DYNAMIC QUENCHERS IN FLUORESCENTLY LABELED MEMBRANES

Theory for Quenching in a Three-Phase System

GENEVA M. OMANN AND MICHAEL GLASER
Department of Biochemistry, University of Illinois, Urbana, Illinois 61801

ABSTRACT The theory for quenching of fluorescently labeled membranes by dynamic quenchers is described for a three-phase system: a fluorescently labeled membrane, a nonlabeled membrane, and an aqueous phase. Two different experimental protocols are possible to determine quenching parameters. Using the first protocol, partition coefficients and bimolecular quenching constants were determined for a hydrophobic quencher in carbazole-labeled membranes in the presence of an unlabeled reference membrane. These parameters determined for 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) using this three-phase analysis were in good agreement with values determined by a two-phase analysis without the reference lipid. Hence, the theory was verified. In the second protocol, the quencher partition coefficient was determined for unlabeled membranes in the presence of a carbazole-labeled reference membrane. Partition coefficients for DDE determined by this method were the same as partition coefficients determined for carbazole-labeled membranes using the two-phase analysis. The greater ease in determining partition coefficients and bimolecular quenching constants by the three-phase analysis and, in particular, the ability to determine the partition coefficient in unlabeled membranes make the three-phase analysis especially useful. This method was used to study the effect varying the membrane lipid composition has on the partition coefficient. The data indicate that partition coefficients of DDE in fluid membranes are not dramatically dependent upon polar head group composition, fatty acid composition, or cholesterol content. However, partitioning into gel-phase lipids is at least 100-fold less than fluid-phase lipids.

INTRODUCTION

The interaction of hydrophobic molecules with membranes is important for a wide variety of biological phenomena. Recently Lakowicz and co-workers (Lakowicz and Hogen, 1977, 1980; Lakowicz et al., 1977) developed a fluorescence method for determining partition coefficients and bimolecular quenching constants of chlorinated hydrocarbons in carbazole undecanoylphosphatidylcholine-labeled vesicles. This method is based on the dynamic quenching of the carbazole moiety's fluorescence by chlorinated hydrocarbons that partition into the bilayer. The extent of quenching of the carbazole moiety in the bilayer yields information on the concentration and mobility of the quencher in the membrane. This methodology has been used to study model membranes (Omann and Lakowicz, 1982) and cell membranes (Omann and Glaser, 1984). To study cell membranes it was necessary to introduce a fluorescent label into the membranes. This was accomplished by supplementing the growth media of tissue culture cells with 11-(9-carbazole)-undecanoic acid and allowing the cells to biosynthetically incorporate the fluorophore into membrane phospholipids (Omann and Glaser, 1984).

The derivation of the quenching theory assumes a two-phase system, a membrane phase and an aqueous phase. The theory yields the following equation for quenchers that strongly partition into the bilayer (Lakowicz and Hogen, 1977, 1980; Lakowicz et al., 1977):

$$1/k_{app} = \alpha_m/k_{qm} + 1/k_{qm}P, \quad (1)$$

where k_{app} is the apparent bimolecular quenching constant determined from Stern-Volmer quenching curves at a given volume fraction of the membrane, α_m. α_m is proportional to the phospholipid concentration and is calculated assuming a lipid density of 1 g/cc. k_{qm} is the bimolecular quenching constant of the quencher in the membrane, and P is the partition coefficient, defined as $[Q]_{membrane}/[Q]_{water}$. When $1/k_{app}$ is plotted against α_m, the bimolecular quenching constant is equal to the reciprocal of the slope of the line, and the partition coefficient is equal to the slope divided by the intercept according to Eq. 1. With quenchers that partition strongly into the bilayer, the error in

Dr. Omann's current address is Department of Immunology, Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, CA 92037.

determining the partition coefficient is large because the intercept, which is close to zero, is difficult to determine accurately (Omann and Lakowicz, 1982). Another practical limitation to this method is that only fluorescently labeled membranes can be studied.

Here a theory is presented for determining the partition coefficient and the bimolecular quenching constant in a three-phase system, an aqueous phase and two different membrane populations, one of which is labeled with the carbazole fluorophore. The introduction of a third phase alters the partitioning equation by increasing the intercept and thus allows the partition coefficient to be more easily calculated. In addition, the partition coefficient can be measured for a membrane that does not contain a fluorophore by making measurements on a carbazole-labeled reference lipid in the presence of the unlabeled membrane. These methods were used to determine the DDE partition coefficient in lipid dispersions of different compositions.

THEORY

Definitions and derivation of the partitioning equation in a three-phase system are analogous to that of a two-phase system. Consider two membrane populations, A and B, in an aqueous solution where A contains the fluorophore and B is unlabeled. The partition coefficients are defined as $P_A = [Q]_A/[Q]_W$ and $P_B = [Q]_B/[Q]_W$, where $[Q]$ refers to the quencher concentration in A, B, or water (W). Then $P_A/P_B = [Q]_A/[Q]_B$, or

$$[Q]_B = [Q]_A(P_B/P_A) \text{ and } [Q]_W = [Q]_A/P_A. \quad (2)$$

Conservation of matter requires that $[Q]_{added} V_T = [Q]_A V_A + [Q]_B V_B + [Q]_W V_W$, where V refers to the volume of each phase and V_T is the total volume. $[Q]_{added}$ is the quencher concentration in the total volume of the sample. Dividing both sides of the equation by V_T gives

$$[Q]_{added} = [Q]_A V_A/V_T + [Q]_B V_B/V_T + [Q]_W V_W/V_T. \quad (3)$$

If the volume fractions of the membrane are defined to be $\alpha_A = V_A/V_T$ and $\alpha_B = V_B/V_T$ with $1 = V_A/V_T + V_B/V_T + V_W/V_T$, then $V_W/V_T = 1 - \alpha_A - \alpha_B$. Substituting these and the identities of Eq. 2 into Eq. 3 gives $[Q]_{added} = [Q]_A\alpha_A + [Q]_A P_B\alpha_B/P_A + [Q]_A(1 - \alpha_A - \alpha_B)/P_A$. Rearrangement gives

$$[Q]_A = Q_{added}/[\alpha_A + (P_B\alpha_B + 1 - \alpha_A - \alpha_B)/P_A]. \quad (4)$$

In generating a Stern-Volmer plot for fluorescently labeled A membranes, either the fluorescence intensity, F, or the fluorescence lifetime, τ, can be measured

$$\tau_0/\tau = F_0/F = 1 + k_A\tau_0[Q]_A, \quad (5)$$

where F_0 and τ_0 are the fluorescence intensity and lifetime in the absence of quencher, k_A is the bimolecular quenching constant in phase A, and $[Q]_A$ is the concentration of quencher in phase A. Since $[Q]_A$ cannot be experimentally determined, it is necessary to consider this equation in terms of the experimentally accessible quantity $[Q]_{added}$. Thus, substituting Eq. 4 into Eq. 5 gives

$$\tau_0/\tau = F_0/F = 1 + k_A\tau_0[Q]_{added}/[\alpha_A + (P_B\alpha_B + 1 - \alpha_A - \alpha_B)/P_A] \quad (6)$$

or

$$\tau_0/\tau = F_0/F = 1 + k_{app}\tau_0[Q]_{added}, \quad (7)$$

where k_{app} is the experimentally determined apparent quenching constant

$$k_{app} = k_A/[\alpha_A + (P_B\alpha_B + 1 - \alpha_A - \alpha_B)/P_A]. \quad (8)$$

For compounds that partition strongly into the membrane, the Stern-Volmer plots are generated at low membrane concentrations such that $1 - \alpha_A - \alpha_B \approx 1$. Thus, the equation simplifies to

$$k_{app} = k_A/[\alpha_A + (P_B\alpha_B + 1)/P_A]. \quad (9)$$

If $\alpha_B = 0$ or $P_B = 0$, the equation reduces further to $k_{app} = k_A/[\alpha_A + 1/P_A]$. The reciprocal of this equation is identical to Eq. 1 for a two-phase system. Experimentally there are two options. Membrane A can be the unknown and membrane B a reference membrane, or membrane A a reference membrane and membrane B the unknown.

Option I

Assume that membrane A, the fluorescently labeled membrane, is the unknown membrane, i.e., a membrane for which P_A and k_A are unknown, and membrane B is a reference membrane for which P_B is known. To determine P_A and k_A, Eq. 9 can be inverted to give

$$1/k_{app} = \alpha_A/k_A + (P_B\alpha_B + 1)/k_A P_A. \quad (10)$$

k_{app} can be determined as a function of α_A with a constant α_B. The equation is similar to that derived for a two-phase system (Eq. 1). When $1/k_{app}$ is plotted against α_m, the slope is inversely proportional to k_A but the intercept is multiplied by the constant factor $(P_B\alpha_B + 1)$.

Option II

Assume that membrane A, the fluorescently labeled membrane, is one for which P_A and k_A have been determined (the reference membrane). This can be done using a two-phase system. Then membrane A can be mixed with an unlabeled membrane for which the partition coefficient is not known, unknown B. By determining an apparent quenching constant at a given α_A and α_B, the partition coefficient for membrane B can be calculated since rearrangement of Eq. 9 gives

$$P_B = (P_A/\alpha_B)[k_A/k_{app} - \alpha_a - 1/P_A]. \quad (11)$$

MATERIALS AND METHODS

Dimyristoyl-L-α-phospatidylcholine (DMPC), dipalmitol-L-α-phosphatidylcholine (DPPC), distearoyl-L-α-phospatidylcholine (DSPC), 1-stearoyl-2-oleoyl-L-α-phosphatidylcholine (SOPC), dioleoyl-L-α-phosphatidylcholine (DOPC), dioleoyl-L-α-phosphatidylethanolamine (DOPE), cardiolipin, sphingomyelin, and cholesterol were purchased from Sigma Chemical Co. (St. Louis, MO) at >98% purity and used without further purification. 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) (Aldrich Chemical Co., Milwaukee, WI) was 99% pure. β-(11-[9-carbazole]-undecanoyl)-L-α-phosphatidylcholine (CUA-PC) was synthesized and purified as described by Lakowicz and Hogen (1980) except that the formation of the fatty acid anhydride was done at room temperature (Selinger and Lipidot, 1966), and all reactions were done in toluene instead of benzene. CUA-PC-labeled phospholipid dispersions were made by mixing measured amounts of lipid and probe in methanol or chloroform and then evaporating the solvent. Phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 12.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2) was added and the samples hydrated for 10–15 min at temperatures above the phase transition temperature of the lipids (50°C for DPPC, 70°C for DSPC, 37°C for all others). The samples were vortexed and sonicated in a bath-type sonicator until all the lipid was dispersed from the bottom of the tube. Unlabeled dispersions were made by the same procedure without addition of CUA-PC.

Stern-Volmer quenching plots were generated by titrating 2 ml of a

lipid dispersion with a concentrated solution of DDE in ethanol (Lakowicz and Hogen, 1977, 1980). The total amount of ethanol added did not exceed 1% of the total sample volume. Fluorescence intensities were measured on an SLM 8000 fluorometer (SLM Instruments, Inc., American Instrument Co., Urbana, IL) with Corning 0-52 and 7-60 emission filters (Corning Glass Works, Corning Medical and Scientific, Corning, NY) instead of an emission monochromator. The excitation filter was a Corning 7-54 (Corning Glass Works, Corning Medical and Scientific). The excitation wavelength was 295 nm with a 4-nm excitation bandpass. Fluorescence lifetimes were measured on the cross-correlation phase fluorometer described by Spencer and Weber (1969) with updated electronics by SLM Instruments, Inc., using the same excitation wavelength and filters as above. All measurements were done at 37°C. The apparent quenching constant was calculated as the slope of the Stern-Volmer plot divided by the unquenched fluorescence lifetime of the carbazole moiety according to Eq. 5.

Partitioning data were analyzed using a weighted least-square fit program which assumed a standard deviation of 10% for determination of k_{app}. The error is based on the variability in the slope of the Stern-Volmer plot and the error in the measurement of τ_0.

RESULTS AND DISCUSSION

Option I

Fig. 1 shows partitioning plots for DDE in CUA-PC-labeled DMPC and CUA-PC-labeled DOPC dispersions determined in the absence and presence of unlabeled DOPC. In the presence of unlabeled membrane the slope of the line remained the same, but the intercept increased as predicted by the theory. Table I lists the partition coefficients and bimolecular quenching constants calculated according to Eq. 10. There was excellent agreement for both parameters determined in a two-phase and three-phase system. Thus, the three-phase theory is valid for determining partition coefficients and bimolecular quenching constants. The greatest difficulty in calculating the partition coefficient in a two-phase system results from the difficulty in determining which intercept is close to zero for compounds that strongly partition into the membrane. However, in a three-phase system, one can choose P_B and α_B such that the intercept is larger and therefore determined with greater ease.

Option II

Table II shows partition coefficients determined for unlabeled DOPC and DMPC dispersions measured relative to CUA-PC-labeled DOPC dispersions using Eq. 11. In both cases, there was good agreement between the partition coefficients determined by the three-phase and two-phase methods. Advantages of the three-phase system are as follows: (*a*) The unknown membrane is not labeled with a fluorescent probe, thus labeling of membranes with a fluorophore and the potential effects of such labeling are avoided. (*b*) The fluorescent signal is determined by the concentration of fluorophore in the reference membrane A. The concentration of fluorophore can be as high as necessary to get an adequate signal without worry about the probe-to-phospholipid ratio affecting the measurement as long as P_A and k_A are determined at the same probe-to-phospholipid ratio. (*c*) Once P_A and k_A have been determined, only one titration is necessary to generate a partition coefficient for membrane B.

This three-phase analysis will not distinguish between compounds that partition into the hydrophobic region of the membrane in membrane B and those that associate with the membrane-water interface. In addition, no information is obtained about the bimolecular quenching con-

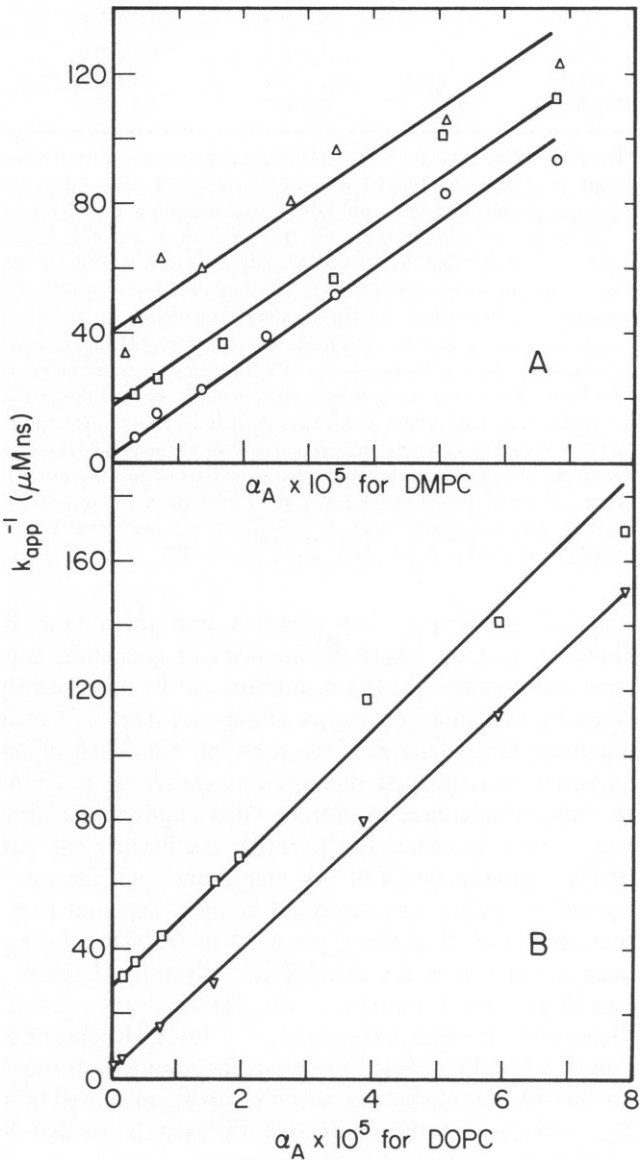

FIGURE 1 Partitioning of DDE into DMPC (*A*) and DOPC (*B*) dispersions. Each apparent quenching constant was determined from a Stern-Volmer plot at a given concentration of either CUA-PC-labeled DMPC (*A*) or CUA-PL-labeled DOPC (*B*) dispersions in the absence of unlabeled lipid (○) or in the presence of unlabeled DOPC dispersions such that α_B for unlabeled DOPC was 1.2×10^{-5} (□) or 2.4×10^{-5} (△). The volume fraction of the membrane was calculated assuming a membrane density of 1 g/ml. Lifetimes of CUA-PC-labeled DOPC and DMPC dispersions were 12.6 and 13.4 ns, respectively. Measurements were made at 37°C.

TABLE I

PARTITION COEFFICIENTS AND BIMOLECULAR QUENCHING CONSTANTS FOR DDE IN EITHER CARBAZOLE-LABELED DOPC OR DMPC DISPERSIONS IN THE PRESENCE OF UNLABELED DOPC DISPERSIONS*

CUA-PC labeled membrane	Unlabeled DOPC α_B	$P \times 10^{-6}$	$k_{qm} \times 10^{-8}(M^{-1}s^{-1})$
DOPC	0	1.2 ± 0.4	5.0 ± 0.4
DOPC	1.2×10^{-5}	1.0 ± 0.4	5.0 ± 0.4
DMPC	0	1.1 ± 0.4	6.8 ± 0.3
DMPC	1.2×10^{-5}	1.2 ± 0.5	7.2 ± 0.6
DMPC	2.4×10^{-5}	1.0 ± 0.4	7.3 ± 0.7

*Partition coefficients and bimolecular quenching constants were determined at 37°C using Eq. 10 with $P_B = 1.2 \times 10^6$. See Fig. 1 for experimental details. DOPC and DMPC are dioleoyl- and dimyristoyl-L-α-phosphatidylcholine, respectively. The value of P in DOPC dispersions (1.2×10^6) using the two-phase analysis was the average of four separate determinations for which the standard deviation was 38%. The remainder of the partition coefficients were calculated from one or two membrane preparations for which the data were pooled in one least-squares fit analysis according to Eq. 10. To estimate the error in the calculation of P for the three-phase system, both the error in determination of the slope-divided-by-the-intercept for that data and the error in P_A had to be considered. Simple propagation of error methods yield standard deviations of ~40% for the three-phase analysis. Thus the error in determination of P was not dramatically increased in the three-phase analysis. The bimolecular quenching constants are unaffected by this consideration and the standard deviations were 4–10%.

stant of compounds that partition into membrane B. However, in cases where the bimolecular quenching constant is of interest and the membrane can be fluorescently labeled, the bimolecular quenching constant is easily obtained. First, determine the partition coefficient of the quencher in unlabeled membranes relative to a CUA-PC-labeled reference membrane (this requires one titration). Then, knowing the partition coefficient, one can choose a concentration of this membrane such that, in a two-phase system, effectively all of the compound partitions into the membrane phase. The fraction of total quencher that is in the membrane is given by (Lakowicz and Hogen, 1977, 1980) $f_m = P\alpha_m/(P\alpha_m + 1 - \alpha_m)$. At high membrane concentrations $f_m \approx 1$. If a high concentration of CUA-PC-labeled unknown membrane is titrated, the bimolecular quenching constant can be calculated from $k_{qm} = k_{app}\alpha_m$. Again, only one titration is needed to generate k_{qm}.

In dealing with a three-phase system, there is a possibility that lipid exchange between the two different membrane populations may occur. The time required to generate an apparent quenching constant is ~15–20 min. For synthetic phospholipid systems, such as those used in this work, lipid exchange is insignificant in this time span. In general, lipid exchange may occur by either spontaneous transfer, fusion, or in biological membranes, enzymatic transfer. Enzymatic transfer seems to be mainly governed

TABLE II

PARTITION COEFFICIENTS OF DDE IN UNLABELED DISPERSIONS DETERMINED RELATIVE TO CUA-PC-LABELED DOPC DISPERSIONS*

	$P \times 10^{-6}$	
Unlabeled lipid	Three-phase analysis‡	Two-phase analysis§
DOPC	0.9 ± 0.6	1.2 ± 0.4
DMPC	0.8 ± 0.5	1.1 ± 0.4

*Measurements were made at 37°C. DMPC and DOPC are dimyristoyl- and dioleoyl-L-α-phosphatidylcholine, respectively. For the three-phase analysis, the standard deviation was approximated at 68%. This takes into account the error in determination of the partition coefficient and bimolecular quenching constant in the reference lipid and assumes a standard deviation of 10% for the determination of k_{app}. Hence, only one titration of the unknown lipid should give a value of P ± 68%.

‡k_{app} was determined by titrating lipid samples containing CUA-PC-labeled DOPC and unlabeled lipid such that $\alpha_A = \alpha_B = 5.9 \times 10^{-6}$. P was calculated from k_{app} with $P_A = 1.2 \times 10^{-6}$ and $k_A = 5.0 \times 10^8$ $M^{-1}s^{-1}$ according to Eq. 11.

§Partition coefficients from a two-phase analysis were determined by the method of Lakowicz and Hogen (1977, 1980).

by cytosolic phospholipid exchange proteins (Zilversmit and Hughes, 1976) and they can be eliminated by using purified membranes. Spontaneous transfer of lipids between membranes and membrane fusion can be rapid or slow depending upon the experimental conditions (Duckwitz-Peterlein et al., 1977; Bloj and Zilversmit, 1977; Nir et al., 1983). Thus, if these processes occur, conditions should be chosen to minimize them.

Application to Studies of DDE-Membrane Interactions

This methodology has been used to determine the partition coefficients of DDE in several different lipid systems

TABLE III

PARTITION COEFFICIENTS FOR DDE IN DISPERSIONS OF DIFFERENT LIPID COMPOSITION AT 37°C*

Lipid	$P \times 10^{-6}$
Dimyristoyl-PC§	0.8
Dipalmytoyl-PC	0.4
Distearoyl-PC	0.01
Stearoyl-oleoyl-PC	0.7
Dioleoyl-PC	0.9
Dioleoyl-PC/cardiolipin (9:1)	1.2
Dioleoyl-PC/sphingomyelin (8.5:1.5)	0.8
Dioleoyl-PC/dioleoyl-PE (1:1)	1.4‡
Dioleoyl-PC/cholesterol (3:1)	1.5

*P was determined by titrating samples containing CUA-PC-labeled DOPC in the presence of the test lipid such that $\alpha_A = \alpha_B = 5.9 \times 10^{-6}$ as described in Table II.

‡P was calculated using the two-phase analysis as described by Lakowicz and Hogen (1977, 1980).

§-PC and -PE refer to the appropriate -L-α-phosphatidylcholine and -L-α-phosphatidylethanolamine lipids.

(Table III). Phosphatidylcholines with saturated, unsaturated, and mixed saturated-unsaturated fatty-acid compositions were tested. For phosphatidylcholines that were above their main transition temperatures (DMPC, DOPC, and SOPC) the partition coefficients were similar ($\sim 10^6$). For DPPC, which is slightly below its phase transition temperature at 37°C, the partition coefficient was approximately twofold less, although the error in the data is too large to conclude that this is a significant difference. However, partitioning into DSPC dispersions at 37°C was 100-fold less than the other lipids. This is probably because at 37°C DSPC dispersions are well below their transition temperature (Hinz and Sturtevant, 1972). DOPC was mixed with other classes of lipids at ratios approximating their content in membranes. Addition of cardiolipin, sphingomyelin, phosphatidylethanolamine, or cholesterol did not significantly affect the partitioning of DDE into the dispersions. Hence it appears that for membranes above the phase transition temperature, partitioning behavior is not highly dependent upon the fatty acid composition, polar head group composition, or cholesterol content of the membrane. Since most cell membranes are near or above their phase transition temperatures at physiological temperatures, it is not likely that compounds such as DDE preferentially partition into membranes based on their lipid composition. However, if cell membranes contain areas of lipid in the gel phase, small molecules such as DDE may be excluded from these areas.

We thank Dr. J. R. Lakowicz for initial discussions on a three-phase analysis and Dr. Gregorio Weber and his group for use of their fluorescence lifetime instrument.

This work was supported by a National Institutes of Health grant GM 21953 and by a National Institutes of Health Postdoctoral Fellowship CA 06775 to G. M. Omann.

Received for publication 14 May 1984 and in final form 29 October 1984.

REFERENCES

Bloj, B., and D. B. Zilversmit. 1977. Complete exchangeability of cholesterol in phosphatidylcholine/cholesterol vesicles of different degrees of unsaturation. *Biochemistry.* 16:3943–3948.

Duckwitz-Peterlein, G., G. Eilenberger, and P. Overath. 1977. Phospholipid exchange between bilayer membranes. *Biochim. Biophys. Acta.* 469:311–325.

Hinz, H.-J., and J. M. Sturtevant. 1972. Calorimetric studies of dilute aqueous suspensions of bilayers formed from synthetic L-α-lecithins. *J. Biol. Chem.* 247:6071–6075.

Lakowicz, J. R., and D. Hogen. 1977. Diffusional transport of toxic materials in membranes studied by fluorescence spectroscopy. *In* Membrane Toxicity. M. W. Miller and A. E. Shamoo, editors. Plenum Publishing Corp, New York. 509–546.

Lakowicz, J. R., and D. Hogen. 1980. Chlorinated hydrocarbon-cell membrane interactions studied by the fluorescence quenching of carbazole-labeled phospholipids: Probe sysnthesis and characterization of the quenching methodology. *Chem. Phys. Lipids.* 26:1–40.

Lakowicz, J. R., D. Hogen, and G. Omann. 1977. Diffusion and partitioning of a pesticide, lindane, into phosphatidylcholine bilayers: A new fluorescence quenching method to study chlorinated hydrocarbon-membrane interactions. *Biochim. Biophys. Acta.* 471:401–411.

Nir, S., J. Bentz, J. Wilshut, and N. Duzgunes. 1983. Aggregation and fusion of phospholipid vesicles. *Prog. Surf. Membr. Sci.* 13:1–124.

Omann, G. M., and M. Glaser. 1985. Biosynthetic incorporation of fluorescent carbazolylundecanoic acid into membrane phospholipids of LM cells and the determination of quenching constants and partition coefficients of hydrophobic quenchers. *Biochemistry.* 23:4962–4969.

Omann, G. M., and J. R. Lakowicz. 1982. Interactions of chlorinated hydrocarbon insecticides with membranes. *Biochem. Biophys. Acta.* 684:83–95.

Selinger, Z., and Y. Lipidot. 1966. Synthesis of fatty acid anhydrides by reaction with dicyclohexylcarbodiimide. *J. Lipid Res.* 7:174–175.

Spencer, R. D., and G. Weber. 1969. Measurement of subnanosecond fluorescence lifetimes with a cross-correlation phase fluorometer. *Ann. NY Acad. Sci.* 158:361–376.

Zilversmit, D. B., and M. E. Hughes. 1976. Phospholipid exchange between membranes. *Methods Membr. Biol.* 7:211–259.