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On the Environment and the Rotational Motion of Amphiphilic Flavins in Artificial Membrane Vesicles as Studied by Fluorescence

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Summary. The incorporation of four amphiphilic flavins ("amphiflavins") as fluorescence markers bearing C_{18}-hydrocarbon chains at various positions of the chromophore into artificial membrane vesicles has been investigated. The vesicles utilized were made from three different saturated phospholipids. The stability of the flavin-charged vesicles was found to be good over several days, depending somewhat on the temperature, the pH, and their concentration. A marked increase of the fluorescence quantum yield near the vesicle phase transition (crystalline → liquid crystalline) was found which was taken to indicate that the flavin nuclei are imbedded more deeply into the hydrophobic portion of the membranes. This is further supported by a hypsochromic shift of the near flavin UV-peak and the increase of absorbance at 450 nm upon melting. Rotational relaxation times of the various amphiflavins bound to the different vesicles are obtained from measurements of the fluorescence polarizations as a function of temperature. From these data, the microviscosities in the region of the chromophors are calculated. Measurements of the fluorescence polarization as a function of the solvent viscosity and vesicle phase (crystalline-liquid crystalline) indicate that below the phase transition the flavin nucleus is protected from the suspension medium by a lipid-water interphase, which softens above phase transition. The dependence of the flavin orientation and microenvironment on the position of the substitution of the aliphatic chain is reflected in the differences of the fluorescence yields and the shape of the emission spectra.

It is common practice in membrane biology to incorporate fluorescence probes into biological and artificial membranes to investigate their structure and dynamics [14, 17, 38, 47]. There is much information available concerning this subject using relatively modest equipment for static experiments. Information may be obtained from fluorescence quantum yield, the fluorescence intensity tensor, the fluorescence emission and the fluorescence polarization spectra [1, 2, 3, 38], and the concentration dependence of polarization [48].

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For such studies a probe should be a small molecule in order to disturb its environment as little as possible. In this respect we describe the investigation of the binding of amphiflavins to artificial membrane vesicles as well as the assessment of their localization and mobility. However, our purpose in this investigation was not simply to add another fluorescence label to the list of those already existing [1].

There are three specific aspects for these probes which allow for specificity not seen with other labels. Firstly, the hydrophobically substituted flavin nucleus is an excellent membrane label because it exists in five different forms (oxidized neutral molecule, neutral radical, radical anion, fully reduced flavin and fully reduced anion). This allows comparative studies distinct from those described for other molecules. The oxidized form of the label can be analyzed by absorption and emission, the half-reduced form by absorption and EPR, and the fully reduced form by absorption and kinetics. Secondly, the membrane can serve as an excellent aid in flavin chemistry (as comparison see the micellar catalysis of flavin reactions as described by Shinkai [44]). In "isotropic" flavin chemistry, i.e., the chemistry of the free flavin nucleus in solution, the flavin "self-contact" is always the fastest reaction (flavin nuclei in isotropic solution are known to interact with each other nearly diffusion controlled; e.g., the dismutation

$$Fl_{ox} + H_2Fl_{red} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [Fl_{ox} - H_2Fl_{red}] \underset{k_4}{\overset{k_3}{\rightleftharpoons}} 2FlH^{\cdot}$$

proceeds with rate constants of $k_1 > 10^9$, $k_2 > 10^6$, $k_3 \approx 5 \times 10^3$ and $k_4 \approx 10^7\ mol^{-1}\ s^{-1}$ [5]). This can, however, be ignored in flavoproteins to which the flavin nuclei are bound in a highly anisotropic manner ("anisotropic" flavin chemistry) and can also be minimized by loading flavin nuclei in a low area density onto vesicles. Thirdly, it is well established that the flavin nucleus has four catalytic, so-called "active centers" (positions C4a, N5, C8 and C10a, which are known to mediate nearly exclusively all flavin (redox-) reactions [15, 21, and further literature there]). It is possible to anchor the flavin by means of long aliphatic chains attached to positions N3, C7 and C10 within a membrane, thereby exposing these four active flavin centers optionally and allowing for a new correlation of reaction modes and active centers.

In biological systems, flavin nuclei are more or less tightly bound to proteins and a large number of flavoproteins appear to be strongly membrane oriented under conditions where steric restrictions are liable to control the specific flow of flavin substrates [21]. With these concepts

before us, it is obvious that a vesicle-bound flavin may serve as a simple model system.

In addition, there is good evidence available that the physiological blue-light photoreceptor in a great variety of organisms is a membrane-bound flavin as well [13, 26, 27, 42, 43]. Up until now, however, it is unclear whether these photoreceptors are specifically flavoproteins or simply flavin chromophors controlling membrane properties. The first approach taken in the understanding of anisotropic flavin (photo-) chemistry by means of a membrane model system was undertaken by Trissl [46], and by Frehland and Trissl [20]. These authors incorporated different amphiflavins into artificial flat bilayer membranes and obtained information regarding the flavin area concentration by fluorescence analysis. Using a simplified model for the spacial distribution of the transition dipolemoment, they concluded by polarized fluorescence analysis that the rotational relaxation time $T_{\Delta E}$ is much shorter than the fluorescence lifetime τ. From this they estimated an upper limit for the viscosity of the membrane of about one poise.

However, using a flat bilayer, compared to vesicles, as models for biological systems [4] has only a rather limited advantage: The normal vector of the membrane area in which the chromophors move is for the flat lipid bilayer a well-defined parameter. To provide for this system, *n*-decane has to be introduced as a ternary membrane-stabilizing component. Moreover, even the highest possible flavin area concentration is too low to permit analytical methods other than fluorimetry. Temperature experiments concerning the influence of the membrane phase on the flavin, as reported here, are not feasible under these conditions. Summarizing, it would appear worthwhile to use either of the described systems for future flavin research as models of anisotropic flavin, depending upon the given problem.

Survey

This paper has been planned to initiate a series of photochemical investigations on vesicle-bound flavins[1]. In the first part, the methods of optical measurements, preparation, loading properties with respect to flavins, and the stability of the vesicles are explored in more detail. The

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second part deals with fluorescence experiments on those vesicle-bound flavins. The following major observations are discussed:

1) The fluorescence quantum yield increases significantly upon the phase transition from the crystalline to the liquid crystalline state, which is temperature reversible with appreciable hysteresis.

2) The fluorescence quantum yield and the wavelength of the near UV-absorption peak of amphiflavins are dependent upon solvent polarity. This suggests that the chromophore sinks deeper into the more hydrophobic parts of the membrane upon phase transition from the crystalline to the liquid crystalline state, and vice versa.

3) On the basis of fluorescence polarization measurements the *Perrin* formula provides information of the rotational motility of the flavin chromophore in the membrane as well as of the viscosity of its microenvironment.

4) The vesicle-bound flavin chromophore is well protected, whenever the membrane is in the crystalline state, from the influences of the viscosity of the medium in which the vesicles are suspended.

Materials and Methods

Chemicals

The phospholipids L-β,γ dimyristoyl-α-lecithin (DML,N 42803), L-β,γ dipalmitoyl-α-lecithin (DPL,N 42556) and L-β,γ distearoyl-α-lecithin (DSL,N 43698), puriss., were purchased from Fluka, Buchs, Neu-Ulm. Egg phosphatidylcholin was synthesized by K. Janko in the lab of Prof. P. Läuger, Konstanz. The lipids were stored as solutes in spectroscopical ethanol in the freezer (DML,DPL: 25 µM, DSL: 12.5 µM). The amphiphilic flavins 7,8,10-trimethyl-3-octadecyl-isoalloxazin (AFl 3), 3,8,10-trimethyl-7-octadecyl-isoalloxazin (AFl 7), 3,7,8-trimethyl-10-octadecyl-isoalloxazin (AFl 10,7-8-CH_3) and 7-8-nor-3-methyl-10-octadecyl-isoalloxazin (AFl 10,7-8-H) were synthesized by Dr. W.R. Knappe. The structure and their hypothesized localization within the membrane are given in Fig. 1. The angle of 60° of the chromophore against the membrane surface was proposed by Frehland and Trissl [20]; the chromophor's depths are suggested from the results reported here. All four compounds were purified by column chromatography on neutral alumina and gave correct elemental analyze for C, H and N. All flavins are insoluble in water ($< 5 \times 10^{-11}$ M) and for better experimental reproducibility stored as solutes in spectroscopic ethanol at room temperature (0.1–0.4 mM).

Tritium labeled phages P22 were a gift of Prof. Knippers, Konstanz.

N,N-dimethyl-3-nitroanilin as a fluorescence standard was purchased from EGA-Chemie, Steinheim, W. Germany. The following (photochemically inert) buffers were used to investigate the vesicle stability depending on the pH; all as sodium salts, plus 0.1 M NaCl: pH 2, sulfat; pH 4, acetate; pH 6, pH 8, phosphate; pH 10, borate.

Glycerol and ethylene glycol were purchased from Merck, Darmstadt.

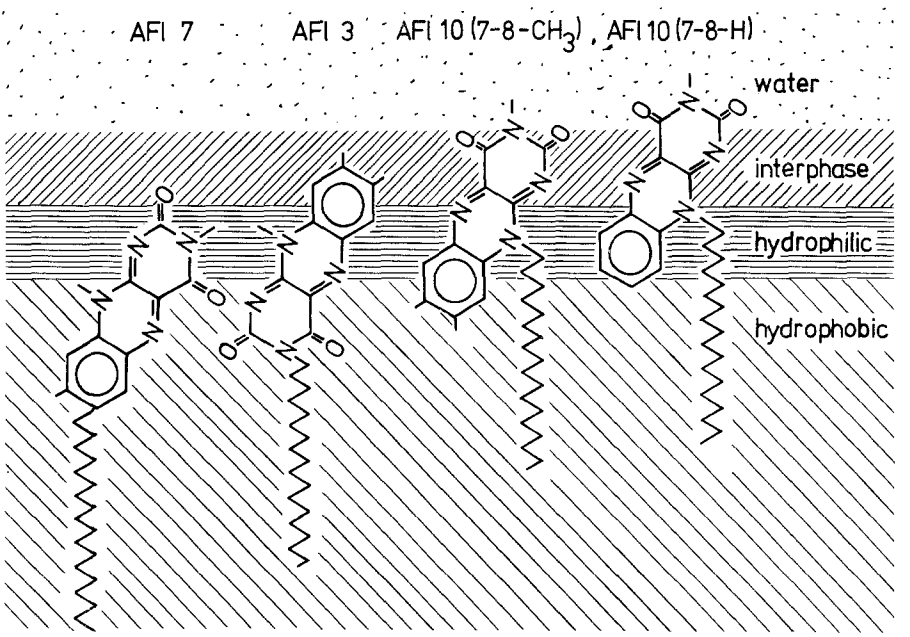

Fig. 1. Structures and hypothetical localizations of the various amphiphilic flavins within the vesicle membrane. The angle of the chromophor's midaxis with the membrane surface has been suggested by Frehland and Trissl [20]; the different depths are proposed from the data reported here. Taking the nomenclature for the flavin molecule into account, abbreviations are self-evident and used throughout the text

Vesicle Preparation

Vesicles were prepared by following a modified procedure of the one described by Huang [22]. Appropriate volumes (*see* below) of solubilized flavins and lipids were mixed, the ethanol then was completely evaporated under vacuum at room temperature in a revolving 10-ml round-bottomed flask. Phosphate buffer (pH 8.2±0.2), 5 ml, was added to the dry lipid/flavin film.

This suspension was sonicated under argon in a thermostat-controlled water bath at a temperature above the lipid phase transition (DML: 30 °C; DPL: 50 °C; DSL: 65 °C) with a MSE-sonifier and microtip at a power setting of 70 W (20 kHz). The progress of vesicle formation was spectroscopically monitored using the extinction at 300 nm as assay. Following a 35 min sonication the extinction levels fell to a minimum and this time was taken as a standard (a more prolonged sonication disrupts the vesicles to much smaller micellar particles, as judged by the column chromatography elution profile). Egg lecithin on the other hand had to be sonicated for 120 min at 0 °C [22].

For separation of vesicles from lipid- and flavin-micelles the 5-ml sonicated suspension was chromatographed (using the suspension buffer as eluting agent) on Sepharose 4B or Sephadex G 50, respectively, in a column of 45 cm length and 2.5 cm inner diameter under dim red safety light. The elution profile was monitored at 250 nm with a LKB-Uvicord. Lipid recovery (DPL/AFl 3) within the vesicle peak was 86±4% for the Sephadex G 50, and 69±4% for the Sepharose 4B column, as determined by the method of Bartlett [6]. While this indicates a better selection of vesicle size by the Sepharose 4B

column, the separation on Sepharose 4B takes 6 hr as compared to 30 min for Sephadex G 50. The Sepharose 4B procedure was chosen therefore only for the loading curve (Fig. 2).

Spectroscopy

Absorption spectra of vesicle-bound flavins were measured in 1-cm cuvettes with a Cary 118 spectrophotometer, using, as the scattering reference, flavin-free vesicles prepared in the same manner as the loaded vesicles. For measurements, the absorbance of the 450-nm flavin band was always adjusted to less than 0.1.

Fluorescence was measured in 1-cm cuvettes using the Perkin Elmer fluorescence spectrophotometer, model MPF-3. Extinction at 450 nm was kept below 0.1 in order to remain in the linear concentration range of the flavin fluorescence. Both extinction and emission slits were set to 10 nm. The red extended type R928 photomultiplier (Hamamatsu) was used.

To measure quantum efficiencies of fluorescence for the different vesicle-bound flavins, corrected emission spectra were evaluated, i.e., spectra in which the wavelength dependency of the lamp, the monochromator, the photomultiplier, etc., were eliminated. The correction curve for the fluorimeter between 440 and 780 nm was obtained by a comparison of the reported fluorescence emission spectrum of N,N-dimethyl-3-nitroanilin [19] with that actually measured. Calculations and plots were performed with a small computer (Hewlett Packard, model 9820). Quantum yields were obtained by referring to the (averaged) reported fluorescence quantum efficiency for lumiflavin of 28.6% [12, 31]:

$$\phi_{\mathrm{AFl}}=\frac{\phi_{\mathrm{LFl}}\times F_{\mathrm{AFl}}\times n^2_{\mathrm{LFl}}}{F_{\mathrm{LFl}}\times n^2_{\mathrm{AFl}}}$$

with

ϕ = quantum efficiency

$F=\int_{14286}^{21276}$ fluorescence intensity $\times\, dk$

k = wavenumber = wavelength $[\mathrm{nm}]^{-1}\times 10^7$

n = refractive index of the solvent [35].

Polarized fluorescence intensities were measured with the polarizer vertically and the analyzer (Spindler & Hoyer KG, Göttingen, No. 036320) either horizontally (I_{90}) or vertically (I_0) oriented. Prior to the calculation of the polarization $P=(I_0-I_{90})/(I_0+I_{90})$, these intensities were computer-corrected for the intrinsic polarization of the fluorimeter. Taking into consideration that the rotational relaxation of N,N-dimethyl-3-nitroanilin is much faster than its fluorescence lifetime at 20 °C, differences between the unpolarized and the polarized fluorescence emission spectra are therefore exclusively due to the intrinsic polarization of the apparatus which can be taken into account. Scattering from vesicle suspension did not measurably contribute to the fluorescence signal or effect the polarization values.

Air had no measurable effect on the fluorescence quenching of flavins, and all experiments were performed under aerobic conditions. Cuvette temperature was controlled within ± 0.5 °C by circulating thermostated water through the copper cellholder. Warming and cooling occurred at about 2 °C/min.

Fluorescence as a function of temperature was monitored continuously by feeding the fluorescence signal to the Y-input of an $X-Y$ recorder. The temperature signal was

transduced into voltage by an iron-constantan thermocouple with icewater reference and fed to the X-input simultaneously.

Results and Discussion

Vesicle Preparation

To estimate the diameter of the vesicles made according to Huang [22] from DML, DPL, DSL and egg lecithin, their elution profile from the Sepharose 4B column was compared with that of tritium-labeled phages P22, which have a diameter of 600 Å and a mol wt of 30×10^6 daltons. Both particle types were mixed and charged onto a Sepharose 4B-column. Taking the countrates for the radioactive labeled phages and the absorption at 450 nm for the flavin-loaded vesicles as assays, two separated single peaks are obtained. The elution profiles of DML, DPL, and DSL vesicles are similar to those given by Pohl for dextran blue and DPL vesicles [37]. Assuming that the peak of the phage elution marks the void volume of the column, a diameter of the vesicles of 465 Å is calculated by the formula of Marrink and Gruber [34]. Vesicles made from egg lecithin had a calculated diameter of 230 Å, in good agreement with the value of 250 Å by Huang [22].

Loading Properties

The question of how many flavin molecules can be loaded on the vesicles was answered as follows: Suspensions of constant amount of DPL (54 μmol) and increasing amounts of amphiflavins (0.54 to 4.32 μmol) were sonicated and chromatographed on a Sepharose 4B column. From the relative respectively absolute flavin concentrations within the vesicle peak, the highest possible flavin load of the DPL vesicles is calculated (AFl 10,7-8-H/DPL $\simeq$ 1:100 and AFl 3/DPL $\simeq$ 2:100), Fig. 2. Assuming that one lipid molecule occupies $\simeq 48$ Å^2 on the vesicle surface [18], a vesicle contains about 22000 lipid, and 220 (AFl 10,7-8-H) and 440 (AFl 3) flavin molecules, respectively, taking the inside and outside surface into account. From these data, a flavin area concentration of 2 to 4 times 10^{12} molecules per cm^2 can be calculated. This value is in good agreement with that of 10^{11} to 10^{13} obtained by Trissl [46] for flat bilayer membranes. From the data presented here, a mean distance of 50 Å between the AFl 3-molecules and 70 Å between the AFl 10,7-8-H-

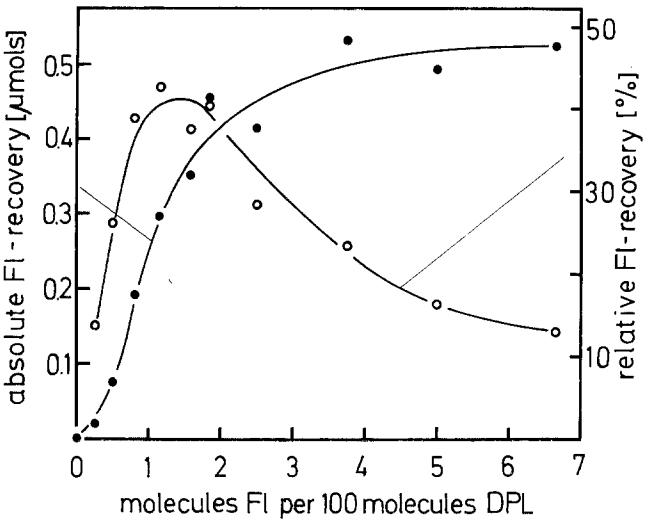

Fig. 2. Absolute (●) and relative (○) recovery of vesicle-bound flavin on a Sepharose 4B column as a function of the concentration ratio AF1 10 (7-8-H)/DPL in the suspension before sonication and chromatography. The AF1 3/DPL system shows the same feature except that the optimum load is 2 flavin molecules per 100 molecules DPL

molecules can be estimated. In comparison, Trissl calculated for the flat bilayer membrane a mean distance of 45 to 58 Å. The loading properties with respect to the other flavin/vesicle combinations have not been worked out as thoroughly but appear to be comparable. Summarizing, we find that vesicle- and flat bilayer membranes show very similar loading properties for amphiflavins.

Vesicle Stability

The dependence of the stability of flavin-loaded vesicles on the pH and the temperature is shown in Fig. 3 (DPL/AF1 10, 7-8-H). At room temperature there is sufficient stability over a number of days independent of the pH between 2 and 10. Transfer to 4 °C causes rapid conglomeration, especially near physiological pH's. The reason for the stability at 25 °C and instability at 4 °C remains unexplained.

There is also a significant dependence of the vesicle stability on their concentration. The conglomeration of freshly prepared vesicles to larger aggregates, as measured by the change in the apparent optical density at 300 nm, is almost proportional to the vesicle concentration. The apparent optical density at 300 nm of a typical sample was about 0.2 and maintained a stability of structure for at least several days at room temperature.

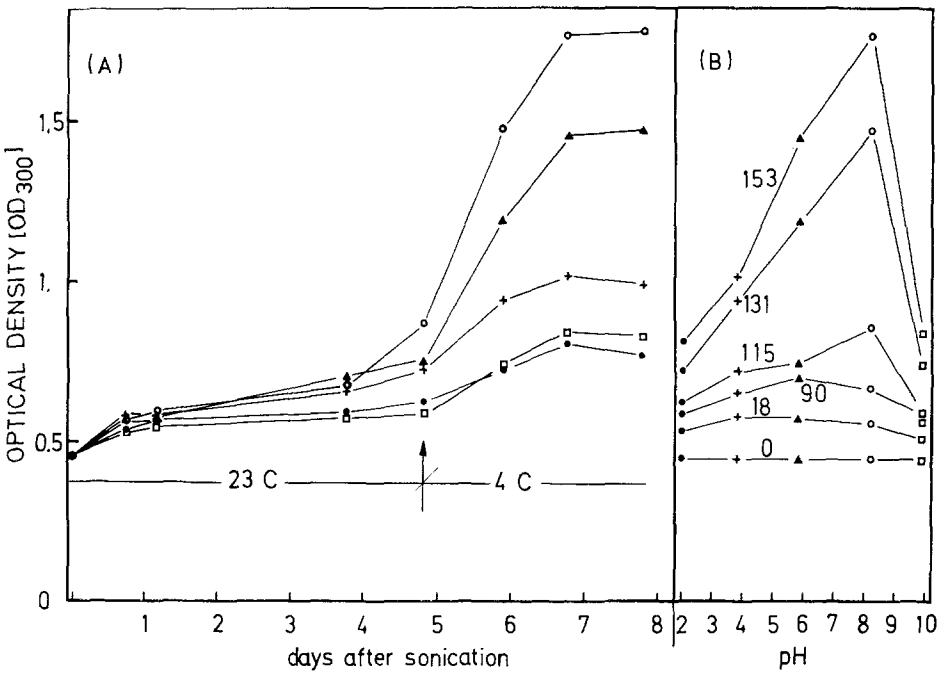

Fig. 3. Dependence of the stability of flavin-loaded vesicles (DPL/AFl 10 (7-8-H)) as assayed by the apparent optical density at 300 nm. (*A*): Kinetic behavior, (●), pH 2; (+) pH 4; (▲), pH 6; (○), pH 8; (□) pH 10. The arrow indicates the time at which the samples were transferred from 23 to 4 °C, inducing a rapid conglomeration. (*B*): The same data as represented in (*A*) with the curve parameter (pH) and abscissa (time) interchanged. The strongest destruction is observed in the vicinity of physiological pH's

Fluorescence as a Function of Temperature

The dependence of the flavin fluorescence as a function of temperature for lumiflavin (LFl) and for flavin/vesicle systems is depicted in Fig. 4. While buffered lumiflavin shows a normal temperature fluorescence quenching, all vesicle-bound flavins exhibit a marked increase of fluorescence near the phase transitions from the crystalline to the liquid crystalline state (DML, 23 °C; DPL, 41 °C; DSL, 58 °C), the extent depending strongly on the specific combination of flavin and lipid (Table 1). There are marked hysteresis effects, sometimes "normal" (e.g., DPL/AFl 3, DML/AFl 7), sometimes "negative" (e.g., DPL, DSL/AFl 10, 7-8-H), but in all cases very reproducible (measuring errors are ± 0.5 °C and $\pm 2\%$ fluorescence). There is nearly no dependence on the velocity of temperature change or the number of temperature cycles. In some cases a broad prephase transition can be seen (e.g., DSL, DPL/AFl

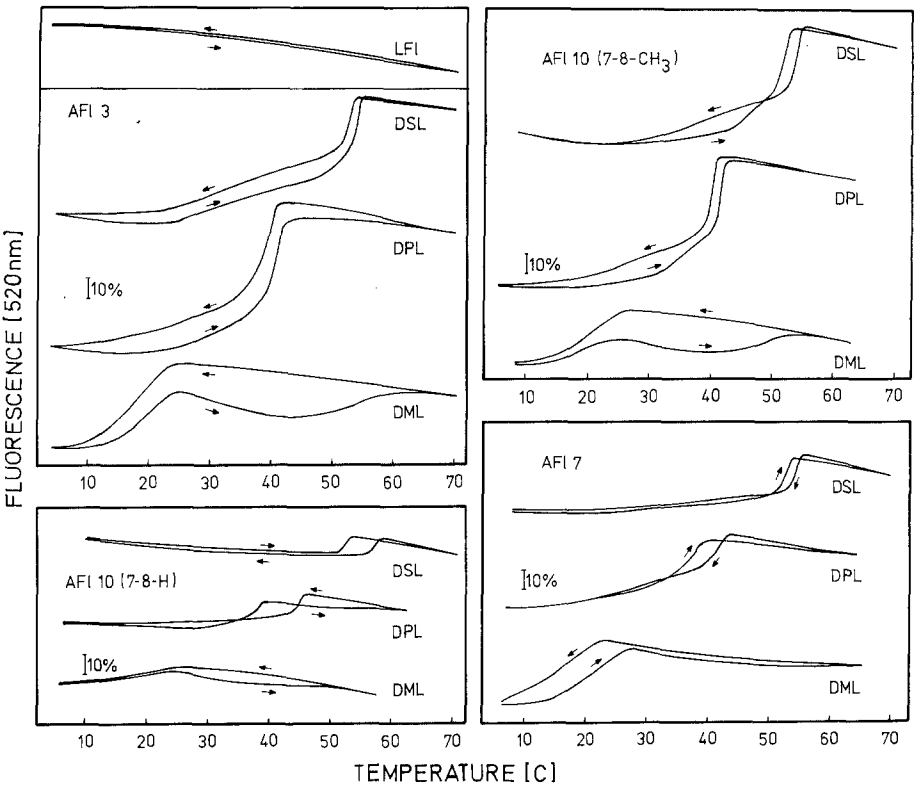

Fig. 4. Dependence of the flavin fluorescence for lumiflavin and the flavin/vesicle systems upon changes in temperature. Temperature change occurred at about 2 °C/min. A variety of complicated but reproducible hysteresis effects is exhibited with negligible influence on the scanning velocity or the number of temperature cycles. Fluorescence at 10 °C on the increasing temperature trace was normalized to 100 %. Measuring errors are ±0.5 °C and ±2 % fluorescence

10,7-8-CH_3; DSL/AFI 3) resembling that described earlier by Hinz and Sturtevant [24] using microcalorimetry. The most pronounced hysteresis is exhibited by the DML/flavin systems. For increasing temperature scans, a second increase of fluorescence intensity is detected between 50 and 60 °C, possibly indicating a second phase transition for the DML membrane. This second phase transition has not been reported previously.

A possible explanation for the increase in fluorescence yield upon membrane melting is obtained from the following reasoning: It is well known that the fluorescence yield of chromophores often increases with decreasing polarity of the solvent [30]. This was confirmed for AFI 3 in solvents of different dielectric constants compared to lumiflavin in buffer

Table 1.

Combination		Pos. S2[b] (nm)		$\Delta\phi$ at T_i[a]	$\phi T < T_i$[a]	ΔA(450 nm), S1[c] at T_i[a]	Micro-viscosity at 60 C (cp)	Rotational relaxation time at 60 C (nsec)
Flavin	Lipid	$T < T_i$[a]	$T > T_i$[a]	[%]	[%]	[%]		
AFl 3	DML	345	343	28	18.7	4		
	DPL	346	344	76	19.7	8	49	18
	DSL	346	344	63	20.6	9		
AFl 7	DML	345	340	27	16.5	13		
	DPL	346	343	31	17.5	6	26	10
	DSL	349	345	26	14.6	10		
AFl 10 ($7\text{-}8\text{-}CH_3$)	DML	348	345	18	26.4	0		
	DPL	350	347	65	23.0	2	23	9
	DSL	349	347	56	21.8	5		
AFl 10 (7-8-H)	DML	Split peak		8	21.5	–	31	12
	DPL			13	21.7			
	DSL			8	22.2			
Lumiflavin	In buffer	366		Ref.: 28.6 (lit.)		–	1.03	0.38
AFl 3	Isopropanol	347		–	36.7	–	–	–
AFl 7		343			36.5			
AFl 10 ($7\text{-}8\text{-}CH_3$)		347			33.5			

[a] Temperature at phase transition.
[b] Near UV-peak of flavin.
[c] Visible peak of flavin.

(Fig. 5, Table 1). The position of the near UV peak of amphiflavins can serve as indicator of the solvent polarity as well (Fig. 6, Table 1). From this it follows that the flavin nuclei are imbedded more deeply into the more hydrophobic portion of the membrane upon phase transition (gel → liquid crystalline). While it is not possible to evaluate a quantitative relationship, the above explanation is supported by the facts that the flavin absorption at 450 nm increases up to 13% upon phase transition (Table 1), presumably caused by an increased scattering or by an increased refractive index. For the isotropic case, the flavin absorption at 450 nm is known to be independent of the solvent polarity (W. Schmidt, *unpublished;* for comparison *see* [37]). Similar to the UV-absorption peak (S2), the fluorescence emission peak exhibits a slight hypsochromic shift of about 5 nm and an increased emission at the phase transition (for comparison *see* [37]).

The hypsochromic shift of either the fluorescence emission peak and the near UV-absorption peak (S2) of the membrane-bound flavins upon

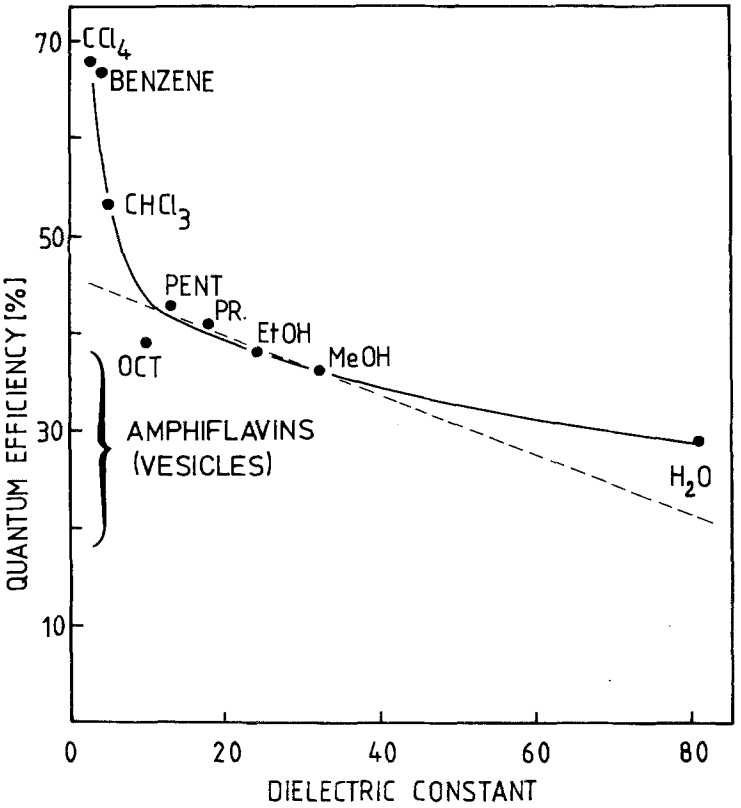

Fig. 5. Fluorescence quantum efficiency of flavin (AFl 3) in solvents of different polarities: CCl 4, carbon tetrachloride; CHCl 3, chloroform; OCT, octanol; PENT, pentanol; PR, propanol. The value for lumiflavin of 28.6 % as averaged from the literature [12, 31] is taken as a reference. The dashed line indicates the quantum efficiencies of riboflavin tetrabutyrate obtained by Kotaki and Yagi [30] for different solvent polarities. In their investigations [30] a strong increase in fluorescence yield was not obtained at small dielectric constants. The fluorescence yield of vesicle-bound flavins is quenched to a range of values indicated by the bracket (for precise values, *see* Table 1)

phase transition can be explained on another basis. Bayliss and McRae [7, 8] discussed the origin of solvent effects and pointed out that all organic electronic spectra in solution are subject to a generalized *polarization red shift* due to solvent polarization by the transition dipole of the solute. Consistently, above the phase transition we found a decreased molecular polarizability α of DML-, DPL- and DSL-vesicles, whether they were loaded with flavins or not, using *Rayleigh* scattering as an assay (the molecular polarizability α is proportional to the square root of *Rayleigh* scattering; Fig. 7). Moreover, a comparison of Figs. 4 and 7 might suggest a relationship of solvent polarizability and fluorescence

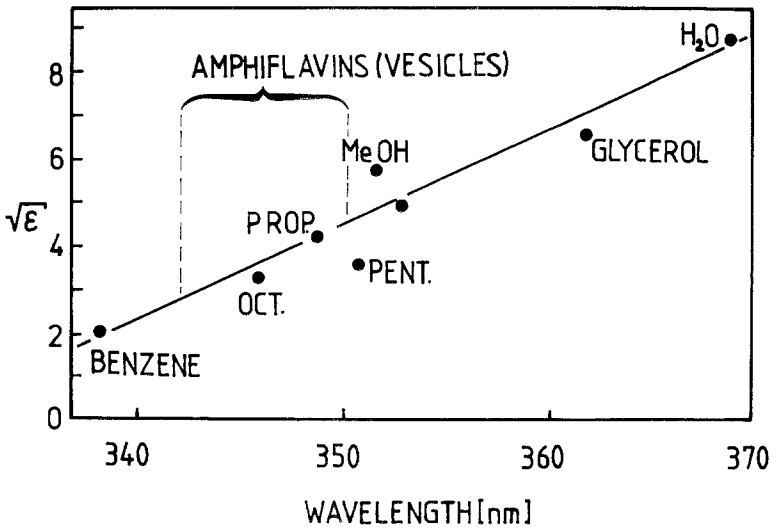

Fig. 6. Empirical linear dependency of the S2 peak position of AF13 and lumiflavin (in H_2O) on the square root of the dielectric constant. OCT, octanol; PROP, propanol; PENT, pentanol. The S2 peak of the vesicle-bound flavins occurs in the wavelength range underneath the bracket corresponding to dielectric constants between 10 and 30 (for precise values, *see* Table 1)

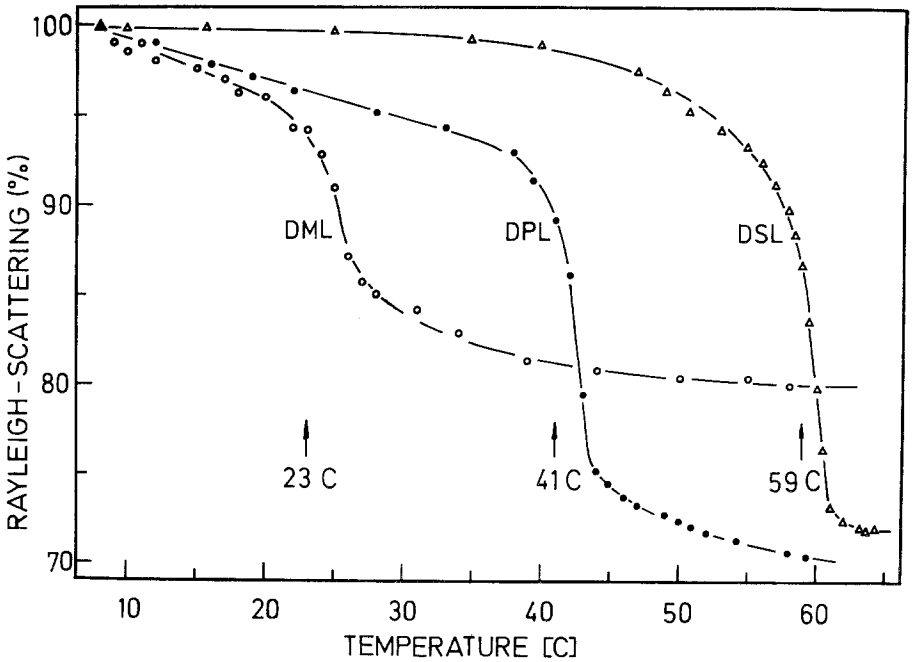

Fig. 7. Rayleigh scattering of the three used vesicle types as a function of temperature, measured at 300 nm within the fluorimeter (i.e., 90 degree scattering). The scattering at 8 °C is normalized to 100 %

quantum yield of flavins; however, no further experimental results are available. In all likelihood the changes in electronic transition energy and intensity, upon the membrane phase transition, depend on many factors, such as dipole-dipole, dipole-polarization, hydrogen bonding, and transition dipole interactions, in a badly understood way.

Träuble [45] observed a similar increase of fluorescence yield of membrane-bound ANS (1-anilino-8-naphthalinsulfonate) by a factor of two upon phase transition. Parallel to this fluorescence increase, he found a twofold decrease of the extinction coefficient of the absorption indicator BTB (bromthymolblue). Both effects were convincingly explained by an increase in the number of binding sites for both ANS and BTB. Such an explanation cannot hold in our case since all amphiflavins are completely insoluble in water and because there is no observed change in the flavin extinction coefficient at 450 nm with the solvent polarity (i.e., all flavin molecules are irreversibly bound to the vesicles).

Rotational Motion, Microviscosity, and Intrinsic Polarization

Information about the rotational motion of flavin chromophores within the membrane was obtained from polarization measurements. Surprisingly, the temperature dependencies of the (corrected) I_0 and I_{90} fluorescence components are clearly not proportional to each other, as expected, with characteristic deviations for the individual flavin/vesicle systems (which excludes trivial artifacts). This effect is not well understood and further work is in progress (no figures shown).

Nevertheless, from these data the fluorescence polarization for all vesicle/flavin combinations was calculated, again as a function of temperature (Fig. 8). The polarization decrease by a factor two to three near the phase transition temperature indicates a strong decrease in the rotational relaxation time of the chromophores within the vesicles. Similar results were obtained with different dyes in lecithin vesicles by Pohl [37] and Lussan and Faucon [33]. This rotational relaxation time can be evaluated quantitatively if (i) the effective Stoke-Einstein radius $\bar{R}$ and (ii) the intrinsic polarization p_o are known. Space-averaged values for $\bar{R}$, Φ and ρ were obtained using the formulae by

Perrin
$$\left(\frac{1}{p}-\frac{1}{3}\right)=\left(\frac{1}{p_o}-\frac{1}{3}\right)\left(1+\frac{3kT\tau}{4\pi R^3}\,\Phi\right) \tag{1}$$

and

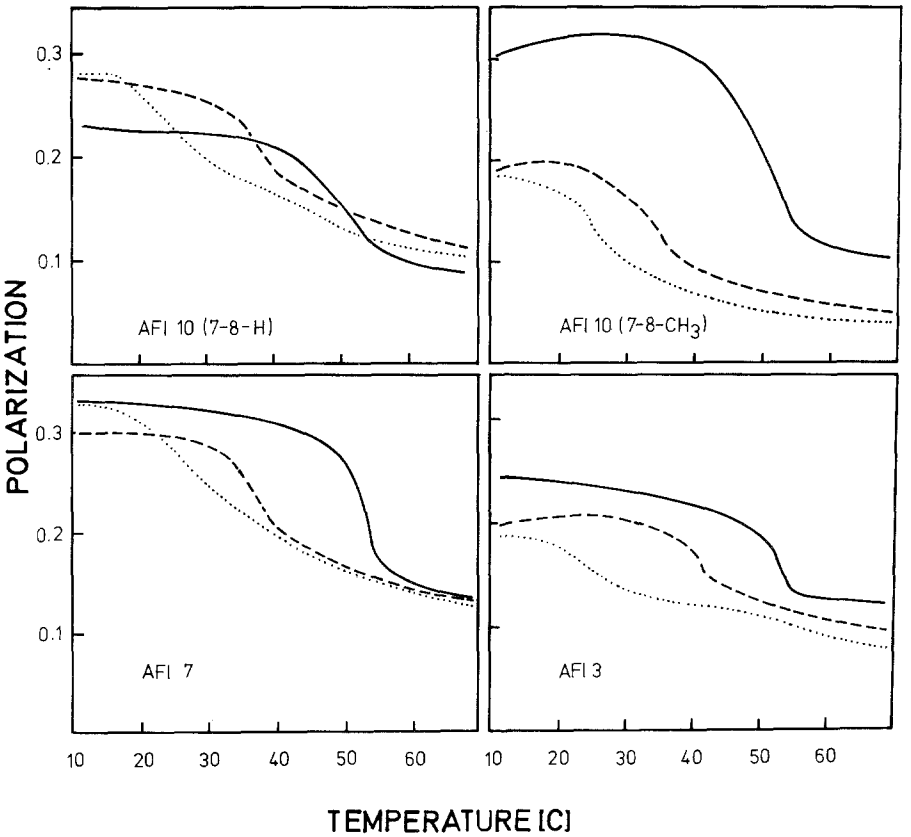

Fig. 8. Apparent flavin fluorescence polarization for all flavin/vesicle combinations as calculated from the temperature dependence of the respective 90° and 0° fluorescence emission components

Stoke-Einstein $$\rho = \frac{4\bar{R}\pi}{kT\Phi} \tag{2}$$

with: p, apparent polarization; k, Boltzmann's constant; T, degree Kelvin; τ, fluorescence lifetime (4.5 nsec [32]); Φ, fluidity (rhe) $= 1/\eta$, $\eta =$ viscosity; and ρ, rotational relaxation time.

The apparent polarizations of lumiflavin in phosphate buffer/glycerol mixtures of different viscosities were measured for comparison. Plotting $1/p$ *vs.* Φ results in a straight line evaluated from least squares fit as shown in Figs. 9*A* and 10. By extrapolation to zero fluidity, an intrinsic polarization for lumiflavin of $p_o = 0.30$ is obtained. Using this value and extrapolating the straight line to the value of $1/p$ at $\Phi = 100$ (i.e., fluidity

of water) an effective radius of $\bar{R}=5.2$ Å and a rotational relaxation time of 0.38 nsec of lumiflavin in water of 20 °C are obtained.

The analogous parameters for AFl 3 were measured in the same way, except that ethylen glycol was taken as the solvent since it provided for better solubility. Fluidities between 2.0 and 20 rhe were obtained by varying the temperature from 0 to 60 °C [28]. From Fig. 10 the intrinsic polarization p_o is evaluated to be 0.28 and the effective radius 5.0 Å, a value which cannot be distinguished from $\bar{R}=5.2$ for lumiflavin within the measuring error.

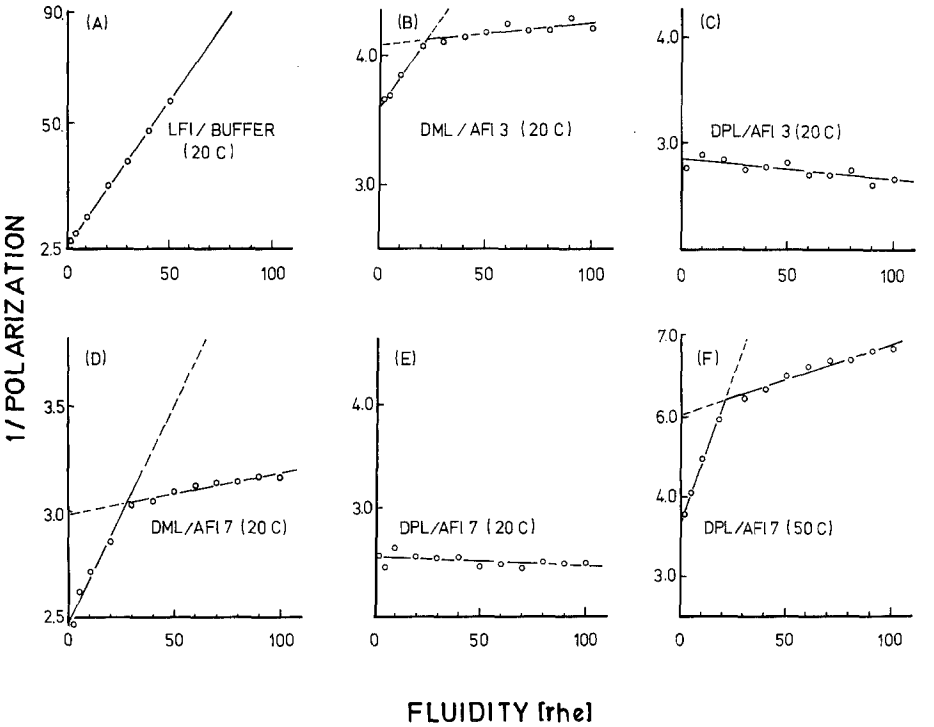

Fig. 9. Least squares fits of reciprocal flavin polarization *vs.* fluidity (Perrin, Eq. (1)). The fluidity was adjusted by different glycerol/buffer mixtures. (*A*): Isotropically dissolved LFl (lumiflavin) exhibits a straight line up to 50 rhe (for a close-up, *see* Fig. 10). (*B*): The measured points are best described by two straight lines. *Note:* DML at 20 °C is mainly in the liquid crystalline state. (*C*): The measured points are best described by a single straight line with a slight negative slope. *Note:* DPL at 20 °C is in the crystalline state. (*D*): The measured points are best described by two straight lines. *Note:* DML at 20 °C is mainly in the liquid crystalline state. (*E*): The measured points are best described by a single straight line with a slight negative slope. *Note:* DPL at 20 °C is in the crystalline state. (*F*): The measured points are best described by two straight lines. *Note:* DPL at 50 °C is mainly in the liquid crystalline state

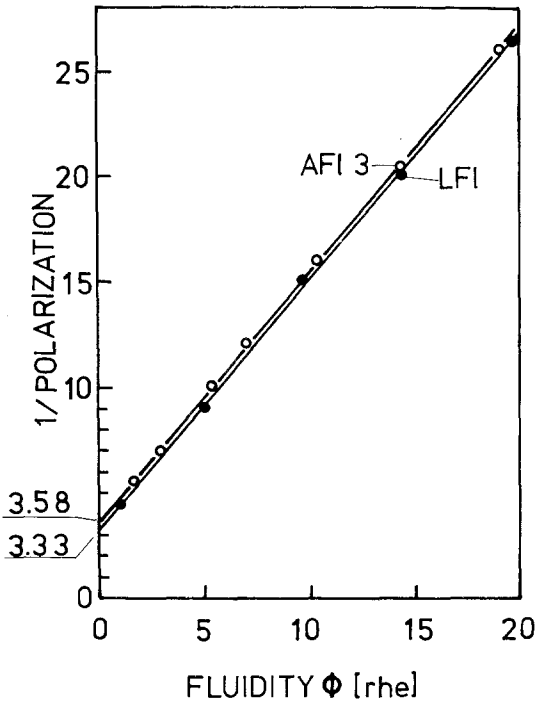

Fig. 10. Least squares fit of the reciprocal polarization *vs.* fluidity. From the extrapolation to zero fluidity an intrinsic polarization p_o for AF13 of 0.28 and for lumiflavin (LFl) of 0.30 is obtained (Perrin [25]). The data for LFl are a close-up of those shown in Fig. 9*A*. The fluidity of the LFl solution was adjusted by different glycerol/buffer mixtures. AF13 was dissolved in ethylen glycol, and the fluidity was adjusted between 0 and 20 rhe by changing the temperature from 0 to 60 °C

As a first quantitative approach, it is reasonable to assume that an effective radius of $\bar{R}=5.1$ Å holds for all amphiflavins as well. Combining Eqs. (1) and (2) the rotational relaxation time can be calculated if the intrinsic polarizations p_o for the different amphiflavins within the vesicle membranes are known. Unfortunately, these cannot be determined by the methodology utilized for the isotropic cases (*see* below). However, a closer inspection of Fig. 8 shows that the apparent polarizations already exhibit an asymptotic behavior near 10 °C, indicating that the polarization values at 10 °C are at least similar to the intrinsic polarizations p_o. Taking those p values as p_o (which are at least lower limits of p_o) and combining Eqs. (1) and (2), the lower limits of the rotational relaxation times of the different flavin/vesicle systems as a function of temperature were calculated (Fig. 11). The rotational mobility is significantly hindered

by the lipid environment in the crystalline state with the rotational relaxation times being in the 55 to 100 nsec range depending on the specific flavin/vesicle combination. However, beyond the phase transition the rotational relaxation times of the different flavins level at about the same value of 10 to 20 nsec independent of the position of substitution of the aliphatic chain. This is consistent with the hypothesis brought forward above, that the flavin nuclei sink deeper into the hydrophobic part of the membrane upon phase transition. The rotational relaxation times are equivalent to the microviscosities as seen by the chromophores [Eq. (2)]. These are 120 to 200 cp below phase transition and 23 to 49 cp above phase transition (Table 1).

However, the most direct method of estimating the viscosity of the lipid phase is based on spin labels and on the rates at which lipid soluble molecules diffuse through membranes. Spin labels seem to encounter

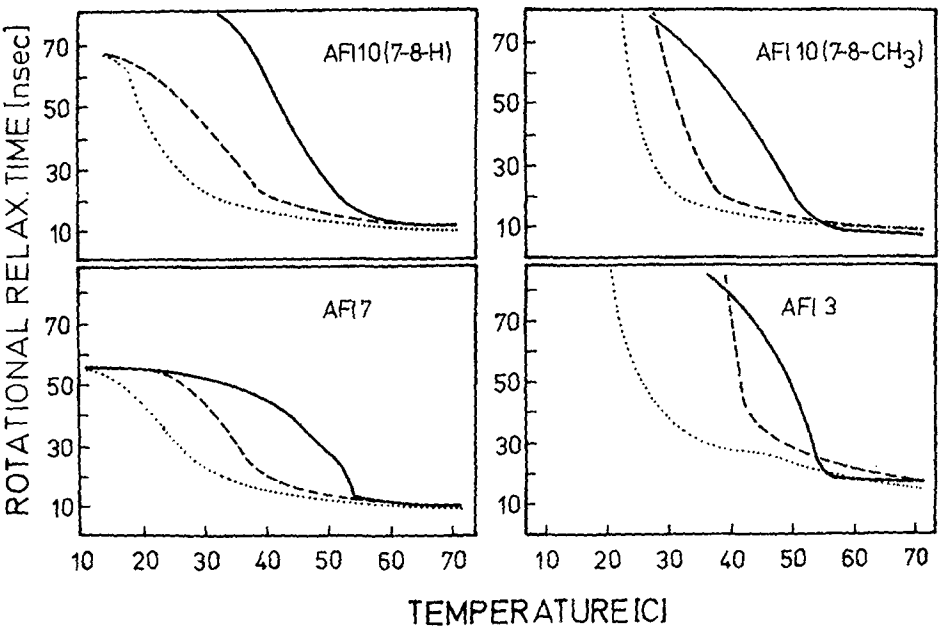

Fig. 11. Rotational relaxation time of the vesicle bound flavins as a function of temperature for all vesicle/flavin-combinations, as calculated and drawn by the computer under the assumption of a fluorescence lifetime of 4.5 nsec [32], an effective chromophore radius of 5.1 Å, and intrinsic polarizations as deduced from Fig. 10. Solid lines: DSL; dashed lines: DPL; dotted lines: DML. The rotational relaxation times level towards the same value between 10 to 20 nsec, independent of the position of fixation of the aliphatic chain

viscosities of 10 to 100 cp in the membranes of mitochondria and nerve axons, which is in good agreement with our data [10, 16, 23, 29]. Preliminary EPR data (electron paramagnetic resonance) on DML-, DPL- and DSL-bound AFl 3 radicals reveal viscosities from 30 to 80 cp (respectively rotational relaxation times of 10 to 35 nsec) as well, depending on temperature (H. Michel and W. Schmidt, *unpublished*).

The values for the viscosities reported here are also consistent with the estimate of microviscosity of 100 cp as an upper limit for the bilayer membrane as given by Frehland and Trissl [20]. However, their conclusion from model calculations that the rotational relaxation time of the flavin nucleus within the bilayer membrane is much smaller than the flavin fluorescence lifetime is in strict contradiction to the results for the vesicle-bound flavins reported here. That is possibly due to the fact that they observed the rotational motion averaged in two dimensions, whereas for the vesicle system a space-averaged value is obtained (i.e., we have to assume that the mobility around different axes is quite different). Moreover, they report for the three amphiflavins which they bound to the bilayer membranes p_o-values of 0.5. Using the formula by *Perrin* [36]

$$\Gamma = \arccos\left[(1+3p_o)/(3-p_o)\right]^{1/2} \tag{3}$$

they conclude that the angle Γ between the transition moments for absorption and emission is 0°, which in turn is an essential prerequisite for their model calculations. However, the theoretically highest possible value for p_o of 0.5 appears to be rather exceptional. When trying to reproduce their p_o value for AFl 3 ("flavin 1" in [46]) a value of 0.28 was obtained which corresponds to an angle of $\Gamma = 34.7°$, which is close to the value $p_o = 0.30$ as obtained for lumiflavin (*see* above). This means their suggested tilt-angle (Fig. 1) of the chromophore within the membrane is questionable.

The temperature dependence of the rotational relaxation (Fig. 11) can be described to a good approximation by a constant activation energy E_a. For example, from the Arrhenius plot for the AFl 10/DML-systems, values of $E_a = 15.5$ and $E_a = 12.5$ kcal/mol, respectively, are obtained. These values are about two times higher than the activation energy for the rotational diffusion of the nitroxid radical, which is 6.8 kcal/mol [40]. This is probably due to the different localization and size of the labels. Such differences in localization are also indicated by different microviscosities (23–49 cp, Table 1, and 4.6–5.7 cp for nitroxide, respectively [39]).

Interphase and Inside/Outside Problem

As mentioned above it was not feasible to determine the intrinsic polarizations for the vesicle-bound flavins as is possible for the isotropic cases. Extrapolation to zero fluidity results in quite different values of p_o for one specific flavin and the same vesicle type, depending on the particular phase of the membrane (Fig. 9*B–F*). Apparently the microviscosities affecting the flavin nucleus within the vesicle is quite different from that of the suspension, the latter being adjusted by different percentages of glycerol and buffer. A few examples are given in Fig. 9 to demonstrate a general feature of the flavin/vesicle systems. With vesicles above the phase transition, the plotted values ($1/p$ *vs.* Φ) are best described by two inclined straight lines rather than a single linear function (least squares fit, Fig. 9*B*, DML/AFl 3,20 °C; Fig. 9*D*, DML/AFl 7,20 °C; Fig. 9*F*, DPL/AFl 7,50 °C). Whenever the membrane is in the crystalline state there is no influence of the macroviscosity, as indicated by a single horizontal line which has a slight negative slope (least squares fit, Fig. 9*C*, DPL/AFl 3,20 °C; Fig. 9*E*, DPL/AFl 7,20 °C).

"There exists, on both sides of the membrane, a physical medium," that Blumenthal, Changeux and Lefever [11] refer to as *equilibrium layer* (boundary layer, interface), "in which the activity and diffusion of a ligand might be different from both that of the bulk solution and that of the membrane phase. In this layer, the concentration of ligand depends on its rate of adsorption on the membrane surface... and on its rate of diffusion from the bulk solution." (quotation taken from [11]).

Even if the exact definition is not possible, the existence of such an interface appears now to be generally accepted, because it is indispensable for most membrane models especially for the description of transport processes.

A straight-forward explanation for the dependency of flavin access on the membrane phase is based on the assumption that the flavin nucleus bound to the vesicle is completely separated from the suspension medium by such an interface. Beyond the phase transition this interface is softened, allowing glycerol molecules to penetrate and to influence the flavin mobility. Although the membrane phase transition takes place mainly within the hydrophobic portion, it is well known that to a smaller extent the polar region of the membrane is involved in the melting process and consequently affects the interface [45]. However, even above the phase transition the influence of the glycerol on the microviscosity around the flavin nuclei is much smaller than in the isotropic case (*compare* Fig. 9), demonstrating its still highly restricted access to the flavin.

This behavior is opposite to that of EDTA (ethylenediamine-tetraacetic acid), known to be an excellent photosubstrate for flavins [41]. Above the melting point of the membrane, EDTA ($<10^{-2}$ M) has no access to the flavin at all, whereas below this temperature it has (W. Schmidt, M. Petzuch, P. Hemmerich, *unpublished*). Both effects have to be attributed to special properties of the membrane/glycerol/cholin phosphate/water interface.

For the flavin/membrane system in the liquid crystalline state (i.e., beyond the phase transition), the break in the plot of $1/p$ *vs.* Φ always takes place between a fluidity of 20 to 30 rhe. This breakpoint, indicating the existence of two phases, probably reflects the fact that the inside-bound flavins are affected differently from those outside by the viscosity of the suspension medium. This hypothesis is supported by preliminary photochemical experiments on vesicle-bound flavins. Oxidized flavin under anaerobic conditions is readily photoreduced in the presence of EDTA (*see* above). Under isotropic conditions (lumiflavin/buffer) the kinetics are strictly first order. However, in the anisotropic case (AFl 10/DPL) it is best fitted by a superposition of a "fast" (outside) and a 10 times "slower" component (inside) (W. Schmidt, M. Petzuch and P. Hemmerich, *unpublished*).

Two remarks are pertinent. Firstly, the temperature of the vesicle phase transition has been proved to be completely independent of the suspension viscosity, and, secondly, the rotational relaxation time of the vesicles themselves in water at room temperature is 37 msec, some five orders of magnitude slower than the rotational relaxation time of the chromophore and can therefore be ignored.

Fluorescence Spectra, Quantum Efficiencies, and Localization

As mentioned above, the fluorescence emission spectrum shows a slight hypsochromic shift with the phase transition. A similar feature is exhibited when different amphiflavins are bound to one vesicle type (below phase transition). From Fig. 12 one can judge that AFl 7 is deepest, AFl 3 less, and AFl 10 least deeply anchored in the DPL membrane (*see* Fig. 1), as reflected by the different peak positions. Because of the different localizations of the chromophore, the averaged quantum efficiencies below the phase transition are 16.2 % for AFl 7, 19.7 % for AFl 3, and 23.7 % for AFl 10 (Table 1). These differences cannot be caused by the different positions of the hydrocarbon chains *per se*, as indicated by the quantum efficiencies of the different amphiflavins in isopropanol (isotropic case). On the other hand, the shapes of the emission spectra of AFl 7 in DML, DPL,

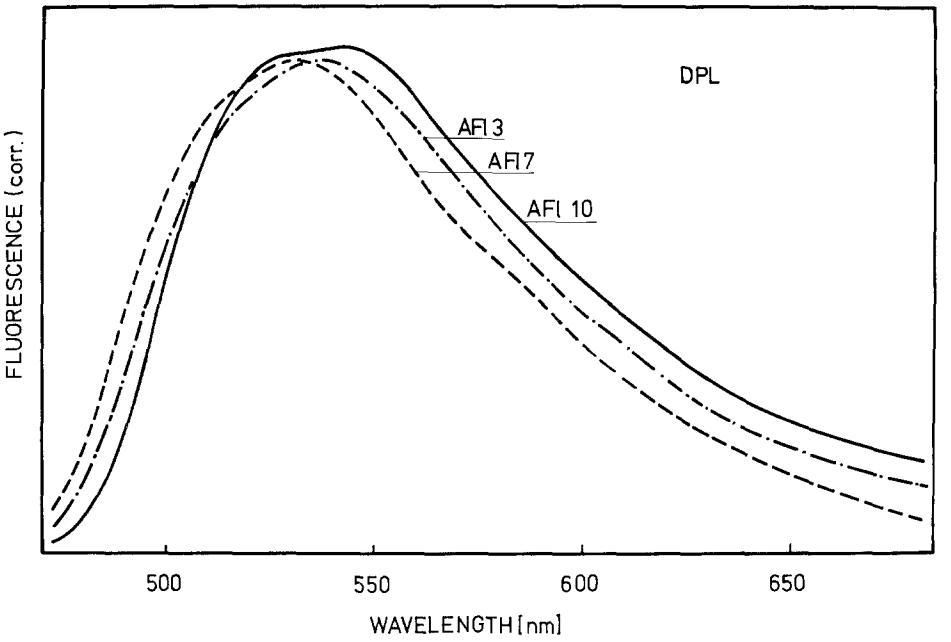

Fig. 12. Corrected fluorescence emission spectra of AFl 3, AFl 7, and AFl 10, 7-8-CH_3, bound to DPL vesicles at 23 °C. Excitation was at 444 nm. Both excitation and emission slits were set to 10 nm. Clearly the peak positions are relatively shifted to each other, indicating highest polarity of the microenvironment for AFl 10, medium polarity for AFl 3, and smallest polarity for AFl 7, as indicated in the hypothetic localization in Fig. 1. This is consistent with the S2-peak positions (below phase transition) as given in Table 1

and DSL vesicles cannot be distinguished (all are similar to AFl 7/DPL of Fig. 12), and the quantum efficiencies are not significantly different (Table 1). This suggests that the localization is independent of the vesicle type. However, the increase in the quantum efficiency upon melting is strongly dependent on the vesicle type (Table 1, Fig. 4).

As depicted in Fig. 1, for the case of AFl 7 the hydrophobic benzene portion of the flavin molecule seems to be buried within the hydrophobic portion of the membrane with positions 2 and 3 exposed in analogy with the riboflavin binding egg white flavoprotein [9]. On the other hand, AFl 3 may serve as a model system for flavodoxins in which the positions 2 and 3 are buried within the binding site and the positions 7 and 8 are exposed [25]. In addition, methylation of positions 7 and 8 of AFl 10 results in a strongly increased fluorescence change upon phase transition (Fig. 4) and a marked change in rotational mobility below phase transition (Fig. 11).

Conclusion

The DML, DPL, and DSL vesicles can be loaded with reasonable amounts of amphiphilic flavins and are stable over several days under suitable conditions. The rotational mobility, localization, and microenvironment of the chromophore within the membrane show resemblance to biological systems. Amphiflavins substituted with aliphatic chains at different positions when bound to vesicles can serve as biological model systems. Different analytical methods allow comparative measurements. EPR and photochemical experiments on vesicle-bound amphiphilic flavins (-radicals) seem to support the data presented here (W. Schmidt and H. Michel, *unpublished*).

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