Biochimica et Biophysica Acta 896 (1987) 136–152
Elsevier

BBA 73187

The interaction of potential-sensitive molecular probes with dimyristoylphosphatidylcholine vesicles investigated by ^{31}P-NMR and electron microscopy

Brad P. Bammel, Joy A. Brand, Robert B. Simmons, Diane Evans and J.C. Smith

Department of Chemistry and Laboratory for Microbial and Biochemical Sciences, Georgia State University, Atlanta, GA (U.S.A.)

(Received 19 September 1986)

Key words: Bilayer; Surface charge; Potential-sensitive dye; Dimyristoylphosphatidylcholine vesicle

The effect of a number of commonly employed potential-sensitive molecular probes on the ^{31}P-NMR properties of dimyristoylphosphatidylcholine vesicles at two field strengths has been investigated in order to obtain information on the location and effect of these probes on the membrane bilayer. In comparison to the control dye-free vesicle spectrum, the probes diS-C_3-(5) and diS-C_4-(5), when added to a vesicle suspension, cause a substantial broadening of the ^{31}P resonance with no detectable chemical shift within an uncertainty of $\pm$0.05 ppm at 24 MHz. The spin-lattice and spin-spin relaxation times are also reduced when the cyanines are present by well over 20% relative to those of the control vesicle preparation. The addition of anionic probes, including several oxonol derivatives and merocyanine 540, causes no chemical shift, line broadening, or changes in the relaxation times. Possible explanations for the failure of the anionic probes to alter the vesicle ^{31}P-NMR properties include charge repulsion between these dyes and the phosphate group that prevents the probes from penetrating the bilayer to a depth sufficient to alter the local motion of the phosphate moiety. The ^{31}P resonance broadening and reduction in the relaxation times caused by the two cyanines is at least in part due to an increase in vesicle size as judged by electron microscopy measurements, although an inhibition of the local phosphate motion as well cannot be completely eliminated. The cyanine-mediated increase in vesicle size appears to be due to an irreversible vesicle-fusion process possibly initiated by the screening of surface charge by these probes. The implications of these observations in relation to functional energy-transducing preparations is discussed.

Introduction

A number of dyes belonging to the polyene class (oxonols, merocyanines, rhodamines and cyanines) have proved capable of functioning as optical indicators of charge separation in a large number of biological preparations ranging from phospholipid vesicles across which diffusion potentials are established, black lipid membranes [1–4], mitochondrial and related respiring preparations [5–11], erythrocytes [12–15], and several applications to excitable tissues such as the giant axon from the squid *Loligo peali* [16–20], perfused heart tissue [21–23] and the exposed cerebral cortex of the rat and gerbil [24–26]. Reviews

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Correspondence: J.C. Smith, Department of Chemistry and Laboratory for Microbial and Biochemical Sciences, Georgia State University, Atlanta, GA 30303-3053, U.S.A.

dealing with the application of potential-sensitive molecular probes to a variety of biological systems have appeared [1,27–31]. In many cases, the polyene probes can be calibrated for quantitative measurements of membrane potentials; the time-course of the probe optical signal and the mechanisms responsible for it are, in many instances, at least partially understood.

Since, in contrast to intrinsic indicators of charge separation such as the carotenoids that are present in chloroplast membranes [32–36], molecular probes such as the polyenes must be added to the preparation of interest; the questions of the effect on the membrane and the location that the probes assume in the membrane are of considerable importance. The location of such probes in part determines the nature of charge separation processes that they can sense, i.e., surface potentials, intramembrane or localized potentials [37], or transmembrane potential differences [38]. For example, in sarcoplasmic reticulum vesicles under conditions of massive Ca^{2+} flux across the membrane, a substantial portion of the optical signal for several oxonol, cyanine, and merocyanine probes is due to changes in the membrane surface potential and, furthermore, the portion of the signal due to surface potential alterations is not readily distinguishable from that due to transmembrane charge gradients, the former contribution to the optical signal apparently arising from probe sites at or near the membrane/bulk phase interface region [39,40]. The location of charge shift electrochromic extrinsic probes [3] in a bilayer membrane is particularly critical, since these indicators can respond only to that portion of an electrical potential difference that falls across the molecular dimension of the probe. Malpress [41] has recently developed a theoretical model in which a surface potential derived from point surface charges intimately associated with electron carriers is the effective intermediate in mitochondrial energy transduction. Kell [42] has proposed that the effective charge gradient in the mitochondrial energy coupling scheme falls essentially across the unstirred layer adjacent to the mitochondrial inner membrane. Kinally et al. [43] have suggested that substrate-induced spectral changes of cyanine and carbocyanine probes in mitochondria suspensions are due to alterations in the membrane surface

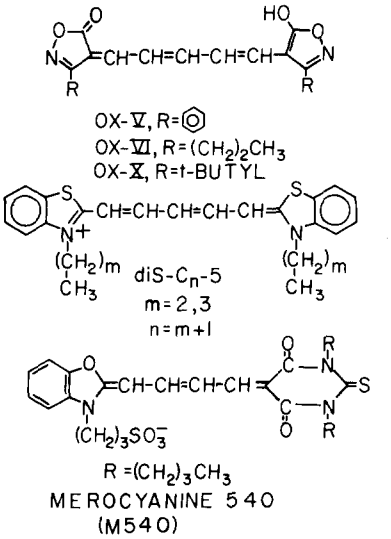

Fig. 1. The structure of the molecular probes employed in this investigation. Since the hydroxyl proton of the oxonols has a pK_a of approx. 4, the dyes exist virtually exclusively as anions under the conditions of the investigations described herein. Merocyanine 540 and diS-C_n-(5) were obtained as the sodium and iodide salts, respectively.

potential; Tedeschi [44] has also discussed this issue in a review. Laris et al. [45], however, have used one of these type probes diS-C_3-(5), in measuring the membrane potential in hamster liver mitochondria and have obtained a value similar to that from independent techniques such as permeant ion distribution.

In order to gain insight into the effect on the membrane and the location of potential-sensitive probes in the membrane bilayer on which many of the preceding issues depend, the effect of a number of commonly employed probes on the ^{31}P-NMR spectrum and relaxation times in a well-defined model membrane system consisting of vesicles formed from dimyristoylphosphatidylcholine (DMPC) has been investigated. Corollary work using electron microscopy is also reported. The structures of the probes employed in this series of investigations are given in Fig. 1.

Materials and Methods

Synthetic L-α-dimyristoylphosphatidylcholine (over 99% pure) was purchased from Avanti Polar Lipids, Birmingham, AL. Merocyanine 540 was purchased from the Eastman Kodak Co. and used

without further purification; diS-C_3-(5) and diS-C_4-(5) were the generous gift of Prof. A. Waggoner or supplied by Molecular Probes, and oxonols V and VI were synthesized according to the procedures given by Smith et al. [46]. Oxonol X was the gift of Dr. R. Haugland, Molecular Probes. Hepes and EDTA were purchased from the Sigma Chemical Co. St. Louis, MO, and reagent-grade KCl was supplied by the Fisher Scientific Co.

Vesicle preparation

Vesicles were prepared according to a modification of the procedure of Suurkuusk et al. [47]. The lipid was suspended in a medium consisting of 160 mM KCl/0.1 mM EDTA/and 10 mM Na-Hepes (pH 7.5) or in 160 mM KCl only at pH 6.5 at 10 mg/ml and sonicated under nitrogen at approx. 50 W power using a Heat Systems Ultrasonic Cell Disruptor (40% duty cycle) for 60 min with a 0.5 inch probe at 35°C, well above the gel phase transition temperature of this lipid. The suspension was centrifuged at $130000 \times g$ for 40 min and the supernatant was retained. The maximum volume which could be effectively sonicated was 10 to 12 ml, which yielded 8 to 10 ml. The quality of the vesicle preparation was routinely monitored using electron microscopy; see below. The preceding procedure produces vesicles of approx. 300 Å diameter with a high degree of uniformity. Depending on sonication power, however, some variation in vesicle size was noted between preparations, but each preparation was itself uniform in vesicle diameter. Vesicle suspensions were stored in an oven at 40°C. An investigation using electron microscopy indicated that, although subtle changes in the vesicle morphology occurred within 48 h, these alterations were not detectable in the ^{31}P-NMR spectrum until nearly 4 days after the vesicles were prepared, at which time gross aggregation of the vesicles occurred. Except for studies on the changes induced by diS-C_3-(5) over time, all work, both control measurements and those with various dyes present, was completed before the approximate 4 day time limit of each preparation. Lipid concentrations were derived from phosphate determinations using a colorometric procedure [48].

Since the DMPC sonication times employed were lengthy, vesicles were also prepared using a Carver Laboratory Press fitted with a 1 inch diameter AMINCO pressure cell according to the method of Hamilton and Guo [49]. The total vesicle preparation time using the latter method was nominally 10 min; this procedure involves subjecting the lipids to one pass through the press assembly at pressures of 5000 and 10000 lb/inch^2 and two passes at 20000 lb/inch^2. The probability of vesicle fusion-inducing agents being produced during the latter vesicle preparation procedure was thus substantially reduced. The starting DMPC material as well as the vesicles prepared by both the French press and sonication methods were subjected to thin-layer chromatography analyses with iodine development at 23°C using the following solvent mixtures (v/v): 80% chloroform/20% methanol/1% NH_4OH; 80% chloroform/20% methanol/1% acetic acid; 80% acetone/20% methanol/1% NH_4OH; 80% acetone/20% methanol/1% acetic acid. In all cases where DMPC alone was used, a single band was observed, indicating the absence of degradation products originating from the preparative procedures. As a test of the sensitivity of the thin-layer chromatography methodology, the DMPC material was doped with dimyristoylphosphatidylglycerol (DMPG) prior to vesicle preparation. The presence of the added component could be readily detected as a second band on the Kodak 13179 silica-gel thin-layer chromatography plate to a limit of $1 \cdot 10^{-3}$ mole ratio DMPG/DMPC.

Although precautions were taken to prevent artifacts due to paramagnetic impurities in the vesicle NMR work by using EDTA in the media, additional controls for paramagnetic species were performed using EPR spectroscopy. Spectra of the DMPC vesicles alone, in the presence of both diS-C_3-(5) and diS-C_4-(5), as well as of these dyes in ethanol solution at nominally 1 mM concentration over a g value range of 1.5 to 8 at 23°C and with samples frozen were obtained using an IBM/Brooker ER 200 spectrometer. No EPR signal was observed in any of these control experiments at the limit of the instrument sensitivity. As a test of the sensitivity of this method, the spectra of Mn^{2+} solutions were recorded; an EPR signal from samples as low as 1 μM in Mn^{2+} could be detected.

The effect of diS-C_3-(5) and diS-C_4-(5) on vesicles prepared by the French press procedure

was virtually identical to that on vesicles prepared by the sonication procedure. The data contained in the following sections were obtained using vesicles prepared by sonication.

Electron microscopy

Vesicles were prepared for transmission electron microscopy as follows: a drop of the liposome suspension was placed on a carbon-stabilized formvar coated 300-mesh copper grid and allowed to sediment for 1 min. Excess fluid was drawn off with filter paper. A drop of 1% (w/v) uranyl acetate was then applied and allowed to stain the liposomes for 1 min. The grids were examined in a JEOL 100CX-II transmission electron microscope operating at 60 kV. Size distribution histograms were constructed from manual vesicle diameter measurements using vesicle suspension electron micrographs taken at 27 000 × or 95 000 × magnification similar to that shown in Fig. 2 with the aid of a magnifying glass equipped with an integral fluorescence light. A minimum of 200 measurements were used in the construction of each histogram.

Potential-sensitive dyes were added in solid form to portions of the stock vesicle suspension and the mixture stirred in an oven at 40°C for 5 to 8 hours before use, except for the electron microscope studies of the time dependence of the diS-C_3-(5) effect on vesicles, for which the first micrographs were taken after just 2 h. This procedure allowed substantial amounts of dye to be bound to the membrane without altering the solvent composition. Experimental conditions are given in figure captions and tables.

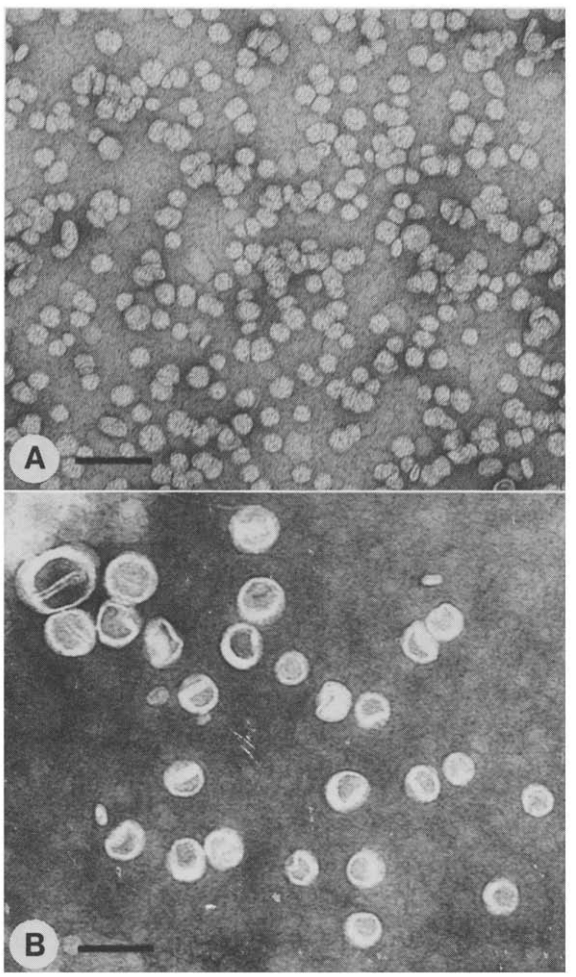

Fig. 2. Typical electron micrograph of the dimyristoylphosphatidylcholine (DMPC) vesicles stained on a carbon coated formvar grid with 1% uranyl acetate. A JEOL JEM-100CX II transmission electron microscope operating at 95 000× magnification was used to obtain the micrograph illustrated in this figure. The scale bars are 1000 Å. (a) Dye-free vesicles, (b) diS-C_3-(5)-to-lipid mole ratio is 0.05.

NMR measurements

^{31}P-NMR studies were carried out at 40°C using JEOL FX60 and GX270 series spectrometers operating at 24.15 and 109.25 MHz, respectively, with broad-band proton decoupling. For linewidth and chemical shift measurements, an external trimethylphosphate reference was employed. For relaxation time investigations, the reference was removed from the sample in order to increase the effective sample concentration and shorten the time required for the T_1 and T_2 measurements. The inversion recovery method and the CPMG pulse sequence were respectively used for the T_1 and T_2 determinations. In both the chemical shift and linewidth as well as the relaxation time work, a control was run on the vesicle sample prior to acquiring data in the presence of the several probes employed in this investigation. In the case of the diS-C_3-(5) and diS-C_4-(5) dyes, a second control was run after the ^{31}P spectrum was obtained in the presence of these probes to demonstrate that the broadening effect of the dyes was

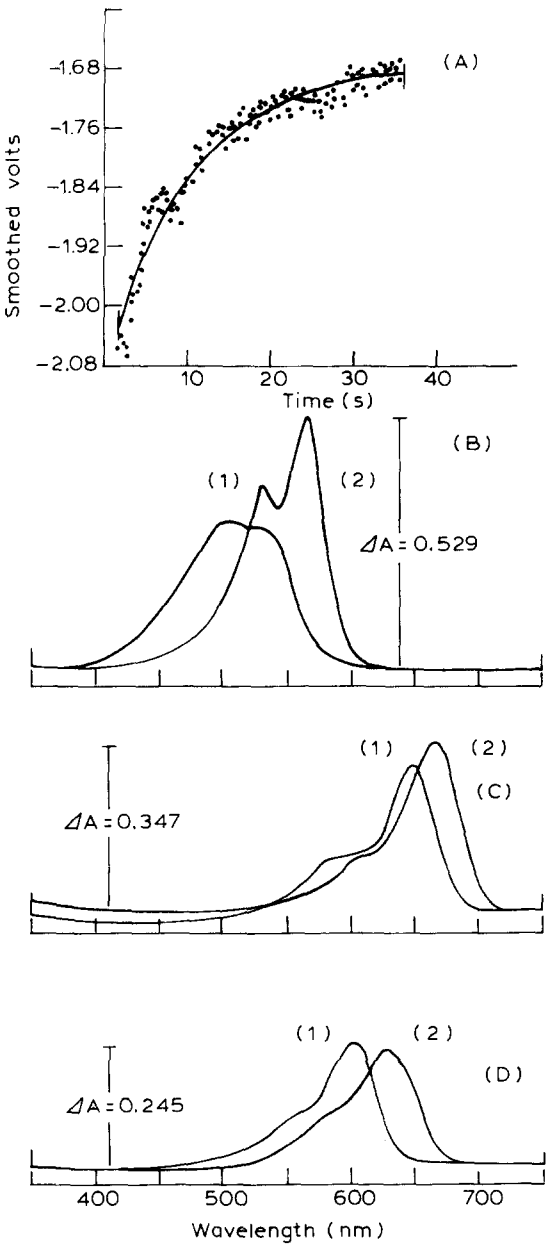

Fig. 3. Typical data obtained using optical spectroscopy. (A) A dilution jump record obtained by the procedures described in Materials and Methods. The solid line is obtained from the fit of the data to a single expoential function; the dots are experimental data points. A large deflection due to the injection of the diS-C_3-(5) vesicle mixture into the optical path of the instrument developed during the time of flow; this spike has been deleted from the record in order to obtain a more expanded scale with which to plot the dye dissociation time-course. The dilution jump work was carried out at 40 ($\pm$1)°C, the same temperature used in the NMR measurements. The

not due to aging of the vesicle suspension. Further evidence for the stability of this preparation is the relative invariance of the T_2 values and the electron microscope histograms of control samples in the studies following the time-course of the diS-C_3-(5) effect. Because of the volume of sample required for NMR spectroscopy, three separate preparations of DMPC vesicles were required for these T_2 experiments. The titration of DMPC vesicles with diS-C_3-(5), monitored by ^{31}P T_2 at 109 MHz, was performed by dividing one vesicle preparation in half and mixing increments of dye into one half, while the T_2 of the other half was measured.

Measurements based on optical spectroscopy

Optical absorption spectra of the dyes employed in these investigations were obtained using a Johnson Foundation DBS-3 scanning double-beam spectrometer. This instrument as well as the MB2 spectrometer employed in the dilution jump procedure to be described subsequently operate on the dual-wavelength principle, which essentially eliminates artifacts due to light scattering from turbid samples. The association of the probes with the DMPC vesicles was accompanied by a red shift of the absorption spectrum, Figs. 3B–D. This signal was used in determining binding constants characterizing the interaction of the probes with the vesicle preparation [50]. A two-step procedure is required to obtain both the maximum number of binding sites, n, and the dissociation constant, K_D. A fixed amount of dye is titrated with aliquots

MB2 spectrometer was calibrated such that 8 mV (one division) on the ordinate corresponds to $\Delta A = 0.0016$. The optical signal was monitored at the 604–640 nm wavelength pair. The MB2 spectrometer RC time constant was 60 ms. Concentrations were 83 μM diS-C_3-(5) and 10.8 mM DMPC. The spectra shown in (B)–(D) are respectively for merocyanine 540, diS-C_3-(5), and oxonol V; in each case trace (1) is the spectrum of the free dye and trace (2) is that of the dye in the presence of the lipid vesicles. The absorption spectrum of the other oxonols and diS-C_4-(5) also red-shifted upon addition of the vesicle preparation to the free dye solution. The spectra were obtained in 160 mM KCl at pH 6.5, one of the two media used in the vesicle formation procedure and the NMR measurements. The reference wavelength was 750 nm. Concentrations are (B) 1.5 μM merocyanine 540, 35 μM DMPC; (C) 3.7 μM diS-C_3-(5), 400 μM DMPC; (D) 7.3 μM oxonol V, 69 μM DMPC.

of a stock vesicle suspension and the binding followed by monitoring the differential absorbance signal that results from the red shift of the dye absorption spectrum at a preselected wavelength pair. An enhancement factor, ε, is obtained from the ratio P_i/P_0 where P_i is the absorbance change resulting from the several additions of the vesicle stock suspension aliquots and P_0 is the initial dye signal in the absence of the vesicles. Under the assumption that a single class of noninteracting binding sites exists, these data are expected to obey the relationship

$$\varepsilon - 1 = \varepsilon_b - 1 - (K_D/n)(\varepsilon_b - 1)/m \quad (1)$$

where m is the vesicle concentration and ε_b is the limiting enhancement factor at infinite vesicle concentration where all dye is bound. A plot of $\varepsilon - 1$ vs. $(\varepsilon - 1)/m$ produces a straight line, the slope of which gives $-K_D/n$ and the intercept $\varepsilon_b - 1$. The second step involves a titration of a fixed amount of vesicle membrane with aliquots from a stock dye solution; an enhancement factor ε is constructed from the ratio of the absorbance signal resulting from the addition of a given quantity of dye to membrane to that corresponding to the same quantity of free probe. The ratio $(\varepsilon - 1)/(\varepsilon_b - 1)$ is the fraction of bound dye. Since the total amount of probe and vesicle lipid present as well as the sample volume are known, it is possible to obtain the free probe concentration [A] and the amount of dye bound per unit weight of lipid, σ. The latter data were then fitted to the Langmuir adsorption isotherm

$$\sigma = \frac{1}{K_D}[n - \sigma][A]_{x=0} \quad (2)$$

or this equation plus a constant using a general nonlinear regression routine [51,52]. The subscript $x = 0$ indicates that the appropriate free dye concentration is that at the aqueous phase/membrane interface. In the analyses used herein, it was assumed that this concentration was the same as the bulk free dye concentration.

The same data used in the Langmuir isotherm analysis were also fitted to the Scatchard equation using the direct linear method [53,54]. In this procedure, K_D and n are obtained from the intercepts in parameter space generated by pairs of line segments drawn through points $(-\sigma_i, 0)$, $(0, [A]_i)$ and $(-\sigma_j, 0)$, $(0, [A]_j)$. For N experimental points there are maximum of $1/2\ N(N-1)$ intersections. A routine was written to generate these intersection values. Any intersections corresponding to negative values of K_D or n were rejected in these calculations since they are not physically significant. In this analysis, the best-fit values of K_D and n are obtained from the median value of the several intersections. A sort routine was used to obtain these values of the binding parameters. The direct liner approach assumes only that the errors in n and K_D are as likely to be positive as negative; no assumptions concerning a normal distribution of errors or a knowledge of experimental-point weighting factors that are required in rigorous least-squares analyses are needed in this case.

Both of the cyanine dyes bind to glass; therefore, acrylic coated cuvettes were used in the diS-C_3-(5) binding studies. However, diS-C_4-(5) appears to bind even to acrylic; no cuvette could be found which did not bind this dye. We were thus unable to determine binding data for it.

Measurements of the rate of diS-C_3-(5) dissociation from the DMPC vesicles were performed using a dilution jump procedure [55]. A Johnson Foundation model E rapid mixing device fitted to a Johnson Foundation model MB2 spinning disk (filter) spectrometer was employed in these measurements. A dye-vesicle mixture at equilibrium was loaded into the minor syringe of the rapid-mixing device. After ascertaining that no air bubbles were present, the contents of the minor syringe were discharged into 160 mM KCl from the major syringe. Since the volume ratio of the major to the minor syringe is 80 : 1, the equilibrium

$$\text{dye} + \text{vesicle} \rightleftarrows \text{complex}$$

is shifted toward the unassociated form of the dye. The dissociation of the dye from the vesicles was followed at 604–640 nm by observing the reversal of the absorption spectrum red shift that occurs when the dye associates with the vesicle preparation; see Fig. 3C. The time-resolved data were acquired, stored, and analyzed by an OLIS 3820 data acquisition system. A typical record is illustrated in Fig. 3A.

Results

Measurements based on optical spectroscopy

The interaction of the several probes employed in these investigations with the DMPC vesicles could be adequately described using a model based on a single class of noninteracting binding sites. The fit of the data for diS-C_3-(5) and oxonol VI as well as that for merocyanine 540 and oxonol V to the Langmuir adsorption isotherm (Eqn. 2) are shown in Figs. 4A and B, respectively. The binding constant values obtained from nonlinear regression and direct linear analyses, described in Materials and Methods, are summarized in Table I, and are in good agreement, indicating that these values are not dependent on the method of data analysis. Experimental conditions are given in the figure legends and tables.

Oxonol V binds to DMPC vesicles with a larger capacity and lower K_D than does oxonol VI. This difference is readily explained on the basis of the isoxazolone ring substituents, which are phenyl and propyl groups, respectively. These groups serve as 'anchors' in the interaction of the oxonols with the bilayer, the strength of the interaction increasing as the hydrophobicity of the side-chain increases [56]. Relatively fewer binding sites appear to be available to merocyanine 540, possibly because of charge repulsion due to the localized negative charge on the sulfonate group, but the probe binds considerably more strongly to the membrane sites, as judged by the lower K_D value, than do the oxonols, possibly because of the two butyl groups present on one end of the ring system of this probe. DiS-C_3-(5) has a capacity similar to that of oxonol V but binds to these sites less strongly than does the oxonol.

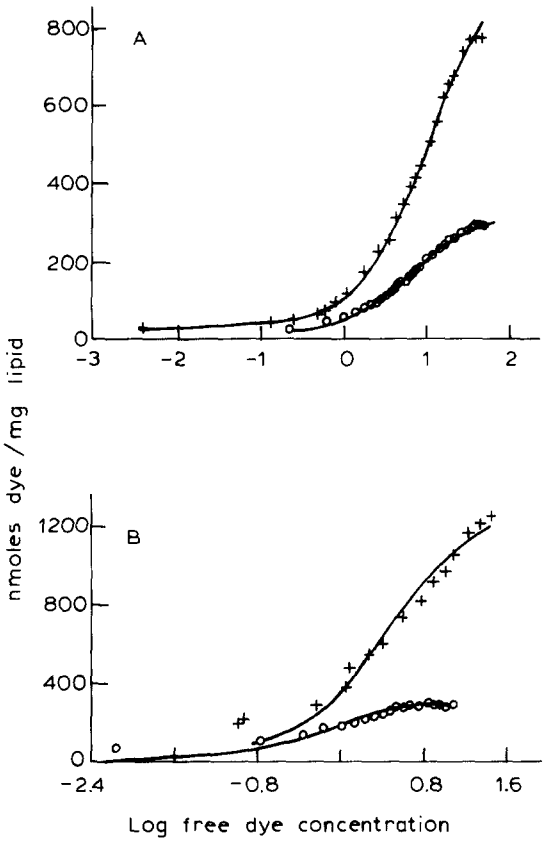

Fig. 4. The fit of probe-DMPC binding data obtained as described in Materials and Methods to the Langmuir adsorption isotherm, Eqn. 2, or this equation plus a constant. In each part of this figure, the solid line is obtained from the nonlinear regression procedure and the several plotting symbols are experimental data points. Data were collected at 40 °C using a medium consisting of 160 mM KCl/0.10 mM EDTA/10 mM Na-Hepes (pH 7.4). The absorbance measurements for titrations with oxonol V, oxonol VI, merocyanine 540, and diS-C_3-(5) were respectively performed at the following wavelength pairs: 630–670 nm; 635–665 nm, 550–650 nm and 675–715 nm. (A) +, diS-C_3-(5); O, oxonol VI. (B) +, oxonol V; O, merocyanine 540. (Free dye concentration in μM.)

Investigations based on NMR spectroscopy

A typical ^{31}P-NMR spectrum of a phospholipid vesicle suspension is illustrated in Fig. 5A with no dye present. Essentially the same spectra were obtained from DMPC vesicles in either of the two media previously described under either control conditions or with probe added. The spectrum is rather sharp and is quite similar to that reported [57] for dioleoylphosphatidylcholine vesicles of 500 Å diameter. The ^{31}P resonance is known to be a composite one consisting of signals from phosphates in both the inner and outer leaflet of the bilayer [58]; in some preparations, a partial resolution of these components could be realized as indicated by a small splitting near the signal maximum. The resonance could be readily separated into two well-resolved signals by rare-earth ions such as Gd^{3+}. In the presence of any of the extrinsic potential-sensitive probes employed in the investigations performed at 24 MHz, a single composite resonance was observed. This is con-

TABLE I

SUMMARY OF BINDING CONSTANTS CHARACTERIZING THE INTERACTION OF POTENTIAL-SENSITIVE MOLECULAR PROBES WITH DMPC VESICLES

The values given for n and K_D are averages ± S.E. Medium: 160 mM KCl/0.1 mM EDTA/10 mM Na-Hepes (pH 7.4). Temperature: 40°C. The absorbance measurements for experiments with oxonol V, oxonol VI, merocyanine 540, and diS-C_3-(5) were, respectively, performed at the following wavelength pairs: 630–670, 635–665, 550–650 and 675–715 nm.

Probe	Non-linear regression analysis		Direct linear analysis	
	n (nmol dye/ mg lipid)	K_D (μM)	n (nmol dye/ mg lipid)	K_D (μM)
Oxonol V	1100 ± 100	3.1 ± 0.26	1065 ± 190	3.1
Oxonol VI	401 ± 75	6.5 ± 0.36	397 ± 68	6.1 ± 0.6
Merocyanine 540	307 ± 18	0.69 ± 0.10	313 ± 12	0.62
diS-C_3-(5)	924 ± 103	9.3 ± 0.66	847 ± 130	8.4 ± 0.67

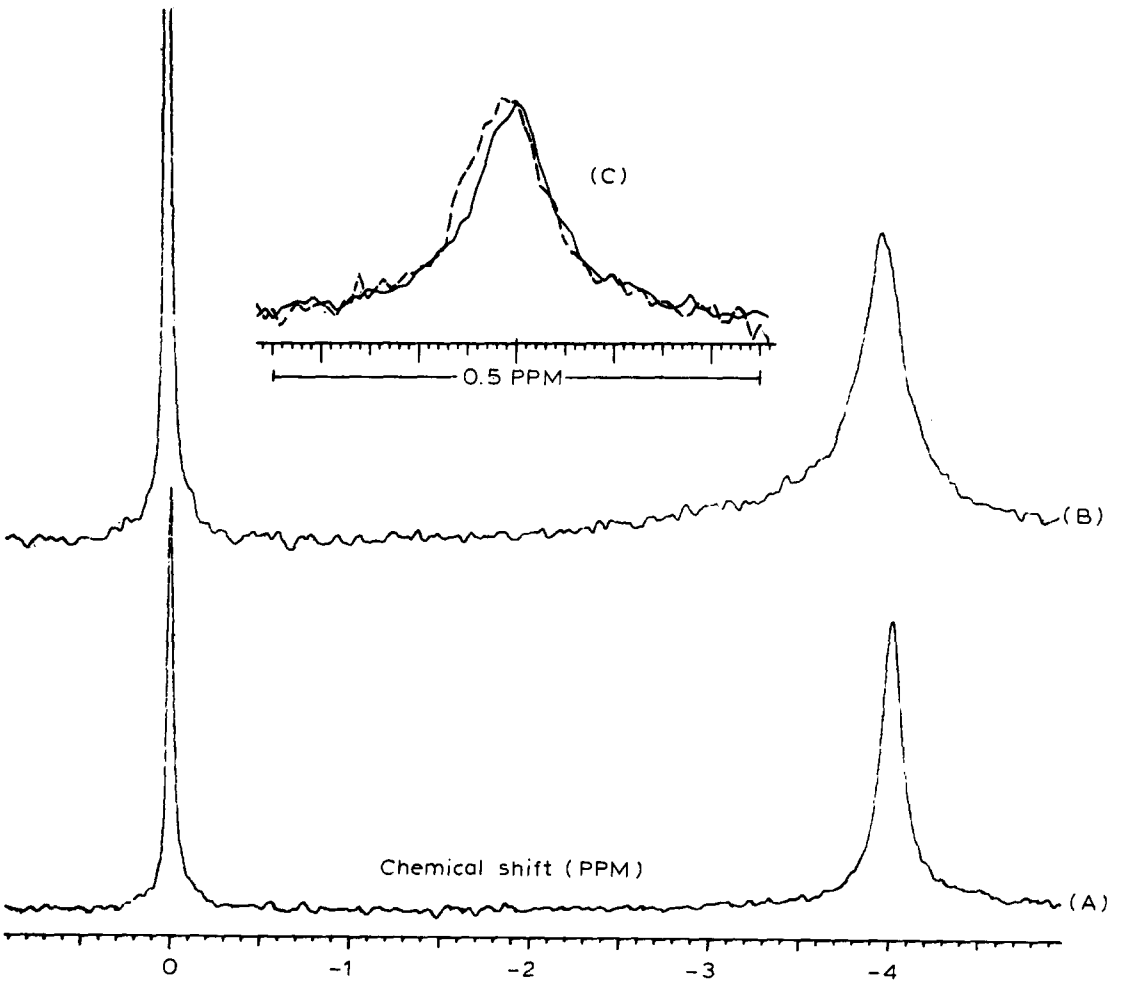

Fig. 5. The ^{31}P-NMR spectrum of dimyristoylphosphatidylcholine vesicles in 160 mM KCl (pH 6.5) at 40°C at 24.15 MHz with complete proton decoupling. Approximately 5000 scans were obtained with 8K data points, a 4 s acquisition time, 8 s pulse delay, and a 0.5 Hz broadening factor. Trace (A) is the vesicle preparation with no dye present; trace (B) is the same preparation with 500 μM diS-C_3-(5) present. Traces (C) were obtained with 1K data points over a 100 Hz span and thus have 0.098 Hz resolution. The dashed line is the spectra obtained in the presence of 500 μM merocyanine 540, whereas the solid line is the control, dye-free vesicle spectrum. Note that the scale in (C) is expanded 10 times relative to that in (A) and (B). Lipid concentration was 8 mM. Chemical shifts were measured relative to an external trimethylphosphate reference. Essentially the same results as those above were also obtained when the NMR measurements were performed using the alternative medium described in Materials and Methods.

TABLE II

SUMAMRY OF ^{31}P CHEMICAL SHIFT AND RELAXATION TIMES IN DIMYRISTOYLPHOSPHATIDYLCHOLINE VESICLES

Abbreviations: DMPC, dimyristoylphosphatidylcholine; OX-V, oxonol V; OX-VI, oxonol VI; OX-X, oxonol X; M540, merocyanine 540. Samples used for T_1 measurements were prepared in 160 mM KCl. Samples used for T_2 measurements were prepared i n160 mM KCl/0.10 mM EDTA/10 mM Na-Hepes (pH 7.4). 10% by volume ^2H_2O was added after the preparative procedure. In all experiments, the dye was added as a solid to vesicle suspensions and stirred 8 to 12 h before NMR measurements were started. With the exception of the measurements using diS-C_4-(5), all of the T values reported were obtained from the same preparation. Because of the time limit over which the stability of the vesicles was considered acceptable, the T_2 measurement with diS-C_4-(5), labeled with *, was performed on a separate vesicle preparation. In the T_2 measurements, a quantity of probes necessary to achieve 140 nmol bound dye/mg DMPL was added. The superscripted letters indicated the elapsed time, in hours, following the preparative procedure completion at which the T_2 measurements were begun: (a) zero; (b) 10; 9c) 20; (d) 30; (e) 40; (f) 50; (g) 60. The DMPC concentration in the T_1 measurements was nominally 8 mM. Temperature: 40°C.

Sample	σ (ppm)	T_1 (s)	Control	σ (ppm)	T_1 (s)
DMPC + 122 μM OX-V	−4.054	1.55	DMPC only	−4.044	1.62
DMPC + 500 μM M540	−4.024	1.65	DMPC only	−4.044	1.85
DMPC + 250 μM OX-X	−4.024	1.53	DMPC only	−4.064	1.66
DMPC + 500 μM diS-C_3-(5)	−4.054	1.23	DMPC only	−4.064	1.55
Sample		T_2 (ms)	Control		T_2 (ms)
DMPC + 460 μM diS-C_3-(5)	–	64.9 [g]	DMPC only	–	79.4 [a] (80.9 [e])
DMPC + 460 μM M540	–	80.4 [d]	DMPC only	–	79.4 [a] (80.9 [e])
DMPC + 480 μM OX-VI	–	79.7 [f]	DMPC only	–	79.4 [a] (80.9 [e])
DMPC + 470 μM OX-V	–	83.3 [b] (78.7 [c])	DMPC only	–	79.4 [a] (80.9 [e])
DMPC + 460 μM diS-C_4-(5) *	–	49.0	DMPC only	–	88.1

sistent with optical studies referenced below which indicate that the probe exchange rate with the lipid should fall under the fast-exchange limit case. However, in light of the small resonance frequency difference for lipids on the inside versus lipids on the outside layer of small bilayer vesicles, it is likely that if the probes induce a frequency shift, it is small. The DMPC vesicle system was also investigated at 109 MHz using either dye-free samples or vesicles in the presence of the diS-C_3-(5) cationic probe that broadens the ^{31}P spectrum. Although the potential resolution is markedly increased when the operating frequency is increased to 109 MHz, the chemical shift anisotropy factor, that increases as the field strength squared, will tend to broaden the ^{31}P-NMR spectrum and to obscure any possible separation that may be obtained between the phosphate moieties existing in the free form and those associated with diS-C_3-(5). The preceding observations are thus consistent with a preparation procedure that results in small single bilayer vesicles.

The effect of adding the widely employed cyanine probe diS-C_3-(5) to a vesicle suspension is to substantially broaden the ^{31}P spectrum as illustrated in Fig. 5B; the dye, however, does not alter the chemical shift within an uncertainty of ±0.05 ppm (Table II). The cyanine diS-C_4-(5) had very similar effects on the ^{31}P resonance properties. In contrast to the effect of diS-C_3-(5) on the ^{31}P vesicle spectrum, oxonols V, VI and X (the *t*-butyl derivative) as well as merocyanine 540 when added to the vesicle suspensions later neither the chemical shift nor the linewidth of the spectrum. Fig. 5C contains a typical result illustrating that at 0.098 Hz resolution, the vesicle ^{31}P-NMR spectrum obtained with merocyanine 540 present (the dashed line) is virtually superimposable on that of the control vesicle sample on a scale expanded 10-times relative to that of parts (A) and (B). These observations suggest a maximum dye-induced chemical shift of roughly 0.01 ppm, well below the reproducibility limit of these measurements. Results using the anionic probes are also summarized in Table II.

Relaxation time measurements

Both the spin-lattice and spin-spin relaxation

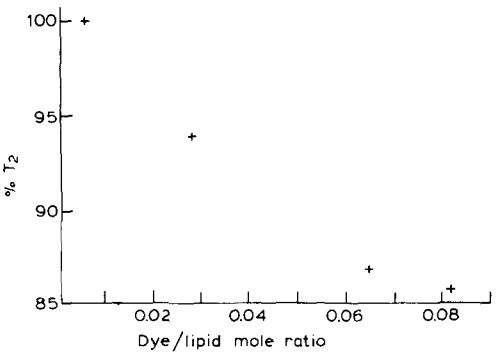

Fig. 6. The percentage decrease in the ^{31}P T_2 relaxation time as a function of the diS-C_3-(5)/DMPC mol ratio. The measurements were carried out at 109 MHz (6.34 tesla) using a JEOL GX series spectrometer to reduce the time requried for each T_2 measurement. 5 h were required for each T_2 measurement. The medium used was 160 mM KCl/0.10 mM EDTA/10 mM Na-Hepes (pH 7.4), at 40°C; 2K data points and a 400 Hz span were employed in these investigations. Proton decoupling was gated to reduce sample heating; decoupling was on only during data acquisition (no nuclear enhancement). 400 acquisitions were taken for each spectra; each point in the plot is a relaxation time from six such spectra.

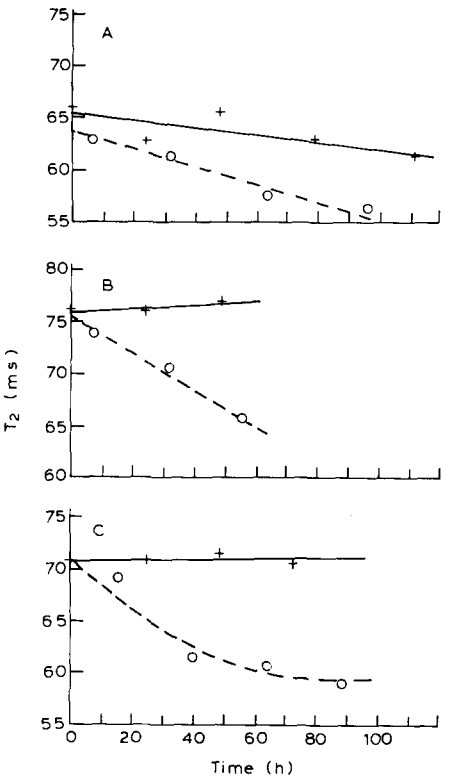

Fig. 7. Representative data from three separate vesicle preparations of the change in ^{31}P T_2 relaxation time as a function of time at various diS-C_3-(5)-DMPC mole ratios. The measurements were carried out at 24.15 MHz (1.41 tesla) using a JEOL FX spectrometer in 160 mM KCl/0.10 mM EDTA/10 mM Na-Hepes (pH 7.4) at 40°C. Six spectra with 400 scans each with a span of 100 Hz, 1K data points and complete proton decoupling, requiring 7.5 h, were used for each T_2 determination. Dye-containing samples were mixed for approx. 8 h before the first T_2 measurement was initiated, after which they were not stirred further. Dye-free control samples are represented by + and dye containing samples by O. Dye-to-lipid mole ratios are $0.93 \cdot 10^{-2}$, $1.8 \cdot 10^{-2}$ and $4.5 \cdot 10^{-2}$ for parts A, B and C, respectively.

times of control vesicle suspensions were reduced by 20% or more by the presence of either diS-C_3-(5) or diS-C_4-(5); results are summarized in Table II. For both T_1 and T_2 measurements, the semilog plots of the signal amplitudes vs. time were linear with minimal variance values, indicating that a single effective relaxation time is being measured. The magnitude of the diS-C_3-(5)-dependent diminution of the ^{31}P T_2 value increased as the dye-to-lipid concentration ratio was incremented until the effect was saturated. A typical plot is illustrated in Fig. 6. Results from monitoring the change in ^{31}P T_2 values with time at various dye-to-lipid mole ratios are summarized in Fig. 7. Clearly even at the lowest mole ratio used, $0.93 \cdot 10^{-2}$, diS-C_3-(5) does affect the phosphate resonance. At high concentration the maximum T_2 reduction is reached more rapidly.

In contrast to the effect of diS-C_3-(5), there was no detectable alteration of the ^{31}P spin-lattice or spin-spin relaxation time by the anionic oxonols or merocyanine 540 within the nominally ± 5% uncertainty of these measurements.

The difference between the readily observed broadening effect of the cyanine dyes and the reduction in the ^{31}P T_1 and T_2 relaxation time values by these probes and the lack of a measurable effect on the ^{31}P-NMR properties by the several anionic probes employed in these investigations has been explored under a number of different conditions. Since the binding constants characterizing the interaction of the probes with the DMPC vesicles have been determined, it has been possible to calculate the dye-to-lipid concentration ratio at which the same quantity of dye/mg lipid is bound. In this case, a value of σ, Eqn. 2, was selected that was less than the smallest value of n, the maximum number of binding sites available to

the probes. Under the preceding conditions, no effect on the ^{31}P resonance properties caused by any of the anionic probes could be detected, whereas an increase in the resonance linewidth and reduction of the ^{31}P T_1 and T_2 values could be readily observed in the presence of the cyanines. The difference in the effect of the anionic and cationic probes on the ^{31}P-NMR resonance thus cannot be ascribed to variations in the amount of dye bound. Since n is substantially larger for

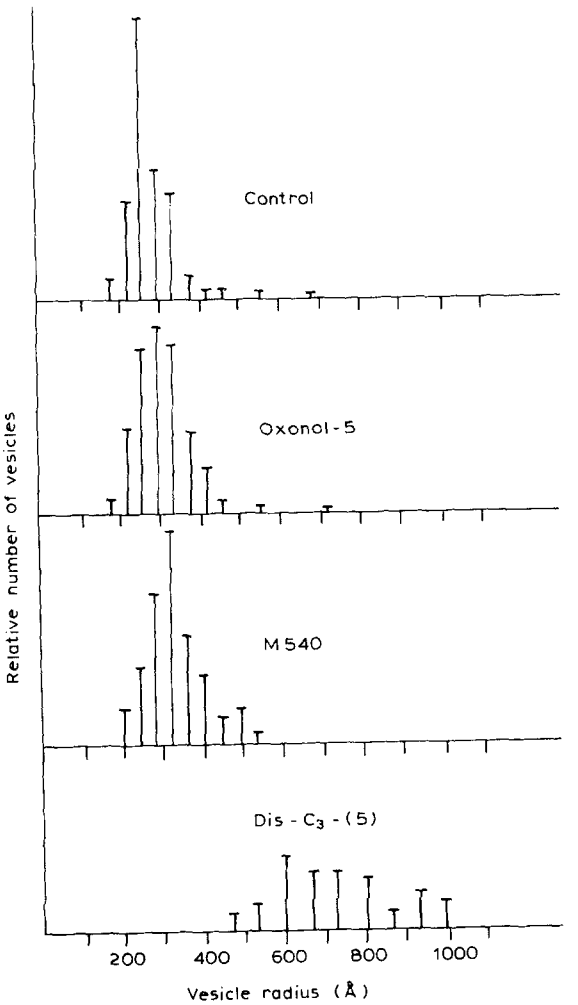

Fig. 8. The effect of potential-sensitive molecular probes on the DMPC vesicle size distribution. In each experiment 140 nmol dye per mg lipid were bound. Vesicles were formed at 40°C in a medium consisting of 160 mM KCl/0.1 mM EDTA/and 10 mM Na-Hepes (pH 7.4) as described in Materials and Methods. Dye was added in solid form and stirred with the vesicles for 12 h at 40°C before the electron micrographs from which the histograms were derived were taken. DiS-C_3-(5) causes a marked shift of the DMPC histogram to larger vesicle diameters. The percentage of available sites occupied was 13, 15 and 46 for oxonol V, diS-C_3-(5) and M-540, respectively.

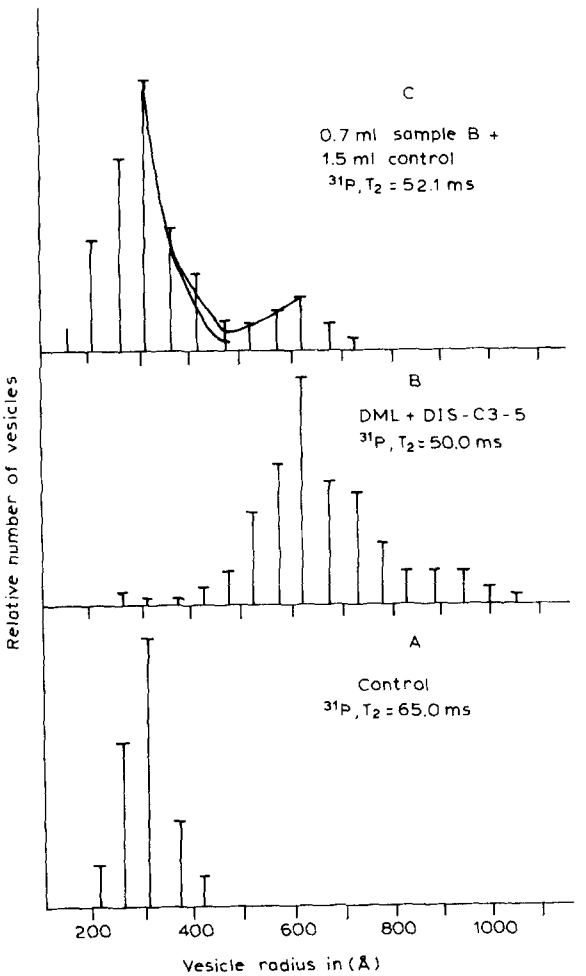

Fig. 9. The effect of the addition of dye-free DMPC vesicles on the vesicle size histogram obtained when diS-C_3-(5) was present. Part (A) is the control histogram; in (B), diS-C_3-(5) was added such that a dye-DMPC mole ratio of 0.06 resulted, and mixed for 12 h. In (C), dye-free vesicles were added to reduce the dye-DMPC mole ratio to 0.02, and mixed for 12 h. The histogram in (C) becomes distinctly bimodal. The lower curve depicts the contribution to the population of the region between 315 Å and 630 Å from the dye-free DMPC based on the height of the peak at 315 Å in (C) and the shape of the distribution in (A). The upper curve depicts the sum of the lower curve and contribution from the vesicles aggregated by dye; estimated from the height of the peak at 630 Å in (C) and the shape of the distribution in (B). The vesicles were formed and the dye added as described in the caption to Fig. 8.

oxonol V and diS-C_3-(5), the percentage of available sites occupied by these probes was not as high as was the case for oxonol VI and merocyanine 540 in the preceding experiments. Dye-to-lipid mole ratios of 0.28 and 0.32 were required to raise diS-C_3-(5) and oxonol V, respectively, to 40% occupancy, the percentage reached by the other dyes in the preceding experiment. At ratios this high, the dyes are a major constituent of the system. Electron micrographs of systems prepared with these ratios do not reveal recognizable bilayers.

Electron microscopy

Typical electron micrographs of a vesicle suspension are shown in Fig. 2. The diameter of the majority of the vesicles in the control sample falls in the 250 Å to 350 Å range with a maximum in the size distribution histogram near 300 Å; Fig. 8 contains histograms of the size distribution of a single vesicle preparation in the presence of several probes. An inspection of this figure indicates that the anionic dyes have little if any effect on the control histogram, whereas diS-C_3-(5) markedly shifts the histogram to larger-diameter values. The maximum in the histogram characterizing the vesicles in the presence of the latter probe is approximately twice that of the control. The effect of diS-C_4-(5) on size distribution is very similar.

In Fig. 9, the effect on the diS-C_3-(5) – shifted histogram caused by supplementing the dye – vesicle system with additional dye-free vesicles from the same preparation is shown. It is readily apparent that the histogram is bimodal and that the position of the original maximum in the size distribution histogram when the probe is present is not affected by the presence of the dye-free vesicles; the maximum in the histogram at the smaller diameter values corresponds to that of the control. By these criteria, the diS-C_3-(5) effect on the vesicle size is irreversible. Also apparent in Fig. 9 is a shift in the population distribution toward larger vesicles. There are more vesicles in the range from 370 Å to 475 Å than expected based on the shapes of the distributions in Fig. 9A and B and the height of the maxima in Fig. 9C.

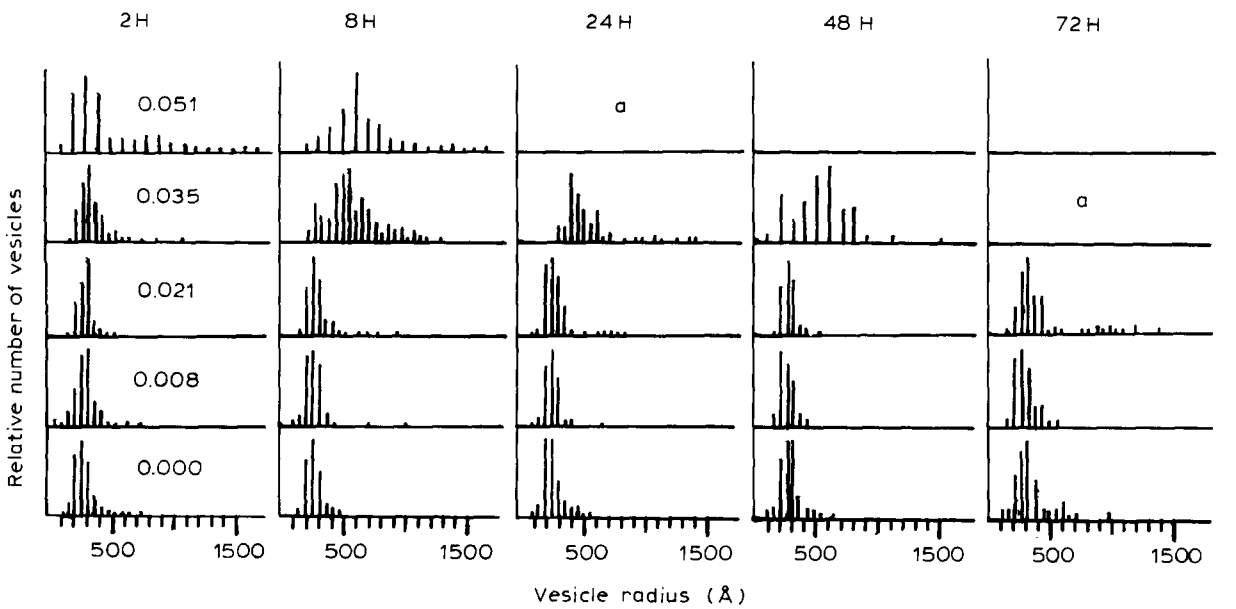

Fig. 10. The change in vesicle size distribution as a function of time at various diS-C_3-(5)-to-lipid mole ratios. Mole ratios are given for each sample in the left-most column. Histograms of the same sample are arranged into rows with the elapsed time after dye addition listed across the top. Vesicles were formed at 40°C in medium consisting of 160 mM KCl/0.1 mM EDTA/10 mM Na-Hepes (pH 7.4) as described in Materials and Methods. Dye was added in solid form and stirred for just 2 h before the first micrographs were taken, and an additional 6 h before the second set of micrographs were taken, after which stirring was ceased. All samples were kept at 40°C at all times. (a) Lipids had aggregated completely; virtually no vesicles remain.

Furthermore, attributing the portion of the vertical lines in the range from 370 Å to 475 Å above the upper curve to the dye-free DMPC added, when the actual volume of bilayer is considered (assuming spherical bilayer vesicles for all sizes above 210 Å and micelles for 210 Å and below), the ratio of small vesicles to large vesicles is unity. The actual ratio mixed was 2. Thus the shift to aggregated vesicles is greater than is apparent.

Histograms showing the change in vesicle size with time at various diS-C_3-(5)-to-lipid mole ratios are contained in Fig. 10. Obvious changes are seen at ratios of 0.051 and 0.035. Changes in the control after 5 days prevent meaningful interpretations of results after more than 4 days when some probable change is seen at a ratio of 0.021. No discernible change is seen at a ratio of 0.008.

Discussion

Theoretical treatments indicate that the phosphate group rotational diffusion tensor can be considered to consist of a parallel component that reflects the composite internal motion of this group, and a perpendicular component that is due to the tumbling of the entire vesicle [57,59]. The observations derived from the NMR investigations using the several potential-sensitive probes will be considered within the framework of the preceding theoretical model. With respect to the internal motion of the phosphate moiety, among the possible explanations for the marked difference in the effect of the anionic probes and the cationic cyanines on the ^{31}P resonance in DMPC vesicle preparations is that, although under the prevailing experimental conditions the lipid headgroup is zwitterionic and bears no net charge, the phosphate moiety is expected to possess a negative charge that is balanced by that on the quaternary amino group. Since the oxonols and merocyanine 540 are anions, charge repulsion may prevent these probes from penetrating the bilayer to a depth sufficient for the motion of the phosphate group to be impeded by these probes, whereas the cationic cyanines are not subject to exclusion from the immediate vicinity of the phosphate group due to charge repulsion and may be able to reduce further the already restricted motion of this group. The lack of a measurable chemical shift change when the cyanine is present, however, requires a minimal perturbation of the local field experienced by the phosphorus species.

An additional contributing factor to the effect of the cyanine probes on the DMPC vesicle ^{31}P resonance properties is that the dye is causing an increase in the vesicle size. The global tumbling rate of the vesicles will be governed by the rotational diffusion coefficient. For a spherical particle in a medium with constant viscosity coefficient and temperature, this coefficient is inversely proportional to the particle radius cubed [60]. An increase in vesicle size caused by diS-C_3-(5) would then be expected to reduce the tumbling rate of the vesicles, which in turn governs the perpendicular component of the phosphate rotational term [57,59].

In view of a possible dye-induced increase in DMPC vesicle size, a series of investigations based on electron microscopy were performed on the same samples that were used in the NMR measurements. The results summarized in Fig. 8 indicate that the anionic probes have little or no effect on the vesicle size distribution histograms, whereas diS-C_3-(5) and diS-C_4-(5) markedly shift the histogram to larger diameter values. These observations suggest that a cyanine-probe-induced increase in vesicle size is occurring and is responsible at least in part for the increase in the ^{31}P resonance linewidth in the DMPC vesicle system. The possibility of additional contributions from an impediment of the internal phosphate group motion by this probe as previously described, however, cannot be eliminated. The marginally greater sensitivty of the ^{31}P T_2 relaxation time to the presence of the cyanine relative to that of the T_1 value (Table II), however, suggests that the increase in vesicle size is the larger contributor to the mechanism(s) responsible for the altered vesicle ^{31}P-NMR properties.

Since the vesicle size is increased by the presence of diS-C_3-(5), the question of the process(es) responsible for the enlargement of the vesicles arises. Among the possibilities are (1) a swelling of the vesicles and (2) a probe-induced fusion of two or more DMPC vesicles. In an effort to distinguish between the preceding two possibilities, the following experiment was performed. The T_2 relaxation time and electron micrographs of con-

trol vesicles suspensions and the same data on the vesicles in the presence of diS-C_3-(5) were obtained. A quantity of dye-free vesicles sufficient to increase the amount of lipid present by a factor of 3 was then added to the DMPC-diS-C_3-(5) system. As shown in Fig. 9, the size distribution histogram becomes bimodal, with the maxima corresponding to that of the control vesicle diameter and that due to the diS-C_3-(5) effect. Since the latter addition of dye-free vesicles was performed under conditions in which virtually all of the diS-C_3-(5) was bound to the vesicles previously added, the bound dye is at high chemical potential after the addition of the dye-free vesicles and would be expected to redistribute to these vesicles and reach a new equilibrium state corresponding to a reduced dye-to-vesicle lipid concentration ratio. Under these conditions, a reversible swelling of the vesicles caused by diS-C_3-(5) would be expected to be diminished at the reduced dye-to-lipid concentration ratio, resulting in a unimodal histogram with a maximum at a diameter intermediate between that of the control vesicles and those originally stained with diS-C_3-(5). The latter effects on the size distribution hisotgram upon addition of dye-free vesicles to the diS-C_3-(5)-DMPC system are clearly not observed, suggesting that the dye-induced increment in the vesicle size is not reversible by the addition of a dye-free aliquot of the same preparation.

It should be emphasized that the bimodal vesicle size distribution histogram does not imply slow dye-vesicle exchange but slow vesicle fusion, since the electron microscope technique is directly sensitive to the vesicle size but clearly not to exchange phenomena. A fusion process would be expected to be essentially irreversible; once formed, the larger vesicles would have little tendency to revert to multiple smaller vesicles, even after the dye in part dissociates from the bilayer membrane. The fusion of similar vesicles would not be expected to alter the local field at the phosphate site and thus is consistent with the observed line broadening and reduction in relaxation times without a change in the ^{31}P chemical shift upon addition of diS-C_3-(5) or diS-C_4-(5).

The T_2 relaxation time of the control DMPC vesicle preparation was decreased by nominally 25% when diS-C_3-(5) was added and increased by approx. 4% when a dye-free vesicle aliquot was supplied that results in the bimodal size distribution histogram previously described. The small increase in the T_2 value (that is likely a weighted average of the dye-free and dye-associated ^{31}P T_2 relaxation times) upon addition of dye-free vesicles is consistent with the shift in population toward larger vesicles seen in Fig. 9C, and both indicate that if there is a threshold dye-to-lipid mole ratio below which dye induced fusion of vesicles does not occur, it is less than 0.02, the ratio in Fig. 9C. Again, the possibility that the cyanine probes are also perturbing the phosphate group locally can not be ruled out.

In terms of a cyanine-induced vesicle fusion model, the monotonic decrease in the T_2 relaxation time value as the dye-to-lipid concentration ratio is increased (Fig. 6) can be explained by postulating that progressively larger vesicles are formed as the amount of dye present is increased, with a concomitant decrease in the T_2 value. Or, the apparent dye-to-DMPC mole ratio dependence of T_2 may be caused by such a dependence of the rate of vesicle fusion. Either process would produce T_2 diminution until overall vesicle tumbling was not significantly slowed by further fusion, and T_2 would exhibit saturation behavior as experimentally observed. The rate of diS-C_3-(5) induced change is dependent on dye concentration as can be seen in Figs. 7 and 10. In the latter figure, a vesicle diameter of nominally 630 Å is reached within 8 h and 48 h at dye-to-lipid mole ratios of 0.051 and 0.035, respectively. The kinetic model is thus favored.

It can be seen in the electron microscope results, Fig. 10, that at high dye concentration, aggregation continues until virtually all vesicles are in the aggregated form while T_2 values reach a minimum relatively quickly (Fig. 7). The rate of vesicle fusion below the mole ratios of $2 \cdot 10^{-2}$ can not be detected by electron microscopy, yet ^{31}P T_2 values, even at the lowest dye concentrations studied, $0.93 \cdot 10^{-2}$, show dye-induced diminution. This may result from a greater sensitivity of NMR to vesicle aggregation, or from other perturbations such as dye-induced restriction of internal motion. An additional contribution to relaxation may also arise from rapid dye exchange between sites at or near the vesicle lipid phosphate

groups and the aqueous phase. Such a process would also produce an apparently single relaxation time that would be the weighted average of that appropriate for dye-free and dye-perturbed phosphate moieties, the contribution of the dye-associated phosphates increasing as the dye-to-lipid concentration ratio is incremented.

The preceding interpretation based on the data contained in Figs. 6–10 is heavily dependent on a new equilibrium state being achieved after the addition of the dye-free vesicle material within a time period that is small compared to that in which the NMR measurements and electron microscopy were performed. The rate at which the new state is reached after the addition of the dye-free vesicles will likely be governed by the dissociation rate of the probe from the vesicles. The association rate is expected to be fast compared to the former process because the dye-membrane association is expected to follow a second-order rate law, the apparent first-order rate of which will be controlled by the local dye concentration, which is likely to be quite high in these experiments, since large quantities of solid dye, 60 to 500 μM, were added to the vesicle suspensions.

The assoication rate of diS-C_3-(5) with similar phospholipid vesicles has been measured as $1 \cdot 10^7$ M^{-1} (dye) $\cdot$ s^{-1} by Smith et al. [8]. Assuming that the dye concentration is 60 μM, near the solubility limit in an aqueous medium, the second-order rate constant previously cited predicts an apparent first-order rate constant of 600 s^{-1} or a half-time of approx. 1 ms. An inspection of Fig. 3(A) indicates that the dye dissociation process has a half-time of nominally 10 s, whereas the NMR and electron microscope work was performed on a time-scale of hours. That the diS-C_3-(5) has not redistributed to the originally dye-free vesicles and a new equilibrium established prior to the electron microscopy and NMR measurements thus appears to be unlikely.

An additional facet of the dissociation kinetics data contained in Fig. 3A is that no faster phase signal could be observed in these measurements. In practice, the on/off exchange from the vesicles is likely to be much faster than suggested by these data; such processes operating on a microsecond scale in work with diS-C_3-(5) and related probes in black lipid membranes have been detected [1]. Events occurring on a microsecond time-scale are not detectable using rapid mixing techniques and even those significantly slower would tend to be masked in the dilution jump work because of the initial absorbance change due to the injection of the dye-vesicle mixture into the light-path of the spectrometer, especially if such processes gave rise to a low-amplitude signal. See Materials and Methods and the caption to Fig. 3A for the details of the dilution jump procedure and associated limitations. The comparatively slowly developing signal detected in these measurements is probably due to the permeation of the bilayer by diS-C_3-(5) that is inside the DMPC vesicles and that would be at a high chemical potential relative to that of dye in the bulk aqueous phase after the dilution jump. In this model, the bilayer permeation process would then be followed by the rapid dissociation of this portion of the vesicle-associated probe to the bulk aqueous phase, a process that is likely to be too fast for detection by the dilution jump technique as previously discussed. This probe is known to cross black lipid membranes [1,61] and to permeate the mitochondrial inner membrane when an electrical potential gradient is present [10].

A number of other probes appear to behave in a similar manner to diS-C_3-(5) in that they also permeate membrane bilayers. The permeation of phospholipid vesicles by oxonol V is manifested by a slow phase in the dye-membrane association time-course profile; the accumulation of the dye in the internal volume of the vesicles can be demonstrated by chromatographic separation procedures [55].

Although the conditions employed in the investigations of the probe-DMPC vesicle interactions described herein differ from those normally employed in functional biological preparations in that significantly lower dye-to-membrane concentration ratios are normally employed in the latter preparations, the model system work is relevant to functional systems for several reasons. DiS-C_3-(5) and a number of structurally similar probes respond to the formation of an electrical potential difference by distributing across the membrane according to the Nernst or Goldman-Katz equation [10–13]. Such a redistribution can lead to the

development of high local concentration of the probes comparable to that employed in work with the DMPC vesicles. High probe concentrations can also exist in the unstirred layer near the membrane surface because of the presence of significant surface charge density [1,27,62]; the presence of these probes at high local concentration could lead to the neutralization of membrane surface charge and thus tend to promote membrane fusion. An additional relevant observation is that the ^{31}P T_2 value is a monotonic function of the diS-C_3-(5) to DMPC concentration ratio (Fig. 6). There appears to be no threshold probe concentration at which the dye-induced T_2 reduction begins to develop, although there is a significant concentration ratio range over which the T_2 could not be measured with sufficient accuracy to distinguish with statistical significance any dye effect on this relaxation time from that of the control dye-free vesicles. The possibility remains, however, that a tendency of diS-C_3-(5) to promote apparent membrane fusion in functional systems may thus be present at significantly lower dye-to-membrane concentration ratios than those employed in the investigations described in this communication.

Acknowledgments

This work was supported by NIH awards GM 30552, RR 07171, Biomedical Research Development Award 09201 to the Laboratory for Microbial and Biochemical Sciences, and by NSF equipment grants CDP-7924717 and PCM-8200262. We thank Mr. Daniel Adams for his assistance in the preparation of the manuscript, Dr. S. Deshmukh for her assistance during the early stages of these investigations, Mr. David McClanahan for performing thin-layer chromatography analyses, and Professors Joseph Hadley and William Nelson of the Georgia State University Physics Department for access to EPR spectrometer facilities.

References

1 Waggoner, A.S., Wang, C.H. and Tolles, R.L. (1977) J. Membrane Biol. 33, 109–140
2 Loew, L.M., Bonneville, G.W. and Surrow, J. (1978) Biochemistry 17, 4065
3 Loew, L.M., Scully, S., Simpson, L. and Waggoner, A.S. (1979) Nature 281, 497
4 Loew, L.M., Simpson, L., Hassner, A. and Alexanian, V. (1979) J. Am. Chem. Soc. 101, 5439
5 Laris, P.C., Bahr, D.P. and Chaffee, R.R.J. (1975) Biochim. Biophys. Acta 376, 415–425
6 Bashford, C.L. and Thayer, W.S. (1977) J. Biol. Chem. 252, 8459–8463
7 Smith, J.C. and Chance, B. (1979) J. Membrane Biol. 46, 255–282
8 Smith, J.C., Frank, S.J., Bashford, C.L., Chance, B. and Rudkin, B. (1980) J. Membrane Biol. 54, 127–139
9 Smith, J.C., Hallidy, L. and Topp, M.R. (1981) J. Membrane Biol. 60, 173–186
10 Bammel, B.P., Brand, J.A., Germon, W. and Smith, J.C. (1986) Arch. Biochem. Biophys. 244, 67–84
11 Akerman, K.E.O. and Wikström, M.K.F. (1976) FEBS Lett. 68, 191–197
12 Hoffman, J.F. and Laris, P.C. (1974) J. Physiol. (London) 239, 519
13 Sims, P.J., Waggoner, A.S., Wang, C.H. and Hoffman, J.F. (1974) Biochemistry 13, 3315–3330
14 Freedman, J.C. and Novak, T.S. (1983) J. Membrane Biol. 72, 59
15 Freedman, J.C. and Miller, C. (1984) Ann. N.Y. Acad. Sci. 435, 541
16 Cohen, L.B., Salzberg, B.M., Davila, H.V., Ross, W.N., Landowne, D., Waggoner, A.S. and Wang, C.H. (1974) J. Membrane Biol. 19, 1–36
17 Ross, W.N., Salzberg, B.M., Cohen, L.B. and Davila, H.V. (1974) Biophys. J. 14, 983
18 Ross, W.N., Salzberg, B.M., Cohen, L.B., Grinvald, A., Davila, H.V., Waggoner, A.S. and Wang, C.H. (1977) J. Membrane Biol. 33, 141
19 Salzberg, B.M. (1984) Biophys. J. 45, 154a
20 Fluhler, E. and Loew, L.M. (1984) Biophys. J. 45, 167a
21 Salama, G. and Morad, M. (1976) Science 191, 484
22 Salama, G. and Morad, M. (1979) J. Physiol. (London) 292, 267
23 Kauppinen, R.A. and Hassinen, I.E. (1984) Am. J. Physiol. 247, H508–H516
24 Bashford, C.L., Barlow, C., Chance, B., Smith, J., Silberstein, B. and Rehncrona, S. (1978) Frontiers of Biological Energetics (Scarpa, A., Dutton, P.L. and Leigh, J.S., eds.), Vol. 2, pp. 1305–1311, Academic Press, New York
25 Mayevsky, M. and Chance, B. (1975) Brain Res. 98, 149
26 Smith, J.C. and Evans, D. (1986) Biophys. J. 49, 95a
27 Waggoner, A.S. (1976) J. Membrane Biol. 27, 317–334
28 Cohen, L.B. and Salzberg, B.M. (1978) Rev. Physiol. Biochem. Pharmacol. 83, 35–88
29 Bashford, C.L. and Smith, J.C. (1979) Methods Enzymol. 55, 569–586, Academic Press, New York
30 Bashford, C.L. (1981) Biosci. Rep. 1, 183
31 Smith, J.C. (1986) Potential-Sensitive Molecular Probes in Energy-Transducing Organelles, in Spectroscopic Membrane Probes (Loew, L.M., ed.), CRC Press, Boca Raton, FL, in the press
32 Schuurmans, J.J., Casey, R.P. and Kraayenhof, R. (1978) FEBS Lett. 94, 405–409

33 Peters, F.A.L.J., Van Spanning, R. and Kraayenhof, R. (1983) Biochim. Biophys. Acta 724, 159–165
34 Peters, F.A.L.J., Van der Pal, R.H.M., Peters, R.L.A., Vredenberg, W.J. and Kraayenhof, R. (1984) Biochim. Biophys. Acta 766, 169–178
35 Galmiche, J.M. and Girault, G. (1980) FEBS Lett. 118, 72–76
36 Bashford, C.L., Chance, B. and Prince, R.C. (1979) Biochim. Biophys. Acta 545, 45–57
37 Williams, R.J.P. (1961) J. Theor. Biol. 1, 1
38 Mitchell, P. and Moyle, J. (1969) Eur. J. Biochem. 7, 471–484
39 Beeler, T.J., Farmen, R.H. and Martonosi, A.N. (1981) J. Membrane Biol. 62, 113–137
40 Russell, J.T., Beeler, T. and Martonosi, A. (1979) J. Biol. Chem. 254, 2047–2052
41 Malpress, F.H. (1984) J. Theor. Biol. 109, 510–521
42 Kell, D.B. (1979) Biochim. Biophys. Acta 549, 55
43 Kinally, K.W., Tedeshi, H. and Maloff, B.L. (1979) Biochemistry 17, 3419
44 Tedeshi, H. (1980) Biol. Rev. 55, 171–206
45 Laris, P.C., Bahr, D.P. and Chaffee, R.R.J. (1975) Biochim. Biophys. Acta 376, 415–425
46 Smith, J.C., Russ, P., Cooperman, B.S. and Chance, B. (1976) Biochemistry 15, 5094
47 Suurkuusk, J., Lentz, B.R., Barenholz, Y., Biltonen, R.L. and Thompson, T.E. (1976) Biochemistry 15, 1393
48 Heinonen, J.R. and Lahti, R.J. (1981) Anal. Biochem. 113, 313–317
49 Hamilton, R.L., Guo, L.S.S. (1984) Liposome Technology (Gregoriadis, G., ed.), Vol. 1, pp. 37–65, CRC Press, Boca Raton, FL
50 Bashford, C.L. and Smith, J.C. (1979) Biophys. J. 25, 81–85
51 Johnson, M.L. and Schuster, T.M. (1974) Biophys. Chem. 2, 32–41
52 Turner, B.W., Pettigrew, D.W. and Ackers, G.K. (1981) Methods Enzymol. 76, 622–628
53 Cornish-Bowden, A. and Eisenthal, R. (1974) Biochem. J. 139, 721–731
54 Porter, W.R. and Trager, W.F. (1977) Biochem. J. 161, 293–302
55 Bashford, C.L., Chance, B., Smith, J.C. and Yoshida, T. (1979) Biophys. J. 25, 63–85
56 Smith, J., Powers, L., Prince, R., Chance, B. and Bashford, L. (1978) Frontiers of Biological Energetics (Scarpa, A., Dutton, P.L. and Leigh, J.S., eds.), pp. 1293–1303, Academic Press, New York
57 Burnell, E.E., Cullis, P.R. and De Kruijff, B. (1980) Biochim. Biophys. Acta 603, 63–69
58 McLaughlin, A.C., Cullis, P.R., Berden, J.A. and Richards, R.E. (1975) J. Magn. Reson. 20, 146–165
59 Campbell, R.F., Melrovitch, E. and Freed, J.H. (1979) J. Phys. Chem. 83, 525–533
60 Tanford, C. (1967) Physical Chemistry of Macromolecules, pp. 433–434, John Wiley, New York
61 Waggoner, A.S., Wang, C.H. and Tolles, R.J. (1977) J. Membrane Biol. 33, 109–140
62 McLaughlin, S. and Harary, H. (1976) Biochemistry 15, 1941–1948