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Lipid Lateral Diffusion by Pulsed Nuclear Magnetic Resonance†

An-Li Kuo and Charles G. Wade*

ABSTRACT: The temperature and hydration dependences of lipid lateral diffusion in model membrane–D_2O multilayers of dipalmitoyl (DPL), dimyristoyl (DML), dilauryl (DLL), and egg yolk (EYPC) lecithins were measured by using pulsed gradient proton NMR spin–echo techniques. Oriented samples were used to minimize anisotropic dipolar interactions and permit formation of a spin–echo. A general discussion of the technique and of the possible errors is included. Significantly lipid lateral diffusion is hydration dependent over the range studied (15–40% D_2O, w/w), varying in DPL over this range, for example, by a factor of 2. For the saturated lipids at the same hydration and temperature, diffusion decreases monotonically as the chain length increases. At a constant hydration of 20% D_2O (w/w), the activation energies (kcal/mol) are as follows: EYPC, 9.0 ± 0.4; DLL, 9.4 ± 0.2; DPL, 18.6 ± 1.3; DML, 15.2 ± 0.3. The results tend to be larger, by factors of 2–5, than the earlier ESR spin-label results, the differences being attributable in part to the differences in hydration and to the absence of probe effects in this work. The value of 5×10^{-8} cm^2/s for DPL (40% D_2O) at 42 °C is slightly larger than the usually reported spin-label value of 2×10^{-8} and than the fluorescence photobleaching recovery measurements which give typically 1×10^{-8} cm^2/s. The results agree with recent photo-spin-label measurements. Cholesterol in small amounts (less than 10 mol %) in DPL *increases* lipid diffusion; its presence in larger concentrations decreases diffusion.

Many fundamental processes in membranes and cells require molecular motions of the membrane components (Singer & Nicolson, 1972; Edidin, 1974; Frye & Edidin, 1970; Wu et al., 1977; Shimshick & McConnell, 1973; Linden et al., 1973; Crick, 1970). In particular, lateral diffusion of lipids and of proteins is important in embryological development (Crick, 1970), in cell fusion (Frye & Edidin, 1970; Wu et al., 1977), in membrane phase separations (Shimshick & McConnell, 1973), and in membrane transport processes (Linden et al., 1973). X-ray diffraction (Levine, 1972), differential calorimetry (Chapman, 1975; Melchior & Steim,

† From the Department of Chemistry, University of Texas, Austin, Texas 78712. *Received November 16, 1978; revised manuscript received March 14, 1979.* The financial support of the Robert A. Welch Foundation (Grant F-370) and the National Institutes of Health (Grant HL-12528) is gratefully acknowledged.

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1976), ESR (McFarland, 1972), and freeze fracture (Branton, 1969; Bullivant, 1969) studies confirm that biological membranes contain regions with a phospholipid bilayer structure. A large body of research indicates that phospholipid–H_2O systems duplicate many properties of natural membranes and can serve as models for biological membranes. Soaps and lipids, when mixed with water at appropriate concentrations, form well-ordered multilayers, i.e., a lamellar phase (Chapman et al., 1967; Eins, 1970), and so are the most widely used systems. Self-diffusion coefficients of these systems have been estimated by using several techniques. These methods often require either photolabeling (FCS, FPR, etc.) (Fahey et al., 1977; Razi-Naqvi et al., 1974; Wu et al., 1977; Sheats & McConnell, 1978; Smith & McConnell, 1978; Rubenstein et al., 1979) or paramagnetic labeling (ESR) (Devaux & McConnell, 1972; Sackman & Trauble, 1972; Gaffney & Chen, 1977; Scandella et al., 1972). Because the label is often rather bulky, it may disrupt the system when it is introduced and can only provide an estimation of the lipid D.[1] Nuclear magnetic resonance relaxation studies can provide an estimation of the diffusion coefficients (Cullis, 1976; Fisher & James, 1978; Lee et al., 1973; Bloom et al., 1978) without the use of added probes, but gross assumptions must often be made to extract a lipid D.

We report results of direct measurements of lipid diffusion with minimal data analysis in oriented lipid–D_2O model membrane multilayers using pulsed NMR CPSE (Carr & Purcell, 1954) techniques. CPSE NMR has been used for two decades to measure D of isotropic liquids. For lipid systems, however, the formation of a CPSE is generally precluded because the anisotropic motions of the lipids result in only a partial averaging of the dipolar interactions (DeVries & Berendsen, 1969; Hemminga & Berendsen, 1972). This problem can be circumvented, however, if oriented multibilayer samples are used. By orientation of the lipid chains at 54° 44′ to the applied field, the dipolar interactions are minimized, a CPSE can be observed, and diffusion can be measured (Samulski et al., 1973; Chien et al., 1974; Roeder et al., 1976; Wennerstrom & Lindblom, 1977; Lindblom et al., 1976; Kuo and Wade, unpublished experiments; Tiddy et al., 1974). Here we extend (Samulski et al., 1973; Chien et al., 1974; Roeder et al., 1976; Kuo and Wade, unpublished experiments) the method to phospholipid diffusion in multibilayers (typically 1000 bilayers) oriented in a conventional manner (Devaux & McConnell, 1972; Wennerstrom & Lindblom, 1977; Lindblom et al., 1976; DeVries & Berendsen, 1969; Hemminga & Berendsen, 1972; Roeder et al., 1976; Kuo and Wade, unpublished experiments) between glass plates. That the diffusion measured is that of lipids parallel to the bilayer is virtually assured by the experimental conditions (Wennerstrom & Lindblom, 1977; Lindblom et al., 1976; Samulski et al., 1973; Chien et al., 1974; Roeder et al., 1976).

Experimental Procedure

Materials. Commercial grade EYPC was purchased from Sigma Chemical Co. and purified by column chromatography. It contained no detectable impurities under thin-layer chromatography carried out on silica gel with a solution of chloroform–methanol–water–acetic acid (65:25:4:1 v/v/v/v). The purified EYPC was dissolved in benzene (~0.1 g/mL), sealed in vials under N_2, and stored at 10 °C. The same batch of EYPC was used for all experiments.

DML and DLL were purchased from Sigma Chemical Co. and Calbiochem, respectively. The DPL used in the hydration experiment was purchased from Serdary Chemical Co. (Canada) and that used in the other experiments was purchased from Sigma Chemical Co. These saturated lipids were checked for purity by the same procedure as described for EYPC above and, if necessary, purified by column chromatography (Kuo, 1979). Cholesterol was purchased from Sigma Chemical Co. and recrystallized twice with methanol before use. The D_2O used was 98% D.

Preparation of Lipid Multilayers. (*a*) *Preparation of Bulk Lipid–D_2O to be Oriented.* All saturated lipids were dried under vacuum (~10^{-5} Torr) for at least 24 h at 90 °C to remove traces of H_2O, and all other manipulations were done in a glove bag under N_2. (The effects of H_2O as an "impurity" are discussed later.) An appropriate amount of D_2O (for example, 80% lipid–D_2O, w/w) was added to the dried lipids by a graduated micropipet in a small vial (8- or 9-mm diameter) with a narrow constriction (DeVries & Berendsen, 1969) in the middle. The mixture was centrifuged back and forth through the constriction at a temperature above the transition point. Equilibrium hydration requires from 7 days to several weeks at room temperature, but about a day at temperatures above about 70 °C (Elworthy, 1961; Kuo, 1979).

The unsaturated lipids in EYPC make this compound sensitive to light and higher temperatures, so extreme care was taken during sample preparation of this compound. In particular, drying was done under conditions where the temperature was less than 50 °C.

Samples of cholesterol and lipids were prepared by first dissolving the compounds in benzene to ensure homogeneous mixing. The samples were then freeze-dried and hydrated under N_2 as described above.

(*b*) *Oriented Lipid–D_2O and Lipid–Cholesterol–D_2O Samples.* Oriented lipid–D_2O multilayers were prepared in an inert atmosphere (N_2) by using the method of DeVries & Berendsen (1969) which results in microscope cover slip "sandwiches", consisting of a liquid crystal layer ~3000–5000 bilayers thick, depending upon the hydration condition of the lipids. The microscope cover slips (Corning cover glass 1) were cut (6 × 24 mm) to fit inside the NMR tubes. To obtain the best sample orientation, the plates must be initially scrupulously clean and free of defects, cracks, and scratches. Cleaning was done by the following procedure. The plates were dipped in and swabbed with benzene and then with acetone. Following this, they were dipped in boiling aqua regia for 5 min, washed with deionized water, washed with D_2O, and then dried with N_2. At this point the surface was hydrophilic and could be wetted completely by water. A treatment of the glass surface with either *p*-xylene or with a surfactant solution was then done. The surfactant treatment, which gave better results, consisted of dipping several times in a 1% solution of hexadecyltrimethylammonium bromide in chloroform and then drying with N_2. This procedure changes the glass surface from hydrophilic to hydrophobic, thus facilitating the interdigitation of the hydrophobic portion of the molecules with the treated glass surface. This orientation technique results in multibilayers with the long lipid axes generally perpendicular to the glass plates. Average molecular orientation was checked by using a polarizing microscope equipped with crossed polaroids and a first-order red retardation plate (DeVries & Berendsen, 1969). A pink color implies a uniaxial sample, and the uniformity of color indicates

[1] Abbreviations used: D, self-diffusion coefficient; CPSE, Carr–Purcell spin–echo; EYPC, egg yolk phosphatidylcholine; DML, dimyristoylphosphatidylcholine; DLL, dilaurylphosphatidylcholine; DPL, dipalmitoylphosphatidylcholine; FCS, fluorescence correlation spectroscopy; FPR, fluorescence photobleaching recovery; T_2, spin–spin relaxation time.

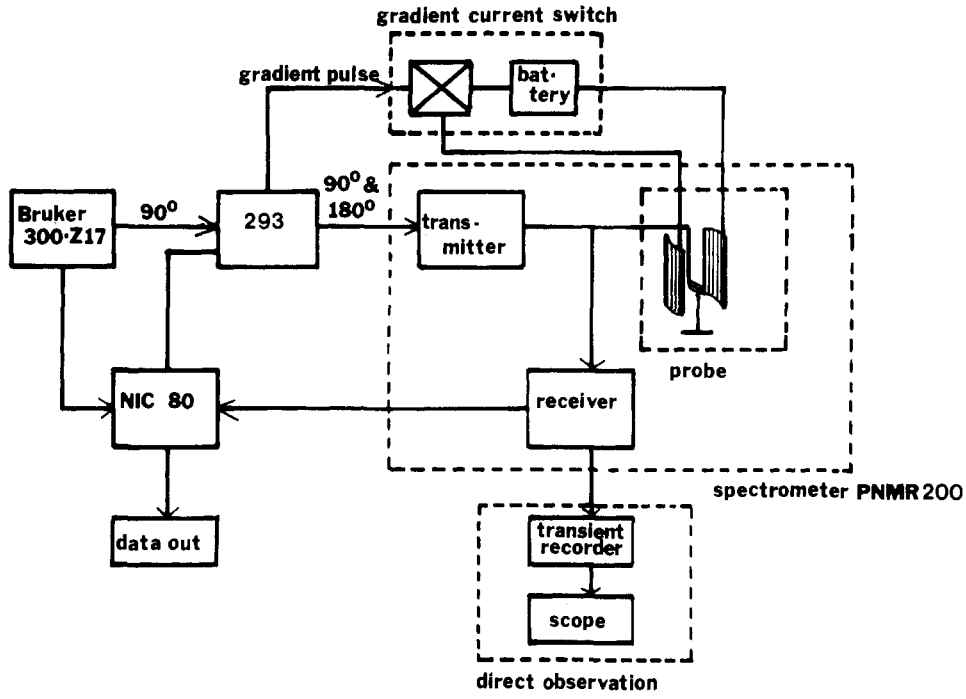

FIGURE 1: Block diagram of apparatus.

the homogeneity of the orientation. Approximately 20–25 of these sandwiches were sealed into a 6 × 6 mm square glass tube (Wilmad Glass Co.) which served as the NMR sample tube. In the probe, the top of this tube was fitted into a simple goniometer which provided sample rotation with a precision of about 1°.

The lipid/D_2O ratio was an important factor in this study. When applicable, the relative humidity in the tube was controlled by using an appropriate salt solution (Hemminga & Berendsen, 1972; *International Critical Tables*, 1926) in order to control the lipid/D_2O ratio. A relative humidity of 100% gives a 21% D_2O system (w/w), and 57% relative humidity gives a 14% D_2O system (Levine & Wilkins, 1971; Hemminga & Berendsen, 1972). For samples with less than 21% D_2O the samples were prepared (under N_2) in the proper ratio and then sealed in NMR tubes with reservoirs containing the appropriate NaCl–D_2O solution to maintain that ratio. Under these conditions, the samples and the D measurements were stable for weeks. For higher concentrations of D_2O, the samples were prepared in the proper ratio and then sealed in tubes containing a 100% relative humidity; these samples were run soon after sealing. In all cases, the samples were analyzed (Kuo, 1979) after measurements for the weight percent D_2O and found equal to the starting concentration within the experimental error of ±5%.

Instrumentation. A high-powered 30-MHz spectrometer described previously (Samulski et al, 1973; Chien et al., 1974) was used with a high-resolution Varian 12-in. electromagnet and associated apparatus as shown in Figure 1. Temperature variation (ambient to 62 °C) was obtained by using a heating tape on the probe. Values of the temperature were measured with a copper–constantan thermocouple and a digital reader.

The conventional 90° – τ – 180° pulse sequence was used (Figure 2) with two gradient pulses. A Nicolet NMR 80 computer was used to control the experiment and to collect and signal average the data. A Bruker digital pulse generator (Model B-KR 300Z17) provided the timing for the initial 90° pulse (~2 μs in duration); the 180° pulse and the gradient pulses were controlled by the Nicolet 293 pulse sequence interfaced to the NMR 80 computer. In the probe, a quadrupolar coil (Odberg & Odberg, 1974) (25 turns of no. 22 wire per quadrant) produced a gradient current of 10.4 G/(cm A). For the gradient source, a Ni–Cd battery pack was used. The resultant gradient was oriented parallel to the bilayers (36° with respect to H_0).

Method and Gradient Calibration. For the present work, diffusion was measured by Fourier transforming the last half of the echo (James & McDonald, 1973) as in Figure 2. Most of the earlier reported diffusion work has been done under low-resolution conditions wherein an average diffusion coefficient is determined by measuring the spin–echo amplitude [Stejskal (1972) and references cited therein] which obeys the Tanner–Stejskal equation (Stejskal & Tanner, 1965) given below. An important advantage of the present technique is that the intensities of all resolved lines in the transformed spectrum follow the same relationship; hence, diffusion coefficients of individual constituents can be measured (James & McDonald, 1973). The method requires that the spectrometer be slightly off resonance in order to perform the Fourier transformation. The advantages of this method with respect to spectrometer instabilities and the importance of the magnitude of spectrometer offset will be discussed elsewhere (M. Hrovat et al., unpublished experiments).

For this work, the spectrometer typically was 700–1000 Hz off resonance. Glycerin was chosen as the compound for gradient calibration since its published D (Fiorito & Meister, 1972) is similar to those of the lipids in this study. The glycerin was carefully dried (10^{-6} Torr at 90 °C for 24 h) to remove H_2O before use. The low-resolution (nonspinning sample) Fourier transformed spectra give two resolved peaks with 1.8-ppm separation representing the backbone protons and the hydroxyl protons. Rather surprisingly, presumably because of proton exchange, they have different diffusion coefficients, particularly at higher temperatures. Such was not obvious in earlier studies [Fiorito & Meister (1972) and references therein] which monitored the spin–echo and thus reported an average D. Our studies indicate that at low temperature (e.g., room temperature), $D_{\text{backbone}} \sim D_{\text{OH}}$ and the published D value (average value of D_{backbone} and D_{OH}) can be used. At higher temperatures, a correction is necessary. A detailed study will

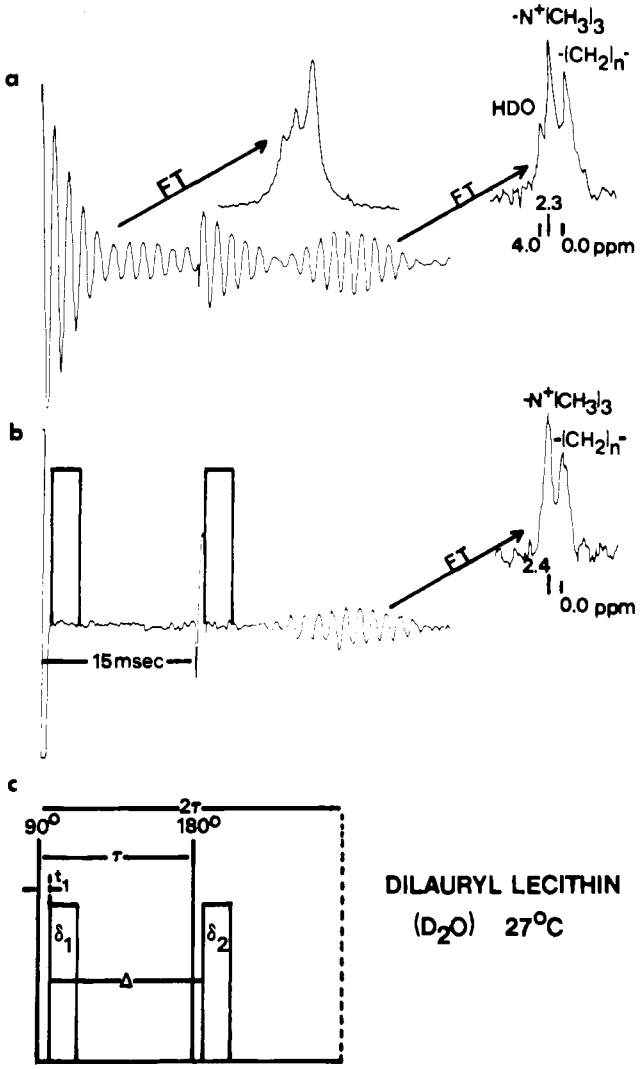

FIGURE 2: Pulse sequence in (c) applied to dilauryl lecithin–D_2O at the magic angle: (a) without gradient pulses and (b) with gradient pulses ($\delta_1 = \delta_2 = 3$ ms). Results shown are in all cases from one pulse sequence except that the FT spectrum in (b) is of five accumulations. Recorder display gain in (b) is 2 times the gain in (a).

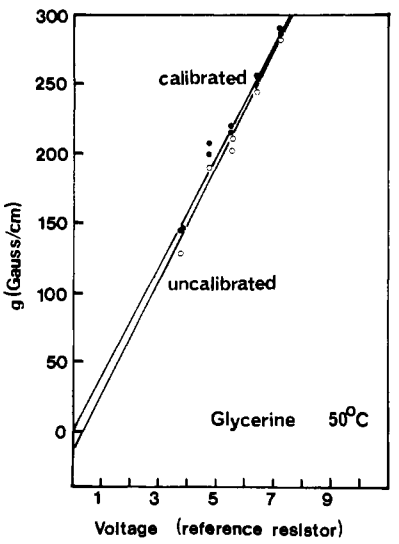

FIGURE 3: Graph used to obtain gradient from precision resistor voltage drop (arbitrary units) measured by boxcar integrator.

be presented elsewhere (M. Hrovat et al., unpublished experiments). As shown in Figure 3, when the corrected value was used for the glycerin D, the calibrated gradient field (determined by varying the current in the gradient coil) went through the origin at zero current flow. If the literature value (averaged) D had been used, a lower calibrated gradient would have been obtained and a larger calculated lipid D would have resulted. Experimentally identical conditions were used for the calibration as well as for the lipid diffusion measurement. All samples were thermally equilibrated for at least 30 min before the data were collected. It was necessary to carefully adjust the gradient pulse widths in order to maximize the spin–echo amplitude; adjustments were made until the peak of the spin–echo occurred at the expected 2 τ after the 90° pulse. Failure to do so would result in incomplete rephasing of the spin–echo and ultimately an apparent lipid D larger than the actual D.

Table I: Pulsed Gradient Spin–Echo NMR Parameters

symbol	definition	typical values	units
t_1	separation of gradient and 90° pulse	1–2	ms
τ	interval between 90° pulse and 180° pulse	20	ms
Δ	interval between gradient pulses	20	ms
δ_1	width of first gradient pulse	5	ms
δ_2	width of second gradient pulse	5	ms
g	amplitude of gradient	100–300	G/cm

Data Collection and Analysis. Gradient calibration and data collection were achieved by varying the current in the gradient coil while holding the parameters t_1, Δ, δ_1, δ_2, and τ (defined in Figure 2 and Table I) constant. The echo response is governed by the Tanner–Stejskal (Stejskal–Tanner, 1965) relation

$$A(\text{on})/A(\text{off}) = e^{-\gamma^2 D g^2 \delta^2 [\Delta - (1/3)\delta]} \quad (1)$$

which assumes a homogeneous static magnetic field H_0. In eq 1, A(on) is the amplitude of the peak in the FT spectrum of the spin echo with the gradient pulses on, A(off) is the amplitude with the gradient pulses off, γ is the nuclear gyromagnetic ratio, $\delta = \delta_1 \simeq \delta_2$, and g is the field gradient (G/cm).

In the experiments, after the echo was optimized by adjusting the timing and pulse-width parameters (Table I), a number of pulses (typically 10–20) were collected with the gradient on, and an equivalent number were collected with the gradient off. For calibration of g, glycerin, which has a known D (vida supra), was used. The gradient current was monitored by measuring (boxcar integrator) the voltage drop across a precision resistor in series with the gradient coil. This current was then assumed to be a measure of g in the lipid experiments.

Resolution. Berendsen and co-workers (Hemminga & Berendsen, 1972; Dijkema & Berendsen, 1974) measured the ^1H NMR spectra of oriented lipid–water and soap–water and found a minimum line width of about 510 Hz at 60 MHz (8.5 ppm) with DPL–cholesterol–water samples oriented at the magic angle. Our spectra had somewhat better resolution for both lipid–D_2O and lipid–cholesterol–D_2O systems. The conventional Fourier transform spectra (Fourier transform of the free induction decay after the 90° pulse) consisted of a broad non-Lorentzian band with splittings (Figure 2) and a total line width $\Delta\nu_{1/2}$ (width at half-height) of about 200 Hz (6.7 ppm) above the phase transition at the magic angle. An even narrower line width is obtained from the Fourier transform of the last half of the spin–echo probably because the spins on lipids poorly aligned are not refocused by the 180° pulse. The line width shows little temperature dependence. It is angularly dependent above the phase transition, varying approximately as $1/2\langle 3\cos^2\theta - 1\rangle$, where θ is the angle

Table II: Composition and Thermal Data on Samples

composition[a]	no. chain carbons	Chol mol %	wt % all components (lipid-chol-D_2O)	transition temp[b] T_c (°C)	temp range (°C)	ΔE_a (kcal/mol)
EYPC-D_2O	16-18	0	80:0:20	<amb	27.1-56.0	9.0 ± 0.4
EYPC-Chol-D_2O	16-18	29.2	62:17.4:20		31.7-57.0	8.2 ± 0.3
DLL-D_2O	12	0	80:0:20	<amb	26.5-51.7	9.4 ± 0.2
DLL-Chol-D_2O	12	28.6	64:16:20		27.0-52.0	9.6 ± 0.4
DML-D_2O	14	0	80:0:20	31.0	31.5-51.0	15.2 ± 0.3
DPL-D_2O	16	0	85:0:15	52.5	52.2-62.4	18.6 ± 1.3
DPL-D_2O	16	0	80:0:20	47.0	49.2-62.3	15.2 ± 0.2
DPL-D_2O	16	0	75:0:25	41.0	47.1-62.3	13.6 ± 0.1
DPL-D_2O	16	0	70:0:30	41.0	44.0-61.9	13.1 ± 0.1
DPL-D_2O	16	0	65:0:35	41.0	43.1-62.2	13.4 ± 0.3
DPL-D_2O	16	0	60:0:40	41.0	43.8-61.0	13.2 ± 0.5
DPL-Chol-D_2O	16	3.5	78.5:1.5:20		36.1-56.2	12.2 ± 0.2
DPL-Chol-D_2O	16	8.8	76.7:3.3:20		39.7-56.3	13.5 ± 0.1
DPL-Chol-D_2O	16	10.6	74.1:5.9:20		46.8-56.9	10.5 ± 0.3
DPL-Chol-D_2O	16	20.5	70.4:9.6:20		34.0-56.4	12.0 ± 0.4
DPL-Chol-D_2O	16	23.5	68.8:11.2:20		28.3-52.8	11.0 ± 0.4
DPL-Chol-D_2O	16	28.3	66.2:13.8:20		34.6-53.7	12.6 ± 0.1
DPL-Chol-D_2O	16	49.0	53.1:26.9:20		35.5-53.2	12.8 ± 0.2

[a] Chol represents cholesterol. [b] Amb represents ambient.

between H_0 and the normal to the plates but is angularly invariant in the gel phase (below the phase transition). In the gel phase, molecular motion is greatly restricted (Urbina & Waugh, 1974; Smith & McConnell, 1978). Whereas the motions above the transition tend to average the dipolar interactions until it has major components along only the molecular axes, in the gel phase motions are more typically those of solids: there is no single common major axis for the dipolar anisotropy. Consequently, in the gel phase there is no sharpening of the signal at the magic angle and no strong spin–echo. Thus, this technique in its present form is not applicable to study diffusion in the gel phase.

A general analysis of $\Delta\nu_{1/2}$ would probably be of doubtful significance because of the limited knowledge of the details of the line shape. Nevertheless, $\Delta\nu_{1/2}$ provides a useful and easy qualitative monitor of the phase transition and of the orientation of the sample.

Because of the relatively low resolution, a separate water peak cannot be observed. If present, H_2O causes serious errors since even in low concentrations in lipids it has a spin–spin (T_2) relaxation time of ~30 ms and a diffusion coefficient of typically 10^{-6} cm^2/s (Inglefield et al., 1976; Rigand et al., 1972). With pure lipid–D_2O systems, the observed T_2 is about 10–15 ms. H_2O, if present, thus dominates both the line width of the spectra and the spin–echo and would obviously cause serious errors in D.

Errors. In general, most of the experimental difficulties encountered in this method tend to result in a D larger than the actual D. Included in this category are spectrometer gradient instabilities (which reduce the echo amplitude) and the presence of H_2O. In the several years these experiments have been done in this laboratory, we have systematically reduced these problems. As discussed earlier, great care is taken to preclude the presence of H_2O. The stability and reproducibility of the gradient pulses is a particular problem since variations of one part in 10^4 can cause echo dephasing (Cooper et al., 1974). We solve this problem by a combination of apparatus design (including battery maintenance), careful observation of the location (in time) of the echo maximum, and repetitive, low-duty cycle operation of the gradient system.

One source of error which would result in a D smaller than the actual D is the presence of restricted diffusion, perhaps caused by variations in the homogeneity of the alignment. Conventional pulsed gradient CPSE tests used to test for this (Stejskal, 1972) indicate no restricted diffusion is occurring over a radius of about 7000 Å.

Two or three freshly prepared samples were used for each D measurement; the standard deviation was typically ±20%.

These procedures and the agreement with other work (see Results and Discussion) indicate that our results tend toward an upper bound on the actual D. We feel that the actual D probably lies within our estimated error of ±30%.

Results and Discussion

The results show lipid D to be dependent upon the chemical structure of the lipid, upon the temperature, upon the extent of hydration, and upon the presence of cholesterol. Insofar as possible, the effect of each variable will be discussed separately, but it should be noted that these are not always independent.

Temperature Dependence. The lower limit of the temperature variation is either the ambient temperature (about 27 °C) or the phase transition temperature (Table II). The latter in general agreed with published phase diagrams (Chapman et al., 1967). All data for lipid–D_2O systems gave linear Arrhenius plots; Figure 4a shows a comparison of lipid D for 20% D_2O. Under similar conditions of hydration, the saturated lipid D data indicate that D decreases as chain length increases (or alternatively, as the transition temperature increases). The activation energies, E_a, from the slopes of the plots (Table II) are monotonic with chain length. A direct comparison of EYPC with the saturated lipids is difficult and of doubtful significance. EYPC is a mixture of lipids with a structure consisting usually of an unsaturated fatty acid chain at the β position of the glycerol backbone and a saturated fatty acid chain at the γ position. The saturated acyl chain is often palmitic while the unsaturated acyl chain often has 16–18 carbons with one or two double bonds. It is a common finding that unsaturation of acyl chains lowers the transition temperature and increases the local short-range molecular interactions (microviscosity) of the molecules as measured by fluorescence (Cogan et al., 1973) and paramagnetic probes. These effects arise in part from the poorer lipid packing possible with chains containing unsaturated bonds. The relationship of unsaturation and of microviscosity to lateral diffusion rates has not been carefully studied.

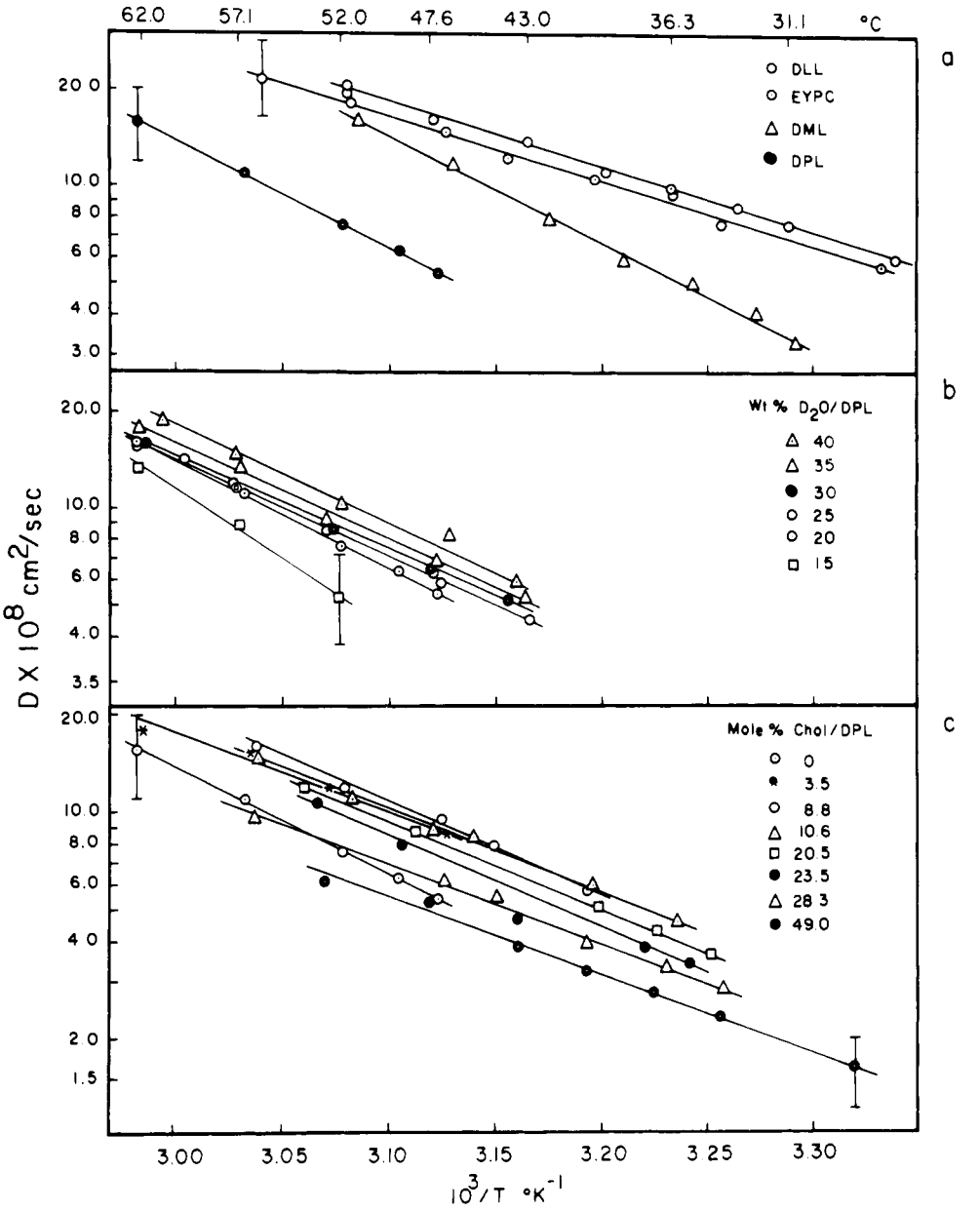

FIGURE 4: Lateral diffusion coefficients vs. temperature for different phospholipids. Points are mean diffusion coefficients obtained from several measurements. Error bars represent maximum spread of these measurements. (a) All samples are 80% by weight lipid in D_2O. (b) Hydration dependence of D in DPL. (c) Diffusion coefficients vs. temperature for various DPL–cholesterol–D_2O systems. All samples are 20% by weight D_2O; the cholesterol/lipid ratio varies.

Hydration Dependence. A significant result of this research is that lipid D is hydration dependent. As shown in Figure 4b and in Table II, D increases and E_a decreases as hydration increases. A qualitative understanding of these trends can be obtained from a consideration of variations of lamellar dimensions with hydration (Levine & Wilkins, 1971; Chapman et al., 1968; LeNeveu, et al., 1976; Elworthy, 1961; Luzatti, 1968) and from thermodynamic considerations. All of the dimensional changes are monotonic with hydration until they level off at about 40% H_2O. As hydration increases, the head group to head group dimension across the hydrophobic region decreases slightly, but the water layer dimensions and the interfacial area per molecular head group increase significantly. In EYPC, for example, a change in hydration from 15 to 40% increases the water channel dimension from about 10 to 27 Å (LeNeveu et al., 1977). Chapman et al. (1968) report that the interfacial area occupied by the DPL head group increases from 60 to 70 Å^2 as the H_2O concentration changes from 20 to 40%. A balancing of forces determines the equilibrium dimensions of the multilayers: attractive forces between the lamellae are opposed by the repulsive electrostatic forces of the head groups (LeNeveu et al., 1977). At lower water content, the decreased bilayer separation and interfacial area per molecule dictate that relatively strong forces are required for the lipids to diffuse. As hydration increases the structure is much less densely packed, and diffusion is easier. Our D and E_a values are qualitatively consistent with these changes.

Comparison with Other Work. A variety of techniques have been used to estimate lipid lateral D and recent reviews (Edidin, 1974; Gaffney & Chen, 1977; Wennerstrom & Lindblom, 1977) indicate the range is 10^{-7}–10^{-9} cm^2/s. Any comparison of experimental measurements confronts several possible complications. Initially, as this work indicates, the extent of hydration is important; hydration has seldom been controlled in earlier work. There is evidence from fluorescence studies that lipid D in single bilayers differs from that in multibilayers and in vesicles (Fahey & Webb, 1978) and evidence from ESR (Brulet & McConnell, 1975) that diffusion

in vesicles may be faster than that in multilayers. Finally, EYPC, an often used lipid, is a mixture whose composition may vary from source to source.

A variety of ESR spin-label estimates of lipid D exist. These tend toward the often quoted value (Gaffney & Chen, 1977) of $\approx 2 \times 10^{-8}$ cm^2/s first measured for spin labels in bis(dihydrosterculoylphosphatidylcholine) at 25 °C (Devaux & McConnell, 1972) and in DPL at 40 °C (Sackman & Trauble, 1972). In general, our results tend to be larger, by factors of 2–5, than the early ESR results. These differences can perhaps be attributed to the absence of probe effects in our experiments with some contribution for differences in hydration. A recent photochemical spin-label experiment of Sheats & McConnell (1978) gave $D = 9.9 \times 10^{-8}$ cm^2/s for DPL (40% H_2O) at 48 °C, in excellent agreement with our data.

FCS and FPR techniques (Fahey et al., 1977; Fahey & Webb, 1978; Wu et al., 1977) give values of D which are dependent upon the probe (Fahey et al., 1977) and on the physical state of the system (Fahey & Webb, 1978). At 41 °C, our D for DPL (40% D_2O) is 5×10^{-8} cm^2/s, smaller than the fluorescence results for single bilayers of $\approx 10^{-7}$ cm^2/s (Fahey et al., 1977; Fahey & Webb, 1978) but larger than the results for DPL multilayers and large vesicles of $\approx 10^{-8}$ cm^2/s (Fahey & Webb, 1978; Wu et al., 1977).

Estimates of D have been obtained by using as a probe the triplet state of anthracene present as a phospholipid head group label. By use of a flash spectrophotometer, the triplet–triplet annihilation kinetics were studied to yield a $D \approx 2 \times 10^{-7}$ cm^2/s in DPL vesicles at 50 °C (Razi-Naqvi et al., 1974).

NMR techniques different from those used in this work give varied results; some (Lee et al., 1973; Bloom et al., 1978; Kroon et al., 1976) provide bounds on D which include the data in Figure 1; some (Brulet & McConnell, 1975; Cullis, 1976) estimate a value consistent with the ESR results of 2×10^{-8} cm^2/s; and one (Fisher & James, 1978) gives even lower values. The one other report using an oriented sample NMR technique similar to ours though without humidity control gives (Wennerstrom & Lindblom, 1977) a D of 1.2×10^{-7} cm^2/s for DLL at 50 °C for a 25% D_2O mixture, in good agreement with our value of 1.8×10^{-7} cm^2/s. Diffusion of DLL and of EYPC in the cubic phases of lipid–sodium cholate–D_2O mixtures has been measured in the same laboratory (Wennerstrom & Lindblom, 1977; Lindblom et al., 1976) and used to estimate lipid D values in the lamellar phases. They find that the two lipid D rates are about the same (consistent with our results) and equal to 2×10^{-8} cm^2/s at 24 °C and 7×10^{-8} cm^2/s at 40 °C (slightly less than our results).

Our E_a of 9.01 kcal/mol for EYPC (20% D_2O) is in general agreement with values reported by others: 11.2 kcal/mol from ^{31}P NMR (Cullis, 1976); 7.3 kcal/mol from fluorescence polarization (Cogan et al., 1973); and 8–10 kcal/mol from FPR (Wu et al., 1977). The variation in EYPC composition might partially account for the variation in experimental values. For DPL, our limiting value of 13 kcal/mol is in serious disagreement with the fluorescence probe measurements which find no temperature dependence (Fahey & Webb, 1978).

We should note that the characteristic time over which diffusion is measured may have an influence on the observed D, especially in mixtures. In the present work, the characteristic time is the time between gradient pulses, typically 40 ms. In this interval, a two-dimensional random walk model predicts a root mean square displacement of $\sim 2(Dt)^{1/2}$ where t is the characteristic time (Devaux & McConnell, 1972). For

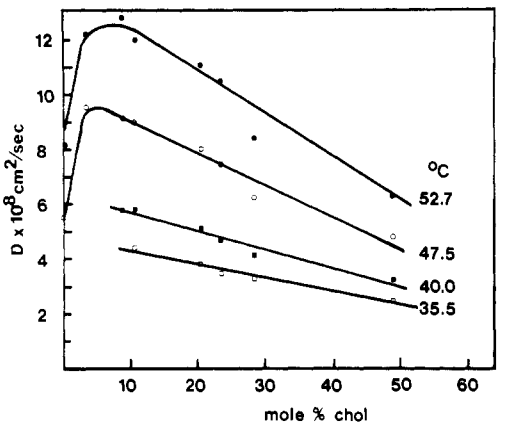

FIGURE 5: Same data as Figure 4c but plotted vs. cholesterol concentration at different temperatures.

DPL at full hydration, for example, this value in our experiments is about 10^4 Å so each molecule moves about 1000 lattice sites by assuming a hexagonal lattice with ~ 70 Å^2 per molecule. It *may* be a significant that the kinetics work, which uses a shorter characteristic time than our work, gives a faster D, whereas the EPR, FCS, and FPR experiments, which measure over time periods of seconds to hundreds of seconds, give in general somewhat shorter values.

Effects of Cholesterol. This three-component system was studied at a constant D_2O concentration of 20% by weight. Because the cholesterol protons could not be resolved from the lipid protons, this experiment measures the effects of diffusion of both molecules. At low cholesterol concentrations, however, the proton signal is completely dominated by the lipid signal and thus accurately measures lipid D.

As noted elsewhere (Kuo & Wade, 1979), at high concentrations (above 25 mol%), cholesterol decreases the lipid D. In EYPC and in DLL, the addition of approximately 30 mol % cholesterol decreases D by 50 and 40%, respectively, but causes little change in E_a (Table II). Similar results have been reported by others (Edidin, 1974; Fahey et al., 1977; Wu et al., 1977; Devaux & McConnell, 1972; Sackman & Trauble, 1972; Gaffney & Chen, 1977; Wennerstrom & Lindblom, 1977; Lindblom et al., 1976; Rubenstein et al., 1979): at high cholesterol concentrations, lipid D decreases as cholesterol content increases. At cholesterol concentrations below 10 mol %, however, our results are very unusual (Figure 4c). For DPL, D *increases* with initial small concentrations of cholesterol. Only at higher concentrations does cholesterol cause an apparent decrease in lipid D (Figure 5). This increased diffusion over the concentration range of ≤ 10 mol % disagrees with recent findings of Rubenstein et al. (1979) using FPR in DML–cholesterol–water oriented multilayers. They found a monotonic decrease in D with increasing cholesterol concentrations; the decrease was very slow up to about 20 mol % cholesterol where a more sudden decrease was noted. Although we cannot specifically account for these differences, it may be significant that their characteristic time (~ 1000 s) is much longer than ours. At dilute concentrations, an average molecule in our experiment samples primarily lipid–lipid interactions, whereas on the longer FPR time scale lipid–cholesterol interactions are also sampled. The only other published result consistent with our observation is an ESR study of Hemminga (1975) of a spin-labeled cholesterol analogue in oriented lecithin. His results showed an increase in the rotational and librational motions of the cholesterol at concentrations less than 10 mol %. Hemminga suggested that the increased motions could be due to a loosening of the lipid

packing and to perhaps a removal of the tilt of the lipids by the small amount of cholesterol. The presence of cholesterol over the concentration used in our study makes substantial changes in lamellar dimensions (Lecuyer & Dervichian, 1969). At higher concentrations, Hemminga's work indicates cholesterol stiffens the membrane, effectively inhibiting the motion of the lipid molecules. Our measurements are of translation; intuitively, however, the rotational motion of individual molecules in a membrane must be qualitatively related to ease of translation. Thus, we consider our observations consistent with Hemminga's conclusions.

Conclusions

This technique provides a measurement of lipid D without the possible interferences of probe molecules. Proton displacements of a few thousand angstroms must occur in a few milliseconds for diffusion to be detected by the methods; consequently, rotational and tilting motions of the lipids will not be observed.

An unusual aspect of the results is that D depends upon the extent of hydration. Because of this, a comparison with previously reported estimates of D is a bit risky. Our method gives results which agree with the new photochemical spin-label technique (Sheats & McConnell, 1978) and with a similar NMR technique applied elsewhere (Wennerstrom & Lindblom, 1977). Our results indicate a somewhat faster D than most earlier ESR reports and than recent fluorescence measurements (Fahey & Webb, 1978). It is speculated that the characteristic time interval used in different types of experiments to measure diffusion may be a factor in the value of D obtained from each type.

For the saturated lipids, at constant hydration, D decreases as the molecular weight increases, an intuitively satisfying result.

The cholesterol results indicate that at concentrations below 10 mol % it increases lipid diffusion; still higher cholesterol concentrations result in a stiffening of the lipid structure with a decrease in lateral mobility.

This technique provides a direct measure of D with a minimum of data analysis. It is applicable to membranes which can be macroscopically oriented.

Acknowledgments

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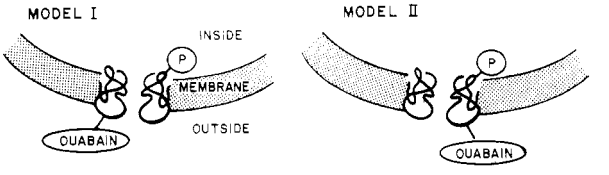
Evidence That Ouabain Binds to the Same Large Polypeptide Chain of Dimeric Na,K-ATPase That Is Phosphorylated from P_i†

Bliss Forbush III* and Joseph F. Hoffman

ABSTRACT: The functional unit of the Na,K-ATPase has previously been shown to include two large polypeptide chains (mol wt ≃ 95 000), only one of which can bind ouabain or be phosphorylated at one time. We have investigated the problem as to whether, when ouabain is bound and the enzyme is simultaneously phosphorylated from inorganic phosphate (P_i), both ligands are on the same large polypeptide chain or if they are on different 95K chains. We covalently labeled Na,K-ATPase purified from pig kidney outer medulla by using [^{3}H]-2-nitro-5-azidobenzoylouabain ([^{3}H]NAB-ouabain) and [^{32}P]phosphate, solubilized it with sodium dodecyl sulfate, and isolated the 95K polypeptide chains by using polyacrylamide gel electrophoresis. Large polypeptide chains labeled with [^{3}H]NAB-ouabain were separated from unlabeled chains by binding to a ouabain antibody and precipitation with immobilized protein A of *Staphylococcus aureus*. It was found that, for each chain thus separated, an equivalent amount of ^{32}P was precipitated. In controls, when two different samples were independently labeled with [^{3}H]NAB-ouabain or phosphorylated from $^{32}P_i$ and mixed before analysis, coprecipitation of ^{32}P with [^{3}H]NAB-ouabain-labeled chains was not observed. The results are thus in quantitative agreement with a model in which [^{3}H]NAB-ouabain binds at the same time to the same 95K polypeptide chain of a Na,K-ATPase functional unit that is phosphorylated from $^{32}P_i$.

The Na,K-ATPase[1] has been shown by electrophoresis on $NaDodSO_4$ polyacrylamide gels to consist of a large polypeptide of mol wt ~95 000, a glycoprotein of mol wt ~45 000, and a proteolipid component of mol wt ~12 000 (cf. Hokin et al., 1973; Lane et al., 1973; Forbush et al., 1978). The 95K polypeptide is phosphorylated from [^{32}P]ATP (Kyte, 1971; Uesugi et al., 1971) or from $^{32}P_i$ (Steckhoven et al., 1976) and forms part of the ouabain binding site (Ruoho & Kyte, 1974; Forbush et al., 1978); the functional roles of the glycoprotein and proteolipid are as yet unknown. The stoichiometry of ligand binding to purified Na,K-ATPase is such that for each pair of 95K polypeptide chains one ouabain molecule or one ATP molecule may be bound (Lane et al., 1973; Jorgensen, 1974b), or one ^{32}P may be incorporated (Hokin et al., 1973; Jorgensen, 1977). Thus the functional unit of the enzyme appears to be a dimer of 95K chains and to contain glycoprotein and proteolipid as well; cross-linking studies are also in agreement with a dimeric arrangement (Kyte, 1975; Giotta, 1976). Since under certain conditions both ouabain and ATP, or both ouabain and phosphate, may be bound to a functional unit of Na,K-ATPase at the same time, the question arises as to whether both ligands are on the same 95K polypeptide chain (model II) or whether they are on separate chains (model I). This problem is depicted in Chart I in which only the large polypeptides are shown traversing the membrane.

Chart I



The binding of [^{3}H]ouabain to Na,K-ATPase occurs most readily in the presence of either Mg, Na, and ATP, or in the presence of Mg and P_i; phosphorylation of the enzyme appears to be involved in both routes (Matsui & Schwartz, 1968;

† From the Department of Physiology, Yale University School of Medicine, New Haven, Connecticut 06510. *Received December 27, 1978.* This work was supported by U.S. Public Health Service Grants AM-17433 and HL-09906.

[1] Abbreviations used: Na,K-ATPase, magnesium dependent, sodium plus potassium stimulated adenosinetriphosphatase (EC 3.6.1.3); 95K chains, the 95 000 dalton polypeptide chains of Na,K-ATPase; P_i, inorganic phosphate; $NaDodSO_4$, sodium dodecyl sulfate; ATP, adenosine triphosphate; NAB-ouabain, 2-nitro-5-azidobenzoylouabain; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetate; K_d, dissociation constant.

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