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On the Permeability of Water Molecules across Vesicular Lipid Bilayers

Rüdiger Lawaczeck

Institut für Physikalische Chemie der Universität Würzburg, Marcusstr. 9/11, 8700 Würzburg, West Germany

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Summary. The permeation of water molecules across single-component lecithin or lecithin-cholesterol bilayers is studied by a new technique. The new technique makes use of the different fluorescence quantum yields of appropriate molecules in D_2O and H_2O. Water-soluble indole derivatives which by experimental manipulation reside almost entirely within the aqueous (H_2O) intravesicular compartment thus can monitor D_2O molecules permeating the bilayer by virtue of an increased quantum yield of the fluorescence. In a stopped-flow instrument, a vesicle solution containing the fluorescent chromophore in the intravesicular space is rapidly mixed with the deuterated solvent. The approach to the "steady state", where the intra- and extravesicular D_2O and H_2O concentrations are equal, proceeds in a single-exponential manner. Consequently, the exchange relaxation time for the D_2O molecules passing the bilayer can be deduced from the time-dependent increase of the fluorescence intensity. The method and results on lecithin and lecithin-cholesterol bilayer vesicles are discussed. The exchange relaxation times of temperature-dependent studies are interpreted within the framework of the solubility-diffusion theory. Below the crystalline to liquid-crystalline phase transition temperature and for cholesterol-free vesicles, the rate-limiting step for the D_2O permeation is attributed to the intracore diffusion. Above the phase transition and for cholesterol-containing vesicles, the intracore diffusion seems not to be rate-limiting. Deviations from the linearity below the phase transition in the Arrhenius-type presentation of the data are related to changes of the partition coefficient of water between the solvent and the lipid phase at the premelting temperature.

Intracellular water is coupled to the external milieu via the permeation of water molecules through the plasma membrane. This permeation is an attracting and substantial process, and different physical methods were developed to measure this step. The water transport across model membranes in the form of bilayer vesicles (Reeves & Dowben, 1970; Andrasko & Forsén, 1974; Haran & Shporer, 1976; Hochster & Prestegard, 1977), multilamellar liposomes (Bangham, DeGier & Greville, 1967; Bittman & Blau, 1972; Blok, van Deenen & DeGier, 1976), and thin lipid films (Huang & Thompson, 1966; Cass & Finkelstein, 1967; Price & Thompson, 1969) has been studied. Measurements on plant cells

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(Collander, 1954; Hüsken, Steudle & Zimmermann, 1978) and erythrocytes (Paganellie & Solomon, 1957; Barton & Brown, 1964; Conlon & Outhred, 1972; Sha'afi & Gary-Bobo, 1973; Morariu & Benga, 1977; Fabry & Eisenstadt, 1978) have also been performed. In principle, three different methods are available. In "tagged" water experiments, the diffusion of $^3H^1HO$ across the membranes in question is monitored in a technically costly manner (Paganellie & Solomon, 1957; Barton & Brown, 1964). Osmotic shrinkage and swelling as a consequence of the changes in the osmolarity can be used to deduce the flow of water from an increase or decrease in volume of the regarded particles (Huang & Thompson, 1966; Bangham *et al.*, 1967; Cass & Finkelstein, 1967; Price & Thompson, 1969; Reeves & Dowben, 1970; Bittman & Blau, 1972; Blok *et al.*, 1976). In NMR experiments the water relaxation time (from either ^1H or ^{17}O) on one side of the membrane is reduced by the addition of paramagnetic ions (usually Mn^{2+}). Measurements of the relaxation times thus allow the determination of the exchange of water molecules between these magnetically different milieus (Conlon & Outhred, 1972; Andrasko & Forsén, 1974; Haran & Shporer, 1976; Morariu & Benga, 1977; Fabry & Eisenstadt, 1978).

Very recently a new technique, which, in principle, allows measurements of the water permeation across vesicular bilayers without creating a severe chemical potential gradient across the membrane in question, was added to the already existing ones (Lawaczeck, 1978*a*). In addition, methods are available to reduce the residual differences in the chemical potentials to those of H_2O and D_2O. The new method is based on the solvent-isotope effect of the fluorescence quantum yield, Φ, which exists for a variety of fluorescent molecules. It is usually found that $\Phi_{D_2O} > \Phi_{H_2O}$ (Stryer, 1966). The entrance of D_2O into an aqueous (H_2O) intravesicular compartment can thus be visualized by an increase in the fluorescence intensity of an appropriate, encapsulated fluorescent chromophore. The method allows routine measurements on model membranes, but it should also be possible to extend the studies to cellular systems. Studies on phospholipid vesicles are of special interest because these vesicles represent a physical model of the lipid part of biological membranes. Besides being a starting point for the understanding of biological membranes, these studies serve to develop and test physical and chemical techniques prior to their applicability to the target object.

In the following, this new technique will be discussed in greater detail. First results on single-component lecithins and lecithin-cholesterol mixtures of (presumably) single-walled vesicles will be interpreted in

terms of the solubility-diffusion theory (Zwolinski, Eyring & Reese, 1949; Hanai & Haydon, 1966; Price & Thompson, 1969; Redwood & Haydon, 1969; Reeves & Dowben, 1970; Träuble, 1971; Cohen, 1975). It has already been noted that the permeation process across lecithin bilayers depends on the thermotropic state of the lipid matrix (Reeves & Dowben, 1970; Andrasko & Forsén, 1974; Cohen, 1975*a*, *b*; Blok *et al.*, 1976, 1977), and it has been postulated that the permeation proceeds by different mechanisms below and above the crystalline to liquid-crystalline phase transition temperature (T_c) (Blok *et al.*, 1976). These results on single-component lipid vesicles can be confirmed with the assumption that the rate-limiting step changes by passing the phase transition temperature. For cholesterol-containing lecithin vesicles the interpretation is not yet straightforward because the partition function of water between the solvent water and the lipid phase cannot be approximated without additional information.

Materials and Methods

Chemicals. L-α-dimyristoyl phosphatidylcholine (DMPC)[1] and L-α-distearoyl phosphatidylcholine (DSPC) were purchased from Calbiochem (San Diego, Calif.), L-α-dipalmitoyl phosphatidylcholine (DPPC) was from Calbiochem, Fluka AG (Buchs, Switzerland) and Nattermann & Cie (Köln, F.R. Germany). These lipids did not reveal detectable impurities as checked by thin-layer-chromatography (TLC silica gel plates from E. Merck (Darmstadt, F.R. Germany), eluent chloroform/methanol/water =65:25:4) and subsequent iodine staining. Palmitic acid (PA) and cholesterol were from Sigma (St. Louis, Mo.). The fluorescent indole derivatives indole-3-acetic acid (E. Merck), tryptamine HCl (3-(2-aminoethyl)-indole HCl, Calbiochem and E. Merck), and 5-methoxy-tryptamine (3-(2-Amino-ethyl)-5-methoxyindole, Sigma) were used without purification. Stock-solutions in the concentration range of 0.3 to 0.5 M in 20 mM $CaCl_2$ were adjusted to pH 7 by the addition of NaOH or HCl, respectively. Inorganic chemicals and deuterium oxide (D_2O) were obtained from E. Merck. In some experiments the tryptophan-residues of pepsin (Sigma) served as indolesource. Throughout the experiments double-distilled or distilled and subsequently membrane-filtered (Millipor-Q water purification system) water was used.

Vesicle preparation. For one-component vesicles the respective lipids (about 20–30 mg) were sonified (Branson Sonifier B 12) in 2 ml of the aqueous (H_2O) solution containing the fluorophore (indole-3-acetic acid, tryptamine HCl or 5-methoxy-tryptamine HCl) at a concentration of 0.3 to 0.5 M and 20 mM $CaCl_2$ at neutral pH. During the continuous sonication (10 min), the solution was protected from over-heating by an

[1] Abbreviations: DMPC, L−α-dimyristoyl phosphatidylcholine; DPPC, L−α-dipalmitoyl phosphatidylcholine; DSPC, L−α-distearoyl phosphatidylcholine; T_c, crystalline to liquid-crystalline phase transition temperature (main transition); NMR, nuclear magnetic resonance; ESR, electron spin resonance; Φ, fluorescence quantum yield; TEMPO, 2,2,6,6-tetramethylpiperidine-N-oxyl.

external water bath. For two-component systems the lipids together with the appropriate amount of cholesterol or palmitic acid were first dissolved in chloroform. The solvent was then removed in a stream of nitrogen followed by an overnight vacuum treatment prior to the addition of the aqueous chromophore-containing solvent. As the actual sonication temperature could not be controlled, the resultant vesicles were routinely annealed at a temperature above their respective crystalline to liquid-crystalline phase transition temperatures (Lawaczeck, Kainosho & Chan 1976). These vesicle solutions were cooled back to room temperature and extensively dialyzed against an aqueous 20 mM $CaCl_2$ solution below T_c for 24–48 hr. In some experiments 0.02% NaN_3 was added to prevent bacterial growth.

As the vesicular bilayer could not withstand the osmotic pressure built up during the dialysis period, the vesicles burst and partly reformed until the bilayer could compensate the residual pressure gradient. Evidently the intravesicular fluorophore concentration was reduced by these opening and reforming processes, especially during the initial stages of the dialysis period. Subsequently the vesicle solutions were centrifuged (Heraeus-Christ Labofuge I) and filtered through a cotton layer to remove multilamellar fragments. Finally, the vesicles were incubated at temperatures above T_c in order to ensure a complete annealing. Additionally this last step reduced air bubble formation during the subsequent stopped-flow measurements. Except for a possible minor leakage of the indole derivatives, these resultant vesicle stock solutions contained the fluorophore almost entirely in the aqueous intravesicular compartment. Prior to the stopped-flow measurements the stock solutions were diluted with degassed aqueous 20 mM $CaCl_2$ to a final volume of about 10 to 20 ml. In some experiments the dialyzed stock solution was passed through a pre-equilibrated Sepharose 4B column (1.5 × 50 cm) (Huang, 1969) and eluted with aqueous 20 mM $CaCl_2$. The column outled was connected to a stirred flow-through fluorescence cuvette, so that the eluation profile could easily be registered. The vesicles were eluted almost as a single peak close to the void volume of the column. This large vesicular size was predominantly a consequence of the vesicle-reforming processes during the reduction of the osmotic pressure in the initial stages of the dialysis period. 20 mM $CaCl_2$ was used throughout the experiments because it is felt that divalent cations seem to stabilize the bilayer of neutral lipids (Lawaczeck *et al.*, 1976).

Physical techniques. For electron micrographs the vesicle solutions were diluted and stained with either uranylacetate or tungsten phosphoric acid (pH 7.4) on a precoated coppergrid. The dried grids were observed in a Zeiss 9A electron microscope.

The stopped-flow measurements were performed on a home-built instrument (Department of Physiological Chemistry) consisting of two 2-ml drive syringes closely connected to the mixing chamber followed by the observation fluorescence cuvette and the stop-syringe. The stop-syringe acts on a microswitch by which the data acquisition system is triggered. The system has a dead time of about 1–2 msec and needs an actual probe volume of about 0.4 ml. The light from a 200 W Xe-Hg lamp is passed through a Bausch and Lomb grating monochromator and then focussed into the observation cuvette. The fluorescent light at 90° is discriminated from the scattered light by cut-off or band-pass filter (λ_{ex} 280–298 nm; $\lambda_{em} \geqq 310$, 340 nm or 310, $340 \leqq \lambda_{em} \leqq 360$ nm). If necessary, part of the incident light could be used as a reference beam to further suppress fluctuations of the lamp intensity. The fluorescence light is voltage-converted by an EMI 9558 QA photomultiplier which is connected to a datalab transient recorder DL 905 working mostly in the pretrigger mode. The stored data – fluorescence intensity *vs.* time – were plotted and redrawn on a logarithmic scale (*see* Fig. 6). The whole system was thermostated by a variable circulating waterflow from a thermobath.

The phase transition profiles were measured by the 90° light-scattering technique at 600 nm and by continuously increasing or decreasing the probe temperature through the

desired ranges. In both the measurements of the phase transition temperature and in the stopped-flow experiments, the temperature of the thermobath was kept constant within ± 1 °C. Ultraviolet and fluorescence spectra were recorded on a Beckman Spectrophotometer ACTA M VI and a Perkin-Elmer fluorescence spectrophotometer 204-A, respectively. The latter served also for the 90° light-scattering measurements.

Outline of the Experimental Technique

1. Principal Idea. Very recently a new technique to measure the permeation of water (D_2O or alternatively H_2O) molecules across vesicular bilayers was published (Lawaczeck, 1978*a*). The principal idea of this new technique is the following: Some fluorescent chromophores are known to show a solvent-isotope effect of the fluorescence quantum yield Φ, with usually $\Phi_{D_2O} > \Phi_{H_2O}$ (Stryer, 1966). By entrapping such molecules entirely inside the vesicular compartment in an aqueous (H_2O) milieu and rapidly changing the extravesicular aqueous composition from pure H_2O to, for example, a one-to-one mixture of H_2O and D_2O, the observed fluorescence intensity will increase as more and more intravesicular H_2O molecules become exchanged by D_2O until the intra- and extravesicular compositions are equal. The experiments are usually performed in a stopped-flow instrument where one syringe contains the respective lipid vesicles with the encapsulated fluorophore in the aqueous (H_2O) solvent and the second syringe is filled with the deuterated solvent.

Due to a 1:1 dilution of the chromophore-containing solutions, the initial fluorescence intensity will drop to half of its original value within the short mixing time. From this point on, the fluorescence intensity will increase because more and more D_2O molecules pass the bilayer and induce an increase in the quantum yield ($\Phi_{D_2O:H_2O} > \Phi_{H_2O}$). The system approaches a "steady state" when the inside and outside solvent compositions with respect to H_2O and D_2O are equal. For a first-order exchange mechanism, the time-dependent increase of the fluorescence intensity is described by

$$I_f(t) = I_0 + (I_\infty - I_0)(1 - \exp(-k_{ex}t)) \tag{1}$$

where I_f, I_0 and I_∞ are the fluorescence intensities at the times t, 0 (pure H_2O) and infinity ($H_2O:D_2O = 1:1$), respectively, and k_{ex} is the exchange relaxation rate. k_{ex} is related to the permeability coefficient, P_d (cm/sec), by

$$P_d = k_{ex} \cdot V/S = k_{ex} \cdot r/3 \tag{2}$$

with the volume, V, surface, S, and vesicular radius, r. For small vesicles where the bilayer thickness d is not negligible with respect to the radius, correction terms must be added to Eq. (2), taking into account that V is the intravesicular volume and S is the external surface.

Before presenting the results for various lipid systems, it is relevant to discuss some aspects concerning these measurements of the water-permeability across vesicular or cellular membranes.

2. The solvent-isotope effect of the fluorescence quantum yield. It is well known to spectroscopists that among other fluorescent molecules indole derivatives show a solvent-isotope effect of the fluorescence quantum yields with $\Phi_{D_2O} > \Phi_{H_2O}$ (Stryer, 1966; Eisinger & Navon, 1969; Kirby & Steiner, 1970; McGuire & Feldman, 1973; Hoss, 1975). Chemical modifications at the indole-ring nitrogen have revealed that neither H- or D-bonds (the crystalline structure of tryptamine HCl shows only a weak hydrogen-bond of

the indole NH to a chloride ion 3.2 Å away (Wakahara, Fajiwara & Tomita, 1973)) nor the H-D exchange of the indole N-proton are solely responsible for the solvent-isotope effect of the fluorescence quantum yield (Eisinger & Navon, 1969). In contrast to the fluorescence spectra, the H-D exchange of the indole-ring imino-proton leads to rather small changes of the UV-absorption spectra for tryptophan molecules (Nakanishi *et al.*, 1978). Though several explanations of the solvent-isotope effect on the quantum yields can be found in the literature (Stryer, 1966; Eisinger & Navon, 1969; Kirby & Steiner, 1970; McGuire & Feldman, 1973), it is felt that a clear-cut mechanism is still missing.

To apply this effect to the measurement of the water permeation across bilayer membranes, the system will be approached from a slightly different point of view. Let us look at one tryptamine molecule in either a D_2O or H_2O environment. For both cases, there are two principal channels deactivating the excited singlet states (S_{1D}, S_{1H}) in a radiative and nonradiative manner. The nonradiative channel should include all possible transitions from the electronically excited state $S_{1(D,H)}$ to the vibrationally excited, electronically relaxed state $S^+_{0(D,H)}$. Thus the following two reactions are assumed to deactivate the excited singlet states:

$$S_{1H} \xrightarrow{k_{fH}} h\nu + S^+_{0H}; \qquad S_{1D} \xrightarrow{k_{fD}} h\nu + S^+_{0D}$$

$$S_{1H} \xrightarrow{k_{nfH}} Q + S^+_{0H}; \qquad S_{1D} \xrightarrow{k_{nfD}} Q + S^+_{0D}.$$

The symbols k_{fH}, k_{fD}, k_{nfH} and k_{nfD} are the rate constants for the fluorescent and non-fluorescent transitions and Q is the dissipated energy. With the help of the above reaction scheme, two quantum yields (Φ_H, Φ_D) are defined as usual.

$$\Phi_H = \frac{k_{fH}}{k_{fH} + k_{nfH}}; \qquad \Phi_D = \frac{k_{fD}}{k_{fD} + k_{nfD}}. \tag{3}$$

For the intermediate case where both H_2O and D_2O are present, it is shown in Fig. 1 that $\Phi_{H,D}$ is a monotonic function of the respective mole fractions x_H, x_D in the bulk phase. It has further been reported that k_{fH} should be equal to k_{fD} (Eisinger & Navon, 1969; Kirby & Steiner, 1970) so that k_{nfH} and k_{nfD} may be interrelated by

$$\begin{aligned} k_{nfH} &= k_{nfD} + k_{nf1} \cdot x_H \\ &= k_{nfD} + k_{nf1}(1 - x_D) \end{aligned} \tag{4}$$

where k_{nf1} can be regarded as the linear coefficient of an expansion series; k_{nf1} is formally equivalent with a second order rate constant. The ratio of the quantum yields is now given by

$$\frac{\Phi_D}{\Phi_H} = 1 + \frac{k_{nf1}}{k_{fD} + k_{nfD}} x_H. \tag{5}$$

By replacing mole fractions with molar concentrations we obtain

$$\frac{\Phi_D}{\Phi_H} = 1 + \frac{k_{nf2}}{k_{fD} + k_{nfD}} c_H \tag{6}$$

where c_H is the molar concentration of water and $k_{nf2} = k_{nf1}/55.56$ (1/mol · sec). Of course Eqs. (5) and (6) are equivalent to the usual Stern-Volmer relation. In Eqs. (5) and (6) the fluorescence lifetime is given by $\tau_f = (k_{fD} + k_{nfD})^{-1}$ (pure D_2O case), and $(k_{nf2} \cdot c_H)^{-1}$ can

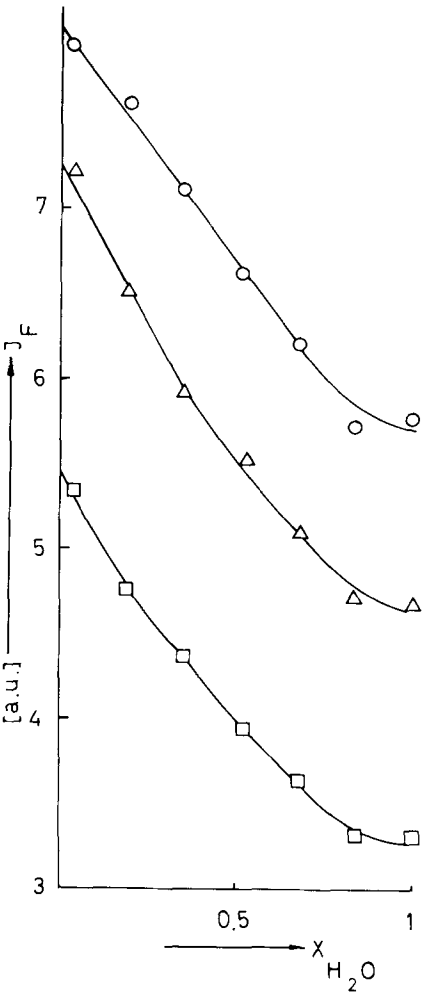

Fig. 1. Tryptamine HCl fluorescence intensity (uncorrected) I_F as function of the mole-fraction of H_2O. Tryptamine HCl 1×10^{-5} M, neutral pH. ○, 18 °C; △, 27 °C; □, 45 °C

be interpreted as an exchange relaxation time leading from, e.g., tryptamine in pure D_2O to a new environment dictated by the bulk D_2O/H_2O composition, c_H. The mixing process is usually assumed to be irreversible, thus one can set the rate constant for the backward reaction equal to zero. As the ratio Φ_D/Φ_H is roughly between 1-2 (*see*, e.g., Hoss, 1975, and Fig. 2) it becomes obvious that for not too small concentrations of H_2O (e.g., $c_H = c_D$) the exchange relaxation time $\tau_{ex} = (k_{nf2} \cdot c_H)^{-1}$ should be in the order of the fluorescence life time. Thus the overall exchange reaction is not diffusion controlled but is several orders of magnitude faster than being detectable by stopped-flow techniques, especially in the case $c_H = c_D$. Indeed, stopped-flow measurements where the two syringes contained tryptamine HCl in H_2O and D_2O, respectively, could not reveal a time-dependent response signal. It should be noted that k_{nf2} is not an intrinsic rate constant for the exchange of one water molecule within the solvation sphere; however, it can be

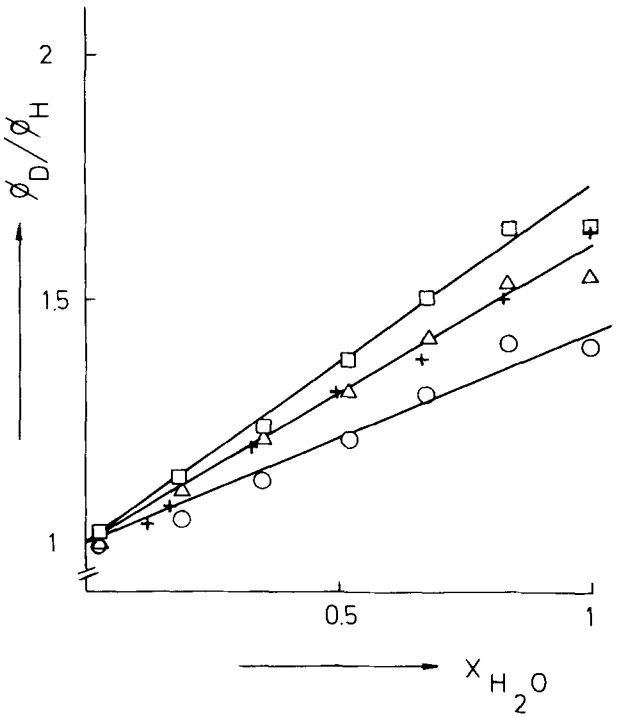

Fig. 2. Ratio of the relative quantum yields $\Phi(D_2O)/\Phi(H_2O)$ *vs.* mole-fraction of H_2O according to Eq. (5). Tryptamine HCl 1×10^{-5} M, neutral pH. ○, 18 °C; △, 27 °C; □, 45 °C; +DPPC vesicles containing tryptamine HCl in the intravesicular compartment at 25 °C

Table 1. Rate constants deduced from Fig. 2

	18 °C	27 °C	45 °C	25 °C (V)[c]
$k_{nf1}/(k_{fD}+k_{nfD})$	0.4	0.6	0.7	0.6
$k_{nf2}/(k_{fD}+k_{nfD})\cdot 10^2$	0.8	1.1	1.3	1.1
$\tau_f=(k_{fD}+k_{nfD})^{-1}$ (nsec)[a]	8.6	7.8	5.7	8.0
k_{nf2} (10^6 l/mol·sec)	0.9	1.4	2.3	1.4
$\tau_{ex}=(k_{nf2}\cdot c_H)^{-1}$ (nsec)[b]	40.4	25.4	15.6	26.0

[a] Calculated data on the basis of Table 1 from Kirby and Steiner, 1970.
[b] For $c_H=c_{H_2O:D_2O=1:1}$.
[c] DPPC vesicle solution containing tryptamine HCl in the intravesicular compartment.

interpreted as a concerted rate constant leading from one to another solvation sphere composition where in the absence of specific interactions the composition of the solvation sphere around the chromophore is equal to the bulk phase composition. One should further consider that due to chemical exchange the species H_2O and D_2O have to

be extended to include HDO. However, this complication will not be taken into account for the following estimates on k_{nf2}. In Fig. 2, the data of Fig. 1 are plotted according to Eq. (5) and in Table 1 values of $k_{nf2}/(k_{fD}+k_{nfD})$ are given together with the fluorescence lifetimes $\tau_f=(k_{fD}+k_{nfD})^{-1}$ and the calculated "second order" rate constants k_{nf2}. From the temperature dependence of k_{nf2}, an activation energy for the exchange process of the solvation shell between 6–7 kcal/mol is calculated. Additionally, apparent exchange relaxation times τ_{ex} for processes where one goes from pure solvents (H_2O or D_2O) to a one-to-one ($H_2O/D_2O=1:1$) composition are included in Table 1. Some points are worth mentioning. The ratio Φ_D/Φ_H increases with increasing temperature. The apparent exchange processes have a low activation energy and they are by no means rate limiting for the processes to be described below. Analogous results as pointed out in Fig. 2 and Table 1 were obtained for indole-3-acetic acid and 5-methoxytryptamine.

3. The location of the probe molecule. As was already discussed in the preceding communication (Lawaczeck, 1978*a*), the question about the location of the probe molecule is a critical one with regard to the validity of the outlined method. This problem could be attacked by measuring the partition-coefficient $\gamma=c_{H_2O}/c_{Lipid}$ of the respective indole derivatives between the aqueous and the lipid phase. However, the realization of these experiments is not easy because the partition-coefficient γ is several orders of magnitude larger than one, so that one has to determine a rather small concentration of the indole derivatives in the lipid phase (c_{Lipid}) in the presence of a very high concentration within the aqueous phase (c_{H_2O}), where especially minor impurities (predominantly neutral indole molecules) would lead to incorrect results. With some restriction one can deduce the real γ-values from comparative studies where an organic solvent could at least mimic the pure hydrophobic part of the lipid bilayer. On an empirical hydrophobicity scale, tryptophan lies between methionine and phenylalanine (Aboderin, 1971). For these zwitterionic amino acids the partition-coefficients between water and various organic solvents were estimated to be greater than 10^4-10^5 (Klein, Moore & Smith, 1971). For the single-charged tryptamine molecules used in the present study the partition-coefficient should be even more in favor of the aqueous phase and thus the permeability through lipid bilayers should be smaller[2]. The interaction of indole-3-acetic acid with lipids in chloroform (Marker, Paleg & Stopswood, 1978), carbon tetrachloride (Weigl, 1969) and water (Weigl, 1969; Zimmermann *et al.*, 1977) has been studied and weak interactions were found both in the nonaqueous and aqueous solvents. For the lipid molecules the interaction area is almost totally restricted to the polar head groups except at high (1 M) KCl concentration. At this high KCl concentration, it is hypothesized that the charged indole-acetic acid can also partition into the hydrocarbon part of the studied black lipid membranes (BLM) (Zimmermann *et al.*, 1977).

In addition to the facts mentioned in the previous communication (the existence of the solvent-isotope effect *per se*, the retainment of a measurable quantity of intravesicular tryptamine molecules even after extended dialysis times, no blue-shift in the fluorescence spectra, no change in the phase transition profile, no break of the fluorescence intensity at T_c), the results of Fig. 2 and Table 1 (last column) reveal that the solvation shell exchange relaxation times for the free and intravesicularly encapsulated tryptamine molecules are almost identical. This rules out that a measurable quantity of the tryptamine molecules is located within the lipid bilayer. In conclusion, one can assume that, especially for $T<T_c$ and the lipids employed, the encapsulated charged indole derivatives are found on the time-average almost entirely within the aqueous phase of the intravesicular compartment.

[2] In the same publication (Klein *et al.*, 1971) efflux rates for methionine and phenylalanine from liposomes of various composition were reported.

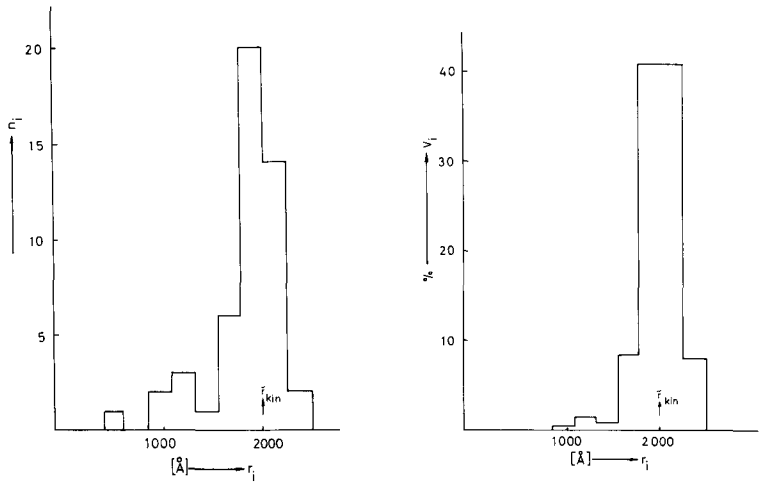

Fig. 3. Vesicular size distribution from an electron-micrograph. (*a*): Number n_i of vesicles *vs.* radius r_i. (*b*): Relative volume V_i/V_{total} in per cent *vs.* radius r_i

The problem about the location and permeability of the probe molecule might become more complex for natural lipid systems. However, it should always be possible to modify the indole derivatives (i.e., add more polar groups) or to use other polar and suitably fluorescent molecules so that the permeability problems could always be overcome.

The permeability coefficients, P_d, of tryptamine and other indole derivatives across bilayers composed of natural lipids have been measured (Bean, Shepherd & Chan, 1968). For charged indole derivatives, P_d is usually small and about two orders of magnitude smaller than $P_d(H_2O)$ for the respective lipid systems (the phase transition temperatures for the lipids studied are usually well below the temperature (30 °C) at which these experiments were performed), so that a competition between a possible efflux of tryptamine and the influx of H_2O, D_2O during the stopped-flow experiments can be neglected.

4. The response signal for a vesicular ensemble of different sizes. So far, vesicles of a uniform size have been considered where, as a result of the experimental manipulation, the fluorescence chromophore is supposed to be homogeneously distributed over the intravesicular compartment. Usually, however, one deals with a size distribution of vesicles, and we must now modify Eq. (1) to take a vesicular ensemble of various sizes into account. Though the size distribution can be narrowed by chromatographic methods, the general problem remains the same. Figure 3 shows a histogram of a fractionated vesicle ensemble, together with the relative volume *vs.* radius distribution, obtained from an electron micrograph. As was already mentioned (*see* methods), these vesicles are large, evidently a consequence of the vesicle preparation. We will now assume a homogeneous intravesicular chromophore concentration over the vesicular ensemble, and we will regard the time-dependent fluorescence intensity given by Eq. (1) as the fluorescence originating from the H_2O to H_2O/D_2O exchange of unit-volume vesicles at the respective chromophore and vesicle concentrations. The total fluorescence intensity aver-

aged over the regarded vesicle ensemble thus becomes

$$I_{av} = \frac{\sum_i \{n_i V_i (I_0 + (I_\infty - I_0)(1 - \exp(-k_{ex} t)))\}}{\sum_i n_i V_i} = I_\infty - (I_\infty - I_0) \frac{\sum_i \left\{ n_i V_i \exp\left(-\frac{3 P_d t}{r_i}\right)\right\}}{\sum_i n_i V_i} \tag{7}$$

with n_i the number of vesicles of volume V_i. In the following we concentrate only on the time-dependent change ($\Delta I(t)$) of I_{av}, which is the accessible quantity of our stopped-flow experiment.

$$\Delta I(t) = I_\infty - I_{av} = (I_\infty - I_0) \frac{4\pi}{3} \frac{\sum_i \left\{ n_i r_i^3 \exp\left(-\frac{3 P_d t}{r_i}\right)\right\}}{\sum_i n_i V_i} = \text{const} \cdot \sum_i \left\{ n_i r_i^3 \exp\left(-\frac{3 P_d t}{r_i}\right)\right\}. \tag{8}$$

By choosing the permeability coefficient $P_d = 1.4 \times 10^{-4}$ cm/sec (Lawaczeck, 1978*a*) and inserting n_i and r_i form the histogram into Eq. (8) one can numerically calculate the time-dependent change of the fluorescence intensity as a consequence of the extravesicularly offered D_2O molecules permeating the vesicular bilayer. The results of these calculations are shown in Fig. 4[3]. Figure 4 and similar examples reveal an almost single-exponential behavior with minor deviations at very short observation times. On the basis of Fig. 4 a kinetically averaged vesicle radius can be deduced which is almost close to the volume-averaged one. The outlined method attaches more weight to the large vesicles for two reasons. First, the fluorescence intensity is proportional to the vesicular volume containing the fluorescent chromophore. Second, the exchange for the D_2O (H_2O) molecules is faster for the small than for the large vesicles. At short observation times the exchange process for the small vesicles is almost completed. Thus for longer times the change of the fluorescence intensity is entirely due to the large vesicles. For the majority of the actual measurements small vesicles obviously contribute to the resultant fluorescence signal at observation times comparable to the dead time of the stopped-flow instrument. In some cases (especially in samples of unfractionated vesicles) minor deviations from single-exponential behavior such as shown in Fig. 4 have been observed.

A second question is concerned with a possible multilamellar shell structure of the vesicle ensemble. It is evident that both these problems, i.e., heterogeneity in the sizes and a possible multiwalled structure are shared with all other methods of measuring the permeability of water molecules across vesicular bilayer membranes. However, the single-exponential approach towards a "steady state" of the fluorescence intensity leads us to assume that we are dealing with a narrow size distribution of single-walled bilayer

[3] It is assumed that the diffusional mixing of D_2O and H_2O in the intravesicular compartment proceeds much faster than the time required for the D_2O molecules to permeate the bilayer.

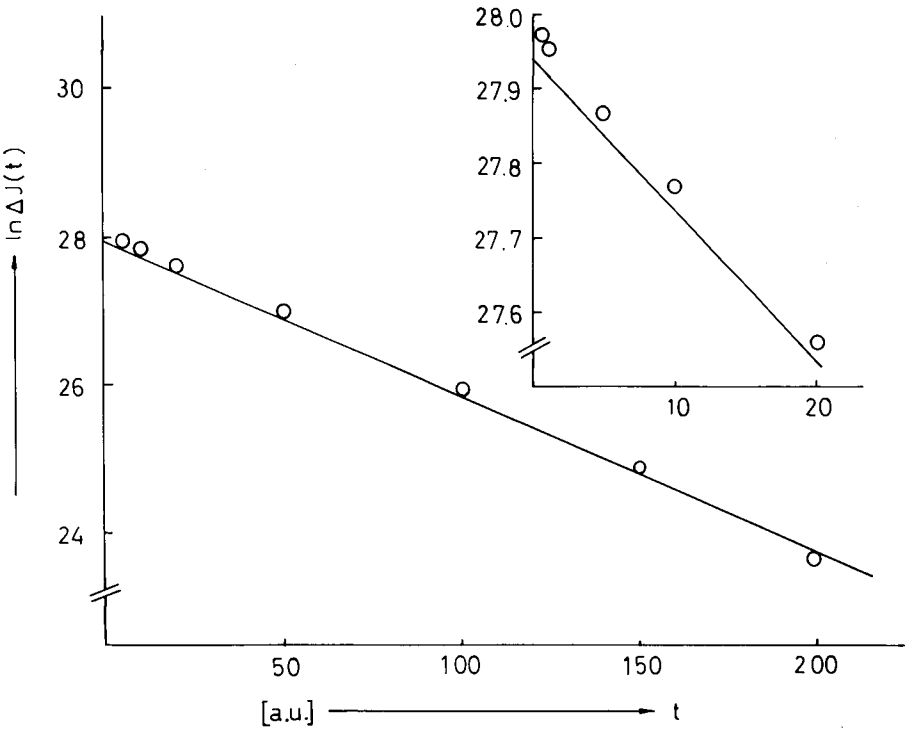

Fig. 4. Time-dependent difference of the fluorescence intensity ΔI_F according to Eq.(8) and inserting the data from Fig. 3 on a logarithmic scale *vs.* time (*see* text)

vesicles in the majority of cases. Additional s pport comes from electron micrographs of uranyl acetate stained vesicles, which show the typical folding structures as a consequence of the drying process of single-bilayer vesicles (Deamer & Bangham, 1976). Electron micrographs further reveal that the vesicular size prior to and after the stopped-flow experiments is practically not altered.

5. *The magnitude of the measuring effect.* In Fig. 5 the magnitude of the measuring effect is demonstrated. For that purpose the time-dependent response signal is compared with the "steady state" signal, i.e., the signal where the extra- and intravesicular D_2O/H_2O compositions are equal. First the fluorescence intensity of the mixing (starting at 1 sec) of the H_2O vesicle solution with the D_2O solvent was registered, and then after a waiting period of about 20 sec the "steady state" signal was written on the same chart paper and with the same sensitivity. At about 4 sec the shutter of the excitation beam was closed to record the dark current signal. It is clearly seen from Fig. 5 that the initial response of the measuring signal is about 20% of the total fluorescence intensity. In Fig. 6 a typical response signal at higher sensitivity together with the logarithmic plot of ΔI_f is presented. From the logarithmic plot of the data it is obvious that the approach to the "steady state" follows the expected single-exponential behavior within experimental error.

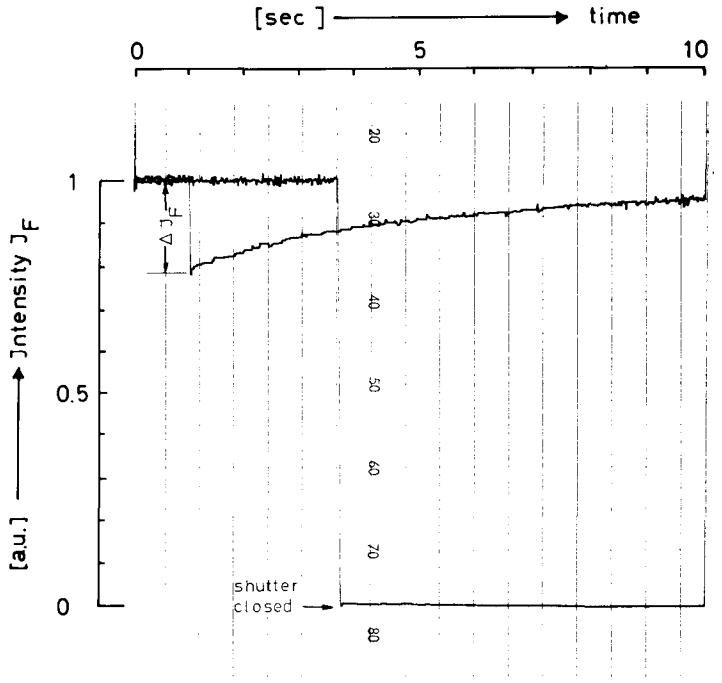

Fig. 5. Magnitude of the measuring effect ΔI_F compared to the "steady state" signal $I_F = 1$ in arbitrary units (a.u.) *vs.* time for DPPC vesicles containing 4 mol/100 mol palmitic acid at 19.5 °C (*see* text). The mixing occurs at 1 sec

Control tests where the second syringe also contained H_2O showed no time-dependent signal response. The following observations, however, are worthwhile. In some experiments we have looked not only at the fluorescence intensity but also at the intensity of the scattered light at 400 nm. When both syringes contained H_2O as the solvent no signal could be detected apart from the time-independent light scattering; thus vesicular fusion processes were almost absent during the observation time (Lawaczeck, 1978*b*) in accord with our results on electron micrographs. However, when the second syringe was filled with D_2O in some experiments a signal was observed which qualitatively corresponds to the equivalent fluorescence signal with respect to the time characteristic. This time-dependent intensity of the scattered light is presumably a consequence of the different indices of refraction of D_2O and H_2O[4]. In short, one has to compare the scattered light from the inside and outside bilayer surfaces during the stopped-flow experiments. At the initial stage of the experiment (just after the short mixing period) the extra- and intravesicular solvents differ in their indices of refraction. This small difference in the indices of refraction is reduced as more and more D_2O (or alternatively H_2O) permeate the membrane and thus the two solvents become optically equal. In principle,

[4] The indices of refraction for D_2O and H_2O at 589 nm and 20 °C are 1.33844 and 1.33299 (Handbook of Chemistry and Physics, 55th edition, The Chemical Rubber Company 1974–1975). Under the same conditions an index of refraction for DPPC (extrapolation to 100% lipid) of 1.475 can be approximated (from Yi & MacDonald, 1973).

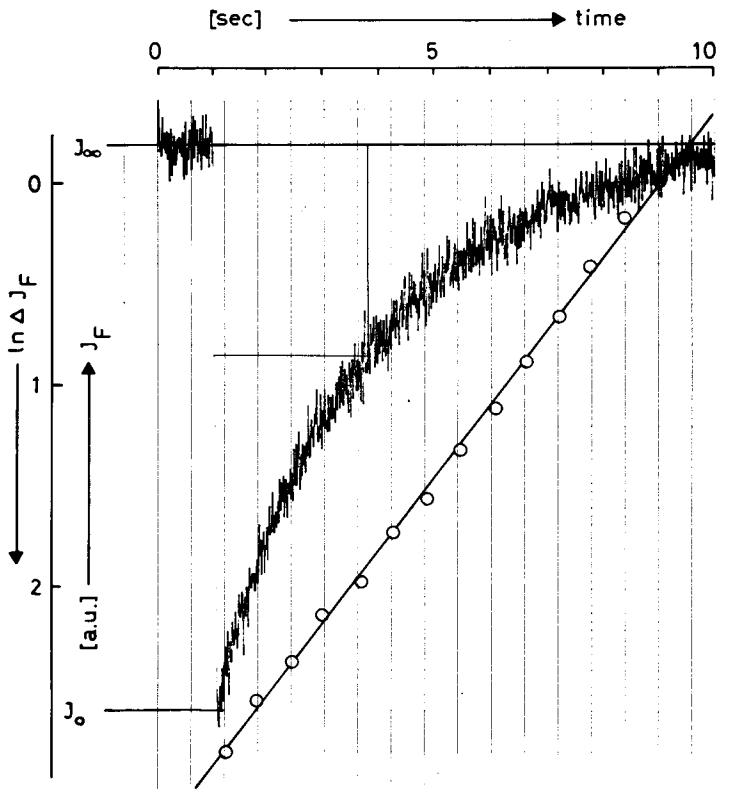

Fig. 6. Response signal. Fluorescence intensity I_F and time-dependent change of the fluorescence intensity ΔI_F on a logarithmic scale (○) *vs.* time for DPPC vesicles containing 4 mol/100 mol palmitic acid at 19.9 °C. The mixing occurs at 1 sec

these rather preliminary results and explanations could offer a "probe-free" method to study permeation processes. However, a full explanation of this promising possibility must await further experiments and will be considered in a subsequent communication.

Results

In this section results are reported on the permeability of water molecules across single component (DMPC, DPPC, DSPC) and two component (DPPC+cholesterol, DPPC+palmitic acid) vesicular lipid bilayers. The measurements were performed with the probe molecule 5-methoxytryptamine HCl, because the additional methoxy-group enhances the polarity and makes 5-methoxytryptamine more suitable than the other indole derivatives tested. 5-methoxytryptamine is not photo-

labile like serotonine, which otherwise would be a perfect candidate as probe molecule. It should be emphasized again that the label molecule only probes the aqueous milieu of the intravesicular compartment and is not supposed to exert an influence on the vesicular bilayer except for an osmotic gradient. However, this osmotic gradient is almost (only in terms of the differences of the chemical potentials of H_2O and D_2O) not altered during the stopped-flow experiment; furthermore experiments are conceivable without these residual osmotic pressure differences by using suitable dialysis solutions. Tests with used and redialyzed samples confirm that the vesicles stay intact during the stopped-flow experiments and that the label remains in the intravesicular compartment.

Being primarily interested in the temperature-induced changes of the permeability, we did not determine for all samples the vesicle size

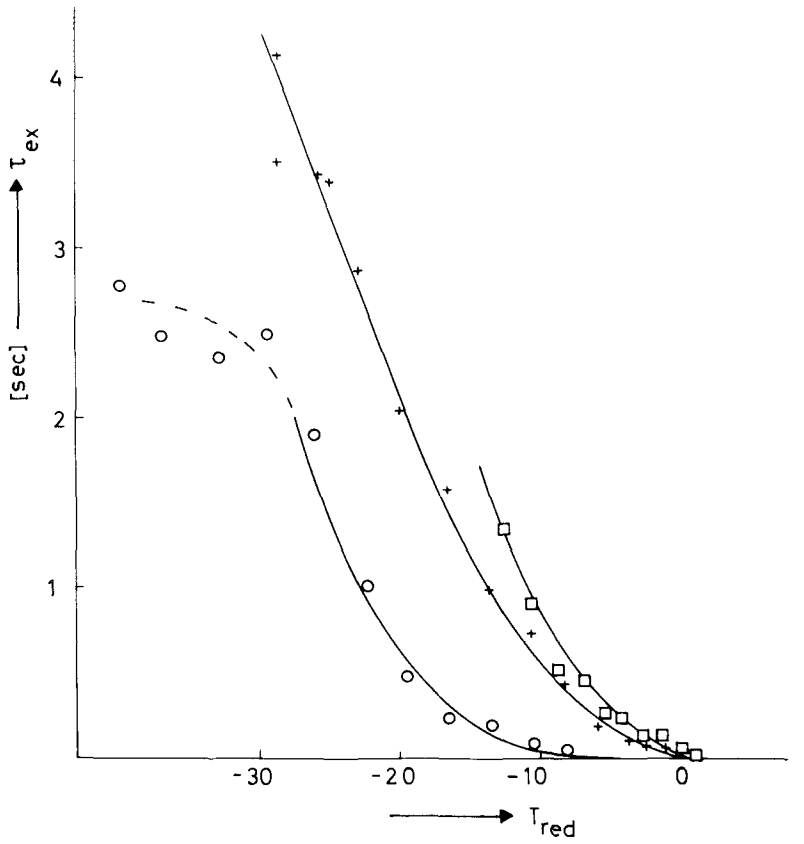

Fig. 7. Exchange relaxation time τ_{ex} as a function of the reduced temperature $T_{red} = T - T_c$ for vesicles composed of (□) DMPC, (+) DPPC, and (○) DSPC

distribution; thus only exchange relaxation rates k_{ex} instead of permeability coefficients P_d will be compared. Within one vesicle preparation and so within one set of experiments the vesicle ensemble is not supposed to change in size; however, for the comparison of different vesicle ensembles only the temperature dependence will be considered and no absolute values of the exchange relaxation rates. Another problem arose by increasing the temperatures to values above T_c. First of all the exchange relaxation times became only a few msec above T_c, and secondly an increased air-bubble-formation interfered with the measurements of the exchange processes. Though these problems can be circumvented, we will in the following concentrate on studies from several degrees below to just a few degrees above T_c.

For the single-component lecithin vesicles the results are summarized in Fig. 7, where the exchange relaxation times ($\tau_{ex}=r/3P_d$) for lecithins which differ in the fatty acyl chain-length are plotted as function of the

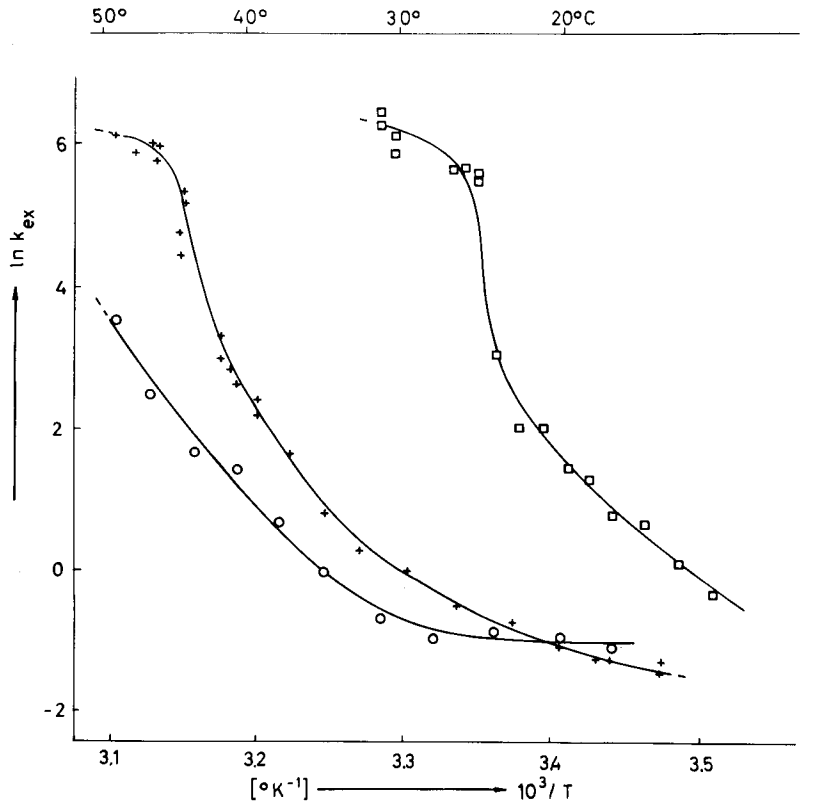

Fig. 8. Exchange relaxation rate k_{ex} on a logarithmic scale *vs.* $1/T$ for (□) DMPC, (+) DPPC, and (○) DSPC vesicles

reduced temperature $T_{red} = T - T_c$[5]. With some deviations for the DSPC system at lower temperatures and having a possible difference of the vesicular sizes in mind, Fig. 7 indicates the same tendencies in the approach of the phase transition temperature for these three lipids. In Fig. 8 the same data are presented as the logarithmus of the exchange relaxation rate *vs.* the reciprocal temperature. This presentation of the data has become common in order to deduce an Arrhenius-type activation energy E_a. However, Fig. 8 clearly demonstrates that it is rather difficult to deduce one activation energy from the respective data in the temperature range studied. It will further be outlined below that this

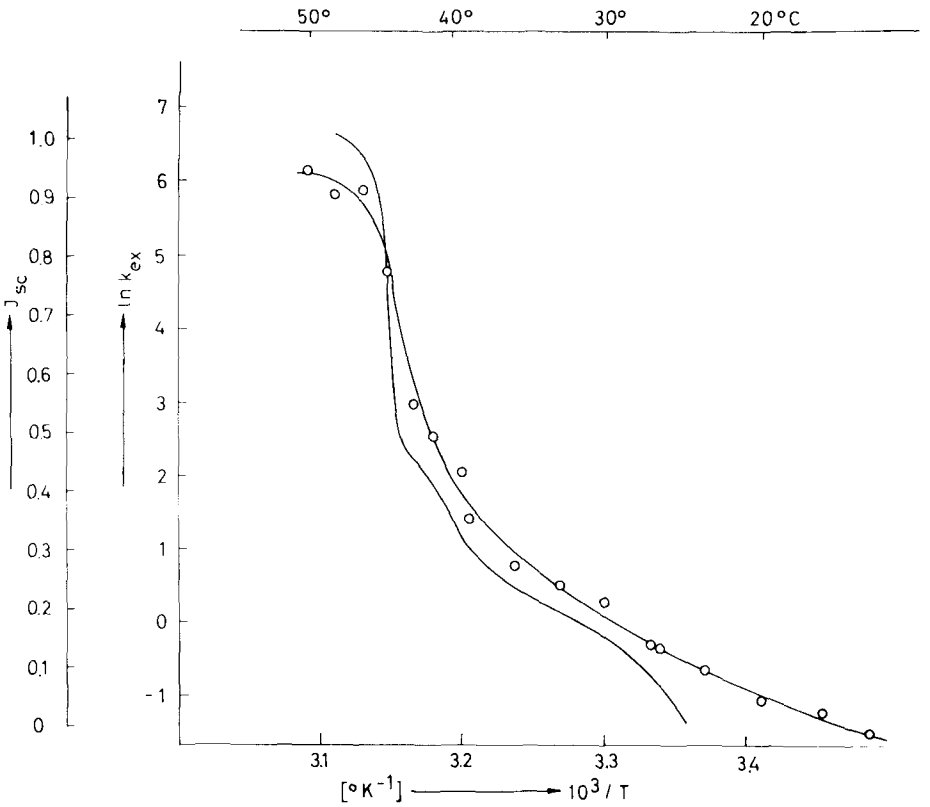

Fig. 9. Exchange relaxation rate k_{ex} (○) on a logarithmic scale and relative intensity of the scattered light at 600 nm I_{sc} *vs.* $1/T$ for DPPC vesicles containing 4 mol/100 mol palmitic acid

[5] As was already discussed in the previous communication (Lawaczeck, 1978*a*) the T_c's are about 1–2° higher than usually observed. This might be due to weak interactions between the probe molecules and the polar head groups of the lipid bilayer at still high concentrations of the probe molecules.

must not be the case because the permeability P_d or the overall exchange rate $k_{ex}(=3P_d/r)$ are usually composite parameters.

Permeability data deduced from osmotic shrinkage experiments on multilamellar liposomes of lecithin containing 4% palmitic acid are available (Blok *et al.*, 1976), and these data can be compared with our results on vesicles of the same composition (Fig. 9). The most striking difference is the deviation of our data from linearity in the Arrhenius-type presentation below T_c. In addition to the exchange relaxation rate, the phase transition profile from light scattering is included in Fig. 9. From the similarity of the two curves in the approach of T_c it becomes evident that the permeability and the phase transition profile are closely

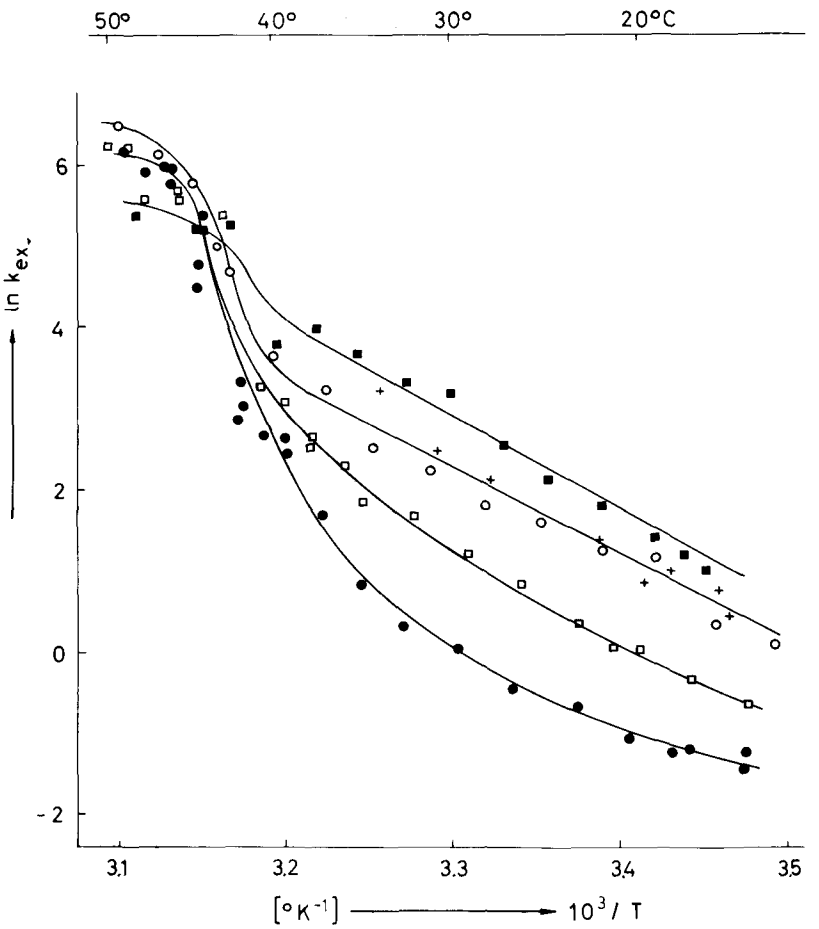

Fig. 10. Exchange relaxation rate k_{ex} on a logarithmic scale *vs.* $1/T$ for DPPC and DPPC-cholesterol vesicles. ●, DPPC; □, DPPC +5 mol/100 mol cholesterol; ○, + DPPC +10 mol/100 mol cholesterol; ■, DPPC +20 mol/100 mol cholesterol

related. This point will be discussed below in greater detail. Furthermore, the comparison of Figs. 8 and 9 shows that the incorporation of 4% palmitic acid into the lecithin matrix does not really reduce the non-linearity of our data in the Arrhenius plots. However, the incorporation of cholesterol leads to one apparent activation energy below T_c. This is indicated in Fig. 10 where samples with increasing amounts of cholesterol are compared with single-component DPPC lecithin vesicles. It can be seen that the previously curved parts (Fig. 8) become straightened for the cholesterol-containing vesicles. The lines for the different cholesterol compositions stay almost parallel up to a point where the straight lines join the steep increase in k_{ex} prior to T_c. This "bending-off" is obviously shifted closer to T_c with increasing cholesterol concentration. The almost parallel lines in the Arrhenius-type presentation allow the deduction of one apparent activation energy between 21.5 and 22.7 kcal/mol. The differences in the k_{ex}-values just below and above T_c are diminished with increasing cholesterol concentration.

Discussion

Throughout the previous sections it was assumed that the flow, J (in moles/cm^2 sec), of D_2O over the bilayer membrane can be described by

$$J_{D_2O} = P_d \Delta c \tag{9}$$

where the concentration gradient $\Delta c = c_a - c_i$ over the bilayer decreases in time and the "steady state" concentrations ($c_a = c_i$) are approached in a single-exponential manner, i.e.

$$c_i(t) = c_a(1 - \exp(-k_{ex} t)). \tag{10}$$

c_i and c_a (=constant under usual conditions where the intravesicular is negligible with respect to the extravesicular volume) are the intra- and extravesicular D_2O concentrations. Equation (1), which seems valid on the experimental basis, is a consequence of Eq. (10).

The water permeability across lipid bilayers and biological membranes has been discussed in terms of three models, i.e., large pores, solubility diffusion, and small pores (Price & Thompson, 1969). On the basis of the given arguments it is felt that the solubility diffusion is the adequate model to describe the water permeation (Price & Thompson, 1969; Reeves & Dowben, 1970). For single-component lipid bilayers a

static pore model is not in accord with the lipid mobilities, especially above T_c. Below T_c pores could be attributed to lattice dislocations or errors in thermodynamic equilibrium with the crystalline matrix. Large pores and grain boundaries can be ruled out for annealed vesicles (Lawaczeck *et al.*, 1976) even below T_c. The phase transition range itself is increasingly more discussed in terms of a coexistence of crystalline and liquid-crystalline domains (Marsh, Watts & Knowles, 1976; Tsong, Greenberg & Kanehisa, 1977; Lee, 1977*a*, *b*; Papahadjopoulos *et al.*, 1973) where the boundaries between these domains are supposed to facilitate a passive diffusion of solutes (Papahadjopoulos *et al.*, 1973; Marsh *et al.*, 1976). However, in contrast to studies on the ^{22}Na permeation across lecithin bilayers (Papahadjopoulos *et al.*, 1973), the water permeability does not show a maximum at T_c; instead, it reveals an almost discontinuous break.

In the following discussion only the solubility diffusion model (Zwolinski *et al.*, 1949; Price & Thompson, 1969) is considered, especially also because of the intuitive character of the parameters entering into this theory. Within this scheme the permeability coefficient is expressed by

$$\frac{1}{P_d} = \frac{2\lambda}{D_{sm}} + \frac{\delta}{D_m K_p}. \tag{11}$$

λ is the length of a single diffusional step from one to a neighbor potential-minimum. D_{sm} and D_m are the diffusion coefficients from the aqueous solvent into the bilayer and within the hydrocarbon core, respectively. δ is the thickness of the hydrocarbon core part of the bilayer and K_p is the partition coefficient of a solute between the lipid bilayer and the aqueous solvent. The permeability coefficient P_d thus depends on membrane and permeant specific parameters which characterize the resistance to carry a molecule from the solvent into the membrane and the subsequent diffusion within the membrane. In the literature the general case described by Eq. (11) is usually considered under conditions where either the first step (i.e., the overcoming of the barrier from the solvent into the membrane) or the second term (the diffusion step through the membrane core) is rate limiting (Zwolinski *et al.*, 1949; Price & Thompson, 1969; Reeves & Dowben, 1970; Diamond & Katz, 1974)[6]. A discrimination on a theoretical basis is so far rather speculative,

[6] In principle, these assumptions imply a homogeneous membrane structure which is a simplification in terms of the polarity and mobility gradients. This was already pointed out (Diamond & Katz, 1974).

especially as separate measurements of, for example, the partition coefficient K_p and the core-diffusion constant D_m are missing. The problem is even more complex as the lipid bilayer is surrounded by water layers with different properties than those of the bulk water (Rigaud, Gary-Bobo & Lange, 1972; Salsbury, Darke & Chapman, 1972; Cornell, Pope & Troup, 1974; Finer & Darke, 1974; Finch & Schneider, 1975; Inglefield, Lindblom & Gottlieb, 1976; Jendrasiak & Mendible, 1976; Keith, Snipes & Chapman, 1977). The penetration depth of the adjacent water layer is almost unknown on a quantitative basis and may be a function of the temperature, the lipid, and additionally, in natural membranes, of the protein composition. In a qualitative description deduced from spin-label studies, the polarity across a lipid bilayer was approximated by a trapezoidal-shaped barrier (Griffith, Dehlinger & Van, 1974). The H_2O penetration into micellar systems has been studied (Menger, Jerkunica & Johnston, 1978); however, even here a quantification of how deep water molecules enter into the nonpolar core seems to be rather difficult.

As an estimate, the partition coefficient K_p between the lipid and the solvent phase is usually deduced from the H_2O partitioning into an organic phase (Collander, 1954; Reeves & Dowben, 1970; Träuble, 1971; Cohen, 1975*b*). A comparison of K_p (organic phase) with the real K_p (lipid phase) might be valid above T_c; however, in the temperature range below T_c this comparison must be questioned. Usually the temperature-dependence of K_p is described by

$$K_p(T) = \exp(\Delta G^{\S}/RT) \tag{12}$$

where $\Delta G^{\S}$ is the difference of reference chemical potentials for water in the bulk and in the lipid phases. These reference states do not contain a concentration-dependent term; however, they include all possible kinds of "solvent-solute" interactions (Kauzmann, 1959; Tanford, 1973). It is thus obvious that $\Delta G^{\S}$ is especially changed by raising the temperature through the pre- and main-phase transition. This behavior makes the deduction of K_p from the comparison with "structure-less" organic solvents in the studied temperature region rather questionable. Instead, K_p (lipid) may be approximated by

$$K_p(\text{lipid}) = f(T)\, K_p(\text{organic phase}). \tag{13}$$

In Eq. (13) $f(T)$ describes the major temperature dependence due to the thermotropic lipid phases with $0 \leqq f(T) \leqq 1$, and K_p (organic phase) is temperature-independent (taken at a reference-temperature). The func-

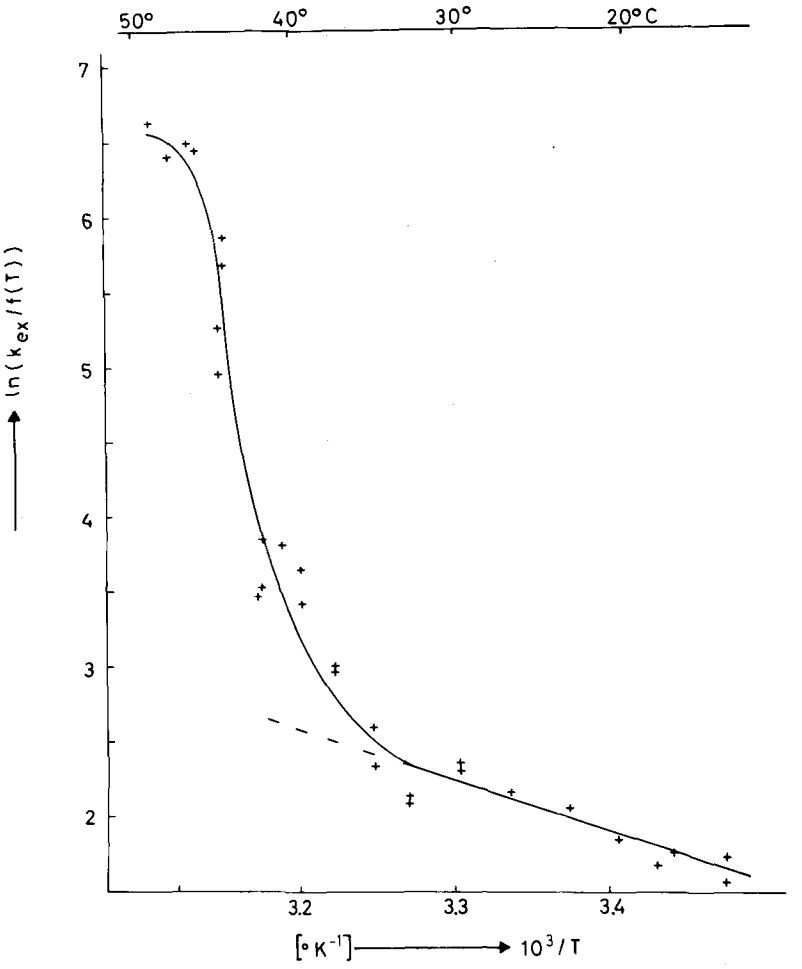

Fig. 11. Logarithmus of $k_{ex}/f(T)$ *vs.* $1/T$ for DPPC vesicles. The exchange relaxation rates k_{ex} are from Fig. 8 and the function $f(T)$ is from Fig. 3 of Polnascek *et al.*, 1978

tion $f(T)$ could be determined from phase transition profiles measured by suitable techniques. In this respect the different spectroscopic response of the ESR probe molecule TEMPO has been thoroughly studied and was recently reviewed (Schreier, Polnascek & Smith, 1978). Indeed, these data are commonly presented in the form of a temperature-dependent partition coefficient for the probe molecule between the lipid and the aqueous phases (Shimshick & McConnell, 1973; Schreier *et al.*, 1978; Polnascek *et al.*, 1978)[7]. In Fig. 11, $k_{ex}/f(T)$ for DPPC vesicles is

[7] Strictly speaking, the function $f(T)$ is not a partition coefficient according to our definition.

plotted on a logarithmic scale *vs.* $1/T$, where the k_{ex}-values were taken from Fig. 8 and the function $f(T)$ from TEMPO partitioning studies (Polnascek *et al.*, 1978). Most strikingly, the previously curved lines (for comparison *see* Fig. 8) become in Fig. 11 a linear function of the reciprocal temperature, and an activation energy E_a of 6.6 kcal/mol is now calculated below T_c. This activation energy E_a can be attributed to the diffusion step through the membrane interior. Our calculated activation energy is about twice the value measured for the diffusion of water through a hexadecane layer (Schatzberg, 1965). The value of 6.6 kcal/mol for E_a of the intracore diffusion-step below T_c is also comparable to estimates from the kink-diffusion model, i.e., 4.8 kcal/mol (Träuble, 1971) and 3–4 kcal/mol[8] (Kroon, Kainosho & Chan, 1976).

At the phase transition the function $f(T)$ (*see* Fig. 3 of Polnascek *et al.*, 1978), which is here supposed to describe the temperature dependence of the water-partitioning into the lipid bilayer, changes from values of 0.3 below to about 0.7 above T_c. However, the exchange relaxation rate k_{ex} increases in the same temperature range by about 2–3 orders of magnitude. Thus within the scheme of our interpretation, the predominant step at T_c occurs at the intracore-diffusion level.

The above considerations lead to the assumption that below T_c the diffusion through the hydrocarbon core is rate limiting where the permeability coefficient is given by $P_d = D_m K_p/\delta$. Taking a mean vesicular radius of 2000 Å for these samples and K_p (organic phase) $= 0.64 \times 10^{-4}$ as an estimate for the partition coefficient (Schatzberg, 1965) above T_c and setting the intracore dimension $\delta = 30$ Å, the permeability and diffusion coefficients given in Table 2 can be approximated.

The permeability coefficients of Table 2 are similar above T_c, but below T_c our values for P_d are lower than reported data for DPPC vesicles (Andrasko & Forsén, 1974). Above the phase transition temperature our results on P_d for saturated lecithins can be compared with measurements of the permeability coefficients on egg yolk lecithin bilayers. However, the literature estimates on P_d lie between $0.4-0.5 \times 10^{-4}$ cm/sec at 25 °C (Reeves & Dowben, 1970; Fettiplace, 1978) and 8×10^{-4} cm/sec at 22 °C (Haran & Shporer, 1976).

Apart from the experimental value k_{ex}, four parameters enter into the expression for the diffusion coefficient D_m using our assumption. From this set of parameters the vesicular radius r can be deduced from electron

[8] The value of 3–4 kcal/mol for E_a corresponds to lipid bilayers above T_c. Below T_c the expectation numbers of kinks per fatty acyl chain should very small and the kink-diffusion should be hindered by the rigidity of the lipid bilayer matrix.

Table 2. Permeability and self-diffusion coefficients for water across DPPC vesicular bilayers

T °C	P_d^a (cm/sec)	$k_{ex}/f(T)^b$ (sec^{-1})	D_m^c (cm^2/sec)
26.5	4.2×10^{-6}	8.8	2.8×10^{-7}
37	3.5×10^{-5}	19.9	6.2×10^{-7}
46	2.4×10^{-3}	586.5	1.8×10^{-5}

[a] $P_d = k_{ex} r/3$ with k_{ex} from Fig. 8.
[b] $f(T)$ from Fig. 3 of Polnascek *et al.*, 1978.
[c] $D_m = k_{ex} r \delta/(3 f(T) K_p$ (organic phase)).

micrographs and the intracore thickness δ from electron density profiles across lipid bilayers (Torbet & Wilkins, 1976). The two remaining, coupled parameters are submitted to the largest uncertainty with respect to the reliability of the proposed comparison, where $f(T)$ and K_p describe the temperature-dependent and independent contributions of the water-partitioning into the lipid bilayer. The choice of 0.64×10^{-4} for K_p (the value of the partition coefficient for water between *n*-hexadecane and the solvent-water at 35 °C (Schatzberg, 1965)) is supposed to be an upper value in the temperature range regarded. The deduction of $f(T)$ from the partitioning of the ESR probe TEMPO must be regarded as an estimate for the so-far experimentally inaccessible temperature dependence of the real water partitioning into the lipid bilayer. The TEMPO molecules are preferentially located in the polar region of the lipid bilayer (Polnascek *et al.*, 1978), i.e., almost that part of the bilayer which might also be accessible to water molecules. The principal advantage in describing the temperature-dependence of the water-partitioning by the introduced function $f(T)$ over the usual thermodynamic relation given by Eq. (12) lies in the fact that $f(T)$ responds to the thermotropic behavior of the lipid bilayers.

Though the values for D_m of Table 2 might be subjected to large experimental errors, these data should reflect the right tendencies and orders of magnitude. The diffusion coefficients, D_m, of Table 2 have some implications for the rate-limiting step. Below T_c the D_m-values are lower than the water self-diffusion coefficient in the bulk phase (e.g., Wang, Robinson & Edelman, 1953) roughly by a factor of 100. However, above T_c the water self-diffusion constant in the solvent and in the membrane core are of the same order of magnitude. Thus in the fluid state the membrane core would not act anymore as a diffusion barrier for the

H_2O (D_2O) molecules[9], and the rate-limiting condition of this step seems invalid above T_c. This implies that in principle the relation used to calculate the values of Table 2 is no longer applicable above T_c. The water self-diffusion coefficient through perfluoro octonoate bilayers was estimated to be about one quarter of the solvent water self-diffusion coefficient (Tiddy *et al.*, 1974). The water self-diffusion coefficient through a hexadecane layer at 45 °C is about 6×10^{-5} cm^2/sec (Schatzberg, 1965), i.e., the same order of magnitude as the D_m-value above T_c in Table 2 and the solvent-water self-diffusion coefficient.

The diffusion of oxygen through the intracore region of lipid bilayers possibly proceeds by the same mechanism as the water diffusion. On the basis of experiments where the fluorescence quenching of pyrene molecules by oxygen was monitored, isotropic diffusion coefficients of oxygen in a variety of lipid bilayers were reported (Fischkoff & Vanderkooi, 1975). These oxygen diffusion coefficients nicely correspond to the D_m-values of Table 2 above T_c, though the changes at T_c are not thus pronounced for the oxygen diffusion. This is possibly a consequence of the fact that lipid-phase-specific partition coefficients were not taken into account; e.g., it is known that the oxygen-solubility in the lipid bilayer changes at the phase transition (Peters & Kimmich, 1978).

Figure 11 reveals that the diffusional step is obviously not affected by passing the prephase transition in contrast to the function $f(T)$ (*see* Fig. 3 of Polnascek *et al.*, 1978, and similar presentations). Early observations had attributed the existence of the premelting to changes at the polar head groups and/or a rearrangement of the surrounding waterstructure (Salsbury, Chapman & Parry Jones, 1970; Salsbury *et al.*, 1972). This early interpretation was later revised for multilamellar systems in terms of X-ray, electron-microscopic and NMR results which are in accord with changes of the molecular organization of the bilayer from $L_{\beta'}$ to $P_{\beta'}(L_\beta)$ below and above the premelting temperature (Rand, Chapman & Larsson, 1975; Janiak, Small & Shipley, 1976; Larsson, 1977; Luna & McConnell, 1978; Seelig, 1978)[10]. For vesicular systems, especially for small single-walled vesicles, tilted (i.e., with a fixed angle of tilt ($L_{\beta'}$)) and rippled bilayer conformations ($P_{\beta'}$) are rather difficult to imagine without the creation of domain-like structures. Indeed, the annealing-type experiments (Lawaczeck *et al.*, 1976) point in the direction that such defect-

[9] The permeability coefficients for D_2O and H_2O across eggyolk lecithin-cholesterol mixtures were measured by the osmotic shrinkage method with $P_d(H_2O) \simeq 2 P_d(D_2O)$ (Bittman & Blau, 1972).

[10] For nomenclature *see* (Tardieu, Luzzati & Reman, 1973).

structures could exist prior to the annealing process. However, after the annealing has taken place even below T_c the angle of tilt should be zero (L_β phase) or continuously variable in terms of geometric considerations for homogeneous and small vesicles in contrast to the preferred orientation in multilamellar and large systems. These possibly different phases for multilayers and small vesicles were already noted (Hui, 1976) and might shed some light on the observed hysteresis at the premelting (Lentz, Freire & Biltonen, 1978) and the differences in the phase transition profiles measured by various techniques (e.g., Marsh *et al.*, 1977; Mitaku, Ikegami & Sakanishi, 1978). The situation is still ambigious and further studies are required.

It can be concluded from the results of Fig. 11 and Table 2 that major defects like grain boundaries can be ruled out as "permeation-channels" because the permeation through large pores is supposed to have a lower activation energy (Price & Thompson, 1969). However, small lattice dislocations in the thermodynamic equilibrium with the ordered matrix might contribute to the observed water permeation if the activation energy for this step is similar to the observed one. The treatment of our data (P_d or k_{ex}) in terms of a hydrophobic-core diffusion step and a partition coefficient, of which the temperature-dependence is deduced from the partitioning of the TEMPO probe by the function $f(T)$, seems to be consistent below T_c. The fact that the premelting behavior is only manifested in the function $f(T)$ indicates that in vesicular systems either a reorganization of the core part (if it exists for single-walled vesicles) induces changes in the water partitioning without affecting the diffusion-step through the hydrocarbon core, or only the polar part of the bilayer is influenced with respect to a change in mobility but not in conformation (*see also* Seelig, 1978). Another possibility can be discussed with respect to changes of the adjacent water layer at certain characteristic temperatures where structural and/or motional changes of the water layer might trigger structural and/or motional variations of the bilayer (Drost-Hansen, 1971; Peschel, 1976). Further experiments using trivalent cations like La^{3+} in order to induce changes in the polar head group conformation (Brown & Seelig, 1977) or ions which are supposed to have an impact on the water structure are under current investigation. So far all experiments were performed in aqueous 20 mM $CaCl_2$. A discussion, however, on the influence of Ca^{2+} ions on possible phase separations of mixed bilayers containing negatively charged lipids or small amounts of palmitic acid will be suppressed until more data are available.

Within the proposed model and estimates it seems almost certain that the rate-limiting step from a core-diffusion controlled behavior below T_c has changed to a situation where the diffusion through the hydrocarbon part is no longer rate limiting above T_c. For the studied saturated lipids it was shown that the activation energy for the over-all water permeation (deduced from osmotic shrinkage experiments on multilayers) are almost independent of the fatty acyl chain-length (25–28 kcal/mol and about 11 kcal/mol below and above T_c, respectively) (Boehler, DeGier, van Deenen, 1978). Within the kink-diffusion model (Träuble, 1971) the activation energy for the kink formation is independent, whereas the number of kinks should depend on the chain length of the lipid molecules (Kainosho *et al.*, 1978). Thus one should observe a chain-length dependence of P_d or D_m below, but not above, T_c.

The incorporation of cholesterol into the lecithin matrix influences the adjacent water layer, the polar and nonpolar part of the lecithin bilayer (e.g., Oldfield & Chapman, 1971; Hinz & Sturtevant, 1972: Engelman & Rothman, 1972; Bittman & Blau, 1972; Kroon, Kainosho & Chan, 1975; Keith *et al.*, 1977; Oldfield *et al.*, 1978). The picture which emerges from a large number of studies on the cholesterol-lecithin interactions within the lamellar phase is still not straightforward and is partly contradictory (for recent review *see* Lee, 1977*b*). For binary systems like DPPC-cholesterol the use of marker molecules to probe the bilayer architecture could lead to unavoidable complications. The marker molecules themselves might not only induce mutual interactions, especially in these delicately balanced bilayer systems, but might also attach different importance to possible domain-like structures. Furthermore, it seems at the present moment too early to couple the temperature dependence of the partition coefficient (introduced by the function $f(T)$) to marker-free measurements of the phase transition profiles for cholesterol-lipid mixtures. However, even without explicitly knowing the function $f(T)$ for the cholesterol-containing vesicles, the following interpretation of the data of Fig. 10 is possible. It is known that the incorporation of cholesterol fluidizes the lecithin bilayer core below T_c and rigidifies the bilayer above T_c with respect to the cholesterol-free system. This effect has been discussed in terms of an intermediate fluid state (Oldfield & Chapman, 1971). For the present case and below T_c the extra-mobility is confined to those lipids which surround the cholesterol molecule on the time-scale which the water molecules need to penetrate into the bilayer. In other words, in a dynamic picture all those lipid

molecules which the cholesterol meets on its diffusion through the bilayer are influenced on a time-scale which for our purpose is defined as the water-barrier time. The fluidizing effect of cholesterol below T_c facilitates the diffusion of water molecules through the intracore of the bilayer (Bittman & Blau, 1972). This happens in the same directions as the temperature-raising through T_c for the cholesterol-free lecithin bilayers. Thus in the regarded cholesterol concentration range the step in k_{ex} at T_c is continuously reduced with increasing cholesterol concentration. This decrease in the pseudo-discontinuity of k_{ex} at T_c with increasing cholesterol content was already observed (Blok *et al.*, 1977) and is in accord with our data. However, within experimental error the data of Fig. 10 do not reveal a simultaneous reduction of the overall activation energy of k_{ex} below T_c from 28.3 to 24.2 kcal/mol for 0 to 33 mol/100 mol cholesterol, respectively (Blok *et al.*, 1977). Instead, we observe an almost constant activation energy (between 21.5 and 22.7 kcal/mol) of the exchange relaxation rate k_{ex} below T_c for 5 to 20 mol/100 mol cholesterol incorporated into a DPPC matrix. It has been observed that the incorporation of cholesterol into the DPPC matrices removes the prephase transition (e.g., Grupe *et al.*, 1976; Estep *et al.*, 1978; Mabrey, Mateo & Sturtevant, 1978; Suckling *et al.*, 1979) and evidently leads to domain-like structures, poor and rich with respect to the cholesterol content (Estep *et al.*, 1978; Mabrey *et al.*, 1978). Consequently, the Arrhenius-plots of Fig. 10 become linear in the temperature range below T_c with increasing amounts of cholesterol in contrast to the cholesterol-free bilayers (Fig. 8 and 9). For these cholesterol-induced "mobility-permeation-channels" through the intracore below T_c, the rate-limiting step might be attributed to the partitioning of the water molecules from the solvent into the lipid phase.

The observed discrepancies between our data (especially the curved lines in Figs. 8 and 9) and the literature data might be a function of the different techniques used. The osmotic shrinkage method (Price & Thompson, 1969; Reeves & Dowben, 1970; Bittman & Blau, 1972; Blok *et al.*, 1976, 1977; Fettiplace, 1978; Boehler *et al.*, 1978) measures a consequence of the water permeation, while we directly monitor the D_2O or alternatively H_2O permeation across the lipid bilayer. With respect to NMR results, these experiments were usually performed with small vesicles (Andrasko & Forsén, 1974; Haran & Shporer, 1976) of about 250 Å diameter. However, it was shown that predominantly due to packing constraints the permeability coefficients for small and large vesicles (diameter $\geqq 1000$ Å) are different (Chan *et al.*, 1973).

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