Biosynthetic Incorporation of Fluorescent Carbazolylundecanoic Acid into Membrane Phospholipids of LM Cells and Determination of Quenching Constants and Partition Coefficients of Hydrophobic Quenchers†

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ABSTRACT: A fluorescence quenching method was developed for determining partition coefficients and diffusional rates of small molecules in cell membranes. This method involves quenching the fluorescence of carbazole-labeled membranes by hydrophobic molecules that partition into membranes. Cell membrane phospholipids of mouse LM cells in tissue culture were biosynthetically labeled with the carbazole moiety by supplementing the growth media with 11-(9-carbazolyl)undecanoic acid. Plasma membranes, microsomes, and mitochondria were isolated free of nonmembranous neutral lipids, and the incorporation of the fluorescent probe was characterized. Quenching studies of the carbazole moiety by a series of N-substituted picolinium perchlorate salts showed that the carbazole moiety was located in the hydrophobic interior of the membrane bilayer. The carbazole fluorescence also was quenched by the hydrophobic quenchers lindane, methoxychlor, and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene, indicating that these compounds partitioned into the membrane. Stern–Volmer quenching constants determined by fluorescence lifetime and intensity measurements were identical, as expected for dynamic quenching. The effects of different lipid compositions on quenching constants and partition coefficients were determined by comparing different membrane fractions. These parameters also were measured in membranes from cells in which the phospholipid composition was altered by substituting ethanolamine for choline in the growth medium. Changes in the lipid composition produced changes in the bimolecular quenching constants. For example, bimolecular quenching constants for 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene were higher in mitochondrial membranes than in plasma membranes and microsomes. They were also higher in dispersions made from membrane phospholipids as compared with intact membranes or total lipid dispersion. These differences were due primarily to the relative absence of sterol from mitochondria as compared with the other membranes and the absence of sterol from phospholipid dispersions as compared with intact membranes and total lipid dispersions. Partition coefficients for a given quencher were similar in intact membranes and in dispersions made from total lipid and phospholipid extracts. The results show that the partition coefficients depend on the nature of the quencher but do not depend to any significant extent on differences in the membrane lipid composition, the presence of proteins, and other features of intact membranes.

Partitioning and diffusion of amphipathic and hydrophobic molecules in membranes are important for a variety of cellular phenomena. The major objective of this study was to develop a sensitive fluorescence method to measure partition coefficients, in particular, and to examine how this parameter is affected by changes in the lipid composition, protein content, and other properties of cellular membranes. Mouse LM cells were selected for this work since they have been extensively used for studies on animal cell membranes and it is relatively easy to manipulate their lipid composition by changing selective components in the growth medium. The phospholipid polar head group composition can be altered by growing the cells in medium with various choline analogues replacing choline (Glaser et al., 1974; Blank et al., 1975), or the fatty acid composition of the phospholipid can be altered by growing the cells in media supplemented with exogenous fatty acids (Wisnieski et al., 1973; Williams et al., 1974; Ferguson et al., 1975; Doi et al., 1978) or both simultaneously (Glaser et al., 1974). In addition, the cholesterol content can be reduced by using LM cell mutants, which are deficient in cholesterol synthesis (Saito et al., 1977), or by depleting membrane cholesterol (Heiniger et al., 1976; Chen et al., 1978; Welti et al., 1981).

A novel fluorescence quenching method has been used to measure the partition coefficients and diffusional rate of chlorinated hydrocarbons in synthetic phospholipid vesicles (Lakowicz et al., 1977; Lakowicz & Hogen, 1980). The method employs a fluorescent probe, β-[11-(9-carbazolyl)-undecanoyl]-L-α-phosphatidylcholine (CUA-PC),[1] where the carbazole moiety is located in the hydrophobic region of the bilayer. The presence of chlorinated hydrocarbon in the bilayer is detected as quenching of the carbazole moiety's fluorescence lifetime. This phenomenon was applied to study the interaction of chlorinated hydrocarbons with synthetic phospholipid vesicles (Omann & Lakowicz, 1981, 1982). Quenching constants, which are related to the rate of diffusion of the quenchers, and partition coefficients depended on the lipid composition of the vesicles. Partition coefficients are also parameters of recent interest since the work of Conrad & Singer (1979, 1981) suggested that some molecules that partition into liposomes do not partition to any great extent into biological membranes.

In this paper we show that the quenching methodology can be applied to biological membranes. LM cells incorporated

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[1] Abbreviations: CUA, 11-(9-carbazolyl)undecanoic acid; CUA-PC, β-[11-(9-carbazolyl)undecanoyl]-L-α-phosphatidylcholine; CUA-PE, β-[11-(9-carbazolyl)undecanoyl]-L-α-phosphatidylethanolamine; PBS, phosphate-buffered saline; lindane, γ-1,2,3,4,5,6-hexachlorocyclohexane; methoxychlor, 1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl)ethane; DDE, 1,2-dichloro-2,2-bis(*p*-chlorophenyl)ethylene; DPPC, dipalmitoyl-L-α-phosphatidylcholine; DMPC, dimyristoyl-L-α-phosphatidylcholine; DOPC, dioleoyl-L-α-phosphatidylcholine; POPC, palmitoyloleoyl-L-α-phosphatidylcholine; DPPC, dipalmitoyl-L-α-phosphatidylcholine.

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carbazolylundecanoic acid (CUA) into their membrane phospholipids when CUA was added to the medium. The carbazole moiety was located in the hydrophobic region of the bilayer. This method was used to determine partition coefficients and bimolecular quenching constants of three quenchers, lindane, methyoxychlor, and DDE, in different membrane fractions and isolated lipids. The results are discussed in relation to the effects of phospholipid composition, sterol content, and protein content.

Materials and Methods

Materials. DDE (Aldrich Chemical Co., Milwaukee, WI) was 99% pure. Commercial-grade lindane was obtained from Sigma Chemical Co. and recrystallized 3 times from methanol. Methoxychlor was obtained from Sigma Chemical Co. at 98% purity. 11-(9-Carbazolyl)undecanoic acid (CUA) was obtained from Molecular Probes, Inc. β-[11-(9-Carbazolyl)-undecanoyl]-L-α-phosphatidylcholine (CUA-PC) was synthesized and purified as described by Lakowicz & Hogen (1980) except that the formation of the fatty acid anhydride was done at room temperature (Selinger & Lipidot, 1966) and all reactions were done with toluene instead of benzene as the solvent. The corresponding phosphatidylethanolamine (CUA-PE) was formed from CUA-PC by the action of phospholipase D (Sigma Chemical Co.) as described by Yang et al. (1967). *N*-Methyl-, *N*-benzyl-, and *N*-hexadecyl-4-picolinium perchlorates were synthesized by the method of Shinitzky & Rivnay (1977).

Tissue Culture Methods and Membrane Fractionation. LM cells were grown in suspension culture in Higuchi's medium supplemented with choline or ethanolamine as described previously (Glaser et al., 1974; Esko et al., 1977). Two days after splitting the cells into fresh media, the media were supplemented with CUA as a bovine serum albumin complex (Spector & Hoak, 1969). The cells were harvested 18–22 h later. LM cell plasma membranes, mitochondria, and microsomes were isolated as previously described (Gilmore et al., 1979).

All membranes were suspended in PBS (137 mM NaCl, 2.7 mM KCl, 12.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2). Membrane samples were stored on ice and in the dark until used in quenching experiments, usually within 2 days. Protein concentrations were determined by the method of Lowry et al. (1951).

Lipid Extraction and Characterization. Membrane lipids were extracted by the method of Bligh & Dyer (1959) and described by Ames (1968). This lipid extract, containing neutral lipids and phospholipids, is referred to as the total lipid fraction. Neutral lipids and phospholipids were separated by silicic acid chromatography on Unisil columns (Clarkson Chemical Co.) as described by Ferguson et al. (1975). The different phospholipid species were isolated by two-dimensional thin-layer chromatography on silica gel G as described previously (Esko et al., 1977). The fluorescent spots were scraped and then the plates stained with I_2 to detect the nonfluorescent spots. Phospholipids were eluted from the silica gel by using $CHCl_3$–methanol–acetic acid–H_2O (5:5:1:1). The amount of CUA present was determined by measuring the absorbance in $CHCl_3$ with a molar absorptivity of 3560 cm^{-1} M^{-1} at 333 nm or by determining the carbazole fluorescence intensity of a methanol solution relative to a standard curve. Phospholipid phosphate was determined by the method of Bartlett as described by Kates (1972).

Formation of Lipid Dispersions. An aliquot of extracted lipids in chloroform was added to a test tube and the solvent evaporated under a stream of N_2 at 40 °C. PBS was added, and the samples were hydrated at 40 °C and then vortexed. The samples were sonicated in a bath-type sonicator for two 1-min bursts or until all the solid phospholipid was dispersed.

Fluorescence Instrumentation. Fluorescence intensity measurements were performed on an SLM 8000 fluorometer without polarizers or emission monochromators. Lifetimes were measured on the cross correlation phase fluorometer described by Spencer & Weber (1969) with updated electronics by SLM Instruments, Inc., using a modulation frequency of 18 MHz. Intensities and lifetimes were measured with a Corning 7-54 excitation filter and Corning 0-52 and 7-60 emission filters. The excitation wavelength was 328 nm unless otherwise stated. Fluorescence emission spectra were recorded on the spectrofluorometer described by Wehrly et al. (1976) and Jameson et al. (1977). Measurements were done at 37 °C unless otherwise noted.

Quenching of Carbazole-Labeled Membranes by Titration with Ethanol-Solubilized Quenchers. Two milliliters of fluorescently labeled cell membranes were placed in a fluorescence cuvette, and the fluorescence intensity was monitored while titrating the sample with microliter additions of quencher in ethanol solution (Lakowicz & Hogen, 1980). The final ethanol concentration in the sample did not exceed 1%. After each addition, the sample was mixed and then allowed to equilibrate for approximately 2 min before measuring the fluorescence intensity. Samples that were allowed to equilibrate longer showed no significant change in intensity, indicating that partitioning into the membrane was complete after 2 min. An identical sample was titrated with ethanol only to correct for small time-dependent changes in the carbazole moiety's fluorescence that were observed at low lipid concentration. Background fluorescence from the membranes was determined by measuring comparable concentrations of membranes without CUA-labeled phospholipids. This procedure will be referred to as the "standard titration method".

The carbazole moiety absorbs maximally at 295 nm. However, in cell membranes, proteins absorb in this region and the protein fluorescence overlaps with the carbazole emission. Thus, an excitation wavelength of 328 nm was used. This resulted in a Raman scatter peak in the region of the carbazole fluorescence. At very low concentrations of membrane, this background was as much as 50% of the total intensity. Hence, it was difficult to determine Stern–Volmer plots from lifetime data. The background signal was very stable and not affected by addition of the quenchers in ethanol. Thus, it was easily subtracted from fluorescence intensity measurements. As a result, Stern–Volmer plots were generated by using intensity measurements, and the unquenched fluorescence lifetime was measured in membrane samples of sufficiently high CUA concentration such that background was insignificant.

Fluorescence Quenching and Partitioning Theory. Quenching of fluorescence by dynamic quenchers is described by the Stern–Volmer equation (1919)

$$F_0/F = 1 + K[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of the quencher, [Q] is the quencher concentration, and K is the quenching constant. K is equal to $k_q\tau_0$, where k_q is the bimolecular quenching constant and τ_0 is the fluorophore's lifetime in the absence of the quencher. For a fluorophore imbedded in a membrane, an apparent bimolecular quenching constant, k_{app}, is observed because the amount of quenching depends upon the partition coefficient of the quencher between the aqueous and membrane phases. This

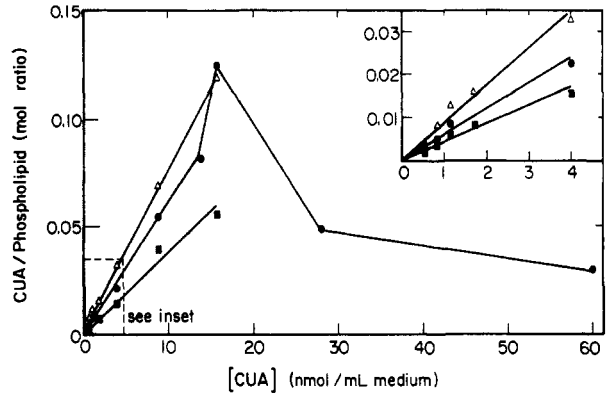

FIGURE 1: Incorporation of CUA into LM cell plasma membranes, microsomes, and mitochondria. Cells were grown for 48 h and then CUA was added as a bovine serum albumin complex. Cells were harvested after 18 h of growth in CUA-supplemented medium, and various membrane fractions were isolated. Phospholipids from microsomes (●), mitochondria (▲), and plasma mambranes (■) were isolated and characterized as described under Materials and Methods.

dependence is described by the linear equation (Lakowicz & Hogen, 1977; Lakowicz et al., 1977)

$$\frac{1}{k_{app}} = \left[\frac{1}{k_{qm}} - \frac{1}{k_{qm}P}\right]\alpha_m + \frac{1}{k_{qm}P} \quad (2)$$

where P is the ratio of the concentration of quencher in the membrane to the concentration of quencher in the buffer, α_m is the volume fraction of the membrane, and k_{qm} is the bimolecular quenching constant in the membrane. By measurement of the apparent bimolecular quenching constant at various volume fractions of the membrane, k_{qm} and P can be determined from eq 2. The apparent bimolecular quenching constant was calculated as the slope of the Stern–Volmer plot (eq 1) divided by the unquenched carbazole fluorescence lifetime. The error in reproducibility of the Stern–Volmer plots and the error in determination of the lifetime resulted in a maximum estimated standard deviation of 10% for k_{app}. For calculation of partition coefficients and bimolecular quenching constants in a given membrane, apparent quenching constants were determined for several lipid concentrations and several membrane preparations. The data were pooled in a weighted least-squares fit analysis, and estimated standard deviations were calculated for P and k_{qm}.

The quenching data can also be analyzed according to the modified Stern–Volmer equation proposed by Lehrer (1971)

$$\frac{F_0}{F_0 - F} = \frac{1}{[Q]f_a K} + \frac{1}{f_a} \quad (3)$$

where f_a is the fraction of fluorophores accessible to the quencher. In this case, a plot of $F_0/(F_0 - F)$ vs. $[Q]^{-1}$ yields a straight line. An intercept of 1 indicates 100% accessibility of the fluorophore to quencher.

Results

Characterization of CUA-Labeled Cells. CUA was incorporated into the phospholipids of LM cells in a concentration-dependent manner. For quenching studies plasma membranes, microsomes, and mitochondria from LM cells were purified to avoid interference by nonmembranous triacylglycerols and alkyldiacylglycerols (Esko et al., 1977; Pessin et al., 1978). Figure 1 shows the concentration dependence of CUA incorporation into these three membranes. Incorporation of CUA into the membrane phospholipids was linear up to 16 nmol of CUA/mL of medium. Greater amounts of CUA were incorporated into mitochondria and microsomes than into plasma membranes. These cells had been growing in CUA-supplemented medium for 18–22 h. Studies of the time dependence of CUA incorporation into these cells showed that incorporation of CUA was complete by this time (data not shown) as found for other fatty acids under these conditions (Ferguson et al., 1975; Hale et al., 1977; Pessin et al., 1978). Quenching experiments generally were done at CUA to phospholipid ratios of less than 1:125. This corresponded to a supplementation concentration of approximately 1 nmol of CUA/mL of medium. When membrane neutral lipids and phospholipids were isolated, a small amount of fluorescence was detected in the neutral lipid fraction. This may have resulted from incomplete removal of cellular triacylglycerols and alkyldiacylglycerols during the fractionation procedure. However, at low supplementation concentrations (<2 nmol of CUA/mL of medium) the fluorescence in the neutral lipid fraction was insignificant.

Table I: Incorporation of CUA into Different Phospholipid Species[a]

membrane	% CUA in phospholipid species		
	PC	PS + PI	PE
plasma membrane	71.5 (0.170)	13.6 (0.062)	14.9 (0.042)
microsomes	81.4 (0.080)	2.1 (0.053)	16.6 (0.067)
mitochondria	81.9 (0.276)	3.8 (0.041)	14.3 (0.048)

[a] LM cells were grown and supplemented with CUA, the membrane fractions isolated, and their phospholipids extracted. The different phospholipid species were resolved by two-dimensional thin-layer chromatography, and the percent of total CUA found in that species was calculated. Phospholipid phosphate was determined and the ratio of CUA to phospholipid calculated. The number in parentheses indicates the mole ratio of CUA to phospholipid for that phospholipid species. PC = phosphatidylcholine; PS + PI = phosphatidylserine plus phosphatidylinositol; PE = phosphatidylethanolamine.

The absorbance and fluorescence emission spectra of CUA-labeled LM cell membranes were the same as spectra of CUA in methanol. Thus, the carbazole moiety was not metabolized or chemically altered upon incorporation into the cell lipids.

Table I shows the amount of CUA incorporated into the various phospholipid species. Most of the CUA was incorporated into phosphatidylcholine not only because phosphatidylcholine was the predominant lipid species (Gilmore et al., 1979) but also because the ratio of CUA to phospholipid was highest in phosphatidylcholine.

The location of the carbazole moiety in the membranes was determined by using the picolinium perchlorate salts described by Shinitzky & Rivnay (1977). *N*-Methyl-, *N*-benzyl-, and *N*-hexadecyl-4-picolinium perchlorates were equally efficient quenchers of the carbazole moiety in ethanol solution with bimolecular quenching constants of approximately 2×10^{10} M^{-1} s^{-1}.

In aqueous suspensions of membranes, *N*-methyl-4-picolinium perchlorate quenches fluorophores in the aqueous phase, *N*-benzyl-4-picolinium perchlorate is soluble in the aqueous phase and presumably accumulates at the hydrocarbon–water interface, and *N*-hexadecyl-4-picolinium perchlorate partitions into the lipid bilayer (Shinitzky & Rivnay, 1977). Figure 2 shows Stern–Volmer plots and modified Stern–Volmer plots for quenching in membranes. In plasma membranes, microsomes, mitochondria, and dispersions of whole cell phospholipids, no quenching was seen with the *N*-methyl-4-picolinium perchlorate or *N*-benzyl-4-picolinium perchlorate. Thus, the carbazole moiety was not located in the aqueous phase or at the head group–water interface. In

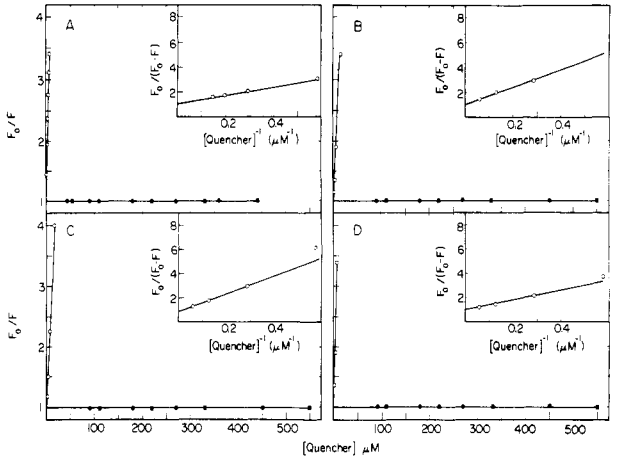

FIGURE 2: Localization of the CUA moiety in (A) whole cell phospholipid dispersions, (B) plasma membranes, (C) microsomes, and (D) mitochondria. CUA-labeled LM cells were grown and membranes isolated as described in Figure 1. The fluorescence intensity of the carbazole moiety was measured at 37 °C as the membrane samples were titrated by addition of *N*-methyl- (■), *N*-benzyl- (●), or *N*-hexadecylpicolinium perchlorate (○). The inset in each panel is a modified Stern–Volmer analysis (eq 3) of the data for *N*-hexadecylpicolinium perchlorate.

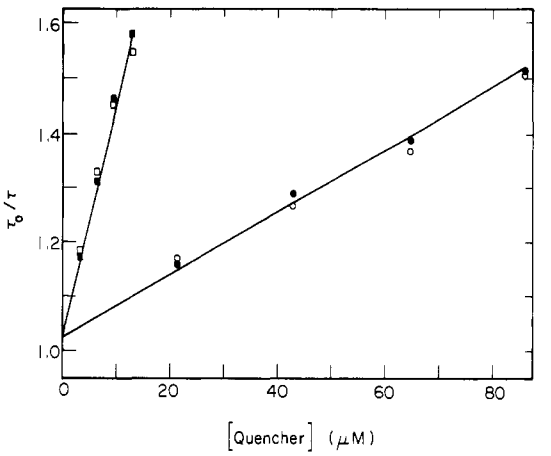

FIGURE 3: Reversibility of uptake in microsomes. CUA-labeled microsomes (0.1 mg of protein/mL) were incubated with either 86 μM lindane or 12.6 μM DDE for 15 min at 37 °C. These microsomes were then mixed with different amounts of unlabeled microsomes (0.1 mg of protein/mL) containing no CUA or quencher to decrease the total quencher concentration while maintaining a constant lipid concentration. These mixtures were incubated for 45 min at 37 °C, and then the fluorescence lifetimes were determined. The points shown in the figure represent the final quencher concentrations [(○) lindane; (□) DDE]. Quenching as monitored by lifetime measurements was also determined for the microsomes (0.1 mg of protein/mL) by the standard titration method described under Materials and Methods [(●) lindane; (■) DDE]. The excitation wavelength was 328 nm, and the ratio of CUA to phospholipid was 1/26.

contrast, quenching by *N*-hexadecyl-4-picolinium perchlorate was substantial. The concentration of *N*-hexadecyl-4-picolinium perchlorate used in this experiment was below the concentration that causes membrane disruption (Gilmore, 1980). Essentially 100% of the fluorophores were accessible to quenching by the *N*-hexadecyl-4-picolinium perchlorate as determined by using eq 3. Thus, these data suggest that the carbazole moiety was located in the internal bilayer region.

Because calculation of k_{app} from intensity data required knowledge of the unquenched probe lifetime, it was necessary to determine τ_0 for the CUA-labeled phospholipids in membranes. The carbazole moiety's lifetime was determined in whole cell phospholipid dispersions of different CUA to phospholipid ratios. τ_0 was 13.2 ns and did not vary within experimental error when the CUA to phospholipid ratio was varied over a range of 1:60 to 1:240. Nor did the lifetime differ in phospholipid dispersions labeled with synthetic CUA-PC or CUA-PE or biosynthetically labeled phospholipids. The lifetimes of the carbazole moiety in plasma membranes and microsomal membranes were also the same. However, the lifetime in mitochondria was slightly lower. This was also true for total lipid and phospholipid dispersions made from the membranes. For calculation of k_{app}, an average modulation lifetime of 13.2 ns was used for the plasma membrane, microsomal membrane, and their total lipid and phospholipid dispersions. A value of 11.5 ns was used for the mitochondrial membrane and its total lipid and phospholipid dispersions. Supplementation of cells with ethanolamine in place of choline did not change the lifetime either. The error in the measurement of the lifetime was 0.5 ns.

Characterization of Quenching by Chlorinated Hydrocarbons in Membranes. The vehicle used to add the quencher to the membrane suspension was ethanol. Previous studies with model membranes showed that small amounts of ethanol did not affect the interaction of the chlorinated hydrocarbon quencher with synthetic phospholipid membranes (Lakowicz & Hogen, 1980; Lakowicz et al., 1977). Evidence that ethanol did not affect the quencher–membrane interaction in cell membranes is as follows. If ethanol was added to a membrane sample at a concentration of 0.5% and then the sample titrated with a quencher such as DDE or lindane by the standard titration method, the quenching constant was the same as that of a sample without 0.5% ethanol added prior to titration (data not shown). In addition, an aliquot of ethanol added to a membrane sample containing quencher did not alter the fluorescence intensity, indicating that the added ethanol did not cause the quencher to move in or out of the membrane or alter its diffusional rate. In total, these experiments indicate that ethanol had not altered the quencher–membrane interaction and therefore was a convenient vehicle for doing the titrations.

Figure 3 confirms that membrane uptake of the quenchers was reversible. CUA-labeled membranes were incubated with quencher and then mixed with unlabeled membranes at the same membrane concentration. If uptake was reversible, the quencher would distribute itself equally among all the lipid present. Thus, upon addition of unlabeled membranes to CUA-labeled membranes with quencher, the quencher would move out of the CUA-labeled membranes causing an increase in the fluorescence lifetime. Figure 3 shows that Stern–Volmer plots determined by this method were identical with the standard titration method. Although not shown in Figure 3, the same result was observed in mitochondria and plasma membrane. Thus, the partitioning observed was a true equilibrium process. This experiment also demonstrates that transfer of quencher between membranes is rapid, since the reequilibration is complete within the 45-min incubation period allowed.

Because previous work with carbazole as a probe for quenchers utilized fluorescence lifetime measurements, it was necessary to confirm that intensity data gave the same result. Apparent quenching constants were determined for the same membrane sample by measuring both fluorescence intensities and lifetimes (Figure 4). For both lindane and DDE, the apparent quenching constants determined by lifetime measurements and intensity measurements were the same. Hence, the quenching process was dynamic. Methoxychlor was not tested in this experiment. Because its quenching efficiency is low, the absolute change in lifetime upon quenching is small relative to the 0.5-ns error in determination of the lifetime.

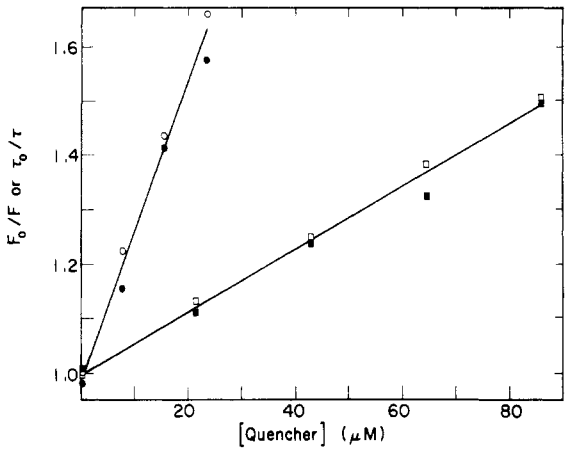

FIGURE 4: Equivalence of quenching curves determined by measurements of fluorescence lifetimes and intensities. Whole cell phospholipid dispersions were titrated with DDE (○, ●) or lindane (□, ■) by the standard titration method described under Materials and Methods. Fluorescence intensities (○, □) or phase lifetimes (●, ■) were monitored. Measurements were made at 37 °C using an excitation wavelength of 328 nm. The ratio of CUA to phospholipid was 1/26, and the phospholipid concentration was 200 nmol/mL.

However, the quenching can be accurately determined by intensity measurements.

Since the different membrane fractions differed in the distribution of CUA among the different phospholipid species, it was necessary to determine if the quenching constant differed depending upon the phospholipid species to which the CUA was attached. Phospholipid dispersions of whole cell phospholipids were mixed with a small amount of either synthetic CUA-PC, CUA-PE, or biosynthetically CUA-labeled phospholipids. The quenching constants for DDE, methoxychlor, and lindane in CUA-PC- and CUA-PE-labeled dispersions were the same as for dispersions containing biosynthetically CUA-labeled phospholipids (data not shown). Thus, variation of the CUA distribution among the different phospholipid species of the membranes had no effect on the interaction of the quenchers with the membrane. Since the different membrane fractions had different CUA to phospholipid ratios, it was also necessary to determine if the CUA to phospholipid ratio had an effect on the observed quenching constant. For these studies, phospholipid dispersions were made from whole cell phospholipids. In these dispersions, the CUA to phospholipid ratio was varied by mixing phospholipids from cells grown with CUA and cells grown without CUA, but the total lipid concentration was constant. Analysis of the phospholipid composition of the CUA-labeled and nonlabeled cells showed identical patterns (data not shown). There was also no change in the quenching constant for lindane, methoxychlor, and DDE over a CUA to phospholipid range from 1/100 to 1/1200. This was consistent with data for quenching constants of lindane in synthetic dipalmitoylphosphatidylcholine vesicles (Lakowicz & Hogen, 1980). For all studies of the interaction of quenchers with membranes, a lipid to probe ratio of greater than 125/1 was used.

Partitioning and Diffusion of Quenchers in Membranes. Figure 5 shows representative Stern–Volmer plots for DDE quenching of various concentrations of LM cell microsomes. In Figure 6, the reciprocal of the apparent quenching constants calculated from data such as shown in Figure 5 is plotted vs. the volume fraction of the membrane. The linearity of these partitioning plots for all three quenchers over a 100-fold range of lipid concentration demonstrated excellent agreement with the theory (eq 2) and indicates that a true partitioning process was being observed.

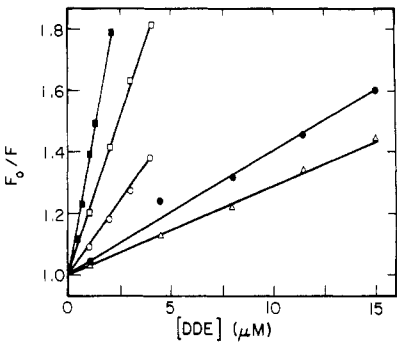

FIGURE 5: Stern–Volmer plots for DDE partitioning into LM cell microsomes at 37 °C. Samples were prepared at different membrane concentrations and titrated with DDE as described under Materials and Methods. Microsomal phospholipid concentrations were 182 (△), 122 (●), 61 (○), 24 (□), and 11 (■) nmol/mL.

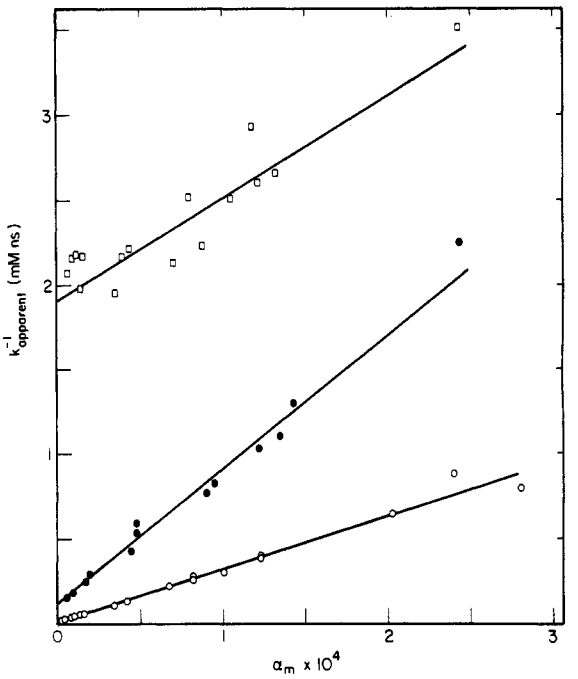

FIGURE 6: Partitioning plot for lindane (□), methoxychlor (●), and DDE (○) in CUA-phospholipid-labeled LM cell microsomes. CUA-labeled LM cells were grown in medium with choline, and microsomes were isolated. Apparent quenching constants at various lipid concentrations were determined by adding quencher to the samples while monitoring the fluorescence intensity and using eq 1. Measurements were done at 37 °C, the fluorescence excitation wavelength was 328 nm, and the CUA to phospholipid ratio was less than 1/125. The volume fraction of the membrane (α_m) was calculated after measuring the phospholipid concentration of the membrane sample and assuming an average phospholipid molecular weight of 790 and a membrane density of 1 g/cm^3.

When the data were analyzed according to the modified Stern–Volmer analysis (eq 3), the results indicated that essentially 100% of the carbazole moieties were accessible to the quenchers.

When cells were grown in normal medium containing choline, plasma membrane and microsomes had higher amounts of sterol, sphingomyelin, and phosphatidylinositol plus phosphatidylserine than mitochondria (data not shown). To compensate for this, mitochondria had more cardiolipin than plasma membranes or microsomes. The same differences in the membranes were true for cells grown in medium supplemented with ethanolamine, although the ethanolamine-supplemented cell membranes had higher amounts of phosphatidylethanolamine, primarily at the expense of phosphatidylcholine. These results were consistent with a more extensive

Table II: Partition Coefficients of Chlorinated Hydrocarbons in Membranes from Choline- and Ethanolamine-Supplemented LM Cell Membranes[a]

quencher	membrane fraction	partition coefficient: whole membrane	total lipid	phospholipid
	Choline-Supplemented Cells			
lindane	plasma membrane	3×10^3	4×10^3	3×10^3
	microsomes	3×10^3	3×10^3	5×10^3
	mitochondria	4×10^3	3×10^3	
methoxychlor	plasma membrane	7×10^4		
	microsomes	8×10^4		
	mitochondria	8×10^4		
DDE	plasma membrane	3×10^5	5×10^5	6×10^5
	microsomes	6×10^5	5×10^5	6×10^5
	mitochondria	5×10^5	7×10^5	5×10^5
	Ethanolamine-Supplemented Cells			
DDE	plasma membrane	10×10^5[b]	7×10^5	5×10^5
	microsomes	5×10^5	9×10^5	7×10^5
	mitochondria	10×10^5[b]	7×10^5	9×10^5

[a] For total lipid and phospholipid dispersions the excitation wavelength was 328 or 295 nm, but for whole membranes only 328-nm excitation was used to avoid interference by protein fluorescence. The CUA to phospholipid ratio was always less than 1/125. The standard deviation of the partition coefficients was estimated to be 25% unless otherwise noted. Measurements were done at 37 °C. Data was pooled for from one to seven separate membrane preparations for each partition coefficient. [b] The standard deviation was approximately 50% in these experiments.

analysis previously published (Gilmore et al., 1979).

Table II shows partition coefficients determined by the carbazole quenching method in membrane fractions from choline- and ethanolamine-supplemented cells. In addition, partition coefficients were determined in dispersions of total lipids and phospholipids isolated from these membranes. The partition coefficient depended on the properties of the particular quencher in the experiment. However, there were not large differences between the partition coefficients of a given quencher in the three membranes. Also, there was not a significant difference in partitioning of DDE into membranes between choline- and ethanolamine-supplemented cells. Thus, the partition coefficient was not highly sensitive to differences in the lipid composition of the membranes. It should be noted that the estimated standard deviation for the calculation of the partition coefficients was approximately 25%. Thus, slight variations in the partition coefficients may not have been detected. There also were no significant differences in partitioning between whole membranes and dispersions of total lipid or phospholipids. This suggested that proteins and neutral lipids in the membranes were not major factors in determining the partition coefficient. If the partition coefficients for a given quencher were averaged over all the types of membranes, the partition coefficients for lindane, methoxychlor, and DDE were 4×10^3, 8×10^4, and 7×10^5, respectively.

Table III shows the bimolecular quenching constants of the quenchers in the various membrane fractions and dispersions of total lipids and phospholipids given in Table II. For lindane and methoxychlor, there were no obvious differences between the membranes, or for lindane in whole membranes, total lipids or phospholipids. However, bimolecular quenching constants for DDE in choline-supplemented cell membranes were higher in mitochondria than in plasma membranes and microsomes and were also higher in mitochondrial total lipid and phospholipid dispersions than in lipid dispersions from the other membranes. The values from phospholipid dispersions for a given membrane were larger than in total lipid extracts or in the membrane itself. Similar trends also were seen in membranes and lipid dispersions from ethanolamine-supplemented cells, but the bimolecular quenching constants were lower than in choline-supplemented membranes and dispersions.

Table III: Bimolecular Quenching Constants (k_{qm}) of Chlorinated Hydrocarbons in Membranes from Choline- and Ethanolamine-Supplemented Cell Membranes[a]

quencher	membrane fraction	bimolecular quenching constant $\times 10^{-8}$ (M^{-1} s^{-1}): whole membrane	total lipid	phospholipid
	Choline-Supplemented Cells			
lindane	plasma membrane	1.7	1.2	2.1
	microsomes	1.7	1.8	1.6
	mitochondria	1.9	2.2	
methoxychlor	plasma membrane	1.3		
	microsomes	1.2		
	mitochondria	1.2		
DDE	plasma membrane	3.3	2.8	4.1
	microsomes	3.2	3.3	4.3
	mitochondria	3.7	3.9	5.2
	Ethanolamine-Supplemented Cells			
DDE	plasma membrane	2.1	2.5	4.1
	microsomes	2.6	2.5	3.4
	mitochondria	2.5	3.4	4.8

[a] The experimental details were the same as in Table II. The standard deviations calculated for lindane, methoxychlor, and DDE were approximately 20%, 10%, and 5%, respectively.

Discussion

CUA was readily incorporated into the phospholipids of LM cells when the media were supplemented with CUA as a bovine serum albumin complex. Quenching of the carbazole moiety in membranes by aqueous and lipid soluble quenchers shows that the probe is located within the bilayer and therefore is a detector of quenchers that partition into the bilayer. This is an important difference from the more commonly used centrifugation or filtration methods of determining partition coefficients where it is not clear if the compound actually partitions into the interior of the bilayer or if it is only loosely attached at the membrane interface [see Conrad & Singer (1979, 1981)]. Quenching was not affected when the CUA to phospholipid ratio was varied below 1/100 or when the probe polar head group composition was varied. The uptake of quencher into the membrane was shown to be reversible. Thus, the carbazole quenching method appears to be an excellent technique for determining partition coefficients and diffusional rates of quenchers in biological membranes.

In previous studies using the carbazole quenching method in synthetic phospholipids, quenching was detected by measuring the fluoroscence lifetime (Lakowicz et al., 1977; Omann & Lakowicz, 1982). In the present studies, fluorescence intensities were measured to detect quenching, and τ_0 was determined in samples of higher CUA content. Measurement of fluorescence intensities allowed for greater sensitivity because the intensity instruments used for this study were more sensitive than the available lifetime instrument. The relative error in measurement of intensities was significantly less than that of lifetime measurements. In addition, it was much easier to subtract background light from intensity measurements than from lifetime measurements. This allowed lower concentrations of lipid and lower ratios of CUA to phospholipid to be used in the measurement of the Stern–Volmer plots. Comparison of Stern–Volmer plots determined by lifetimes and intensities (for samples at sufficiently high lipid concentration and CUA to phospholipid ratio so τ could be accurately measured) were the same. Thus, the quenching mechanism was dynamic.

It was expected that changes in the lipid composition of the membrane would result in changes in the partition coefficient and bimolecular quenching constant of the quenchers. However, there was essentially no difference in partitioning of a given quencher into plasma membrane, microsomes, or mitochondria in spite of their different phospholipid compositions and sterol contents. Membranes of enhanced phosphatidylethanolamine content had the same partition coefficients as normal membranes, and partitioning was the same in whole membranes and dispersions of total lipids or phospholipids extracted from the membranes. Thus, the partition coefficient in membranes depended primarily on the nature of the quencher and was not highly sensitive to variations in the membrane composition. This is surprising in light of model membrane studies where partitioning of chlorinated hydrocarbons differs depending upon the lipid system studied (Omann & Lakowicz, 1982). These authors showed that partitioning of several chlorinated hydrocarbons is 3-fold higher in DOPC than in DPPC vesicles at 25 °C. The α and δ isomers of 1,2,3,4,5,6-hexachlorocyclohexane partition 2–3-fold more strongly into DPPC above the phase transition temperature than below the phase transition temperature. The most dramatic alteration of partitioning was observed when a 5-fold reduction of the lindane partition coefficient resulted from adding cholesterol to DPPC vesicles below the phase transition temperature. However, these authors did not report the effects of cholesterol above the phase transition temperature of DPPC. It is probable that partitioning into the gel state is more sensitive to the effects of different lipid components than the liquid-crystalline state. This would be in accord with previous data that show that partitioning of lindane into DOPC, DMPC, and POPC (13 400, 10 500, and 11 200, respectively) above their phase transition temperatures was similar despite their different fatty acyl chain compositions (Omann & Lakowicz, 1982). All of the lipid systems in the present study were in the liquid-crystalline state at 37 °C, the temperature at which the measurements were taken.

Membrane proteins and features of intact membranes such as asymmetry had little if any effect on partitioning since similar results were obtained in whole membranes, total lipid dispersions, and phospholipid dispersions. Recent studies have suggested that partitioning of amphipathic molecules is different for cell membranes and lipid dispersions (Conrad & Singer 1979, 1981). The results show that this was not true for hydrophobic quenchers used here. In addition, the amphipathic quencher *N*-hexadecyl-4-picolinium perchlorate readily partitioned into whole membranes as well as lipid dispersions for which the partition coefficient was approximately 10^4 (G. M. Omann and M. Glaser, unpublished observation). These data are consistent with a recent study by Gaffney et al. (1983) that showed that membrane proteins do not have a large effect on the partitioning of spin-labeled compounds into membranes as determined by electron paramagnetic resonance techniques.

In an ideal case, the bimolecular quenching constant is related to the diffusion coefficient of the quencher by the Smolochowski equation (1917)

$$k_q = \gamma 4\pi\sigma_{fq} N(D_f + D_q)/1000$$

where σ_{fq} is the sum of the molecular radii of the fluorophore and quencher, D_f and D_q are the diffusion coefficients of the fluophore and quencher, N is Avogadro's number, and γ is the quenching efficiency of the quencher–fluorophore pair. Since the carbazole moiety was anchored to a phospholipid, it is reasonable to assume that $D_f < D_q$ and $D_f + D_q \approx D_q$. Thus, changes in k_q reflect changes in the diffusion rate of the quencher (Lakowicz & Hogen, 1977; Lakowicz et al., 1977). If we assume the values of γ and σ_{fq} reported by Omann & Lakowicz (1982) and take the values of the plasma membrane bimolecular quenching constant in Table III, the calculated diffusion coefficients for lindane, methoxychlor, and DDE are 1.3×10^{-6} cm^2/s, 2.2×10^{-6} cm^2/s, and 1.5×10^{-6} cm^2/s, respectively. These values are in the same range as the diffusion coefficients of the compounds in synthetic phospholipid vesicles (Omann & Lakowicz, 1982).

The bimolecular quenching constants for DDE were higher in phospholipid dispersions than in total lipid extracts or intact membranes. The quenching constants were also higher in mitochondrial membranes and lipid extracts as compared with the respective samples from the plasma membrane and endoplasmic reticulum. These results parallel measurements for 1,6-diphenyl-1,3,5-hexatriene rotational rates (Gilmore et al., 1979). This suggests that the absence of sterol might be an important factor responsible for the higher quenching constants since there is little or no sterol in mitochondrial membranes and phospholipid extracts. To confirm this, cholesterol was added to DOPC in a 1/3 mole ratio. The bimolecular quenching constant for DDE went from 4.5×10^8 M^{-1} s^{-1} to 3.7×10^8 M^{-1} s^{-1}, which is consistent with a difference in the sterol content being one factor in determining the bimolecular quenching constant.

These studies show that the carbazole–quencher methodology for determining partition coefficients and bimolecular quenching constants is valid in LM cell membranes. In addition, CUA has been incorporated into membrane phospholipids of chicken embryo fibroblasts and baby hamster kidney cells grown in tissue culture medium supplemented with CUA (G. M. Omann and M. Glaser, unpublished observation). Carbazole-labeled phospholipids should also be incorporated into membranes by the use of phospholipid exchange proteins and other methods. Although measurement of partition coefficients and bimolecular quenching constants is limited to compounds that quench the carbazole fluorescence, the number of compounds to be studied could be greatly increased by substituting other fluorophores that are quenched by other compounds [for examples, see Lakowicz & Hogen (1977) and Omann & Lakowicz (1982)]. Thus, the quenching methodology should be applicable to a wide variety of membranes and partitioning compounds.

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Registry No. DDE, 72-55-9; CUA, 73025-00-0; methoxychlor, 72-43-5; lindane, 58-89-9; choline, 62-49-7; ethanolamine, 141-43-5.

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Influence of Phospholipid Peroxidation on the Phase Behavior of Phosphatidylcholine and Phosphatidylethanolamine in Aqueous Dispersions†

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ABSTRACT: The influence of oxygen-induced phospholipid peroxidation on the phase behavior of aqueous dispersions of both egg phosphatidylcholine (egg-PC) and egg phosphatidylethanolamine (egg-PE) has been investigated. Phospholipid peroxidation was followed via malondialdehyde formation and analyses of acyl chain compositions. ^{13}C nuclear magnetic resonance spectroscopy (NMR) and the amino-indicating probe trinitrobenzenesulfonic acid were used to study the effect of peroxidation on the chemical structure of hydrated egg-PE. The macroscopic organization of the phospholipids was monitored by ^{31}P NMR and small-angle X-ray diffraction. Differential scanning calorimetry was employed to study the influence of peroxidation on the thermotropic behavior of egg-PE. The results show that egg-PE is more sensitive to the effects of peroxidation than egg-PC. In the latter, no changes in the macromolecular organization were observed. However, peroxidation strongly influenced the polymorphic phase behavior of PE. Initial peroxidation stabilized hydrated egg-PE in a lamellar system up to 70 °C, presumably by modification of the head group. Such modifications were confirmed by ^{13}C NMR experiments, which indicated the formation of Schiff bases between PE head groups and aldehydes. Furthermore, quantitative analyses of trinitrobenzenesulfonic acid reactable egg-PE and the corresponding fatty acid compositions revealed the presence of cross-links between the ethanolamine head groups, likely involving the bifunctional malondialdehyde. Prolonged peroxidation of egg-PE resulted in a loss of order in the system, possibly by the formation of intermediate nonbilayer structures.

Lipid peroxidation in biological membranes and its consequences for different cellular processes are areas of growing interest [for a recent review, see Ramasarma (1982)]. The complexity of the various peroxidation mechanisms and the resulting physiological changes, which often appear to involve changes in membrane function, has resulted during the last decade in intensive studies on the peroxidation of phospholipids in more simple, model membrane systems.

Considerable progress has been made concerning the oxygen radical initiation and the mechanism of peroxidative reactions (Frankel, 1980; Porter et al., 1980a). The chemical structure of different products derived from peroxidized phosphati-

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