (EPR) spectra were recorded between 0 and 20 °C. The spin-labels included nitroxide derivatives of stearic acid (16-doxylstearic acid), androstane, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidic acid (PA). The phospholipid spin-labels had 16-doxylstearic acid in the *sn*-2 position. All the spectra showed two components corresponding to a relatively mobile bilayer component and a motionally restricted "protein-perturbed" component. The relative amounts of mobile and perturbed components were quantitated by spectral subtraction and integration techniques. The mobile/perturbed ratio was somewhat temperature dependent, and the results are discussed in terms of exchange between mobile and perturbed environments. Plots of the mobile/perturbed ratios vs. lipid/protein ratios at 0 °C gave straight lines from which the relative binding affinity of each spin-label and the number of perturbed lipids per receptor protein could be calculated. All the spin-labels gave similar values for the number of perturbed lipids (40 ± 7), a number close to the number of lipids that will fit around the intramembranous perimeter of the receptor. The affinities of the spin-labeled lipids for the receptor relative to DOPC were androstane (K = 4.3) ≃ 16-doxylstearic acid (4.1) > PA (2.7) > PE (1.1) ~ PC (1.0) ~ PS (0.7). The lipids having the highest affinity for the acetylcholine receptor were also those that have the largest effects on the ion flux functional properties of the receptor, and the results are discussed in terms of lipid effects on receptor function.

The study of lipid–protein interactions in biological and model membranes has been widespread and controversial (Parsegian, 1982). A key problem is to relate the elegant biophysical measurements of lipid and protein dynamics to specific functional properties of membranes. The integrated approach of Fleischer and colleagues (Fleischer et al., 1979; Seelig et al., 1981), as applied to the Ca^{2+}-ATPase[1] from sarcoplasmic reticulum, offers a good model for correlating biophysical and biochemical data.

We have focused our efforts on the nicotinic acetylcholine receptor (AChR) from the electric ray *Torpedo californica*. The *Torpedo* AChR is a multisubunit, transmembrane protein involved in cholinergic synaptic transmission. It binds acetylcholine and transduces the binding into a large increase in cation permeability. Considerable progress has been made recently in defining the molecular mechanism of AChR function (Karlin, 1980; Changeux, 1981; McNamee & Ochoa, 1982). The AChR can be isolated and purified from *Torpedo* tissue and the purified receptor can be reconstituted into lipid bilayer vesicles with complete recovery of ligand binding and ion permeability properties (McNamee & Ochoa, 1982).

In addition to the advantages outlined above, the isolated AChR is an ideal molecule for both detailed biophysical and functional analysis of lipid–protein interactions since receptor function appears to be quite sensitive to the lipid environment. The receptor must be maintained in the presence of phospholipids at all stages of purification in order to retain ion flux properties (Huganir et al., 1979; Anholt et al., 1981), and changes in the lipid composition of reconstituted membranes containing AChR and defined lipids appear to dramatically affect AChR function (Kilian et al., 1980; Dalziel et al., 1980; Ochoa et al., 1983; Criado et al., 1983). Previous studies of AChR function in native membranes prepared from *Torpedo* electroplax have shown that AChR-mediated ion flux is very sensitive to perturbations of the lipid environment achieved either with phospholipase A_2 (Andreasen et al., 1979) or with local anesthetics (Changeux, 1981).

For the experiments described here, the electron paramagnetic resonance (EPR) technique of spin-labeling is used to probe the dynamics of lipid–protein interactions in both native membranes and in reconstituted membranes containing purified AChR and defined lipids. The EPR spectra of spin-labeled lipid probes are sensitive to the molecular motions and polarity of the membrane bilayer region, and the EPR technique has been widely used in membrane studies (Jost & Griffith, 1982; Thomas et al., 1982; Marsh et al., 1982; Watts et al., 1982). There is considerable evidence for motionally restricted lipids associated with membrane proteins, and the presence of such "boundary lipids" have been assumed to play an important functional role in lipid–protein interactions (Sandermann, 1978). Although most NMR evidence suggests that nearly all lipids in membranes can undergo fairly rapid exchange (Brown et al., 1977; Rice et al., 1979), the functional effects of lipids on membrane proteins are most likely due to those lipids in direct contact with the protein.

Previous EPR analyses of native *Torpedo* membranes using spin-labeled fatty acid, phospholipid, and sterol analogues have shown the presence of a motionally restricted lipid component

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[1] Abbreviations: AChR, acetylcholine receptor; EPR, electron paramagnetic resonance; BAC, bromoacetylcholine bromide; DOPC, dioleoylphosphatidylcholine; [^{125}I]-α-BgTx, iodinated (^{125}I) α-bungarotoxin; Carb, carbamoylcholine chloride; ATPase, adenosinetriphosphatase; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride, SDS, sodium dodecyl sulfate.

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in addition to the typical bilayer lipid component (Marsh & Barrantes, 1978; Rousselet et al., 1979; Marsh et al., 1981; McNamee et al., 1982). However, quantitative analysis of the results has been hampered by the compositional heterogeneity of the native membranes.

The use of reconstituted membranes in the present study permits an unambiguous and systematic analysis of lipid interactions with the acetylcholine receptor. The effects of different spin-labels and different lipid to protein ratios on the amount of restricted component provide a major advance over previous studies of AChR–lipid interactions. The lipid interaction models developed by Brotherus et al. (1981) are used as a framework for analyzing the EPR data in terms of specific lipid affinities.

Materials and Methods

Preparation of Acetylcholine Receptor Rich Membranes. A crude membrane fraction was prepared from liquid nitrogen frozen *Torpedo californica* electroplax essentially as described by Ochoa et al. (1983). In most cases, the final pellet from 600 g of tissue was resuspended in 75 mL of buffer A [100 mM NaCl, 10 mM 3-(*N*-morpholino)propanesulfonic acid (Mops), 0.1 mM EDTA, and 0.02% sodium azide, pH 7.4] at a protein concentration of 10–15 mg/mL and then stored frozen in liquid nitrogen.

In some cases, the pellet was resuspended in 28% w/w sucrose to a protein concentration of about 10 mg/mL, and more highly purified membranes were obtained by sucrose density gradient centrifugation. Briefly, 8-mL aliquots of the membranes in 28% sucrose were layered on top of discontinuous gradients consisting of 10 mL of 30% sucrose, 12 mL of 35% sucrose, and 7 mL of 41% sucrose. All sucrose solutions were a weight to weight ratio and contained 10 mM sodium phosphate and 0.02% sodium azide, pH 7.0. The samples were centrifuged at 27 000 rpm in a Beckman SW 27 rotor for 4 h at 4 °C. The membranes at each interface were collected with a syringe and their specific α-bungarotoxin binding activities were measured. The membranes from the 30–35% interface were further purified by removing peripheral membrane proteins by using an alkaline extraction procedure (Neubig et al., 1979). The membranes were then suspended in buffer A to a final protein concentration of 5 mg/mL and stored in liquid nitrogen.

Preparation of Acetylcholine Affinity Column. The procedure was adapted from Karlin et al. (1976); 50 mL (packed volume) of Affi-Gel 401 (Bio-Rad, Richmond, CA) was mixed with 50 mL of 20 mM dithiothreitol in 200 mM Tris-HCl (pH 8.0) in a 2.5 × 40 cm column. The mixture was agitated for 20 min at room temperature, and the column was washed with 5 column volumes of buffer B (100 mM NaCl, 20 mM sodium phosphate, and 0.02% sodium azide, pH 7.0) and then resuspended to a volume of 90 mL with buffer B. Bromoacetylcholine bromide (0.45 g) (Damle et al., 1978) was added to the suspended gel, and the column was agitated for 20 min. The gel was washed with 3 column volumes of buffer B and resuspended to 90 mL, and then 0.45 g of iodoacetamide was added to block any remaining sulfhydryl groups. The column was washed extensively with unbuffered 0.02% sodium azide and stored at 4 °C until used. Assay of free sulfhydryl groups before and after derivatization with bromoacetylcholine showed that 95% of the sulfhydryl groups were derivatized. The gel was stable for approximately 2 months in the presence of 0.02% sodium azide. The column was equilibrated with buffer A containing 1% sodium cholate and 1 mg/mL dioleoylphosphatidylcholine (DOPC; Avanti Polar Lipids, Birmingham, AL) immediately before use.

Purification and Reconstitution of Acetylcholine Receptor. All steps were carried out at 0–4 °C. Crude membranes having a total protein content of 600 mg were suspended in 600 mL of buffer A, and solid sodium cholate was added to give a final concentration of 1% cholate (w/v). The mixture was gently stirred for 30 min and then centrifuged at 35 000 rpm in a Beckman Type 35 rotor for 1 h. The supernatant was filtered through four layers of cheesecloth and then applied to the acetylcholine affinity column. The column flow rate during this and all other steps was 2 mL/min, and all solutions containing DOPC were saturated with argon. The column was washed with 1.5 column volumes of buffer A containing 1 mg/mL DOPC and 1% cholate. The column was further washed with 1 column volume of the same buffer containing 2.5 mg/mL DOPC and allowed to equilibrate overnight in order to ensure maximum equilibration of native lipids with the DOPC. Following two additional washes with the high DOPC lipid concentration, the column was washed with 2 column volumes of buffer A containing 0.1–0.4 mg/mL DOPC and 0.5% cholate. The lipid/protein ratio of the final preparation could be adjusted at this stage by the choice of the DOPC concentration. Pure AChR was eluted from the column with a solution containing 10 mM carbamoylcholine, 0.1–0.4 mg/mL DOPC, and 0.5% cholate in buffer A (with an NaCl concentration of 80 mM rather than 100 mM). Protein concentration in the 2-mL fractions was monitored by absorbance at 280 nm, and fractions containing the AChR were pooled and dialyzed for 40–60 h against 4 × 2 L of buffer A. Typically, the volume of pooled fractions was 25 mL and the protein concentration of the reconstituted membranes ranged from 0.4 to 1 mg/mL as determined by the procedure of Lowry et al. (1951). The membranes were stored in liquid nitrogen until used.

Extraction of Lipids from Membranes and Preparation of Liposomes. Lipid was extracted from membranes by the Bligh & Dyer procedure (1959). All solvents were saturated with argon, and extracted lipids were concentrated and stored as 1 mg/mL solutions in $CHCl_3$ at −20 °C. Phosphate assays before and after extraction revealed that 100% of the phospholipid was extracted from both native AChR membranes and reconstituted membranes.

Liposomes were prepared in either of two ways: (a) An aliquot of lipid was dried under argon, and the last traces of organic solvent were removed under vacuum for 12 h. The lipid was then hydrated with buffer A and suspended by vortex mixing. (b) A solution of lipids in 0.5% cholate in buffer A was dialyzed for 48 h against 4 × 2 L of buffer A. All liposomes were stored in liquid nitrogen.

Preparation and Incorporation of Spin-Labeled Lipids into Membranes and Liposomes. 2-(14-Carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy (16-FASL; fatty acid spin-label, also known as 16-doxylstearic acid), and 17β-hydroxy-4′,4′-dimethylspiro[5α-androstane-3,2′-oxazolidin]-3′-yloxy (ASL; androstane spin-label) were purchased from Syva Co., Palo Alto, CA. Egg phosphatidylcholine was purchased from Avanti Polar Lipids and was converted to lysophosphatidylcholine by the procedure of Molotkovsky & Bergelson (1980). Phosphatidylcholine containing 16-FASL in the *sn*-2 position (PCSL) was prepared by the method of Boss et al. (1975) as modified by Bergelson (1980) with the following exceptions. The silicic acid column was first eluted with $CHCl_3$ and then 5% MeOH in $CHCl_3$ (v/v). The MeOH concentration was increased by 5% (v/v) increments in successive elution steps. Pure PCSL was eluted from the silicic acid column with 25% MeOH in $CHCl_3$ (v/v). Phosphatidic

acid, phosphatidylethanolamine, and phosphatidylserine containing 16-FASL in the *sn*-2 position (PASL, PESL, and PSSL) were prepared by transphosphatidylation with phospholipase D according to the method of Comfurius & Zwaal (1977). The spin-labeled phospholipids eluted from the CM-52 cellulose column in the same order as native phospholipids. The concentration of methanol in chloroform required to elute a specific phospholipids was somewhat dependent upon the elution history of the column, with lower concentrations of methanol being sufficient if the column was washed extensively. A complete separation of PASL and PSSL was not achieved, although some fractions containing pure PASL and pure PSSL were obtained, and these fractions were used in the EPR studies. Spin-labels were stored in ethanol (16-FASL, ASL, PESL, and PCSL) or chloroform (PASL and PSSL) at −20 °C.

16-FASL was incorporated into membranes and liposomes by two procedures. (a) A total of 15 nmol (1.5 μL) of 16-FASL was put into a glass tube, and the ethanol was removed by drying under a stream of argon. Native membranes containing 5 mg (about 1 mL) of membrane protein or liposomes containing 3 μmol of lipid were added to the tube, and the mixture was incubated for 15 min at room temperature. The membranes were then pelleted in an SW-60 rotor by spinning at 40 000 rpm for 15 min. The pellet was put into a 50-μL capillary tube, and the EPR spectra were recorded. (b) The second procedure was the same as procedure a except that the spin-label in ethanol was added to the membrane while they were vortexing. EPR spectra of native membranes labeled with 16-FASL by procedure a or b were identical. ASL and PCSL were incorporated into native membranes and receptor lipid liposomes by procedure b. Lipid to spin-label ratios were always >150 mol/mol, and the amount of added ethanol was always less than 1% of the total volume.

The procedures used to incorporate spin-labels into reconstituted membranes and DOPC liposomes were similar to those described above with some exceptions. All membranes and liposomes made by the cholate dialysis technique were frozen in liquid nitrogen and thawed to room temperature 6 times in order to increase vesicle size (Ochoa et al., 1983). The increase in size made it possible to rapidly pellet the membranes and liposomes in the ultracentrifuge. Membranes and liposomes were concentrated to a protein concentration of about 5 mg/mL (or to a liposome phospholipid concentration of about 3 mM) by centrifugation and resuspension.

A decrease in EPR spectral intensity due to spin-label reduction was observed upon incorporation of spin-labels into reconstituted membranes containing AChR; reduction was most apparent in membranes having the lowest lipid to protein ratio (100/1 mol/mol) and at temperatures in excess of 15 °C. In order to increase EPR signal strength without increasing the spin-label to lipid ratio, the membranes were treated in the following way. 16-FASL was incorporated into membranes as in procedure a above except that after addition of spin-label, the membranes were allowed to sit overnight at 4 °C. An equal volume (about 1 mL) of 1 mM fatty acid free bovine serum albumin (BSA) in buffer A was then added to the membranes, and this mixture was incubated at room temperature for 15 min. The membranes were pelleted by centrifuging at 40 000 rpm in an SW-60 rotor for 15 min and washed once with buffer A. The BSA treatment completely removed the 16-FASL from reconstituted AChR membranes and DOPC liposomes as judged by EPR spectroscopy. Pretreatment with 16-FASL followed by removal with BSA was carried out on all reconstituted membranes containing AChR. Spin-label reduction in these membranes after further incorporation of spin-label was greatly reduced compared to the initial reduction, and there was no effect on subsequent measurements of ion flux activity.

16-FASL, PASL, and PSSL were incorporated into pretreated reconstituted membranes and DOPC liposomes by procedure a above. After membranes and liposomes were added to the dried PASL or PSSL, it was necessary to scrape the bottom of the tube with a spatula, vigorously vortex the tube for 3 min, and then freeze in liquid nitrogen and thaw 6 times in order for almost complete incorporation of spin-label to occur. PCSL, PESL, and ASL were incorporated into reconstituted membranes by procedure b above. Before the final pelleting step the membranes were frozen in liquid nitrogen and thawed to room temperature 6 times to aid spin-label incorporation. EPR spectra of membranes or liposomes containing PASL, PESL or PSSL occasionally exhibited a broad, single peak component due to PASL, PSSL, or PESL vesicles. If this component constituted <20% of the total spectral intensity, it was removed by subtraction. (If the broad component contributed greater than 20% of the total spectrum, the sample was discarded since subtractions were unreliable for such samples.)

EPR Spectroscopy. EPR spectra were recorded and processed with a Bruker EPR 200 D spectrometer equipped with a VT-1 temperature control device and a Nicolet 1180 data system. The uncertainty in the temperature was ±1 °C. Instrumental parameters were midfield magnetic field strength = 3363 G, microwave frequency = 9.39 GHz, microwave power = 10 mW, modulation frequency = 100 KHz, and modulation amplitude = 1.00 G. Measurement of spectral widths and amplitudes, spectral subtractions, and double integrations were carried out with Nicolet NTCESR programs. Criteria outlined by Jost & Griffith (1978) were satisfied when performing spectral subtractions. Pairwise subtractions were done by the method of Brotherus et al. (1980).

In some cases EPR spectra of the spin-labels in organic solvents at low temperatures were used to simulate the protein-perturbed component in two-component spectra. A collection of spectra in ether–pentane–ethanol (1:1:3 v/v/v) were recorded over a temperature range to give all observed values of the outermost spectral splittings. In the case of 16-FASL, the temperature range was −125 to −108 °C, and for ASL the range was −143 to −127 °C.

Other Procedures. Thin-layer chromatography techniques were used to assess the lipid composition of the reconstituted AChR membranes. Lipid extracted from reconstituted membranes was chromatographed on type S chromorods (Iatron Laboratories, Inc.). The solvent systems used were $CHCl_3$–MeOH–H_2O (65:25:4 v/v) and $CHCl_3$–MeOH–H_2O–NH_3 (65:25:4:1 v/v). After chromatography, lipids were detected with an Iatroscan TH-10 [Iatron Laboratories, Inc.; see Ackman (1981) for further description of chromorod chromatography and the Iatroscan TH-10]. Peak identification was done by comparing experimental mobilities with those of standards. Quantitation of peak areas was done by cutting out the peaks and weighing them. Cholesterol was assayed with a Boehringer-Mannheim cholesterol assay kit. Prior to assay, lipids were dissolved in 2-propanol.

Sucrose density gradient centrifugation was used to characterize the density heterogeneity of the reconstituted AChR membranes. Continuous sucrose gradients (20–55% w/w in 10 mM Mops, 0.1 mM EDTA, and 0.02% azide, pH 7.4) containing reconstituted membranes were prepared in Beckman SW-60 cellulose nitrate tubes. Less than 1% of the AChR

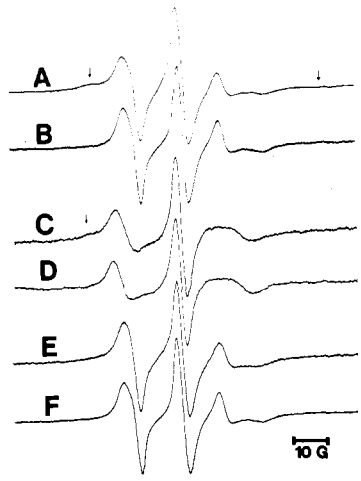

FIGURE 1: EPR spectra of spin-labeled lipids in native AChR membranes (AChRM) and in liposomes prepared from extracted AChRM lipids. (A) 16-Fatty acid spin-label (16-FASL) in AChRM; (B) 16-FASL in liposomes; (C) androstane spin-label (ASL) in AChRM; (D) ASL in liposomes; (E) phosphatidylcholine spin-label (PCSL) in AChRM; (F) PCSL in liposomes. Spectra A, B, E, and F were recorded at 0 °C, and spectra C and D were recorded at 23 °C. Arrows indicate regions where protein-associated spectral intensity is clearly evident.

α-toxin binding sites were labeled with [^{125}I]-α-BgTx before loading the membranes on the gradients. Membranes were loaded at the top and at the bottom of separate gradients. Gradients were centrifuged for 24 h at 55000 rpm in a SW-60 rotor; 200-μL fractions were collected manually. Fraction densities were measured with a Bausch & Lomb refractometer. AChR was monitored by measuring the [^{125}I]-α-BgTx cpm in a Packard gamma scintillation spectrometer. Aliquots of gradient fractions were diluted with water and lyophilized, and the phosphate content was measured (McClare, 1971).

Equilibrium binding of iodinated (^{125}I) α-bungarotoxin ([^{125}I]-α-BgTx) to AChR was measured as described by Walker et al. (1981). Influx of $^{86}Rb^+$ into reconstituted AChR membranes was measured by the manual mixing technique described by Walker et al. (1982). Membranes were frozen in liquid nitrogen and thawed several times before $^{86}Rb^+$ influx assays.

Protein was assayed by the Lowry method (Lowry et al., 1951). Polyacrylamide slab gels (7.5%) were prepared, run, and stained according to the procedures of Ames (1974). Quantitative densitometry of the gels was done with a Zeineh soft lasar scanning densitometer (Model SL-504-XL).

Results

Analysis of Spin-Labeled Lipids in Native Membranes. Highly purified membranes were prepared from the electroplax of *Torpedo californica* by a combination of differential and sucrose gradient centrifugation followed by alkaline extraction of peripheral membrane proteins. The membranes had a specific activity for α-bungarotoxin binding of approximately 2 nmol/mg of protein, indicating that about 25% of the total protein was AChR. These membranes have been shown previously to retain all the functional properties of the receptor, including ligand binding and ion flux properties, and serve as an excellent model system for AChR in its native membrane environment (McNamee & Ochoa, 1982). The lipid to protein ratio in highly purified native AChR membranes is about 170/1 (mol/mol), and the cholesterol to phospholipid ratio is about 1/1 (mol/mol) (Gonzalez-Ros et al., 1982).

The EPR spectra of spin-labeled lipids incorporated into the native membranes and into liposomes prepared from lipid extracts of the native membranes are shown in Figure 1. The

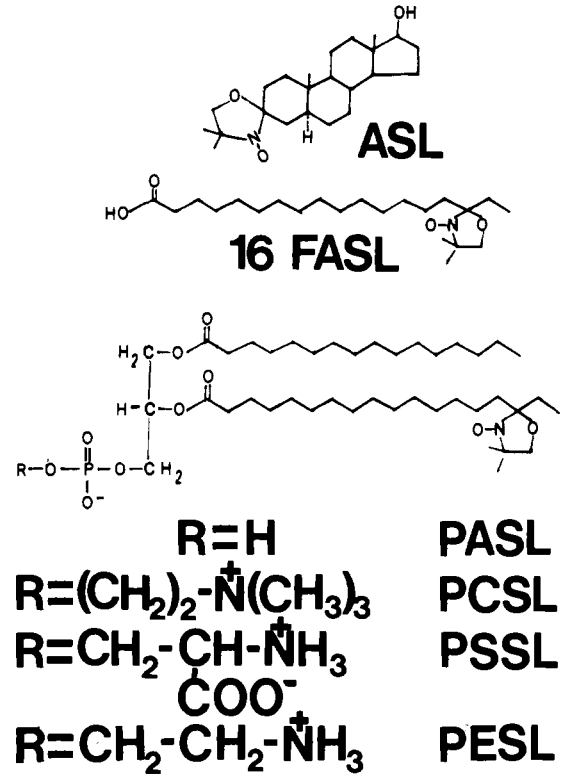

FIGURE 2: Structures of spin-labeled lipids.

methods used for incorporation of the individual spin-labels into membranes and into liposomes prepared from extracted lipids are detailed under Materials and Methods and the structures of the spin-labels are shown in Figure 2. The presence of a two-component spectrum is clearly illustrated in the case of 16-FASL in native membranes by the presence of spectral intensity in the outer regions of the EPR spectrum. The absence of such a component in the corresponding liposome spectrum is evidence for the effect of the proteins on the molecular motion of a fraction of the spin-labels.

In order to analyze the spectra quantitatively, efforts were made to obtain model spectra that matched both the protein-perturbed component and the lipid bilayer component. For the protein-perturbed component, it was necessary to create an environment that mimicked both the polarity and effective viscosity of the label environment. Attempts to use liposome spectra at low temperatures were unsuccessful because there is a large polarity difference between the liposome bilayer center and the protein-perturbed environment. In addition, the molecular motions of the lipid probes in a bilayer environment may be more anisotropic than the motion of the label in the protein-perturbed environment. Similar observations have been made by Griffith & Jost (1976). Successful matching of the perturbed component was obtained empirically by using spectra of the 16-FASL in organic solvent mixtures at low temperatures. Selection of appropriate solvent mixtures was based on the work of Griffith et al. (1974), and ether–pentane–ethanol mixtures were used as described under Materials and Methods. Subtraction of an organic solvent spectrum of 16-FASL (Figure 3B) from the spectrum of 16-FASL in native membranes (Figure 3A) gave a resultant spectrum (Figure 3C) that was very similar to the spectrum of 16-FASL in liposomes prepared from extracted *Torpedo* lipids (Figure 3D).

In order to quantitate the two components present in the 16-FASL–AChR membrane spectrum, it was doubly integrated before and after subtraction of the protein-perturbed component. The double integrations indicated that 38% of

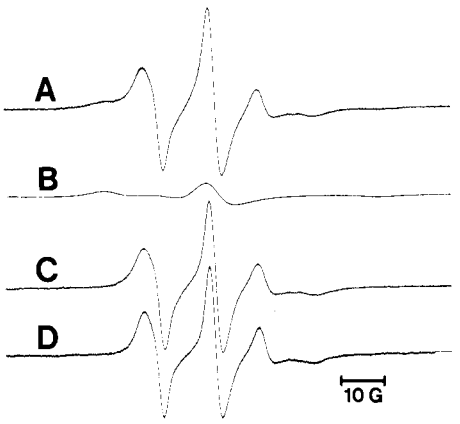

FIGURE 3: (A) EPR spectrum of 16-FASL in native AChR membranes at 0 °C. (B) Spectrum of 16-FASL in ether–pentane–ethanol (1:1:3 v/v/v) at −120 °C (simulation of the protein-perturbed environment). (C) Spectrum A minus spectrum B. (D) EPR spectrum of 16-FASL in extracted *Torpedo* lipids at −5 °C.

these spin-labels were strongly perturbed by the protein and 62% were not strongly perturbed. The spectrum obtained by subtraction of the protein-perturbed component (Figure 3C) was slightly broader than the liposome spectrum (Figure 3D), indicating that the protein had a small effect on the more mobile component of the membrane spectrum. Because this effect is quite small, use of the term "protein perturbed" will refer only to the component that can be removed by subtraction; the other component will be referred to as the mobile component and is representative of spin-labels in a lipid bilayer.

From the spectrum, the upper limit for the exchange rate of spin-labels between protein-perturbed and relatively mobile environments can be calculated. The maximum rate is equal to the difference between the hyperfine splittings of the two spectral components (Marsh et al., 1982). Calculation of this difference yielded an exchange rate of $<5 \times 10^7$ s^{-1} similar to values obtained by Marsh & Barrantes (1978). The apparent rotational correlation time of the spin-label of the protein-perturbed 16-FASL was calculated in two ways by using techniques developed by Freed and co-workers [see Freed (1976)]. Values of 15–16 ns were obtained by both techniques. In contrast, the rotational correlation time for the mobile component was 1 ns (see Appendix for details).

The relative amounts of perturbed and mobile spectral components in native membranes depended on the purity of a given membrane preparation, and a detailed quantitative comparison of the relative affinities of different spin-labeled lipids for AChR was not attempted. However, from inspection of Figure 1 and from results presented previously (McNamee et al., 1982) it is clear that 16-FASL gave rise to a more perturbed component than PCSL. For the PCSL it was often difficult to see the perturbed component in the composite spectrum due to the dominance of the sharper mobile lipid lines. Reconstituted membranes offered the opportunity for a more complete analysis as described below.

Spin-Labeled Lipids in Reconstituted Membranes. Reconstituted membranes containing purified acetylcholine receptor were prepared by the cholate dialysis procedure originally described by Epstein & Racker (1978). In the experiments presented here highly purified phospholipids were used at all stages of purification and reconstitution. The lipid to protein ratio of the final reconstituted membranes was controlled by equilibrating the receptor with the appropriate concentration of lipids while it was still on the affinity column. Approximately 65 ± 8 (n = 8) mol of phospholipid/mol of AChR remained associated with the AChR during purification as judged by the excess lipid recovered after eluting AChR with a known lipid concentration. This value was included when estimating the amount of lipid to be used during the washing procedure. The final lipid to protein ratio was always confirmed by phosphate and protein assays. Nearly all native lipids could be removed from AChR by the washing steps. In most cases, however, a small amount of an unidentified lipid component copurified with the AChR. (The unidentified component migrated in thin-layer chromatography solvent systems as a neutral, nonpolar lipid.)

Table I: AChR-Mediated Cation Flux into Reconstituted Membranes

membrane preparation[a]	$^{86}Rb^+$ influx[b] (cpm × 10^{-3}) no carbamoylcholine	$^{86}Rb^+$ influx[b] (cpm × 10^{-3}) +1 mM carbamoylcholine
1	1.9 ± 0.5	5.1 ± 0.7
2	5.3 ± 1.0	9.5 ± 1.6

[a] The lipid/AChR (mol/mol) of both membrane preparations was 200. The AChR concentrations were the following: preparation 1, 0.65 mg/mL; preparation 2, 1.03 mg/mL. [b] Assays were done by the method of Walker et al. (1982) by using 50 μL of membranes for each assay.

The protein composition of the reconstituted AChR membranes was analyzed by SDS–polyacrylamide gel electrophoresis and showed only the four AChR subunits (α, β, γ, and δ). Integration of densitometer traces indicated that >99% of the protein on the gel was AChR with the expected subunit ratios of $2\alpha/1\beta/1\gamma/1\delta$. The specific [^{125}I]-α-BgTx binding activity of the reconstituted membranes was 6–8 nmol/mg of protein, consistent with the expected value of 8 nmol/mg for pure AChR (McNamee & Ochoa, 1982).

The ion flux properties of the membranes were measured by the Rb^+ influx technique as described by Walker et al. (1982). A typical flux result is given in Table I. In general, the flux response in reconstituted membranes used here was relatively small due primarily to the small internal volume of the vesicles. In order to confirm that the AChR remained fully functional at all stages of the experiments, reconstituted membranes were mixed with an excess of asolectin and cholate and rereconstituted. In these cases the flux response at the high lipid to protein ratios was typical of fully functional receptor.

Reconstituted membranes with lipid to protein ratios of 112/1 and 202/1 (mol/mol) were run on 20–55% sucrose gradients (Figure 4). Each membrane sample was loaded at the top or the bottom of separate gradients. All gradients exhibited a single peak of α-bungaratoxin binding activity and total phosphate, indicating that AChR was firmly associated with the lipid vesicles.

Analysis of EPR Spectra. Figure 5 shows spectra of PSSL, PCSL, PESL, PASL, 16-FASL, and ASL in reconstituted AChR membranes at 0 °C. There is intensity present in the high- and low-field regions of the AChR-membrane spectra that is not present in the corresponding spectra of each spin-labeled lipid in DOPC liposomes. The top spectrum shows PCSL in DOPC liposomes, and the bottom spectrum shows ASL in DOPC liposomes. All the other phospholipid spin-labels gave spectra in DOPC liposomes identical with that of PCSL. Qualitatively, the difference between reconstituted AChR membrane and liposome spectra is the same as observed between spectra of spin-labels in native membranes and the corresponding spectra in liposomes prepared from extracted native membrane lipids. The protein clearly perturbed the motion of some of the spin-labels. The exchange rate between

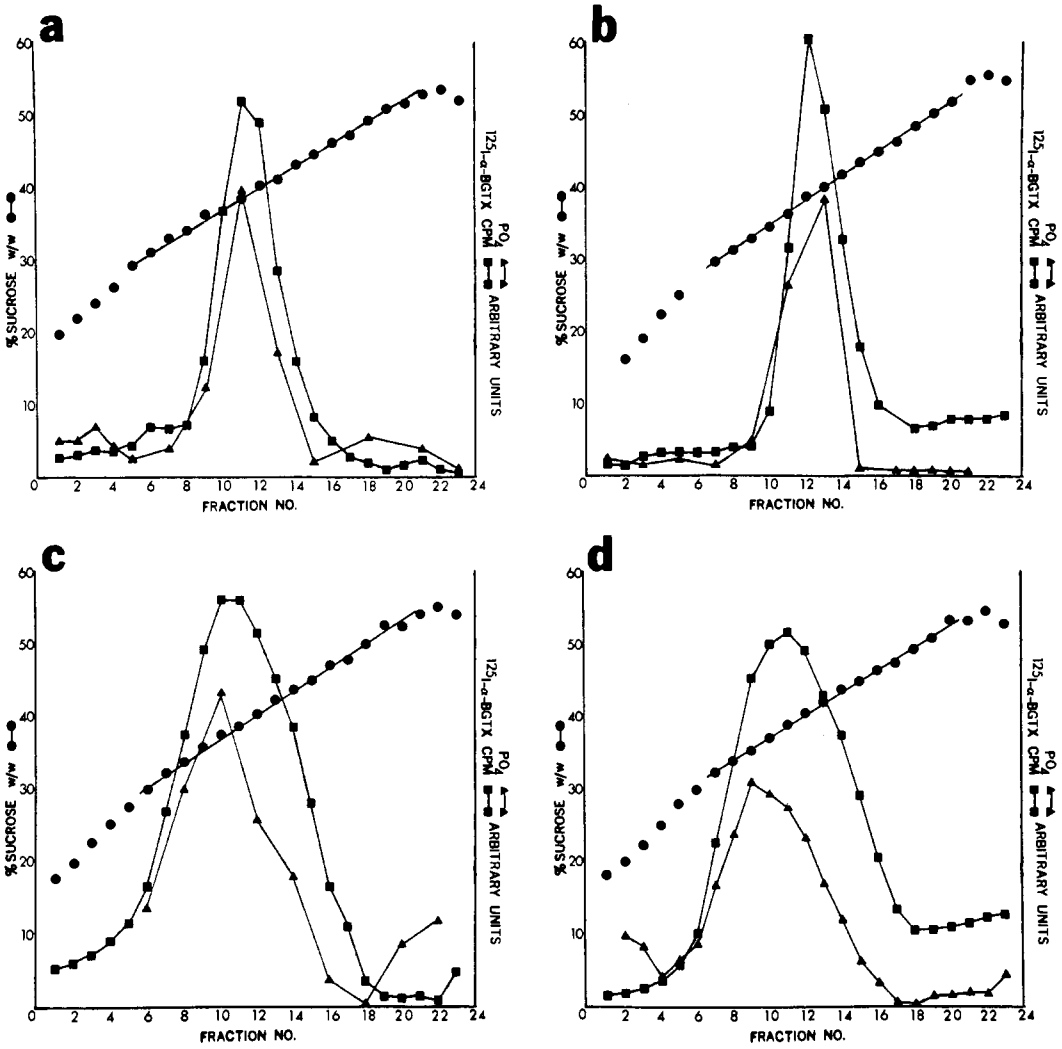

FIGURE 4: Sucrose gradient analysis of reconstituted AChR membranes. (■) [^{125}I]-α-BgTx (cpm); (▲) phosphate (arbitrary units); (●) % sucrose (w/w). (a) Lipid/AChR = 112 mol/mol; membranes were loaded at the top of the gradient; (b) lipid/AChR = 112 mol/mol; membranes were loaded at the bottom of the gradient; (c) lipid/AChR = 202 mol/mol; membranes were loaded at the top of the gradient; (d) lipid/AChR = 202 mol/mol; membranes were loaded at the bottom of the gradient.

protein-perturbed and relatively unperturbed environments was $<5 \times 10^7$ s^{-1}, determined by calculating the difference between the hyperfine splittings of the two components in reconstituted AChR membrane spectra. Visual comparison of the amplitude of the low-field peak (due to the perturbing effect of the protein) with the relatively unperturbed low-field lipid peak (Figure 5) indicates that the fraction of spin-labels perturbed decreased in the following order: ASL ~ 16-FASL > PASL > PESL ~ PCSL ~ PSSL.

Three methods of spectral subtraction, followed by double integration, were performed in order to quantitate the protein-perturbed and mobile components from the reconstituted AChR membrane spectra.

(a) Spectra of spin-labeled lipid in DOPC liposomes were used to subtract the relatively mobile component from two-component spectra [spin-labeled lipid refers in (a) and (b) to 16-FASL when two-component spectra of PSSL, PCSL, PESL, PASL, and 16-FASL are considered and to ASL when two-component spectra of ASL are considered]. DOPC liposome spectra were recorded over a temperature range that was larger than the range used when two-component spectra were recorded. The liposome spectra that best removed the mobile component from two-component spectra were those recorded at 5 °C lower than the two-component spectra. Figure 6A shows the spectrum of 16-FASL in reconstituted AChR membranes at 0 °C. Figure 6B shows the spectrum of 16-FASL in DOPC liposomes at −5 °C. The mobile component was removed from Figure 6A by subtracting the liposome spectrum from it, and the resulting protein-perturbed component is seen in Figure 6C.

(b) Spectra of spin-labeled lipid in organic solvents (see Materials and Methods) were used to subtract the protein-perturbed component from two-component spectra. The appropriate spectra for subtraction were selected by empirically matching the maximum splittings of the organic solvent and the protein-perturbed spectra.

(c) The final method used was the pairwise subtraction method devised by Brotherus et al. (1980). In this method pairs of spectra with different relative amounts of the two components are manipulated to eliminate one of the components. For this method to work it was essential to select spectra in which the line shapes of the paired components were identical.

Quantitation of components was done by (1) comparing double integrals before and after subtractions described in (a) and (b) above and (2) inserting double integrals of spectra A and B and amplitude factors obtained during subtractions described in (c) into equations derived by Brotherus et al. (1980). Results obtained by using the different quantitation methods are shown in Table II. The very good agreement

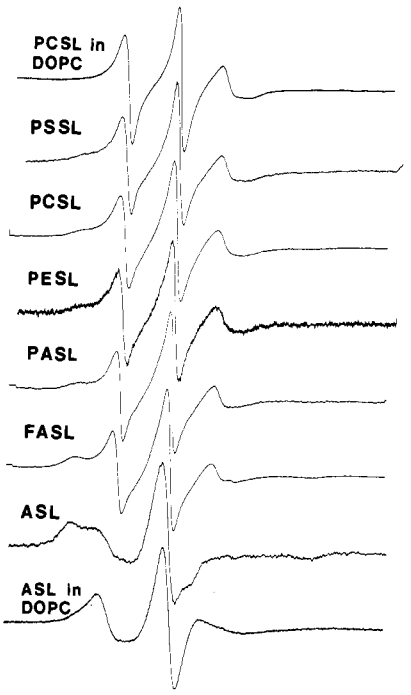

FIGURE 5: EPR spectra of spin-labeled lipids in reconstituted AChR membranes and in DOPC liposomes (top and bottom spectra). The reconstituted membrane spectra are ordered from top to bottom according to each spin-label's relative affinity for the AChR. For each sample, the lipid to protein mole ratio (L/P) and the percent perturbed component is given. In descending order the spectra are the following: PCSL in DOPC liposomes (represents 0% perturbed component or a completely mobile environment); PSSL in AChR membranes (L/P 142/1, 21% perturbed); PCSL in AChR membranes (L/P 142/1, 34% perturbed); PESL in AChR membranes (L/P 151/1, 32% perturbed); PASL in AChR membranes (L/P 142/1, 54% perturbed); FASL in AChR membranes (L/P 142/1, 60% perturbed); ASL in AChR membranes (L/P 155/1, 59% perturbed); ASL in DOPC liposomes (represents 0% perturbed for ASL spectral subtractions).

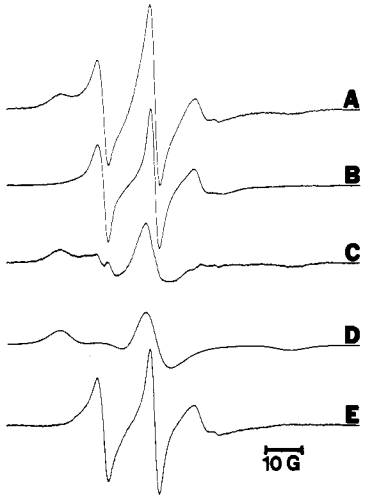

FIGURE 6: Component subtraction method. (A) EPR spectrum of 16-FASL in reconstituted AChR membranes at a lipid to protein ratio of 142/1. Spectrum recorded at 0 °C is representative of the usual two-component spectrum. (B) 16-FASL in DOPC liposomes at −5 °C serves as the mobile component. (C) The resultant perturbed component spectrum obtained by subtracting spectrum B from spectrum A. This spectrum represents 60% of the total spin-label concentration. (D) Model perturbed component spectrum of 16-FASL in ethyl ether–*n*-pentane–ethyl alcohol (1:1:3 v/v/v) at −122 °C. This solvent was chosen because it appears to closely approximate the polarity of the lipid–protein interface (see text). (E) The mobile component spectrum obtained when (D) is subtracted from (A). This spectrum represents 40% of the total spin-label concentration.

Table II: Comparison of Techniques Used To Quantitate Mobile and Protein-Perturbed Components

lipid/protein ratio (mol/mol)	subtraction method[a]	% mobile component
100	A	28
	B	30
	C	27
142	A	40
	B	41
	C	41
198	A	48
	C	48

[a] The following are the subtraction methods: (A) two-component spectrum minus simulated protein-perturbed component [simulation achieved using 16-FASL in ether–pentane–ethanol (1:1:3) at low temperature]; (B) two-component spectrum minus mobile-component spectrum obtained from protein-free liposomes; (C) pairwise subtraction [for details see Brotherus (1980)]. In all cases the membranes contained 16-FASL in reconstituted membranes with AChR and DOPC.

among the different quantitation methods and the similarity in line shape between (1) a protein-perturbed component obtained by subtraction of the mobile component from a two-component spectrum (Figure 6C) and (2) an appropriate organic solvent spectrum (Figure 6D) suggest that the organic solvent mixture at an appropriate temperature is a good model for the protein-perturbed environment. Organic solvent spectra and procedure b above were used to do all further quantitation of components.

Although the three different subtraction techniques yielded similar results for some two-component spectra, each technique could not be used for all two-component spectra. Subtraction of mobile components from two-component spectra could not be done at temperatures >10 °C because the mobile component line shape became sharper as temperature increased, and it was extremely difficult to match. Also, if a mobile component was >65% of the total two-component spectral intensity and was subtracted, the resulting protein-perturbed component was quite noisy and difficult to quantitate. The same problems (mismatch of mobile components and poor quality of the protein-perturbed component obtained by subtraction of the mobile component when this component was >65% of the two-component spectrum) were encountered when the pairwise subtraction technique was used. These problems were mainly technical and do not indicate fundamental inconsistencies among the subtraction techniques.

Effect of Lipid to Protein Ratio on EPR Spectra. Spin-labeled lipids were incorporated into reconstituted AChR membrane preparations having lipid to protein ratios between 100 and 400 to 1 (mol/mol). The protein-perturbed and mobile components of spectra recorded at 0 °C were quantitated and the percent mobile component/percent protein-perturbed component for each spectrum was calculated and plotted vs. the lipid to protein ratio (mol/mol) (Figure 7). The data for each spin-label were fit to the following equation derived by Brotherus et al. (1981):

$$y = x/(NK_{av}) - 1/K_{av} \quad (1)$$

where y = % mobile component/% protein-perturbed component, x = [membrane lipid]/[AChR] (mol/mol), N = number of spin-labeled molecules strongly perturbed per AChR molecule, and K_{av} is the equilibrium constant for the following equilibrium:

$$PL + L^* \rightleftharpoons PL^* + L$$

where PL is DOPC immediately adjacent to protein, L* is

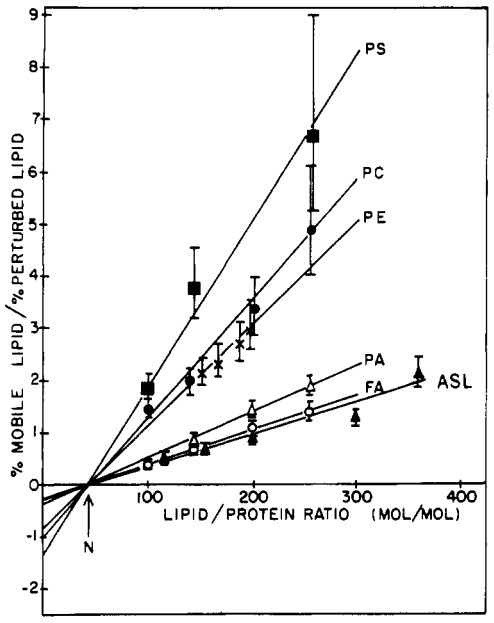

FIGURE 7: Plot of % mobile lipid/% perturbed lipid as a function of the lipid to protein ratio (mol/mol) for different spin-labels in AChR–DOPC-reconstituted membranes. The x intercept is the number of perturbed lipids, and the y intercept is $-1/K_{av}$, where K_{av} is the average affinity of a lipid for the perturbed environment (see text). The lipids corresponding to the spin-label probes are indicated on the figure [PS = phosphatidylserine; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PA = phosphatidic acid; FA = free fatty acid (16-FASL); ASL = androstane spin-label].

Table III: Affinity of Lipid for AChR[a]

spin-labeled lipid	N	K_{av}
PSSL	43	0.7
PCSL	46	1.0
PESL	45	1.1
PASL	44	2.7
FASL	38	4.1
ASL	39	4.3

[a] Data are obtained by linear regression analysis of the plots shown in Figure 7. N is the x intercept and represents the limiting number of lipids associated with the receptor. K_{av} is the average affinity of the spin-labeled lipids for the receptor as compared with DOPC as indicated in the equilibrium P-L + L* $\rightleftharpoons$ P-L* + L where P-L represents DOPC immediately adjacent to AChR, L* is spin-labeled lipid not adjacent to protein, P-L* is labeled lipid immediately adjacent to protein, and L is DOPC not adjacent to protein. The linear relationship between the percent mobile/percent perturbed component and the lipid-protein ratio was derived by Brotherus et al. (1981).

spin-labeled lipid not adjacent to protein, PL* is labeled lipid immediately adjacent to protein, and L is DOPC not adjacent to protein. All molecules referred to are intramembranous. Table III shows the values of N and K_{av} obtained by linear regression analysis. Data for PSSL were treated in two ways: (1) N and K_{av} were determined as described above, and (2) when the linear regression equation was computed, several points with the coordinates ($x = 43$, $y = 0$) were included in the data set; this forced the x intercept to be 43, the average value of N for the other lipids. The correlation coefficient for all lines was 0.98 or greater. The data points for PCSL were also fit to the following equation derived by Hoffman et al. (1981):

$$y^* = x^{*N^*} \quad (2)$$

where y^* = the fraction of the two-component spectrum that is the mobile component, x^* = the fraction of molecules

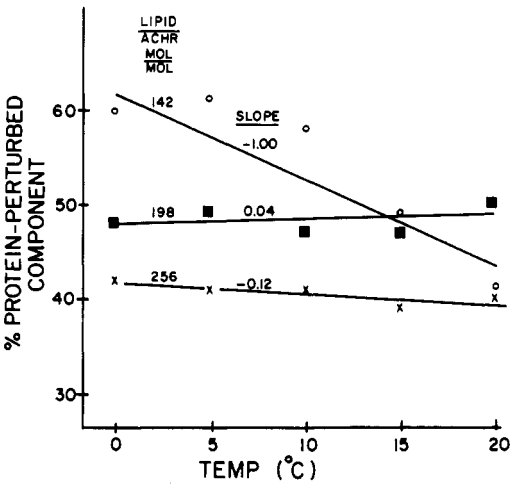

FIGURE 8: Temperature dependence of the protein-perturbed spectral component. The temperature dependence of the amount of perturbed component is shown for 16-FASL in DOPC–AChR membranes at three different lipid to protein mole ratios (142/1, 198/1, and 256/1). Only reconstituted membranes with a lipid to protein ratio less than 150 to 1 showed a significant temperature dependence.

present in the bilayer that are lipid, and N^* is taken to represent the maximum number of lipids that can be associated with the protein. From the PCSL spectra, a value of $N^* = 55$ was obtained. The number of bilayer phospholipids required to surround the intramembrane perimeter of the AChR was calculated to be 43 based on the AChR dimensions obtained by Kistler et al. (1982).[2]

Temperature Dependence. The temperature dependence of the percent protein-perturbed component for 16-FASL in reconstituted AChR membranes at three different lipid to protein ratios is shown in Figure 8. The percent of protein-perturbed component generally decreased as temperature increased for all the different spin-labels, and the trend was the same for all labels. The absolute amount of perturbed component was different for each spin-label as expected from the previous results. The decrease was small in membranes with lipid to protein ratios of 198 and 298 (mol/mol) but was significantly greater in membranes with lipid to protein ratios of 100 and 142. In the case of the ASL-containing membranes, the outer splitting of the two-component spectrum was about 2 G higher at each temperature compared to the other lipid spin-labels. In all cases, the temperature-dependent changes in the relative amounts of the two-spectral components were reversible.

Discussion

Motional and Exchange Characteristics of Highly Perturbed and Relatively Unperturbed Lipid Spin-Labels in Membranes Containing AChR. The protein in native AChR membranes from *Torpedo californica* and in reconstituted AChR membranes significantly perturbs the motion of lipid

[2] The intramembranous portion of the AChR is approximately cylindrical and its radius is 29 Å (Kistler et al., 1982). The membranous AChR is surrounded by phospholipids which are also approximately cylindrical with a radius of 4.9 ± 0.3 Å (Lis et al., 1982). The arrangement of the phospholipids and the AChR in the membrane is assumed to be such that the axes through the top and bottom of the phospholipid and AChR cylinders are parallel. Therefore, the perimeter defined by a shell of phospholipids that surrounds the AChR and is one lipid thick is 2π (29 ± 4.9 Å) = 213 Å. This perimeter is calculated such that it passes through the center of each lipid. The membrane bilayer is composed of two phospholipid leaflets; therefore, the number of phospholipids that would exist in the first shell of phospholipid surrounding each AChR is [213 Å/(2 × 4.9 Å)] × 2 = 43.

spin-labels. The perturbations can be monitored by EPR spectroscopy, and several characteristics of the perturbations (such as lipid exchange rates, lipid molecular motions, and relative lipid binding affinities for protein) can be deduced from the EPR spectra as indicated under Results. A common feature of all the spectra in AChR-containing membranes was the presence of a two-component spectrum. One component was attributed to lipid spin-labels in a normal lipid bilayer environment and the other component to protein-perturbed spin-labels in a more motionally restricted environment. The resolution of two distinct components places an upper limit of 5×10^7 s^{-1} on the exchange rate of lipids between the two environments, based on measurements of hyperfine spectral splittings for 16-FASL in native AChR membranes at 0 °C.

When two different methods were used, it was shown that the rotational correlation times for 16-FASL and for the phospholipid spin-labels (containing 16-FASL in the 2-position) were about an order of magnitude slower in the protein-perturbed environment (16 ns) than in the lipid bilayer environment (1 ns). There was very little difference among the spin-labeled phospholipids in the values for the correlation times. In the case of the androstane spin-label (a cholesterol analogue), the perturbed component gave spectral splittings close to the rigid limit, and it was not possible to make direct comparisons with the other spin-labels.

The independence of the exchange rate upper limit and the motional properties of protein-perturbed components on spin-label structure suggests that the different spin-labels were interacting with the protein in a similar way. Similar observations on the effects of membrane proteins on spin-label motion and exchange have been made in several other studies (Jost & Griffith, 1980; Marsh et al., 1982). A complete description of the motional processes that give rise to the observed correlation times is not possible because the current understanding of acyl chain motion in membranes is incomplete (Brown, 1979). The results of this and other spin-label studies of lipid–protein interactions are consistent with the hypothesis that almost all interactions between lipids and proteins are short-lived. Indeed, EPR and NMR studies indicate that the exchange rate of lipid between protein-perturbed and mobile environments is that expected for simple diffusion on and off the protein surface (Paddy et al., 1981; Marsh et al., 1982).

Methods Used To Quantitate Relative Amounts of Protein-Perturbed and Mobile Components in Spectra. One feature of the EPR of spin-labels in native and reconstituted AChR membranes is the clear dependence of the relative amounts of protein-perturbed and mobile components on spin-label structure (Figures 1 and 5). Although different spin-labeled lipids interacted with membrane proteins in a similar way (see above), the lipids had different affinities for the protein-perturbed environment and/or different numbers of interaction sites per protein. The three methods used here to quantitate the relative amounts of perturbed and mobile component gave self-consistent results. However, as outlined under Results, the best method for a given spectrum depended upon the actual amounts of the two components. In all cases an adequate fit to the experimental data was achieved by using two spectra, although the fits could probably be improved by using additional spectral components to fit the data. We chose to limit the analysis to two components since a molecular description of additional components would rely entirely on speculation at this stage.

The conditions necessary to simulate both the mobile and the protein-perturbed component provide some insight into the molecular environment of probes in each location. For example, it was necessary to use spectra of pure liposomes recorded at a temperature of 5 °C lower than the AChR membrane spectra to achieve a close fit. This observation suggests that the protein slowed down and/or ordered the motion of some or all of the mobile lipids in the membranes to a small extent. Also, the solvent used to mimic the perturbed component indicates that the polarity of the protein-perturbed environment was greater than the polarity near the center of the bulk bilayer. This is not unexpected since detailed structural studies of membrane proteins (Engleman et al., 1980; Ross et al., 1982) have shown that some polar side chains are found in membrane-embedded regions of proteins. In addition, protein backbones are more polar than acyl chains.

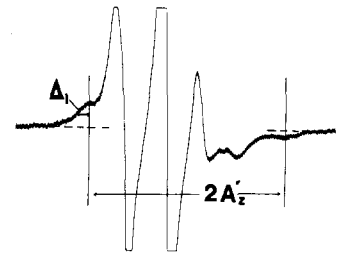

FIGURE 9: Spectrum of 16-FASL in native *Torpedo* AChR membranes at 0 °C. The outer splitting ($2A_z$) is 61.6 G, and the low-field half-line width (Δ_1) is 4.38 G.

Reconstituted Membranes: Rationale for Use and Characterization. As noted above, the differences in the relative amounts of protein-perturbed and mobile components in native and reconstituted membranes can be quantitated. In the native membranes, however, analysis of the data in terms of specific lipid–protein interactions was not feasible because of the complexity of the system. SDS–polyacrylamide gel electrophoresis has shown that a substantial amount of the protein in native membranes is not AChR. In addition, the lipid composition of these membranes is heterogeneous (Schiebler & Hucho, 1978; Gonzalez-Ros et al., 1982). EPR data can be interpreted in a much less ambiguous way by using membranes composed of a single lipid, a single protein, and a spin-labeled lipid. The reconstituted membranes used here fulfill these requirements. As shown under Results, the purification and lipid exchange methods used here resulted in membranes containing pure AChR and over 95% DOPC as the lipid. The residual lipids were carefully analyzed by thin-layer chromatography and revealed the presence of extremely small amounts of cholate and an unknown lipid (lipid X). Lipid X behaved in all solvent systems as a nonpolar lipid but was not cholesterol, free fatty acids, or other common lipids. The amount of lipid X was always proportional to the amount of AChR in the sample, and efforts are continuing to make a definitive identification.

The AChR in the reconstituted membranes was functional as judged by toxin binding studies and by ion flux studies on membranes prepared after addition of a large excess of lipid to the isolated membranes.

Lipid–Protein Interactions and Membrane Molecular Organization. Using reconstituted membranes, it was possible to measure the dependence of the mobile/perturbed ratio as a function of lipid to protein ratio as illustrated in Figure 7. Analysis of the data for each spin-labeled lipid in terms of eq 1 yielded the number of spin-labeled lipid–AChR interaction sites per AChR molecule (N) and the average equilibrium constant (K_{av}) for the spin-labeled lipid–AChR interactions. Values of N determined for all spin-labels were similar (Figure 9) with an average N for these spin-labeled lipids of 43 ± 4.

The similarity of the N values indicates that there are, within error, the same number of sites per AChR for all lipid spin-labels. The average value of N (43) is identical with the number of phospholipids (43) that will fit around the intramembranous perimeter of the AChR.[2] Thus, the environment that gives rise to the protein-perturbed EPR spectral component seems to be the lipid layer immediately surrounding the AChR. The AChR appears to have a relatively large effect on those lipids immediately adjacent to it and a smaller effect on lipids not contacting it. Furthermore, the number of phospholipids (65 ± 8) that copurify with the AChR is similar to the number that will fit around its intramembranous perimeter. This observation is consistent with the idea that when the AChR is purified in the presence of phospholipid, a phospholipid "coat" may be maintained around the intramembranous perimeter of the AChR.

N^*, the maximum number of membrane interaction sites per AChR, was determined to be 55. Only PCSL data could be used to make this determination because eq 2 was derived under the assumption that interactions between intramembranous molecules are random and of equal strength. N^* is the number of lipids that will be relatively highly perturbed by an infinitely dilute protein (i.e., the lipid to protein ratio = ∞). N is the number of lipids that will be relatively highly perturbed in membranes with lipid to protein ratios of 100–400 mol/mol. Therefore, if the AChR–AChR interaction had occurred due to random, reversible contact, the N value for PCSL would have been lower than the N^* value. N was in fact slightly lower than N^* although the values agreed within experimental error. This suggests that, at most, a rather small fraction of the intramembranous AChR perimeter is involved in AChR–AChR interactions in reconstituted membranes with lipid to AChR ratios of 100–400 mol/mol at 0 °C.

From the N and K_{av} values the difference in the relative amounts of protein-perturbed and mobile components seen for the different spin-labeled lipids in the same membranes (Figures 2 and 7) can be attributed to a difference in the average *affinity* of the AChR for the different lipids. A possible minor exception to this is PSSL. Data for PSSL were treated in two ways because there was a rather large standard deviation for the data points. When the least-squares lines were drawn through the data points, values of N = 31 and K_{av} = 1.2 were obtained, indicating that there are fewer sites on the AChR for PSSL than for PCSL (or DOPC) and that the affinity of the AChR for PSSL is a bit higher than for PCSL (or DOPC). If N is forced to be 43, the average of the N's for the other lipids, then K_{av} = 0.72, indicating that the affinity of AChR for PSSL is slightly lower than for PCSL (or DOPC). The fact that the N's calculated for all other spin-labeled lipids are very similar would seem to support the latter treatment of the data. It is unlikely that PSSL would be selectively excluded from some of the sites that could be occupied by the other spin-labeled lipids. In any case, the K_{av} values for PSSL and PCSL are quite similar.

The K_{av} value for PCSL is 1.0, and therefore, PCSL and DOPC have equal affinities for the AChR. Thus, the difference in acyl chain composition of the two PC's does not affect their relative affinities for the AChR, and the presence of the spin-label group on PCSL does not make a significant contribution to the interaction between PCSL and the AChR.

ASL (K_{av} = 4.4), 16-FASL (K_{av} = 4.1), and PASL (K_{av} = 2.7) have significantly higher affinities for the AChR than PESL, PCSL, and PSSL. In the treatment used here, only an *average* affinity can be calculated. It is likely that there exists a spectrum of binding sites with a range of relative binding affinities. There are several general structural properties that may account for the binding differences among the spin-labels as outlined below.

The pK_a of 16-FASL in a membrane is approximately 7.0 (Egret-Charlier et al., 1978; Ptak et al., 1980), and thus both the negative and neutral species exist at the pH of our experiments (7.4). Preliminary experiments (J. F. Ellena, J. Kelly, F. Hagen, and M. G. McNamee, unpublished observations) suggest that the affinity of the AChR for 16-FASL increases when the experimental pH is raised and decreases when the pH is lowered, indicating that the negatively charged species interacts more strongly with the AChR. PASL has a net charge of ~1.5 at pH 7.4. Although head-group charge seems to be important in determining the K_{av} for 16-FASL, no direct relationship between net head-group charge and K_{av} exists when all the spin-labeled lipids are considered. The order of spin-labeled lipid affinity for the AChR is ASL ~ 16-FASL > PASL > PCSL ~ PESL ~ PSSL. The spin-labeled lipid order based only on negative charge at pH 7.4 would be PASL > PSSL > 16-FASL > PESL ~ PCSL ~ ASL.

Another important factor in determining the observed affinity of spin-labeled lipid for the membranous AChR may be dynamic molecular shape. Shapes of lipids can be defined by comparing the diameter occupied by the head-group area of the rodlike lipid molecules in a membrane with the diameter occupied by the opposite end, and a correlation between lipid shape and phase behavior has been observed (Cullis & De-Kruijff, 1979; Israelachvili et al., 1980). It has been suggested that lipids of different shape may be required at the structurally rough or irregular lipid–protein interface in order to form an adequate transmembrane seal (Madden et al., 1983).

Membrane lipid–lipid interactions may also have a significant effect on the experimentally observed affinity of a membrane protein for a certain lipid. The existence of a hydrogen bonded ion pair of the type $-PO_3^-\cdots H-{}^+NH_2-$ between lipid head groups in membranes has received considerable experimental support (Browning, 1981, and references therein). Thus, a competition between electrostatic lipid–lipid and lipid–protein bonding may exist. Also, it has been shown recently that phospholipid head-group conformation can depend on membrane phospholipid composition (Sixl & Watts, 1983). One might speculate that lipid–protein affinity will be dependent on head-group conformation.

No simple physical explanation of the observed affinity difference among lipids exists, and the above discussion indicates that several factors may be involved. At this point, it seems most appropriate to interpret the data in terms of the existence of relatively specific sites on the AChR for certain classes of molecules, and it is interesting to note that those lipids that have the highest affinity for the AChR also seem to be capable of modulating AChR function (see below).

Temperature Dependence of the Percent Perturbed Component. In general, the amount of the perturbed component decreased as the temperature increased. The temperature-dependent decrease was very small for membranes with a lipid/protein ratio greater than 200/1 and was quite pronounced at lipid/protein ratios less than 150/1. Davoust et al. (1979) observed a qualitatively similar temperature dependence in reconstituted membranes containing rhodopsin. Recently, Davoust & Devaux (1982) showed that this temperature dependence was most likely due to partial coalescence of the protein-perturbed and mobile components. As the temperature is increased, the exchange rate between the two environments increases and reaches a value comparable to the inverse of the hyperfine splitting differences of the two com-

ponents. Since our spectral analysis assumes it is possible to achieve a complete separation of the two components, the exchange coalescence will appear to decrease the contribution of the subtracted component to the total spectrum. The stronger temperature dependence at low lipid to protein ratios can be explained since individual lipid exchange events at low lipid to protein ratios are more likely to represent exchanges on and off the protein surface that would lead to spectral coalescence.

The temperature-dependent decreases in the percent perturbed component could also be due to aggregation of receptors with a corresponding loss of lipid–protein contact sites. This explanation is deemed unlikely since nonspecific aggregation is more likely to occur at 0 °C than at 20 °C (Chen & Hubbell, 1973; Kleeman & McConnell, 1976). However, a temperature-induced increase in AChR–AChR affinity cannot be ruled out. Dense patches of AChR are known to exist in the postsynaptic terminals of vertebrate neuromuscular junctions, and one factor that stabilizes these patches may be noncovalent interactions among AChR molecules. In all cases, the temperature-dependent EPR effects were reversible, and the AChR remained functional, thus ruling out temperature-induced denaturation.

Comparison of Present Results with Previous EPR Studies of Lipid–Protein Interactions and Functional Implications. The demonstration that 16-FASL has a higher affinity for AChR than PCSL in both native and reconstituted AChR membranes resolves apparently conflicting results that were previously reported for spin-labeled lipids in AChR membranes. Rousselet et al. (1979) did not observe a perturbed component with PCSL in native membranes whereas Marsh et al. (1981) did. In our studies we had difficulty visually identifying a perturbed component in native membranes, but we could attribute about 15–20% of the spectral intensity to the perturbed component. It is likely that the particular preparation used by Marsh et al. (1981) had a lower lipid to protein ratio than that used by us or by Rousselet et al. (1979). The discrepancies point out the problems in using native membranes to draw quantitative conclusions.

On the basis of analysis of spectra in native membranes, Marsh et al. (1981) suggested that the number of highly perturbed lipids may be significantly greater than the number necessary to surround the AChR with one layer of lipid. However, their analysis was based on the assumption that the FASL and the androstane spin-label had a K_{av} of 1.0. As demonstrated here, FASL and ASL have relatively high affinities for AChR, and the analysis used in this study is necessary to sort out effects due to affinity and to the number of perturbed sites. All of our lipids had about the same number of sites (43) corresponding to one layer of lipid. Our results are in agreement with studies of other membrane proteins for which a single perturbed lipid layer can be detected by EPR techniques (Jost & Griffith, 1982).

The relatively high affinities detected for the fatty acid and the cholesterol-like spin-labels have interesting functional implications. Unsaturated fatty acids have been shown to act as inhibitors of AChR-mediated ion flux (Andreason & McNamee, 1980), and the lipid–protein interface was suggested as the probable site of action. The preferential binding of fatty acids to the AChR lends support to the suggestion. In contrast, cholesterol has been shown to be an important lipid component in maintaining the AChR in an active form in reconstituted membranes (Ochoa et al., 1983). If cholesterol interacts with AChR to the same extent as the androstane spin-label, then the preferential binding of cholesterol may play a role in stabilizing the active conformational state of the AChR.

On the basis of fluorescence quenching data, Simmonds et al. (1982) concluded that fatty acids and cholesterol bind to sites on the (CA^{2+}–Mg^{2+})-ATPase that are unavailable to phospholipids. This may also be true for the AChR. In this study, only average K values were measured, and there may be a small number of quite specific sites for 16-FASL and androstane spin-label that exclude phospholipid. However, the fact that all the N values are similar suggests that none of the spin-labels are selectively excluded from most of the sites at the lipid–AChR interface. A high affinity of cholesterol or cholesterol-like molecules has not been found for other membrane proteins. It will be interesting to determine to what extent the high-affinity binding of cholesterol is correlated with the role of cholesterol in stabilizing AChR function.

Future Work. The present studies have provided new information on lipid–protein interactions in membranes containing AChR. All of the reconstituted membranes have contained only DOPC as the host lipid. The effects of lipid mixtures, particularly those containing cholesterol, on both the spectral properties of incorporated spin-labels and on the functional properties of the membranes will be analyzed in future work. In addition, the effects of local anesthetics on the lipid–protein interactions will be analyzed. Since the anesthetics may be acting at the lipid–protein interface, they may have dramatic effects on the relative affinities of the AChR for specific lipids. In preliminary experiments using a spin-labeled anesthetic, a large protein-perturbed component was observed in DOPC–AChR membranes (Earnest et al., 1983). The ability to measure both functional and structural properties of the AChR continue to make the AChR an ideal membrane protein for a complete analysis of lipid–protein interactions.

Appendix

Empirical methods for the calculation of spin-label rotational correlation times (t_c) in the range $7 \times 10^{-9} < t_c < 10^{-7}$ have been developed by Freed and co-workers (Freed, 1976). Two methods were used to calculate the rotational correlation time of the protein-perturbed component in the 16-FASL–AChRM spectrum recorded at 0 °C (Figure 3A).

(1) Initially, the following equation was used:

$$t_c = a(1 - A_z'/A_z)^b$$

where $a = 5.4 \times 10^{-10}$ s, $b = -1.36$, $A_z' = 30.8$ G, $A_z = 33.6$ G, and thus $t_c = 16$ ns.

a and b are empirical parameters that depend on the type of diffusion that the spin-label undergoes and the spectral line width. All the spin-labels used in this study are expected to reorient by Brownian diffusion (Schreier et al., 1978). The appropriate line-width parameter for the protein-perturbed component was 3.0 G (Hubbell & McConnell, 1971; Marsh & Barrantes, 1978). Appropriate values of a and b were obtained from Goldman et al. (1972). The value of A_z' was obtained by measuring the outer splitting ($2A_z'$) of the 16-FASL–AChRM spectrum and dividing by 2. A_z is half the outer splitting expected for a 16-FASL spectrum obtained under conditions that completely immobilize the spin-label (Gaffney, 1976). Results obtained in this study and that of Griffith et al. (1974) indicate that no polarity correction is required for A_z.

(2) The other equation used was

$$t_c = a'(\Delta_1/\Delta_1^r - 1)^{b'}$$

where $a' = 1.15 \times 10^{-8}$ s, $b' = -0.943$, $\Delta_1 = 4.38$ G, $\Delta_1^r = 2.5$ G, and therefore $t_c = 15$ ns. a' and b' are empirical

parameters that depend on type of diffusion and spectral line width. Again, Brownian diffusion and a line-width parameter of 3.0 G are appropriate. Δ_1 is the low-field half-line width of the 16-FASL–AChRM spectrum (Figure 9). Δ_1' is the low-field half-line width of the spectrum of immobilized 16-FASL, and the value of 2.5 G is based on the work of Hubbell & McConnell (1971). The protein-perturbed spectral component was nearly independent of membrane preparation, temperature, and spin-label structure; therefore, rotational correlation times for all protein-perturbed components were quite similar. The above equations are not applicable to spectra of spin-labels undergoing highly anisotropic motion (Freed, 1976). If the motion of the protein-perturbed spin-labels was highly anisotropic, the two methods for determining t_c would probably not give very similar results.

Registry No. DOPC, 10015-85-7; 16-FASL, 53034-38-1; ASL, 25521-33-9; PASL, 87373-13-5; PCSL, 63321-67-5; PSSL, 87373-14-6; PESL, 87373-15-7.

References

Ackman, R. G. (1981) *Methods Enzymol. 72*, 205–252.

Ames, G. F. L. (1974) *J. Biol. Chem. 249*, 634–644.

Andreasen, T. J., & McNamee, M. G. (1980) *Biochemistry 19*, 4719–4726.

Andreasen, T. J., Doerge, D. R., & McNamee, M. G. (1979) *Arch. Biochem. Biophys. 194*, 468–480.

Anholt, R., Lindstrom, J., & Montal, M. (1981) *J. Biol. Chem. 256*, 4377–4387.

Bergelson, L. D. (1980) in *Lipid Biochemical Preparations* (Bergelson, L. D., Ed.) pp 151–153, Elsevier/North-Holland, New York.

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.

Boss, W. F., Kelley, C. J., & Landsberger, F. R. (1975) *Anal. Biochem. 64*, 289–292.

Brotherus, J. R., Jost, P. C., Griffith, O. H., Keana, J. F. W., & Hokin, L. E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 272–276.

Brotherus, J. R., Griffity, O. H., Brotherus, M. O., Jost, P. C., Silvius, J. R., & Hokin, L. E. (1981) *Biochemistry 20*, 5261–5267.

Brown, M. F. (1979) *J. Magn. Reson. 29*, 203–215.

Brown, M. F., Deese, A. J., & Dratz, E. A. (1982) *Methods Enzymol. 81*, 709–728.

Browning, J. L. (1981) *Biochemistry 20*, 7144–7151.

Changeux, J.-P. (1981) *Harvey Lect. 75*, 85–224.

Chen, Y. S., & Hubbell, W. L. (1973) *Exp. Eye Res. 17*, 517–525.

Comfurius, P., & Zwaal, R. F. A. (1977) *Biochim. Biophys. Acta 488*, 36–42.

Criado, M., Eibl, H., & Barrantes, F. J. (1982) *Biochemistry 21*, 3622–3629.

Cullis, P. R., & DeKruijff, B. (1979) *Biochim. Biophys. Acta 559*, 399–420.

Dalziel, A. W., Rollins, E. A., & McNamee, M. G. (1980) *FEBS Lett. 122*, 193–196.

Damle, V. N., & Karlin, A. (1978) *Biochemistry 17*, 2039–2045.

Damle, V. N., McLaughlin, M., & Karlin, A. (1978) *Biochem. Biophys. Res. Commun. 84*, 845–851.

Davoust, J., & Devaux, P. F. (1982) *J. Magn. Reson. 48*, 475–494.

Davoust, J., Schoot, B., & Devaux, P. F. (1979) *Proc. Natl. Acad. Sci. U.S.A. 14*, 2755–2759.

Egret-Charlier, M., Swanson, A., & Ptak, M. (1978) *FEBS Lett. 89*, 313–316.

Engelman, D. M., Henderson, R., McLachlan, A. D., & Wallace, B. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2023–2027.

Earnest, J. P., Ellena, J. F., James, L., Blazing, M. A., McNamee, M. G., & Wang, H. H. (1983) *Biophys. J. 41*, 364a.

Epstein, M., & Racker, E. (1978) *J. Biol. Chem. 253*, 6660–6662.

Fleischer, S., Wang, C.-T., Hymel, L., Seelig, J., Brown, M. F., Herbette, L., Scarpa, A., McLaughlin, A. C., & Blasie, J. K. (1979) in *Function and Molecular Aspects of Biomembrane Transport* (Quagliariello, E., et al., Eds.) pp 465–485, Elsevier/North-Holland, New York.

Freed, J. (1976) in *Spin Labeling—Theory and Applications* (Berliner, L. J., Ed.) pp 53–132, Academic Press, New York.

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

Goldman, S. A., Bruno, G. V., Polnaszek, C. F., & Freed, J. H. (1972) *J. Chem. Phys. 56*, 716–735.

Gonzalez-Ros, J. M., Llanillo, M., Paraschos, A., & Martinez-Carrion, M. (1982) *Biochemistry 21*, 3467–3474.

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

Griffith, O. H., Dehlinger, P. J., & Van, S. P. (1974) *J. Membr. Biol. 15*, 159–192.

Griffith, O. H., Baumeister, J. C., & Jost, P. C. (1982) *Biophys. J. 37*, 152–154.

Hoffman, W., Pink, D. A., Restall, C., & Chapman, D. (1981) *Eur. J. Biochem. 114*, 585–589.

Hubbell, W. L., & McConnell, H. M. (1971) *J. Am. Chem. Soc. 93*, 314–326.

Huganir, R. L., Schell, M. A., & Racker, E. (1979) *FEBS Lett. 108*, 155–160.

Israelachvili, J. N., Marcelja, S., & Horn, R. G. (1980) *Q. Rev. Biophys. 13*, 121–200.

Jost, P. C., & Griffith, O. H. (1978) *Methods Enzymol. 49*, 369–418.

Jost, P. C., & Griffith, O. H. (1980) *Ann. N.Y. Acad. Sci. 348*, 391–407.

Jost, P. C., & Griffith, O. H., Eds. (1982) *Lipid-Protein Interactions*, Wiley-Interscience, New York.

Karlin, A. (1980) in *The Cell Surface and Neuronal Function* (Poste, G., Nicolson, G. L., & Cotman, C. W., Eds.) pp 191–260, Elsevier/North-Holland, New York.

Karlin, A., McNamee, M. G., Weill, C. L., & Valderrama, R. (1976) in *Methods in Receptor Research* (Blecher, M., Ed.) Part I, pp 1–32, Marcel Dekker, New York.

Kilian, P. L., Dunlap, C. R., Mueller, P., Schell, M. A., Huganir, R. L., & Racker, E. (1980) *Biochem. Biophys. Res. Commun. 93*, 409–414.

Kinsey, R. A., Kintanar, A., & Oldfield, E. (1981) *J. Biol. Chem. 256*, 9028–9036.

Kistler, J., Stroud, R. M., Klymkowsky, M. W., LaLancette, R. A., & Fairclough, R. H. (1982) *Biophys. J. 37*, 371–383.

Kleeman, W., & McConnell, H. M. (1976) *Biochim. Biophys. Acta 419*, 206–222.

Lis, L. J., McAlister, M., Fuller, N., Rand, R. P., & Parsegian, V. A. (1982) *Biophys. J. 37*, 667–672.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Luzzati, V., & Tardieu, A. (1974) *Annu. Rev. Phys. Chem. 25*, 79–94.

Madden, T. D., Hope, M. J., & Cullis, P. R. (1983) *Biochemistry 22*, 1970–1974.
Marsh, D., & Barrantes, F. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4329–4333.
Marsh, D., Watts, A., & Barrantes, F. J. (1981) *Biochim. Biophys. Acta 645*, 97–101.
Marsh, D., Watts, A., Pates, R. D., Uhl, R., Knowles, P. F., & Essmann, M. (1982) *Biophys. J. 37*, 265–274.
McClare, C. W. F. (1971) *Anal. Biochem. 39*, 527–530.
McConnell, H. M. (1976) in *Spin Labeling—Theory and Applications* (Berliner, L. J., Ed.) pp 525–560, Academic Press, New York.
McIntyre, J. O., Samson, P., Brenner, S. C., Dalton, L., Dalton, L., & Fleischer, S. (1982) *Biophys. J. 37*, 53–56.
McLaughlin, A. C., Hrebette, L., Blasie, J. K., Wang, C. T., Hymel, L., & Fleischer, S. (1981) *Biochim. Biophys. Acta 643*, 1–16.
McNamee, M. G., & Ochoa, E. L. M. (1982) *Neuroscience 7*, 2305–2319.
McNamee, M. G., Ellena, J. F., & Dalziel, A. W. (1982) *Biophys. J. 37*, 103–104.
Molotkovsky, J. G., & Bergelson, L. D. (1980) in *Lipid Biochemical Preparations* (Bergelson, L. D., Ed.) p 139, Elsevier/North-Holland, New York.
Neubig, R. R., Krodel, E. K., Boyd, N. D., & Cohen, J. B. (1979) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3057–3061.
Ochoa, E. L. M., Dalziel, A. W., & McNamee, M. G. (1983) *Biochim. Biophys. Acta 727*, 151–162.
Paddy, M. R., Dahlquist, F. W., Davis, J. H., & Bloom, M. (1981) *Biochemistry 20*, 3152–3162.
Parsegian, V. A., Ed. (1982) *Biophys. J. 37*, 1–383.
Pinto da Silva, P., & Kachar, B. (1982) *Cell (Cambridge, Mass.) 28*, 441–450.
Ptak, M., Egret-Charlier, M., Sanson, A., & Bouloussa, O. (1980) *Biochim. Biophys. Acta 600*, 387–397.
Raftery, M. A., Hunkapiller, M. W., Strader, C. D., & Raftery, M. A. (1980) *Science (Washington, D.C.) 208*, 1454–1457.
Rice, D. M., Meadows, M. P., Scheinman, A. O., Goni, F. M., Gomez-Fernandez, J. C., Moscarello, M. A., Chapman, D., & Oldfield, E. (1979) *Biochemistry 18*, 5893–5903.
Ross, A. H., Radhakrishnan, R., Robson, R. J., & Khorana, H. G. (1982) *J. Biol. Chem. 257*, 4152–4161.
Rothman, J. E., & Lenard, J. (1977) *Science (Washington, D.C.) 195*, 743–753.
Rousselet, A., Devaux, P. F., & Wirtz, K. W. (1979) *Biochem. Biophys. Res. Commun. 90*, 871–877.
Sandermann, H. (1978) *Biochim. Biophys. Acta 515*, 209–237.
Schiebler, W., & Hucho, F. (1978) *Eur. J. Biochem. 85*, 55–63.
Schreier, S., Polnaszek, C. F., & Smith, I. C. P. (1978) *Biochim. Biophys. Acta 515*, 375–436.
Seelig, J., Tammn, L., Hymel, L., & Fleischer, S. (1981) *Biochemistry 20*, 3922–3932.
Simmonds, A. C., East, J. M., Jones, O. T., Rooney, E. K., McWhirter, J., & Lee, A. G. (1982) *Biochim. Biophys. Acta 693*, 398–406.
Sixl, F., & Watts, A. (1982) *Biochemistry 21*, 6446–6452.
Thomas, D. D., Biegelow, D. J., Squier, T. C., & Hidalgo, C. (1982) *Biophys. J. 37*, 217–225.
Vik, S. B., & Capaldi, R. A. (1977) *Biochemistry 16*, 5755–5759.
Walker, J. W., Lukas, R. J., & McNamee, M. G. (1981) *Biochemistry 20*, 2191–2199.
Walker, J. W., Takeyasu, K., & McNamee, M. G. (1982) *Biochemistry 21*, 5384–5389.
Watts, A., Volotovski, I. D., & Marsh, D. (1979) *Biochemistry 18*, 5006–5013.
Watts, A., Volotovski, I. D., Pates, R., & Marsh, D. (1982) *Biophys. J. 37*, 94–95.