Pennington, R. M., & Fisher, R. R. (1981) *J. Biol. Chem. 256*, 8963-8969.
Phelps, D. C., & Hatefi, Y. (1981) *J. Biol. Chem. 256*, 8217-8221.
Phelps, D. C., & Hatefi, Y. (1984) *Biochemistry 23*, 6340-6344.
Pick, U., & Racker, E. (1979) *Biochemistry 18*, 108-113.
Sane, P. V., Johanningmeier, U., & Trebst, A. (1979) *FEBS Lett. 108*, 136-140.
Saraste, M., Pentilla, T., & Wikstrom, M. K. F. (1981) *Eur. J. Biochem. 115*, 261-268.
Schatz, G., & Racker, E. (1966) *J. Biol. Chem. 241*, 1429-1438.
Schneider, D. L. (1981) *J. Biol. Chem. 256*, 3858-3864.
Senior, A., & Wise, J. G. (1983) *J. Membr. Biol. 73*, 105-124.
Sigrist-Nelson, K., & Azzi, A. (1980) *J. Biol. Chem. 255*, 10638-10643.
Stone, D. K., Xiao-Song, X., & Racker, E. (1983) *J. Biol. Chem. 258*, 4059-4062.
Stouthamer, A. H. (1980) *Trends Biochem. Sci. (Pers. Ed.) 5*, 164-166.
Vitols, E., & Linnane, A. W. (1961) *J. Biophys. Biochem. Cytol. 9*, 701-710.
Yagi, T. (1986) *Arch. Biochem. Biophys. 250*, 302-311.
Yagi, T., Matsuno-Yagi, A., Vik, S. B., & Hatefi, Y. (1984) *Biochemistry 23*, 1029-1036.
Zhang, F., & Schneider, D. L. (1983) *Biochem. Biophys. Res. Commun. 114*, 620-625.

Charged Anesthetics Selectively Alter Plasma Membrane Order†

William D. Sweet,*,‡ W. Gibson Wood,§ and Friedhelm Schroeder‡,‖

Department of Pharmacology, School of Medicine, University of Missouri, Columbia, Missouri 65212, and Geriatric Research, Education and Clinical Center, Veterans Administration Medical Center and Department of Internal Medicine, St. Louis University School of Medicine, St. Louis, Missouri 63125

Received March 5, 1986; Revised Manuscript Received January 6, 1987

ABSTRACT: Although indirect evidence supporting differential lipid fluidity in the two monolayers of plasma membranes has accumulated, unambiguous demonstration of this difference has been difficult to obtain. In the present study, the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH), selective quenching of fluorescence by trinitrophenyl groups, and differential polarized phase fluorescence techniques were used to directly examine the static (order) and dynamic (rotational rate) components of lipid motion in the exofacial and cytofacial leaflets of LM fibroblast plasma membranes. The limiting anisotropy (0.137), the order parameter (0.590), and the rotational relaxation time (1.20 ns) of DPH in the plasma membranes (inner plus outer leaflet) indicated rapid but restricted probe motion in the lipid environment. However, the statics and dynamics of DPH motion in the individual monolayers were significantly ($p < 0.025$) different. The limiting anisotropy, order parameter, and rotational relaxation time of DPH in the cytofacial monolayer were 0.036, 0.08, and 0.16 ns, respectively, greater than calculated for the exofacial monolayer of the LM plasma membrane. At appropriate concentrations, phenobarbital and, to a lesser degree, pentobarbital preferentially reduced the limiting anisotropy of DPH calculated for the exofacial leaflet while prilocaine reduced the limiting anisotropy of DPH in the cytofacial leaflet of LM fibroblast plasma membranes. In contrast, the putative cytofacial anesthetic procaine failed to show any preference for either leaflet. Arrhenius plots of DPH fluorescence in LM plasma membranes showed a prominent characteristic break point near 30-32 °C. Phenobarbital, pentobarbital, and procaine did not affect this break point while prilocaine selectively abolished it. The break point was therefore assigned to the inner monolayer of the LM plasma membrane.

Over the past decade, it has been well established that the cytofacial and exofacial leaflets of the eukaryotic cell plasma membrane are dissimilar with respect to lipid, as well as protein, composition [reviewed in Schroeder (1984, 1985)]. This compositional transbilayer asymmetry is expected to confer asymmetry of structure between the monolayers, provided that they are not coupled. Indeed, no, or only weak, coupling of lipid motion across the bilayer has been experimentally verified (Hunt & Tipping, 1978; Johnson & Robinson, 1976; Sillerud & Barnett, 1982; Flamm & Schachter, 1982). The only reported exception is sphingomyelin containing long-chain fatty acids (24:0), which illustrated coupling between pure sphingomyelin monolayers (Schmidt et al., 1978). Results obtained with electron spin resonance (Morse et al., 1975; Tanaka & Ohnishi, 1976; Wisnieski & Iwata, 1977; Seigneuret et al., 1984), differential scanning calorimetry (Van Dijck et al., 1976), and fluorescence (Schroeder, 1978, 1980; Schroeder & Kinden, 1979; Cogan & Schachter, 1981; Schachter et al., 1982) were consistent with the interpretation that the motional properties of lipids in the inner and outer

† This work was supported in part by grants from the USPHS, National Institutes of Health Grants GM31651 and CA24339. A preliminary report of this work was presented as an abstract at the annual FASEB Meeting, Anaheim, CA, 1985 (Sweet & Schroeder, 1985).
* Correspondence should be addressed to this author. Present address: Department of Pharmacology, School of Medicine, Oral Roberts University, Tulsa, OK 74137-1297.
‡ University of Missouri.
§ St. Louis University School of Medicine.
‖ Present address: Department of Pharmacology and Medicinal Chemistry, College of Pharmacy, and Department of Pharmacology and Cell Biophysics, College of Medicine, University of Cincinnati Medical Center, Cincinnati, OH 45267-0004.

0006-2960/87/0426-2828$01.50/0

monolayers of plasma membranes differed. However, it is not clear whether these differences were due to static or dynamic parameters of lipid motion.

More than a decade ago, Sheetz and Singer (1974) proposed that the asymmetry in net charge at the surface of the two leaflets of biological membranes could establish an asymmetric transbilayer distribution of charged amphipaths (e.g., charged anesthetics) intercalating into the monolayers. Such a preferential modification, which would selectively fluidize one leaflet of the membrane, could be used to investigate the functional consequences of changes in the fluidity of individual membrane monolayers. The above concept has been tested only indirectly by comparing changes in the characteristic temperatures ("break points") of enzyme activities with known transbilayer orientation in the membrane (Houslay et al., 1980; Houslay & Palmer, 1978; Dipple et al., 1982) or by examining effects of amphipathic drugs on break points in Arrhenius plots of fluorescent or electron spin resonance probe molecule behavior (Salesse et al., 1982). However, break points in Arrhenius plots are not necessarily indicative of membrane lipid fluidity and at best represent an indirect measurement of lipid fluidity.

The objective of this study is 2-fold: (1) to examine both the static and dynamic aspects of 1,6-diphenyl-1,3,5-hexatriene (DPH)[1] motion in LM fibroblast plasma membranes in order to interpret the dynamics and statics of membrane components and (2) to interpret the results of the first objective in terms of the static and dynamic parameters of fluidity in each monolayer in the absence and presence of anionic anesthetic amphipaths (phenobarbital and pentobarbital) and cationic anesthetics (prilocaine and procaine).

Experimental Procedures

Chemicals. Prilocaine was the generous gift of Astra Pharmaceuticals (Södertälje, Sweden, and Worcester, MA). Pentobarbital, phenobarbital, procaine, and 5-doxylstearate were purchased from Sigma Chemical Co. (St. Louis, MO). 1,6-Diphenyl-1,3,5-hexatriene was obtained from Aldrich Chemical Co. (Milwaukee, WI). Other reagents were of the highest commercially available quality.

Cell Culture and Growth Conditions. LM cells, a transformed mouse fibroblast line obtained from the American Type Culture Collection (CCL 1.2), were cultured in suspension in a lipid-, protein-, and serum-free medium as described (Schroeder et al., 1976). Cells were routinely cultured in choline-containing media. Log-phase cells were sedimented before being resuspended in fresh medium at $(0.8–1.0) \times 10^6$ cells/mL. The cells were harvested 3 days later. The cultures were routinely tested for mycoplasmal, bacterial, and fungal contamination.

Trinitrophenylation and Isolation of Plasma Membranes. To determine fluorescence parameters of probe molecules in each membrane leaflet, techniques previously established in our laboratory were used (Schroeder, 1978, 1980; Schroeder & Kinden, 1979; Hale & Schroeder, 1982). Briefly, membranes were prepared either without fluorescence quenching agent or with quencher covalently linked to the outer leaflet of the plasma membranes as follows. Half of each culture was treated with trinitrobenzenesulfonic acid under nonpenetrating conditions (4 °C, 4 mM) as described earlier (Fontaine & Schroeder, 1979). Excess reagent was removed, and plasma membranes were isolated by a modification (Schroeder & Gardiner, 1984) of a published procedure (Schroeder et al., 1976). Microsomes and mitochondria were also routinely isolated in order to determine if any of the trinitrobenzenesulfonic acid penetrated the cell (Fontaine & Schroeder, 1979). (Na^+,K^+)-ATPase and 5′-nucleotidase were assayed as described earlier (Schroeder & Gardiner, 1984) to determine plasma membrane purity. The specific activities of (Na^+,K^+)-ATPase and 5′-nucleotidase were 8-fold and 6-fold, respectively, enriched in the plasma membrane fraction as compared to crude homogenate. No differences in fold purification between untreated vs. trinitrophenylated plasma membranes were noted. Membrane protein concentration was determined by the procedure of Lowry et al. (1951) using bovine serum albumin as standard.

Fluorescence Spectroscopy and Differential Polarized Phase Fluorescence. Absorbance, absorption-corrected fluorescence, relative fluorescence efficiency, and corrected fluorescence emission of DPH in LM plasma membranes were determined concurrently with a computer-centered spectrofluorometer (Holland et al., 1973) as described (Schroeder, 1981a; Hale & Schroeder, 1982; Schroeder et al., 1984). Descending Arrhenius plots were obtained as detailed elsewhere (Schroeder et al., 1984). All fluorescence measurements, except for Arrhenius plots, were made at 37 °C. Fluorescence lifetime, τ, and steady-state fluorescence polarization, P, were obtained with a T-format SLM4800 subnanosecond spectrofluorometer (SLM Instruments Inc., Champaign-Urbana, IL) essentially as described (Hale & Schroeder, 1982). Fluorescence lifetimes were simultaneously measured relative to a reference solution of 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene in absolute ethanol (Lakowicz et al., 1981). The data were fitted to multiple-lifetime analysis by the method of Weber (1981). The fluorescent probe DPH was incorporated into plasma membranes at a concentration of 0.1 μg/100 μg of membrane protein as described (Schroeder et al., 1984). The excitation wavelength for DPH in LM plasma membranes was 362 nm, and fluorescence emission was monitored at 424 nm.

Differential polarized phase fluorometry, as described by Weber (1978) and extended by Lakowicz et al. (1979), was used to obtain the rotational rate (R in radians per second) and the limiting anisotropy (r_∞) of membrane-bound DPH. The limiting anisotropy reflects restriction to probe motion and can be converted to an order parameter, $S = (r_\infty/r_0)^{1/2}$ (Kawato et al., 1981), where r_0, the anisotropy in the absence of motion, is equal to 0.390 for DPH (Lakowicz et al., 1979). This order parameter facilitates comparison to data obtained with electron spin resonance or nuclear magnetic resonance techniques. The rotational rate can be converted to a rotational relaxation time in nanoseconds as follows:

$$\text{rotational relaxation time} = (6R)^{-1}$$

where R represents the rotational rate (radians per second).

Treatment of Plasma Membranes with Charged Anesthetics. Anesthetics were prepared fresh daily in deionized water. The pH was adjusted to 10 with NaOH and, as necessary, with HCl. The drugs were added directly to membranes resuspended in phosphate-buffered saline (50 μg of protein/mL) in the fluorometer cuvette. The pH of the buffered sample was not changed significantly by addition of the anesthetic solution. Measurements commenced usually within 1 min after addition of drug. No effect of longer incubation times was noted.

Electron Spin Resonance Spectroscopy. LM membranes that had been treated with or without TNBS were incubated with 5-doxylstearic acid spin-label for 1 h at 37 °C. Tubes

[1] Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; r_∞, limiting anisotropy; $(6R)^{-1}$, rotational relaxation time in nanoseconds; r, steady-state anisotropy; r_0, anisotropy in the absence of rotational motion; τ, fluorescence lifetime; TNBS, trinitrobenzenesulfonic acid; BSA, bovine serum albumin.

Table I: Effect of Anesthetics on Transbilayer Distribution of 1,6-Diphenyl-1,3,5-hexatriene in LM Fibroblast Plasma Membranes[a]

drug	concn (mM)	% quenching of 1,6-diphenyl-1,3,5-hexatriene
none		54.5 ± 1.8
phenobarbital	0.5	54.7 ± 1.8
phenobarbital	10.0	56.2 ± 2.3
pentobarbital	0.5	60.3 ± 3.4
pentobarbital	10.0	62.4 ± 3.8
prilocaine	1.0	56.0 ± 4.0
prilocaine	10.0	57.0 ± 3.9
procaine	1.0	58.0 ± 3.4
procaine	10.0	59.0 ± 3.9

[a] LM fibroblasts were treated at 4 °C ± trinitrobenzenesulfonic acid, plasma membranes were isolated, 1,6-diphenyl-1,3,5-hexatriene was incorporated, and the absorbance-corrected fluorescence was determined in the absence or presence of anesthetics at the indicated concentrations as described under Experimental Procedures. Values indicate the mean ± SEM (n = 3–6).

were vortexed every 15 min. After incubation, the samples were washed at 105000g for 90 min in order to remove any excess BSA that could distort the spectra. The pellet was drawn up into a 100-μL micropipet, placed in a Varian E 109E EPR spectrometer (Varian Associates, Palo Alto, CA), and read at 37 °C. Spectra due to spin-label bound to BSA were not observed, indicating essentially complete removal of any excess BSA–spin-label by this procedure. Spectra were recorded at X-band frequency using 100-kHz modulation. The microwave power was 5 mW, the modulation amplitude was 2 G, the scan range was 100 G, and the microwave frequency was 9100 MHz for all samples. The 2T was calculated by the method of Hubbell and McConnell (1971). Each sample was scanned 4–5 times.

Results

Trinitrophenylation and Transbilayer Location of Trinitrophenyl Groups in LM Plasma Membranes. The structures of the intact LM plasma membrane (inner and outer monolayer), the exofacial leaflet (extracellular monolayer), and the cytofacial leaflet (cytoplasmic monolayer) are examined with DPH as a fluorescent reporter molecule and trinitrophenyl groups as quenching agents. Trinitrophenylation of the intact LM cell at 4 °C (nonpenetrating conditions) and subsequent isolation of plasma membranes result in covalent attachment of trinitrophenyl quenching agents to the exofacial leaflet. The trinitrobenzenesulfonic acid labeling reaction must be carefully monitored in order to assure that the reagent is not penetrating into the cells and labeling both sides of the plasma membrane. For this purpose, four control procedures are routinely used.

First, as an "internal control", mitochondria and microsomes are isolated from the same cells from which the trinitrophenylated plasma membranes are isolated. If any significant degree of penetration of TNBS into the cell occurred, then these intracellular organelles would also become trinitrophenylated. Only 1.7 ± 0.2% and 1.9 ± 0.4% of microsomal and mitochondrial phosphatidylethanolamine are trinitrophenylated. When the trinitrobenzenesulfonic acid treatment procedure is performed under penetrating conditions (37 °C), 60–80% of the phosphatidylethanolamine in microsomes and mitochondria is trinitrophenylated. If isolated microsomes or mitochondria are treated with trinitrobenzenesulfonic acid at 4 °C, 53% or 63% of the phosphatidylethanolamine is trinitrophenylated, respectively. Treatment of isolated microsomes and mitochondria at 37 °C results in 66% and 79%, respectively, of phosphatidylethanolamine trinitrophenylated. Thus, if trinitrobenzenesulfonic acid had penetrated the intact cell during the treatment procedure, then the intracellular membrane phosphatidylethanolamine would be extensively trinitrophenylated. As noted above, this is not the case. Transbilayer flip-flop of trinitrophenylated phosphatidylethanolamine is insignificant under the conditions used for fluorescence measurement herein; additional plasma membrane phosphatidylethanolamine does not become available for trinitrophenylation.

Second, as shown in Table I, approximately half of the DPH fluorescence per milligram of membrane protein is quenched in the trinitrophenylated membrane. This percent quenching is maximal since addition of trinitrophenylglycine, a water-soluble nonpenetrant quencher, does not further decrease the DPH fluorescence (data not presented). If the trinitrophenylation reaction is performed at 37 °C (penetrating conditions), greater than 90% of the fluorescence of DPH is quenched.

Third, because quenching by trinitrophenyl groups occurs by Förster nonradiative energy transfer (Schroeder, 1980, 1984, 1985; Schroeder et al., 1979a,b), the fluorescence lifetime of DPH should be substantially reduced (Knox, 1968; Kelly & Patterson, 1971). Under the conditions used herein, the fluorescence of DPH molecules located in the outer monolayer is maximally quenched due to a large excess of trinitrophenyl groups covalently attached to the outer monolayer. For example, the fluorescence lifetime of diphenylhexatriene (DPH) is nontrinitrophenylated LM cell plasma membranes can best be described as having a single component (Table II). When the data were fit to a single component, the lifetime was 9.7 ± 0.1 ns (χ^2 value of 0.8). When the data were fit to two components, major 10.5 ± 0.1 ns (96% of fluorescence signal) and minor 0.8 ± 0.5 ns (4% of fluorescence

Table II: Dynamic Properties of 1,6-Diphenyl-1,3,5-hexatriene in LM Fibroblast Plasma Membranes[a]

plasma membrane fraction	polarization	lifetime (ns)	limiting anisotropy	rotational relaxation time (ns)	order parameter
untreated	0.228 ± 0.002	9.71 ± 0.13	0.137 ± 0.002	1.20 ± 0.01	0.590 ± 0.005
TNBS treated	0.258 ± 0.004	7.89 ± 0.06	0.155 ± 0.002	1.28 ± 0.03	0.630 ± 0.006
calcd for outer monolayer	0.198 ± 0.004[c]	11.53 ± 0.13	0.119 ± 0.002[b]	1.12 ± 0.03[b]	0.550 ± 0.006[c]

[a] LM fibroblasts were treated ± trinitrobenzenesulfonic acid under nonpenetrating conditions (4 °C, 4 mM) as described under Experimental Procedures. Plasma membranes were isolated, 1,6-diphenyl-1,3,5-hexatriene was incorporated (1:100 probe:lipid ratio), and fluorescence parameters were determined at 37 °C also as described under Experimental Procedures. Values from untreated cells represent inner + outer monolayer; values from cells treated with trinitrobenzenesulfonic acid represent the inner monolayer; values for the outer monolayer were calculated from a fluorescence intensity normalized average of the inner and outer monolayer. In the latter calculation, the value of each parameter in the inner and outer membrane equals the [(the value of each parameter in the inner monolayer) × (the fraction of total fluorescence due to the inner monolayer)] plus [(the value of each parameter in the outer monolayer) × (the fraction of total fluorescence in the outer monolayer)]. The fractional fluorescence values are not assumed values. Instead, they are experimentally obtained by lifetime heterogeneity analysis of LM plasma membranes trinitrophenylated under nonpenetrating conditions. Individual values represent the mean ± SEM (n = 3). [b] $p < 0.025$ compared to the inner monolayer by the Student's t test. [c] $p < 0.005$ compared to the inner monolayer by the Student's t test.

signal) lifetime components were resolved but without improvement of fit (χ^2 value of 10.9). Thus, at 37 °C, the probe diphenylhexatriene appears to be sampling primarily one lipid phase with perhaps a minor contribution due to a second component. Treatment of LM plasma membranes with TNBS resulted in a decreased lifetime, 7.9 ± 0.1 ns, which was resolved into two components of 9.8 and 2.2 ns, respectively. With only three frequencies (6, 18, and 30 MHz) available in the SLM 4800 fluorometer, it was not possible to resolve the minor component near 0.8 ns. The significant reduction in lifetime of DPH located in the extracellular leaflet of the plasma membrane is consistent with the Förster energy transfer. When fractional fluorescence values, also obtained by heterogeneity analysis, are converted to mole fractions, 49 ± 1 mol % of DPH is localized in the outer monolayer, and 51 ± 1 mol % is localized in the inner monolayer of the LM plasma membrane. However, since the quantum yield of the quenched fluorescence in the outer monolayer is only one-fifth that in the inner monolayer, more than 90% of the measured fluorescence signal of DPH in plasma membranes trinitrophenylated in the outer monolayer is due to DPH located in the inner monolayer. If both monolayers are trinitrophenylated (e.g., at 37 °C), then only the short-lifetime component appears. If as little as 10% of the inner monolayer fluorescence probe molecules were quenched by trinitrobenzenesulfonic acid that had penetrated to the inner monolayer, the fluorescence lifetime in that monolayer would be significantly reduced. The data show that this does not occur. In summary, a large reduction in lifetime is not observed with our LM cells due to the fact that most of the fluorescence of the outer monolayer is quenched, thereby contributing little signal in the trinitrophenylated plasma membrane.

Fourth, the trinitrophenylation of the cell membrane can alter membrane enzyme activities. In contrast to results obtained under penetrating conditions (37 °C), the activity of neither (Na^+,K^+)-ATPase nor 5′-nucleotidase is significantly altered by the TNBS reaction under nonpenetrating conditions (4 °C).

Last, it is important to show that the trinitrophenylation procedure itself did not significantly alter the structure of the plasma membrane. The spin-label probe 5-doxylstearic acid was incorporated into plasma membranes from untreated LM cells and into plasma membranes from LM cells treated at 4 °C with TNBS. The $2T_\perp$ value was 9.800 ± 0.001 before and after treatment with TNBS. Thus, TNBS treatment at 4 °C did not affect the structure of the LM plasma membrane.

Dynamic and Static Properties of 1,6-Diphenyl-1,3,5-hexatriene in LM Fibroblast Plasma Membranes. Previous investigations using fluorescent probe molecules to measure plasma membrane transbilayer fluidity differences (Schroeder, 1978, 1980; Schroeder & Kinden, 1979; Schachter et al., 1982; Cogan & Schachter, 1981) reported only steady-state fluorescence polarization or rotational correlation times calculated according to the Perrin equation (Perrin, 1936; Shinitzky & Inbar, 1974). However, it has been shown that these parameters are comprised of static as well as dynamic components (Lakowicz et al., 1979; Heyn, 1979; Kinosita et al., 1981). Therefore, in the present investigation, the static (limiting anisotropy and order) and dynamic (rotational relaxation time) parameters are resolved in order to clarify which is responsible for the observed differences in probe motion in the two leaflets of the membrane.

The static properties of DPH indicate that at 37 °C there is still considerable restriction to motion of DPH in LM plasma membranes since the limiting anisotropy and order parameter are not zero (Table II). In contrast, the dynamic property, rotational relaxation time, indicates rapid motion of DPH near 1.2 ns. When the exofacial leaflet of LM plasma membranes contains the covalently linked trinitrophenyl quenching groups, the majority of fluorescence in that monolayer is quenched, and most of the remaining fluorescence signal arises from DPH molecules located in the inner monolayer. As shown in the preceding section, the quenching from DPH to trinitrophenyl groups is by Förster nonradiative energy transfer. Nonradiative energy transfer between like molecules reduces fluorescence polarization as well as lifetime (Kelly & Patterson, 1971; Knox, 1968). In the case of unlike molecules (DPH and trinitrophenyl groups), this is not true.

The limiting anisotropy and order parameter of DPH in the TNBS-treated membrane (inner monolayer) (0.155 and 0.630, respectively) are significantly higher than calculated for the outer monolayer (0.119 and 0.550, respectively), as demonstrated in Table II. Concomitantly, the rotational relaxation time of DPH is also longer, 1.28 ns, in the TNBS-treated membrane (inner monolayer) than calculated for the outer monolayer, 1.12 ns. The significance of the magnitude of difference in DPH limiting anisotropy between monolayers, namely, 0.036, can be illustrated by comparison to the effect of temperature on this parameter. The limiting anisotropy of DPH in LM plasma membranes is 0.181 ± 0.002 (n = 3) and 0.137 ± 0.002 (n = 6) at 24 and 37 °C, respectively. Thus, the difference in limiting anisotropy between the inner and outer monolayer is as large as that produced by a 10.6 °C change in temperature. Taken together, these data indicate that the motion of DPH in LM plasma membranes is less rapid and considerably more restricted in the inner than the outer monolayer. This observation is also reflected in the simple measurement of steady-state fluorescence polarization (Table II).

Effect of Anesthetics on Individual Leaflet Structure. As shown above, and consistent with results obtained by others (Lakowicz et al., 1979), the rotational relaxation time of DPH is much less sensitive to alterations in lipid composition (outer vs. inner leaflet of plasma membrane) or temperature than is the limiting anisotropy, a structural parameter. Limiting anisotropy is primarily determined by the degree of limitation to rotation and may vary independently of rotational rate. In order to determine the effect of charged anesthetics on individual monolayer structure, it is first necessary to demonstrate that these molecules do not interact directly with DPH and thereby quench its fluorescence. Quenching of absorbance-corrected fluorescence intensity by anesthetics is not observed over the entire concentration range used for phenobarbital, pentobarbital, procaine, or prilocaine (Table I). Furthermore, if direct quenching of DPH by anesthetics occurred, fluorescence lifetime would decrease. However, the DPH fluorescence lifetime is not changed by phenobarbital in the plasma membrane. The lifetime of DPH in the plasma membrane was 9.7 ± 0.01, 9.6 ± 0.2, 9.7 ± 0.3, 9.5 ± 0.1, and 9.6 ± 0.2 ns at 0.1, 0.5, 1, 5, and 10 mM phenobarbital, respectively. Therefore, direct quenching of DPH fluorescence by phenobarbital is ruled out. Similar results were obtained with pentobarbital, procaine, and prilocaine.

The effect of increasing concentrations of phenobarbital or pentobarbital on the limiting anisotropy of DPH in plasma membranes from LM cells is shown in Figure 1A,B. The limiting anisotropy of the untreated membrane shows a gradual decrease (fluidization) with increasing phenobarbital concentration, with a significant decrease from membranes with no drug noted only at 10 mM (Figure 1A, closed circles). A

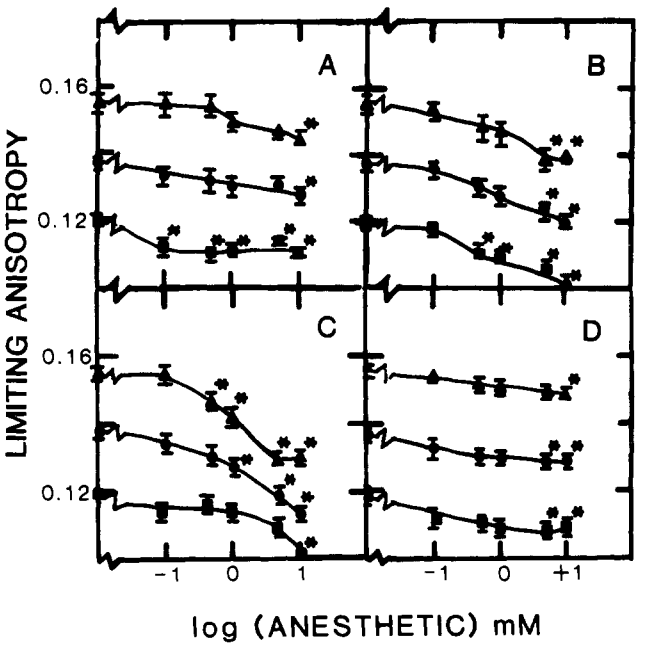

FIGURE 1: Anesthetics alter limiting anisotropy of DPH in LM cell plasma membranes. DPH was incorporated into isolated plasma membranes, and parameters were determined at 37 °C as described under Experimental Procedures. Untreated (inner and outer monolayer, circles); calculated for outer monolayer (squares); TNBS treated (inner monolayer, triangles). Values represent the mean ± SEM (n = 3–4). An asterisk refers to $p < 0.05$ by the Student's t test as compared to no anesthetic. (A) Phenobarbital; (B) pentobarbital; (C) prilocaine; (D) procaine.

Table III: Effect of Anesthetics of DPH Limiting Anisotropy in LM Plasma Membranes[a]

	plasma membrane fraction		
anesthetic (mM)	untreated	calcd for outer monolayer	TNBS treated
none	0.137 ± 0.002	0.119 ± 0.002	0.155 ± 0.002
0.1 mM phenobarbital	0.133 ± 0.002	0.111 ± 0.001[b]	0.155 ± 0.002
0.5 mM pentobarbital	0.130 ± 0.003	0.110 ± 0.002[b]	0.150 ± 0.002
0.5 mM prilocaine	0.131 ± 0.003	0.115 ± 0.002	0.147 ± 0.002[b]
5.0 mM procaine	0.129 ± 0.003	0.108 ± 0.002[b]	0.151 ± 0.002

[a] All conditions were described in the legend to Figure 1. Values represent the mean ± SEM (n = 3–4). [b] $p < 0.05$ as compared to none.

similar pattern is observed for the TNBS-treated membrane (cytofacial leaflet) (Figure 1A, closed triangles). However, the limiting anisotropy of DPH calculated for the exofacial leaflet is significantly decreased at 0.1 mM phenobarbital (Table III and Figure 1A, closed squares), a 10-fold lower concentration than needed for a statistically significant decrease in the cytofacial leaflet. Thus, phenobarbital preferentially increases the range of fluorescent probe motion (fluidizes) in the exofacial leaflet.

The effects of pentobarbital on the limiting anisotropy of DPH in LM cell plasma membrane are shown in Figure 1B. Pentobarbital decreases the limiting anisotropy of DPH in the whole membrane, with significant decreases already at 0.5 mM drug concentration calculated for the exofacial leaflet (Table III and Figure 1B) and at 5 mM drug concentration in the TNBS-treated membrane (cytofacial leaflet) (Figure 1B).

Interpretation of the above results could be obscured if alterations in the distribution of DPH between the leaflets occurs upon addition of phenobarbital or the other anesthetics. Redistribution of DPH would result in a change in the fraction of fluorescence quenched by trinitrophenyl groups. However,

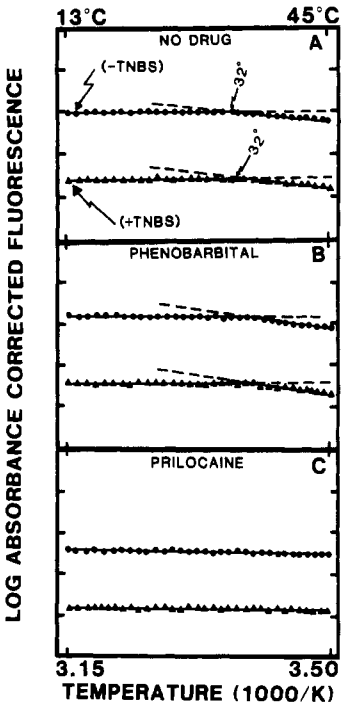

FIGURE 2: Anesthetics alter temperature dependencies of DPH in LM plasma membranes. Arrhenius plots of DPH absorbance-corrected fluorescence were determined as described earlier (Schroeder et al., 1979).

significant changes in DPH fluorescence intensity distribution in the presence of anesthetic are not detected (Table I).

The effects of prilocaine on the limiting anisotropy of DPH in plasma membranes from LM cells are shown in Figure 1C. Again, direct quenching of DPH fluorescence (Table I) or lifetime (data not shown) by prilocaine is not noted. Prilocaine decreases the DPH limiting anisotropy significantly in the TNBS-treated membrane (cytofacial leaflet) at 0.5 mM (Table III and Figure 1C, closed triangles), in the whole membrane at 1 mM (Figure 1C, closed circles), and that calculated for the exofacial leaflet at 10 mM (Figure 1C, closed squares). Thus, prilocaine preferentially fluidizes the TNBS-treated membrane (cytofacial leaflet).

The effects of procaine on the fluorescence parameters, as above, are shown in Figure 1D. Increasing concentrations of procaine do not change the DPH fluorescence quenching by trinitrophenyl groups (Table I) or the DPH lifetime (data not shown), indicating no direct quenching of DPH fluorescence by procaine. Small but significant decreases in limiting anisotropy at 5 mM procaine (Table I and Figure 1D) are calculated for the exofacial leaflet and untreated membrane and at 10 mM in the TNBS-treated membrane (cytofacial leaflet); therefore, procaine does not display selectivity for one plasma membrane leaflet over the other.

Temperature Dependence of DPH in LM Plasma Membranes. The effects of anesthetics on the characteristic temperatures ("break points") of Arrhenius plots of some probe molecules (e.g., fluorescence, NMR, or ESR) or enzyme activities have been commonly employed as measures of changes in fluidity. Arrhenius plots of DPH absorbance-corrected fluorescence in the absence or presence of exofacial trinitrophenyl groups and in the absence or presence of 10 mM phenobarbital, pentobarbital, prilocaine, or procaine with plasma membranes from choline-fed cells are obtained (Figure 2). Characteristic temperatures are found in these plots. With neither drug nor trinitrobenzenesulfonate treatment, a characteristic temperature is shown near 32 ± 1 °C. In the

presence of exofacial trinitrophenyl quenching groups and the absence of anesthetic, the same characteristic break-point temperature (32 ± 1 °C) is present unaltered. This result indicates that the break point is associated with the TNBS-treated membrane (cytofacial leaflet) or that both leaflets have a very similar characteristic temperature. In the presence of phenobarbital, pentobarbital, and procaine, the characteristic temperature was 32 ± 1, 31 ± 1, and 32 ± 2 °C in intact plasma membranes, respectively, and 31 ± 1, 32 ± 1, and 32 ± 1 in trinitrophenylated plasma membranes, respectively. Prilocaine, however, abolishes the break point, resulting in linear Arrhenius plots within the temperature range examined (13–45 °C) for intact or trinitrophenylated membranes. Thus, the characteristic temperature in Arrhenius plots appears to be located in the cytofacial leaflet of the LM plasma membranes. In addition, these results demonstrate that substantial fluidization, directly measured, can be induced without an alteration in the characteristic temperature of Arrhenius plots.

Discussion

A number of potential difficulties in studying molecular aspects of biological membranes must be considered for optimal interpretation of the results presented here. First, lipids in biological membranes are part of a many-component system and may therefore not be in a single phase at the temperature in question. Certainly, temperature studies of diphenylhexatriene fluorescence in LM plasma membranes indicate the presence of at least one break point near 32 °C in Arrhenius plots (Figure 2). Such a break point may be interpreted as indicative of a lipid phase alteration. At 32 °C, it would be difficult to know in advance which of the phases the diphenylhexatriene probe may be sampling. Therefore, in the present work, all data were obtained at 37 °C, well above the putative phase transition temperature of 32 °C. It is still possible, however, that small clusters or domains of phase-separated lipid may exist even at 37 °C. This possibility was examined by heterogeneity analysis of diphenylhexatriene fluorescence at 37 °C in LM plasma membranes. When the data were fit to a single component or to two-component analysis, the probe diphenylhexatriene appears to be sampling primarily one lipid phase with perhaps a minor contribution due to a second component. Treatment of LM plasma membranes with TNBS resulted in the appearance of two components near 9.8 and 2.2 ns. However, with only three frequencies (6, 18, and 30 MHz) available in the SLM 4800 fluorometer, it was not possible to resolve the minor component near 0.8 ns. Treatment of membranes with varying concentrations of anesthetics did not alter the fractional fluorescence due to any of the lifetime components described. Thus, it is unlikely that anesthetics caused an equilibrium redistribution of probe molecules. Second, the effect of proteins on the limiting anisotropy of diphenylhexatriene must be considered (Hoffman et al., 1981). Certainly in a model system, it has been proposed not only that diphenylhexatriene may not report the average disorder of the hydrocarbon chains but also that its proximity to intramembrane proteins may lead to greatly hindered motion (Hoffman et al., 1981). However, tryptophan quenching experiments suggest that diphenylhexatriene is not associated with protein in biological membranes (Klausner et al., 1980), whereas diphenylhexatriene has been shown to bind to hydrophobic regions of proteins in aqueous media (Mely-Goubert & Freedman, 1980). In summary, interpretation of lipid mobility from fluorescence quenching as outlined below is not the only effect that must be considered.

The asymmetric distribution of phospholipids and sterol between the two leaflets ("vertical asymmetry") of eukaryotic plasma membranes is well established for LM cells (Schroeder, 1981a; Hale & Schroeder, 1982; Fontaine & Schroeder, 1979; Sandra & Pagano, 1978), erythrocytes (Hale & Schroeder, 1982; Fisher, 1976), phagosomes (Hale & Schroeder, 1982; Sandra & Pagano, 1978), and membrane-enveloped viruses (Pal et al., 1981). This asymmetric distribution of lipids appears responsible for the difference in fluidity between the two membrane leaflets of red blood cells as determined by ESR order parameters of probe molecules (Tanaka & Ohnishi, 1976; Seigneuret et al., 1984). Break points in Arrhenius plots of ESR probe molecule order parameters in LM fibroblast phagosomes and enveloped viruses have been interpreted to indicate that the exofacial leaflet is more fluid than the cytofacial leaflet of the LM plasma membrane. Fluorescence polarization and trinitrophenyl quenching data were consistent with this interpretation of LM plasma membrane structure (Schroeder, 1978, 1980; Schroeder et al., 1979a,b; Schroeder & Goh, 1979). However, the possibility that the comparison of trinitrobenzenesulfonic acid treated (resulting in covalent attachment of trinitrophenyl groups to lipids and proteins) and untreated membranes may also reflect changes induced by the labeling as well as differences owing to intrinsic hemileaflet asymmetries must be considered. Recently, four different approaches were used to examine this possibility (Schroeder, 1980, 1981a,b; Hale & Schroeder, 1982; and results presented herein). First, a fluorescent glucosamine derivative of *trans*-parinaric acid did not penetrate intact LM cells or phagosomes derived therefrom. Phagosomes may be considered as being surrounded by an "inside-out" plasma membrane surrounding an endocytosed latex bead. The glucosamine–parinaric acid probe was readily accessible to nonpenetrating quenching agents, such as trinitrophenylglycine, that were not covalently linked to the membrane. The exposed membrane monolayer in the phagocytosed latex bead membrane was more rigid than that of the intact LM cell. Second, the fluorescence of *trans*-parinaric acid, 1,6-diphenyl-1,3,5-hexatriene, *N*-phenyl-1-naphthylamine, and dehydroergosterol was quenched either by covalently linked trinitrophenyl groups and/or by adding the water-soluble nonpenetrating quenching agent trinitrophenylglycine. Both agents gave the same results for the direction of the fluidity gradient; i.e., the inner monolayer of the plasma membranes was more rigid than the outer monolayer. Third, if the differences in individual monolayer fluidity were due primarily to the covalent attachment of trinitrophenyl groups, then all LM fibroblasts would be expected to show a more rigid inner monolayer. However, the ratio (inner monolayer/outer monolayer) limiting anisotropy in plasma membranes from LM cells fed choline, *N*,*N*-dimethylethanolamine, *N*-methylethanolamine, or ethanolamine was 1.31, 1.19, 1.08, and 0.97, respectively. Fourth, trinitrophenylation did not affect the order of 5-doxylstearic acid in LM plasma membranes. The $2T_{\perp}$ value of 5-doxylstearic acid in LM plasma membranes was 9.800 ± 0.001 before and after treatment with trinitrobenzenesulfonic acid. Likewise, these differences in spectral shape were not observed. It is considered from these data that even if trinitrophenylation of LM plasma membranes may alter the dynamics and statics of lipid motion reported by diphenylhexatriene, this effect is expected to be small and should not affect the overall interpretation of the results presented here.

Neither steady-state anisotropy nor rotational correlation time of fluorescence probes, calculated according to the Perrin equation in the above reports, adequately describes whether the static (order or restriction to motion) or dynamic (rate of rotation) components of probe motion differ in the two mon-

olayers of the plasma membrane. The results in Table II show that both the restriction to motion and the rotational relaxation time of DPH are greater in the cytofacial leaflet of the LM plasma membrane than in the calculated exofacial leaflet of plasma membranes from LM fibroblasts. Transbilayer distribution of sterol, a primary determinant of lipid structure, in LM plasma membranes is consistent with this observation. The inner monolayer of the LM fibroblast plasma membrane is enriched with sterol (Schroeder, 1981a; Hale & Schroeder, 1982), and the molar ratio of sterol/phospholipid is approximately 0.15 and 0.61 in the exofacial and cytofacial leaflets, respectively. Cholesterol content less than 33 mol % in phosphatidylcholine liposomes did not change the rotational relaxation time of DPH (Lakowicz et al., 1979; Veatch & Stryer, 1977) but increased the limiting anisotropy of DPH (Lakowicz et al., 1979). Thus, DPH in the cytofacial layer of LM fibroblast plasma membranes, being enriched in sterol, should have a much larger limiting anisotropy, and this is confirmed by the data presented here. The data also confirm the prediction (Lakowicz et al., 1979) that changes observed in DPH steady-state anisotropy derive primarily from changes in limiting anisotropy, not rotational relaxation time. The rotational relaxation time was only 14% greater in the inner vs. outer monolayer whereas the limiting anisotropy was 30% greater in the inner vs. outer monolayer.

It has been proposed that predominance of anionic phospholipid head groups on the cytofacial leaflet will preferentially associate with and be fluidized by cationic amphipaths while anionic amphipaths will be excluded and thereby preferentially associate with and fluidize the zwitterionic neutral phospholipid head groups on the exofacial leaflet (Sheetz & Singer, 1974). This concept has been tested by shape changes in erythrocytes (Sheetz & Singer 1974) and by observing changes in activity of asymmetrically disposed membrane-bound enzymes (Houslay et al., 1981, 1980; Dipple et al., 1982; Salesse et al., 1982; Houslay & Palmer, 1978). To our knowledge, the results reported herein demonstrated for the first time that the fluidization part of the Sheetz–Singer hypothesis may be valid for plasma membranes of nucleated mammalian cells. Electron spin resonance methods were recently used to show that the fluidization part of this hypothesis was also valid for the surface membrane of the nonnucleated human erythrocyte (Seigneuret et al., 1984). These investigators reported that the outer leaflet of the red blood cell plasma membrane was more rigid (higher order parameter) than the inner leaflet. This finding was consistent with the transbilayer distribution of cholesterol which appears enriched in the outer leaflet (Fisher, 1976; Schroeder, 1981a; Hale & Schroeder, 1982) and confirmed results on erythrocyte anisotropic fluidity suggested by other investigators (Tanaka & Ohnishi, 1976; Williamson et al., 1982; Chabanel et al., 1985). In contrast, these data disagree with the results of Cogan and Schachter (1982).

The use of inflections ("break points") in Arrhenius plots of some physical measure, e.g., fluorescence or ESR signals, to detect phase transitions is well established for pure phospholipid systems. The temperature at these inflections correlates well with the phase transition as determined by X-ray diffraction or differential scanning calorimetry of the bulk lipids. However, the phase transition temperature may or may not correlate with the viscosity of the liquid state. Despite such observations, characteristic temperatures in membranes have been taken as a fluidity index of different membranes (or one membrane perturbed with anesthetics) (Houslay et al., 1980, 1981; Houslay & Palmer, 1978). According to this rationale, the presence of a characteristic temperature in the cytofacial leaflet (with trinitrobenzenesulfonic acid treatment), but not in the exofacial leaflet, is consistent with, but not proof of, a more rigid cytofacial monolayer. In contrast to the interpretations based only on characteristic temperatures, the data presented here offer direct evidence in support of the hypothesis. It is evident from an examination of Table III and Figure 1 that at certain concentrations there appears preferential fluidization (i.e., decreased fluorescence anisotropy) of LM plasma membrane monolayers in the predicted direction [reviewed in Schroeder (1985)]. Phenobarbital and pentobarbital interact preferentially with the exofacial leaflet while prilocaine interacts preferentially with the inner leaflet. However, little or no preference is exhibited by procaine in these plasma membranes.

It is well established that experimental changes in fluidity of the whole LM cell plasma membrane result in many functional changes including endocytosis (Schroeder, 1981b; Schroeder & Kier, 1983), lectin binding and agglutination (Schroeder, 1982), transport (F. Schroeder, unpublished observation), and adenylate cyclase activity (Engelhardt et al., 1976). Despite these findings, the functional consequences of changing the fluidity of one membrane leaflet are not yet well established. Further, it is not yet known whether, or under which circumstances, the fluidity of each leaflet may be independently regulated by the cell, as during compositional changes to maintain whole membrane viscosity (homeoviscous adaptation). It may be anticipated that charged amphipaths, if employed carefully, can be used to investigate these questions.

ACKNOWLEDGMENTS

We thank Larissa L. Schuster and Gene W. Hubert for their superb technical assistance and Genie Eckenfels and Lindy Corkins for typing the manuscript.

Registry No. Phenobarbital, 50-06-6; pentobarbital, 76-74-4; prilocaine, 721-50-6; procaine, 59-46-1.

REFERENCES

Chabanel, A., Abbott, R. E., Chien, S., & Schachter, D. (1985) *Biochim. Biophys. Acta 816*, 142–152.

Cogan, U., & Schachter, D. (1981) *Biochemistry 20*, 6396–6403.

Dipple, I., Gordon, L. M., & Houslay, M. D. (1982) *J. Biol. Chem. 257*, 1811–1815.

Engelhard, V. H., Esko, J. D., Storm, D. R., & Glaser, M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4482–4486.

Fisher, K. A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 173–177.

Flamm, M., & Schachter, D. (1982) *Nature (London) 298*, 290–292.

Fontaine, R. N., & Schroeder, F. (1979) *Biochim. Biophys. Acta 558*, 1–12.

Hale, J. E., & Schroeder, F. (1982) *Eur. J. Biochem. 122*, 649–661.

Heyn, M. P. (1979) *FEBS Lett. 108*, 359–364.

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

Holland, J. F., Teets, R. E., & Timnick, A. (1973) *Anal. Chem. 45*, 145–153.

Houslay, M. D., & Palmer, R. W. (1978) *Biochem. J. 174*, 909–919.

Houslay, M. D., Dipple, I., Rawal, S., Sauerheber, R. D., Esgate, J. A., & Gordon, L. M. (1980) *Biochem. J. 190*, 131–137.

Houslay, M. D., Dipple, I., & Gordon, L. M. (1981) *Biochem. J. 197*, 675–681.
Hunt, G. R., & Tipping, L. R. H. (1978) *Biochim. Biophys. Acta 507*, 242–261.
Kawato, S., Kinosita, K., & Ikegami, A. (1978) *Biochemistry 17*, 5026–5031.
Kelly, A. R., & Patterson, L. K. (1971) *Proc. R. Soc. London, A 324*, 117–126.
Kinosita, K., Kataoka, R., Kimura, Y., Gotoh, O., & Ikegami, A. (1981) *Biochemistry 20*, 4270–4277.
Klausner, R. D., Kleinfeld, A. M., Hoover, R. L., & Karnovsky, M. J. (1980) *J. Biol. Chem. 255*, 1286–1295.
Knox, R. S. (1968) *Physica (Amsterdam) 39*, 361–386.
Lakowicz, J. R., Prendergast, F. G., & Hogen, D. (1979) *Biochemistry 18*, 508–519.
Lakowicz, J. R., Cherek, H., & Balter, A. (1981) *J. Biochem. Biophys. Methods 5*, 131–146.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Mely-Goubert, B., & Freedman, M. H. (1980) *Biochim. Biophys. Acta 601*, 315–327.
Morse, P. D., Ruhlig, M., Snipes, W., & Keith, A. D. (1975) *Arch. Biochem. Biophys. 168*, 40–56.
Pal, R., Barenholz, Y., & Wagner, R. R. (1981) *Biochemistry 20*, 530–539.
Perrin, F. (1936) *J. Phys. Radium 7*, 1–11.
Salesse, R., Garnier, J., Leterrier, F., Daveloose, D., & Viret, J. (1982) *Biochemistry 21*, 1581–1586.
Sandra, A., & Pagano, R. E. (1978) *Biochemistry 17*, 332–338.
Schachter, D., Cogan, U., & Abbot, R. E. (1982) *Biochemistry 21*, 2146–2150.
Schmidt, C. F., Barenholz, Y., Huang, C., & Thompson, T. E. (1978) *Nature (London) 271*, 775–777.
Schroeder, F. (1980) *Eur. J. Biochem. 112*, 293–307.
Schroeder, F. (1981a) *FEBS Lett. 135*, 127–130.
Schroeder, F. (1981b) *Biochim. Biophys. Acta 649*, 162–174.
Schroeder, F. (1982) *Biochemistry 21*, 6782–6790.
Schroeder, F. (1984) *Prog. Lipid Res. 23*, 97–113.
Schroeder, F. (1985) *Subcell. Biochem. 11*, 51–101.
Schroeder, F., & Goh, E. H. (1979) *J. Biol. Chem. 254*, 2464–2470.
Schroeder, F., & Kinden, D. A. (1980) *Nature (London) 287*, 255–256.
Schroeder, F., & Kier, A. B. (1983) *J. Immunol. Methods 57*, 363–371.
Schroeder, F., & Gardiner, J. M. (1984) *Cancer Res. 44*, 3262–3269.
Schroeder, F., Perlmutter, J. F., Glaser, M., & Vagelos, P. R. (1976) *J. Biol. Chem. 251*, 5015–5026.
Schroeder, F., Goh, E. H., & Heimberg, M. (1979a) *FEBS Lett. 97*, 233–236.
Schroeder, F., Goh, E. H., & Heimberg, M. (1979b) *J. Biol. Chem. 254*, 2456–2463.
Schroeder, F., Goetz, I., & Roberts, E. (1984) *Eur. J. Biochem. 142*, 183–191.
Seigneuret, M., Zachowski, A., Hermann, A., & Devaux, P. F. (1984) *Biochemistry 23*, 4271–4275.
Sheetz, M. P., & Singer, S. J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4457–4461.
Shinitzky, M., & Inbar, M. (1974) *J. Mol. Biol. 85*, 603–615.
Sillerud, L. O., & Barnett, R. E. (1982) *Biochemistry 21*, 1756–1760.
Sklar, L. A., Mantulin, W. W., & Pownall, H. J. (1982) *Biochem. Biophys. Res. Commun. 105*, 674–680.
Sweet, W. D., & Schroeder, F. (1985) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 44*, 1350 (Abstr. 5493).
Tanaka, K.-I., & Ohnishi, S.-I. (1976) *Biochim. Biophys. Acta 426*, 218–231.
Van Dijck, P. W. M., van Zoelen, E. J. J., Seldenrijk, R., Van Deenen, L. L. M., & de Gier, J. (1976) *Chem. Phys. Lipids 17*, 336–343.
Veatch, W. R., & Stryer, L. (1977) *J. Mol. Biol. 117*, 1109–1113.
Weber, G. (1978) *Acta Phys. Pol., A A54*, 859–865.
Weber, G. (1981) *J. Phys. Chem. 85*, 949–953.
Williamson, P., Bateman, J., Kozarsky, K., & Mattocks, K. (1982) *Cell (Cambridge, Mass.) 30*, 725–733.
Wisnieski, B. J., & Iwata, K. K. (1977) *Biochemistry 16*, 1321–1326.