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Further Photophysical and Photochemical Characterization of Flavins Associated with Single-shelled Vesicles

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Summary. This paper is a continuation of a series of physicochemical studies of vesicle-bound flavins. In this study a detailed analysis of the Sepharose 4B elution profile of various sonicated phospholipids is given which demonstrates the presence of three distinct particle populations. Electron microscopy reveals heterogeneous, multilamellar lipoid aggregates of a diameter of more than 500 Å in fraction I, unilamellar, closed vesicles of 200 to 250 Å diameter in fraction II, and previously unreported micellar structures of approximately 50 Å diameter in fraction III. Fraction II represents vesicles very similar to those obtained by the "deoxycholate procedure." Fine structure analysis of corrected fluorescence and fluorescence polarization spectra of amphiphilic flavin (AFl 3) bound to vesicles prepared from various phospholipids demonstrates the specificity of the flavin/membrane interaction. When bound to fractions I, II and III of sonicated egg lecithin, AFl 3 exhibits different temperature (i.e. phase-) dependencies. This demonstrates that the flavin/membrane interaction depends strongly upon the particular lipid *structure* as well. Evidence is presented which defines the type of excited state of flavin involved in photochemical and photophysical reactions (triplet or singlet). Finally, the dependencies of the photoreactions of vesicle-associated flavins on parameters such as pH, temperature and ionic strength are discussed. Comparison of photoreactions of isotropic and anisotropic flavins also reveal clues for the various mechanisms involved.

Key Words flavin · fluorescence · photoreduction · singlet state · triplet state · photo-pK · lipid-phase transition

Introduction

The present work is a continuation of the series of experiments describing our studies of vesicle-bound flavins. In the preceeding papers we studied various physico-chemical properties of amphiphilic flavins anchored in different positions within the vesicle membranes. These membranes were made from synthetic phospholipids with well-defined phase-transition temperatures.[1] In these experiments we have determined that the phase of the lipid is a highly significant parameter capable of controlling virtually all basic physico-chemical properties of the flavin nucleus within the lipoid microenvironment [25, 33, 34, 36, 37, 41].

Solubilized flavins are not ideal models for studying biological redox mechanisms as mediated by flavoproteins since the flavin/flavin interaction is diffusion controlled and always the fastest reaction to occur. However, when the flavin is vesicle bound this interaction is suppressed by 98% and, therefore, presents a good flavin model system [37]. We use the term "anisotropic flavin chemistry" for our experiments with membrane-associated flavins to avoid confusion with the results obtained from measurements made in solution.

In the present work we describe findings on the preparation of single-shelled vesicles made from various phopholipids where flavins are incorporated either in the membranous monolayer or in the lumen. We present evidence for identification of the type of excited flavin state involved in photochemical/photophysical reactions (triplet or singlet). Finally, studies of the dependencies of photoreactions on the pH, temperature and ionic strength are discussed with respect to how these factors reveal clues for the mechanisms involved.

Materials and Methods

Chemicals

A detailed description of the synthesis of amphiphilic flavins has been published previously [24]. 3-methyl-lumiflavin was obtained as a gift from Hoffmann La Roche, Basel. Tetraacetylri-

[1] *Abbreviations and Definitions:* DML: L-β, γ dimyristoyl-α-lecithin, $T_c=23$ °C; DPL: L-β, γ dipalmitoyl-α-lecithin, $T_c=41$ °C; DSL: L-β, γ distearoyl-α-lecithin, $T_c=58$ °C; AFl 3: 7,8,10 trimethyl-3-octadecyl-isoalloxazin; EDTA: Ethylenediaminetetraacetic acid; T_c: Phase-transition temperature; 3-me-LFl: 3-methyl-lumiflavin; LFl-3-aa: Lumiflavin-3-acetic acid; TARF: Tetraacetylriboflavin; DOC: Deoxycholate (sodium salt); MAO: Monoamineoxidase (monoamine: O_2 oxido-reductase, EC 1.4.3.4); "lumen": Inner aqueous space of (single-shelled) vesicles; "vesicle": Single-shelled, closed membraneous aggregate

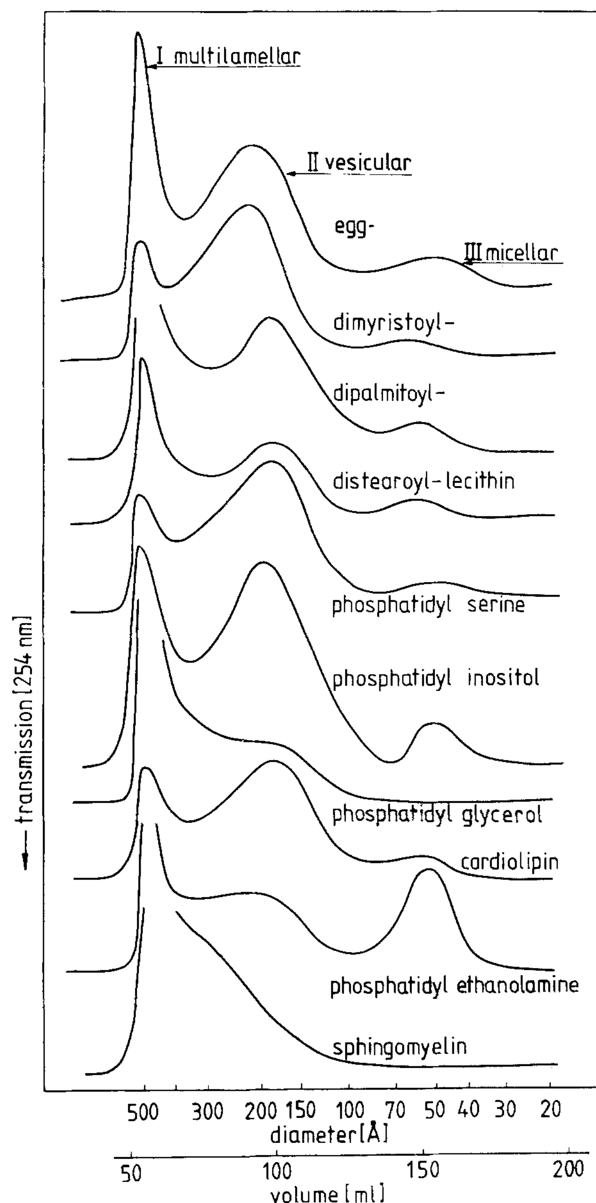

Fig. 1. Elution profile of various sonicated phospholipids on a Sepharose 4B column. Both sonication time and temperature are crucial parameters for the distribution of lipids over the three fractions I, II, III (the identification is sketched in Fig. 2). Since we were mainly interested in single-shelled vesicles, we attempted to optimize the various systems for maximal production of fraction II. Optimal temperatures and sonication times were determined (°C/min): Egg lecithin 30/120; DML 25/120; DPL 45/60; DSL 55/120; phosphatidyl serine 20/30; phosphatidyl inositol 0/60; phosphatidyl glycerol 20/36; cardiolipin 40/100; phosphatidyl ethanolamine 0/80 and sphingomyelin 0/60. The elution volume was calibrated for particle size [23]. These parameters are highly critical with respect to synthetic lipids having well-defined chain lengths and phase transition temperatures T_c (DML, DPL, DSL). If these parameters are not optimized, sonication tends to form predominantly fraction I. Egg lecithin and cardiolipin are least critical and form some fraction II under any sonication condition

boflavin and lumiflavin-3-acetic acid were prepared by K. Bluhme and H. Klenk in our laboratory. The purity of the flavins was tested by thin-layer chromatography (2-butanone/chloroform/methanol = 3:4:6), or by the determination of the extinction coefficient ($\varepsilon = 12{,}200\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$). L-$\beta$, γ dimyristoyl-α-lecithin (N42803), L-β, γ dipalmitoyl-α-lecithin (N42556), L-β, γ distearoyl-α-lecithin (N43698), puriss and sodium deoxycholate (Art 2728) were purchased from Fluka, Buchs, Neu-Ulm, phosphatidyl serine (4-4004), phosphatidyl inositol (4-6005), phosphatidyl glycerol (4-6013), cardiolipin (4-6011), phosphatidyl ethanolymine (4-6101) and spingomyeline (4-6009), were purchased from Supelco Chemical and were obtained in ampules each containing approximately 50 µmol of lipid solubilized in various organic solvents. Ethylenediaminetetraacetic acid (0.1 M Titriplex III, 8431 or 8418), potassium iodide (Art 5043), potassium chloride (Art 6404) and cholesterol (Art 24622) were obtained from Merck, Darmstadt. The experiments were generally performed in 0.01 M phosphate buffer at pH 8.0, containing 0.01 M NaCl. For the investigation of the ph dependencies of fluorescence and photoreduction, pH values where adjusted as follows (0.01 M): pH 1, HCl; pH 2/3, sulfate; pH 4/5, acetate; pH 6/7/8, phosphate; pH 9/10/11, borate; pH 12, phosphate; pH 13 NaOH/KCl. These suspension media are photochemically inert. The present experiments were performed under anaerobic conditions, which were achieved by extensive deaeration (bubbling) of the sample with argon [*cf.* 41].

Spectroscopy

Absorbancies were determined with a Cary 118/120 or a single beam spectrophotmeter [39]. Corrected fluorescence excitation and emission spectra, fluorescence difference spectra, and fluorescence polarization spectra were obtained with a JY 3 CI fluorimeter (Jobin Yvon) optionally equipped with polarizers and connected on-line with a Hewlett-Packard 9825 desktop calculator (for details *see* [37]). The temperature dependence of fluorescence was monitored continuously as described previously [33]. Kinetics of photoreduction were monitored using a self-made dual wavelength spectrophotometer and fluorometer [35], either in the dual wavelength mode with a 150 W xenon lamp (Oriel 6253), yielding blue light of 100 W m^{-2} through a 365 nm interference filter (UV, IL, Schott and Gen, Mainz), or in the fluorometer mode with a 100 W halogen lamp (100 W m^{-2}, broad blue light). Quantum efficiencies of photoreduction and fluorescence are given either in absolute numbers (for details *cf.* [35, 41]), or, for the purpose of comparison (*cf.* Figs. 7 and 8) and the determination of activation energies, in relative units where the greatest quantum efficiency of photoreduction or fluorescence was defined as 100%.

Vesicle Preparation

Vesicles were normally prepared based on the sonification procedure originally introduced by Huang [17]. Approximately 50 µmol of lipid or an entire whole ampule (containing about the same amount; *see Chemicals*) were dissolved in chloroform and/or methanol. The organic solvents were evaporated under vacuum at room temperature in a revolving 10 ml round-bottomed flask and AFl 3 was added at a lipid/flavin ratio of 100:1. Five ml of phosphate buffer (pH 8) were added to the dry lipid or lipid/amphiphilic-flavin film, and the suspension was sonicated in a thermostat-controlled water bath at specific temperatures for a defined period of time (*cf.* Fig. 1 legend) under argon. The clear suspension was charged onto a Sepharose 4B column and the elution monitored by extinction (i.e. essentially scattering) at 250 nm with a LKB-Uvicord (Figs. 1, 2, 4).

For our experiments with vesicle-associated proteins and hydrophilic flavins we chose a method for the preparation of single-shelled vesicles which is based on the solubilization of the lipid by DOC [14] and subsequent removal of the detergent [3]. A clear aqueous suspension, 5 ml containing 50 µmol of egg lecithin, 0.1 g DOC and LFl-3-aa at a saturating concentration was dialyzed for 24 hr at 4 °C against 0.01 M phosphate buffer at pH 7.2. The free LFl-3-aa was then separated from the vesicles by Sephadex G50 chromatography and subsequently eluted on a Sepharose 4B column (45 cm length, 2.5 cm in diameter) (Fig. 4, dashed line).

Unfortunately, however, this method was unsuitable for our present and projected experiments as MAO and cytochrome *c*, the proteins which we hoped to incorporate into vesicles, are irreversibly denatured by DOC. Mimms et al. [26] suggest in a recent paper that the nonionic detergent octyl glucoside, in contrast to the commonly used DOC, does *not* denature proteins. This statement is not valid for MAO and cytochrome *c*, as both proteins are irreversibly denatured.

Results and Discussion

Sonicated Lipids

"Sonication to clearness," which is often taken as the sole criterion for determination whether vesicles are, in fact, single-shelled [1, 4, 6–8, 9, 18, 19, 21, 22, 31], is by no means a sufficient assay for this classification. In recently published studies it was reported that the permeability to hydroxyl ions of "single-shelled vesicles," produced by sonication or by the "ether-injection method," was several orders of magnitude higher [27, 30] than that observed in black lipid membranes [11]. This discrepancy is probably related to an erroneous assumption of what constitutes "single-shelled-vesicles." Therefore, additional criteria are needed [*cf.* 32].

In addition, even if it is assumed that single-shelled vesicles exist, results obtained using these systems are tenuous. A living cell is at least 1 µm in diameter and contains, at pH 7, approximately 50 protons which represents a statistically defined pH [10]. Conversely, a vesicle-lumen with a diameter of 10 nm contains less than a calculated value of 10^{-3} protons! Therefore, based upon statistical considerations, experiments that utilize small vesicles to estimate proton or hydroxyl fluxes, as well as experiments using pH probes [16, 28, 30] remain questionable.

Huang [17] was the first to study in detail the formation and physical properties of phosphatidyl chloine vesicles prepared by sonication. He demonstrated by column chromatography using Sepharose 4B that a clear sonicated suspension does *not* present a population of homogeneously sized single-shelled vesicles, but instead represents two distinct fractions (I, II). Electron microscopy of this suspension showed that fraction I forms large multilamellar aggregates of lipid whereas fraction II contains single-shelled vesicles of approximately 250 Å diameter. We found, for most lipids investigated, a third, but smaller peak in the elution profile of a Sepharose 4B column which we designated "fraction III." This fraction occurs independently of the presence or absence of amphiphilic flavin bound to the lipid (Fig. 1). In contrast to the results of Huang, however, rechromatography of fraction II always resulted in the appearance of smaller amounts of fraction I, which increased upon aging and/or lowering of the temperature (Fig. 2). This is taken to indicate either an equilibrium between fractions I and II, or a kinetic instability of single-shelled vesicles suggesting that eventually all lipids will aggregate to form fraction I. Such a fusion of vesicles shows a strong dependence upon temperature, pH, or mitogenic agents which facilitate mitosis, as has been known some time [21, 33, 46]. When rechromatographed, fractions I and III are eluted identically as before. Using the formula developed by Marrink and Gruber [23], the elution of a Sepharose 4B column and particle size can be quantitatively correlated

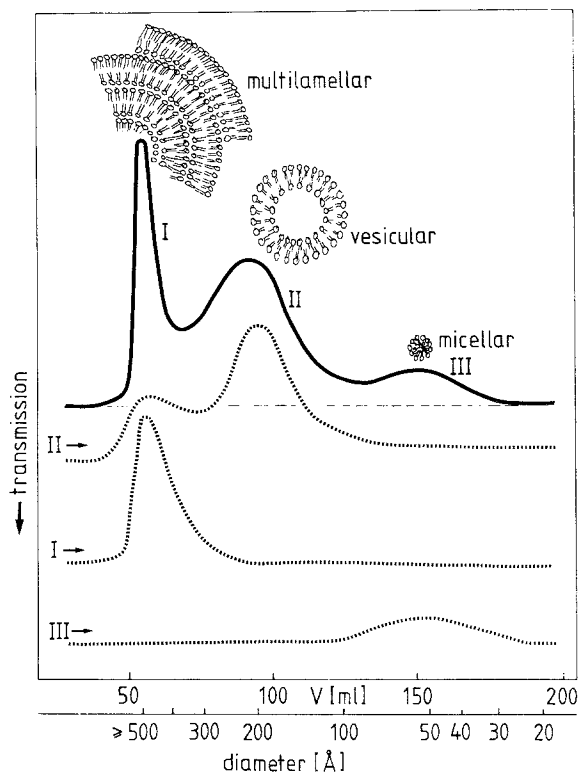

Fig. 2. Reproduction of the elution profile similar to the upper curve of Fig. 1. Immediately after completion of elution at room temperature, the distinct fractions I, II, III were rechromatographed. Part of fraction II reappears as fraction I. Sketches of the electron micrographs taken from the three fractions are included

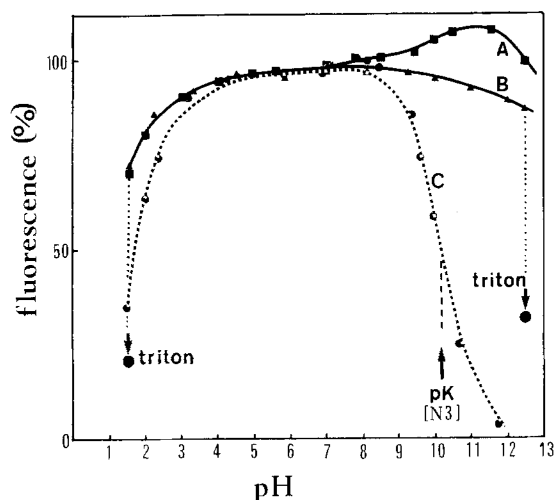

Fig. 3. *B.* Relative pH dependence of fluorescence of TARF (effective concentration 8.5×10^{-7} M) trapped in the lumen of egg-lecithin vesicles. *A.* Same experiment as in *B*, except that 30% cholesterol was incorporated in order to "stablize" the vesicle membrane. *C.* pH dependence of isotropic aqueous TARF. When the vesicles corresponding to curve *B* were treated with one drop of Triton X-100 per standard cuvette (approximately 1% final concentration), fluorescence decreased immediately as indicated. Measurements were performed at 21 °C

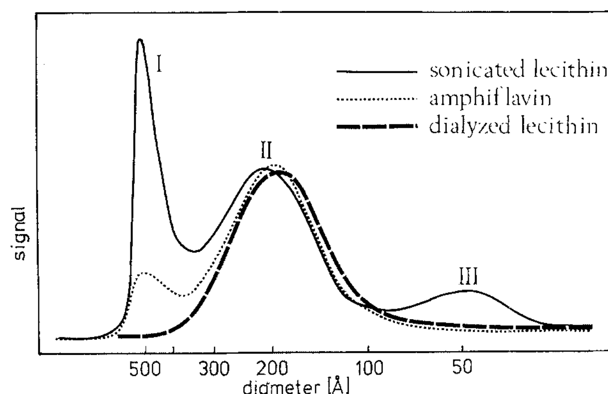

Fig. 4. *Solid line:* Similar elution profile to that shown in Fig. 1, upper curve, except that AFl 3 has been added prior to evaporation so that the binding properties of amphiflavin (AFl 3) to the various fractions could be monitored. *Dotted line:* Elution profile of lipid-bound AFl 3, as monitored by flavin fluorescence (excitation at 450, emission at 525 nm). The bulk of the flavin is incorporated in the vesicle fraction. *Dashed line:* Elution profile of egg-lecithin vesicles prepared by the deoxycholate method [3]. Fractions I and III are essentially absent

(Figs. 1 and 2). According to this, fraction III represents typical micelles (40 to 70 Å diameter [14]). This was verified in cooperation with Drs. Allmann and Plattner, Konstanz, by electron microscopy of sonicated egg lecithin (sketched in Fig. 2). Judged from the elution profiles, the same interpretation can be applied qualitatively for all phospholipids. However, quantitatively significant differences are exhibited (Fig. 1).

A further verification that fraction II represents closed single-shelled vesicles is given in the experiment shown in Fig. 3. The pK of the N3-proton of aqueous TARF was determined to be 10 (curve *C*). The fact that the anionic form shows little or no fluorescence permits its precise determination [15, 41]. Clearly, the encapsuled flavin remains fluorescent up to the point when the pH of the bulk solution equals 13. In addition, the fluorescence is much higher in the acidic range than under isotropic conditions. Application of Triton X-100 (1% final concentration), an efficient detergent for the disruption of membranes [14], immediately diminishes flavin fluorescence.

Some distinct physico-chemical properties of the three lipid fractions as eluted from the Sepharose 4B column (Fig. 4, solid line), can be examined by the specificity of binding of AFl 3 (dotted line). Small concentrations of AFl 3 are found in fractions I and III; however, the bulk flavin coelutes with fraction II. Moreover, if a hydrophilic flavin such as TARF or LFl-3-aa was added prior to sonication, the flavin was carried exclusively by fraction II (elution profile not shown). This finding suggests that the lumen of the lipid aggregates eluted in fraction I (and III) is much smaller than the lumen of single-shelled vesicles, in contrast to the observation of Kano and Fendler [18].

Fluorescence Properties of Vesicle-Associated Flavins

Figure 5*A* shows the fully corrected fluorescence excitation and emission spectra of amphilic flavin (AFl 3) bound to vesicles (fraction II) made from egg lecithin and phosphatidyl serine, respectively. Small, but significant, differences are exhibited in the difference spectrum (Fig. 5*B*, 1), whereas, similar comparisons between difference spectra of egg lecithin and cardiolipin (Fig. 5*B*, 2), phosphatidyl inositol (Fig. 5*B*, 3), and phosphatidyl ethanolamine (Fig. 5*B*, 4) reveal that all are very similar. Conversely, the difference spectrum between egg-lecithin and DPL vesicles is significantly different. As before, the degree of dissimilarity depends strongly upon the phase of the lipid (Fig. 5*B*, 5). For the purpose of comparison of the relative polarities of the flavin microenvironment in different vesicles, Fig. 5*B*, 6 shows the difference of fluorescence spectra of AFl 3 solubilized in benzene (dielectric constant $\epsilon = 4$) with that of ethanol

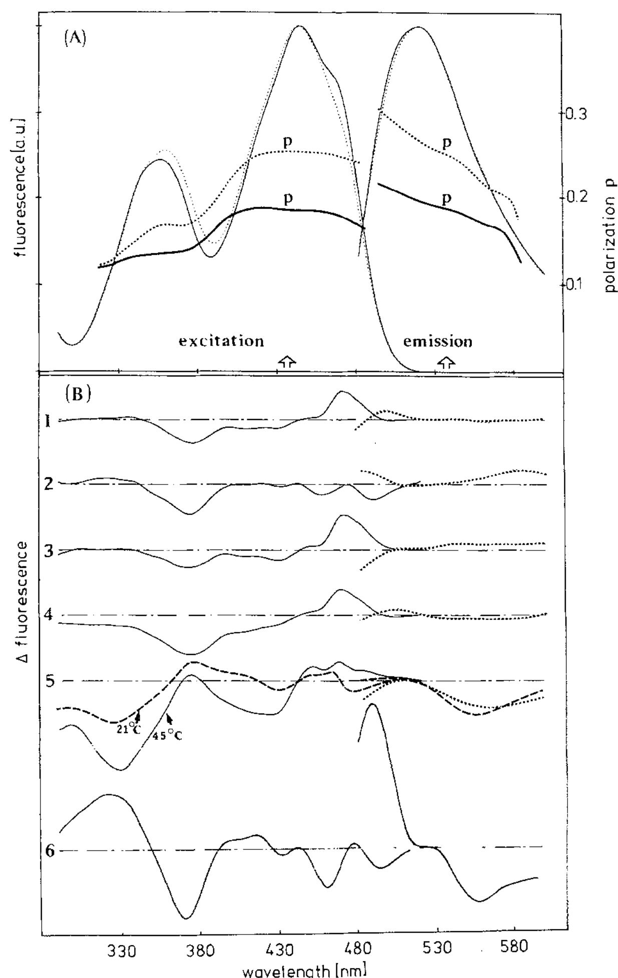

Fig. 5. *A*. Corrected fluorescence excitation and emission spectra, as well as continuous fluorescence polarization spectra of AFl 3 bound to vesicles prepared from phosphatidyl serine (dotted lines) and egg lecithin (solid lines). Wavelengths of excitation and emission are indicated by arrows. Measuring temperature was 21 °C. *B*. curve 1: Difference of the fluorescence spectra shown in (*A*), egg-lecithin minus phosphatidyl serine vesicles; curves 2–5: similar difference spectra, except that phosphatidyl serine was replaced by cardiolipin (2), phosphatidyl inositol (3) or phosphatidyl ethanolamine (4); curve 5 shows similar difference spectra of egg-lecithin and dipalmitoyl lecithin vesicles below (dashed line) and above (solid line) phase-transition temperature T_c; curve 6: difference spectra of *isotropic* AFL 3, in solvents of different polarities, i.e. benzene-ethanol at 21°C

($\epsilon = 23$). From this we conclude that, when compared to egg-lecithin vesicles, the polarity is higher in phosphatidyl serine, cardiolipin, phosphatidyl inositol and phosphatidyl ethanolamine, but lower in DPL vesicles.

Typical fluorescence polarization spectra are given for egg-lecithin and phosphatidyl serine vesicles (Fig. 5*A*). It can be seen that, while the spectra do not differ significantly in shape, there is considerable dissimilarity in height. This reflects a smaller

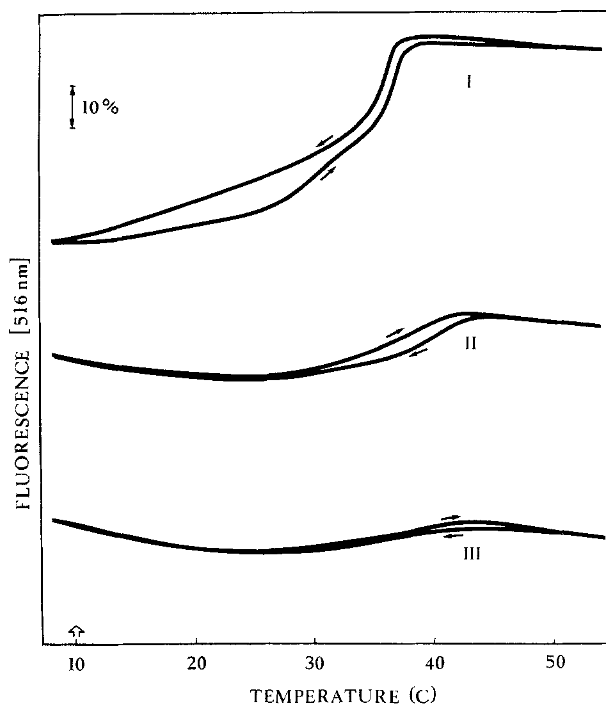

Fig. 6. Dependence of fluorescence of lipid-associated flavin on temperature (for technical detail *see* [33]). AFl 3 bound to fraction I (multilamellar layers), II (single-shelled vesicles) and III (micelles) prepared from egg lecithin exhibit marked differences. The absolute fluorescence signals are quite different (*cf.* Fig. 4) and were normalized at 10 °C to 100%. Measuring errors are ±0.5 °C and ±2% fluorescence. The hysteresis effects were reproducible

rotational relaxation time for the chromophore in egg-lecithin vesicles (20 nsec) than in phosphatidyl serine vesicles (30 nsec) (for details *cf.* [33]).

Figure 6 shows that, in contrast to the corresponding fluorescence *spectra* of fractions I, II and III of AFl 3/DPL, the temperature dependency of fluorescence depends strongly on the fraction used. Fraction I undergoes the strongest change in fluorescence quantum efficiency upon phase transition, whereas fractions II and III display smaller changes. Because the absolute fluorescence signals from the three fractions are very different (Fig. 4), the data from the three fractions were normalized to 100% at 10 °C for these measurements.

Quenching of Vesicle-Associated Flavin by Iodide

Iodide is known to be a highly efficient quencher of excited triplet states, but is ineffective as a quencher of excited singlet states which decay approximately 1000 times faster. This fact permits the use of iodide for the determination of the state which is predominantly involved in a specific pho-

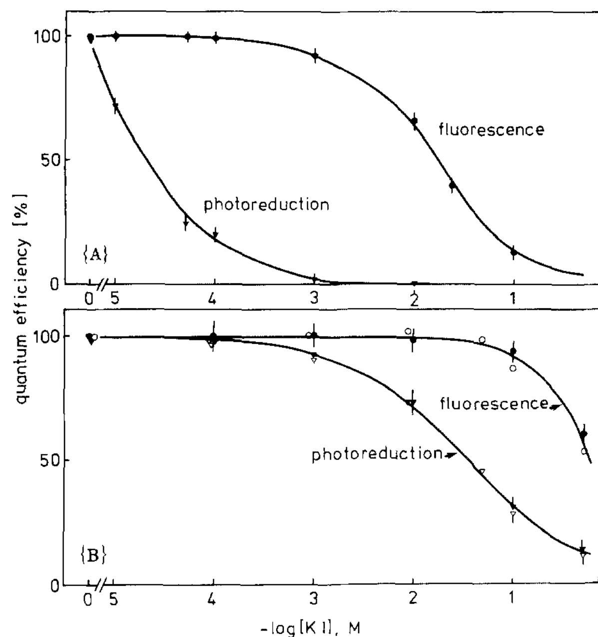

Fig. 7. *A*. Dependence of the quantum efficiencies of photoreduction and fluorescence of aqueous 3-me-LFl (4.4×10^{-6} M) on the concentration of iodide in the presence of EDTA (2.1×10^{-1} M) at 21° C. *B*. Dependence of the quantum efficiency of photoreduction and fluorescence of AFl 3 bound to single-shelled vesicles prepared from phosphatidyl serine (▾, •) or cardiolipin (▵, o) on the concentration of iodide at 21 °C. Photoreduction is exclusively mediated by the *intrinsic* donor. The effective flavin concentrations are 2.5×10^{-6} M for cardiolipin and 1.5×10^{-5} M for phosphadityl serine. All quantum efficiencies were normalized to 100% in the absence of iodide. Bars represent standard errors of the mean

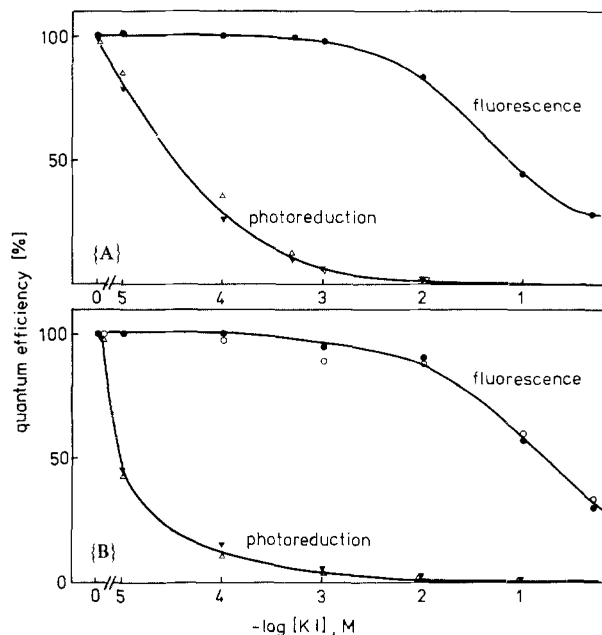

Fig. 8. *A*. Dependence of the quantum efficiencies of photoreduction (▾, ▵) and fluorescence (•) of LFl-3-aa trapped in the lumen of single-shelled vesicles, prepared from DPL, on the concentration of iodide. Prior to the experiment several temperature cycles were applied for proper stabilization of the system (*cf.* Fig. 10*B*). The photoreduction was performed with a 2.5×10^{-3} M concentration of exogeneous EDTA. *B*. Dependence of quantum efficiencies of photoreduction and fluorescence of AFl 3 (effective concentration 3.3×10^{-6} M) bound to DPL vesicles on the exogeneous iodide concentration, as a function of phase (▾, •: 21 °C, *gel;* ▵, o: 45 °C, *liquid crystalline*). The photoreduction in the gel state of the membrane was mediated by the intrinsic donor and in the liquid crystalline state by exogeneous EDTA (2×10^{-1} M). Note, photoreduction by both exogeneous EDTA and the intrinsic donor (*cf.* [41]) follow exactly the same iodide dependence, indicating that the availability of the electron donor is not rate limiting

toreaction. Figure 7*A* shows the inhibition of fluorescence and photoreduction of aqueous lumiflavin by iodide. The fact that the photoreduction is quenched about 1000 times more efficiently than the fluorescence indicates that the photoreduction is exclusively mediated by the *triplet* state. Conversely, the photoreduction of amphilic flavin bound to vesicles prepared from cardiolipin or phosphatidyl serine is only about 10 times more efficiently quenched than fluorescence (Fig.7 *B*). These results suggest that, in this particular case, the photoreduction process is largely mediated by the first excited *singlet* state. This observation is a rare but well-known phenomenon in photobiology and photochemistry [16, 20, 29, 44].

In a previous paper we demonstrated that vesicle-bound amphiphilic flavin could be reduced by an *intrinsic* donor in the *gel* state of the membrane as well as by *exogeneous* EDTA in the *liquid crystalline* state [41]. Surprisingly, both photoreduction mechanisms for the DPL/AFl 3 system, although obviously different, display precisely the same pattern of quenching by iodide (Fig. 8*B*). The differences in the mechanisms of photoreduction by the intrinsic donor between natural lipids (Fig. 7*B*) and synthetic lipids (Fig. 8*B*) introduce further complications which are as yet not resolved. Moreover, photoreduction is 10 times more efficiently quenched than fluorescence in the DPL/AFl 3-system when compared to the isotropic system (Figs. 8*B* and 7*A*). Thus, it appears that the singlet and triplet states are affected differently by the membrane.

Figure 8*A* compares the quenching efficiency of iodide on fluorescence and photoreduction (*see below*) of LFl-3-aa trapped in the lumen of DPL vesicles. The quenching rate constant for photoreduction (Fig. 8*A*, ▵, ▾) is comparable to that obtained for isotropic flavin (Fig. 7*A*, ▾). However, the fluorescence curve is shifted so that the half-maximal quenching rate constant is found at an iodide concentration that is 4 times higher

(Fig. 8*A*, •; Fig. 7*A*, •). This selective shift of the curves describing the photoreduction and fluorescence dependencies indicates a finite permeability of the membrane to iodide. Thus, permeation is the rate-limiting step for the quenching process of the flavin singlet, but not of the flavin triplet state.

Redox Properties of Flavin Bound to the Vesicle Membrane

In a previous paper we investigated the photoreduction of flavins bound to vesicles prepared from synthetic lipids with well-defined phase-transition temperatures [41]. Below phase-transition temperature the flavin is photoreduced by some *intrinsic* electron donor which most probably affects the secondary hydrogen of the glycerol backbone. Above phase-transition temperature only *exogeneous* electron donors like EDTA were found to be effective. Generally, it is assumed that all natural lipids are in the *liquid crystalline* (i.e. fluid) state at physiological or room temperature. Nevertheless, all lipids investigated proved to be good electron donors at this temperature when present in the vesicle form. In contrast, additional potential exogeneous electron donors like EDTA always tended to quench the rate of photoreduction at any concentration.

We have previously reported half-life values [41] for autoxidation rates of dihydro-amphiflavin bound to DPL vesicles of 0.5 sec in the *gel* state, and 0.05 sec in the *liquid crystalline* state. Based on our recent findings, it is neccessary for us to extend and comment upon our previous results. As shown in the stopped-flow experiments conducted above phase-transition temperature with EDTA, autoxidation occurs to the full extent only if the flavin had first been photoreduced by *exogeneous* donors. To our surprise, if the flavin was photoreduced by the *intrinsic* donor, as occurs when it is attached to vesicles made from natural phospholipids or those made from synthetic lipids and photoreduced below T_c, the reduction is essentially irreversible and stable for hours under aerobic conditions. No clue regarding the two essentially different photoredox mechanisms could be obtained from analysis of the absorption spectra. That is, they cannot be distinguished even if their sensitivities to iodide quenching are different (Figs. 7*B* and 8*B*). We suggest that the *irreversible* photoreduction represents some kind of *photoaddition* of the type that is observed in flavin photochemistry [15].

Figure 9 compares the extent of cooperativity of flavin-photoreduction by EDTA under isotropic

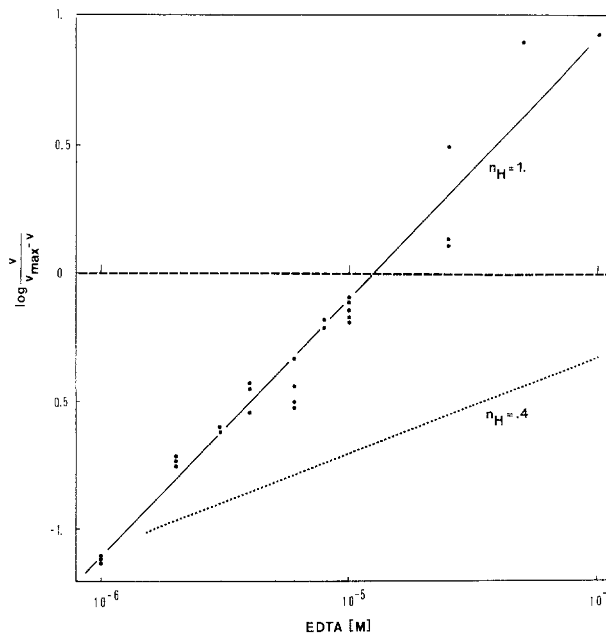

Fig. 9. Hill plot of the initial slopes of the photoreduction of aqueous 3-me-LFl (3.6×10^{-6} M, solid line, "isotropic"), and AFl 3 bound to DPL vesicles (dotted line, data taken from [41]) as function of the exogeneous EDTA concentration. The isotropic condition reveals no cooperativity ($n_H = 1$); however, vesicle-bound flavin is photoreduced by EDTA reflecting a *negative* cooperativity ($n_H = 0.4$)

(3-me-LFl) and anisotropic (DPL/AFl 3) conditions (Hill plot). Under *isotropic* conditions a Hill coefficient of $n_H = 1.0$ is obtained which represents a thermodynamically trivial situation commonly observed in "isotropic chemistry." However, under *anisotropic* conditions, where a Hill coefficient as low as 0.4 can be obtained, *negative* cooperativity is suggested.

Additional data can be derived from the determination of the activation energies of fluorescence and photoreduction under *isotropic* and *anisotropic* conditions (Table). For these determinations the term "activation energy" has to be interpreted operationally because of negative values (*cf.* [45]) that were occasionally obtained from the Arrhenius plots (Table). In strict contrast to the temperature dependency of *photoreduction,* which is 10 times higher under anisotropic than under isotropic conditions, the temperature dependency of fluorescence is quite similar in both systems. This, in turn, appears to be consistent with the finding that the photoreduction under *isotropic* conditions proceeds from the first excited triplet, whereas under anisotropic conditions, it originates largely from the first excited singlet state of flavin. This is true, however, only if natural lipids are used to prepare vesicles (Fig. 7).

Table. Comparison of the activation energies for flavin under isotropic/anisotropic conditions and for flavin trapped in the lumen of single-shelled vesicles prepared from DPL

System	E_A, activation energy (kJ × M^{-1}) Reaction	
	Photoreduction	Fluorescence
isotropic (3-me-LFl)	3.85 (EDTA), ref. [38]	−4.0, from ref. [33]
anisotropic (AFl 3/cardiolipin)	40.0 (intrinsic donor)	−4.4
LFl-3-aa, trapped in the lumen of DPL vesicles	complex, nonlinear Arrhenius plots (not shown)	−1.5 (aged) −2.9 (fresh) (*cf.* Fig. 10)

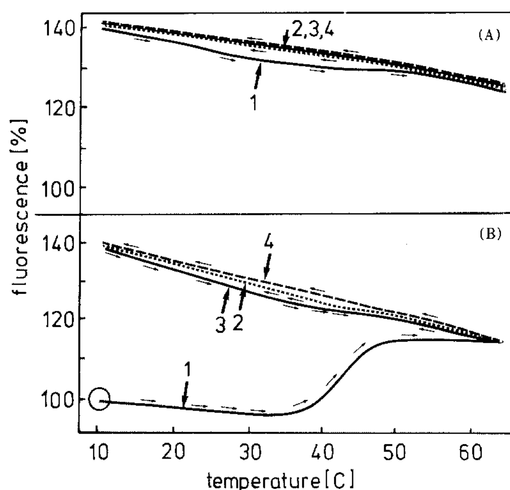

Fig. 10. *A*. Temperature dependence of fluorescence of LF-3-aa, trapped in the lumen of single-shelled vesicles prepared from DPL, as a function of several subsequent temperature cycles as indicated. Prior to the experiment, the vesicles were stored for three days at room temperature in the dark. *B*. Same experiment as in (*A*), except that the vesicles were used immediately after elution from the Sepharose 4B column at room temperature

Fluorescence Properties of Flavin Trapped in the Vesicle Lumen

As described in Materials and Methods, we trapped the highly hydrophilic flavins LFl-3-aa and TARF in the lumen of single-shelled vesicles. In order to further verify that the flavin is really solubilized and freely moving, we monitored the temperature-dependence of fluorescence. In the case of membrane-associated flavin, we would expect a marked fluorescence change at T_c. Indeed, with freshly prepared vesicles we observed a fluorescence increase at T_c, but only upon the very first phase transition (Fig. 10*B*). Conversely, all subsequent temperature runs, whether done with decreasing or increasing temperature, failed to reveal any further changes at T_c. Surprisingly, storage of vesicles that never had been exposed to temperatures above T_c, for several days at room temperature in the dark produced the same effect as a short increase of temperature above T_c (Fig. 10*A*).

When taken at room temperature, fluorescence polarization spectra of fresh or aged vesicles revealed additional clues regarding the interpretation of this phenomenon. The polarization value of 0.08 at 450 nm decreased upon the *first* phase transition to a constant value of 0.03. This is taken to indicate that, regardless of whether the sonication- or the deoxycholate method was used, minute amounts of hydrophilic flavin was incorporated in the membrane by the preparation procedure. Upon the first phase transition or simply by aging below T_c, these flavins are irreversibly extruded. This is consistent with our previous finding that the permeability of the membrane/water interface is strongly dependent on the phase of the membrane [33, 41]. It is also consistent with our previous results that suggest that the *absolute* quantum efficiency of flavin fluorescence is generally higher in water than in the membrane [33]. However, it is interesting that the fluorescence polarization of flavin "freely" moving in the lumen of the vesicles is small, but definitely different from zero. This corresponds to a rotational relaxation time larger than that of 0.38 nsec in aqueous solution [33]. A similar effect was observed recently for vesicle-trapped pyrine [5]. We are just completing work on the photoreduction of vesicle-trapped cytochrome *c* via membrane-bound flavins by exogeneous EDTA. This is an ideal model system for studying the transport of redox equivalents across natural membranes by plasto- and ubiquinones on the basis of well-known isotropic reactions [40].

Ph Dependency of Photoreduction

The dependence of flavin redox reactions under *isotropic* conditions has been extensively studied by Haas [12] and Haas and Hemmerich [13] in this laboratory. Figure 11 shows the pH dependence of photoreduction of aqueous 3-me-LFl by EDTA. This result corresponds to that obtained by Haas and Hemmerich [13] as measured in water/methanol (vol/vol = 1:1). The pK's of EDTA are marked by arrows indicating that they are probably responsible for the observed pH dependence. As previously observed [13], the substrate pK gives rise to an *increase* in the photore-

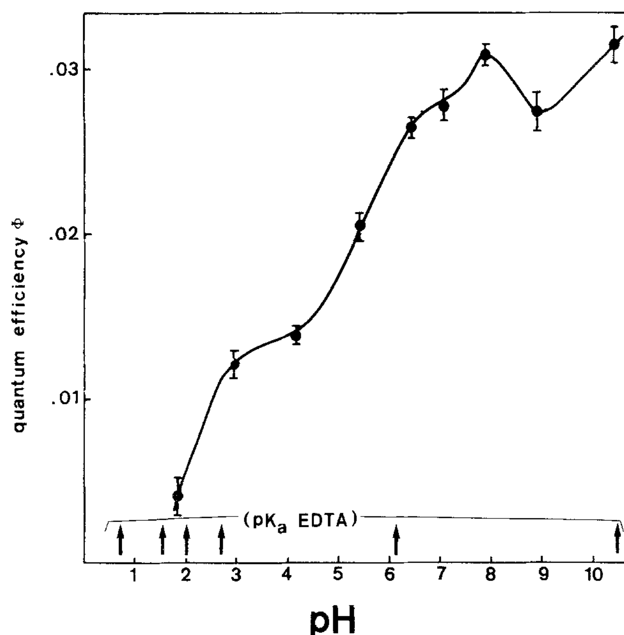

Fig. 11. Dependence of quantum efficiency of photoreduction of aqueous lumiflavin (9.10×10^{-6} M) by EDTA (23×10^{-4} M) on pH. The pK's of EDTA are marked by arrows. Values of pH were adjusted as described in Materials and Methods

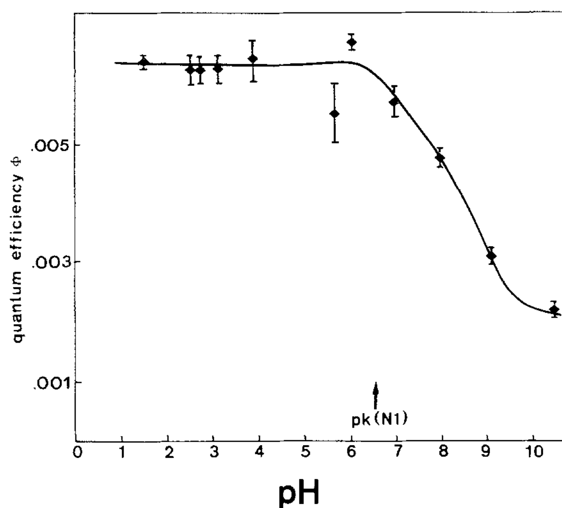

Fig. 12. Analogous to Fig. 11. Quantum efficiency of photoreduction of AFl 3 bound to vesicles prepared from egg lecithin. Measuring temperature 21 °C. The pK of the N1-hydrogen of dihydroflavin determined under *isotropic* conditions is marked by the arrow

duction rate with increasing pH, whereas a *flavin-photo-pK* leads to a *decrease*. In the present case, however, the latter is probably overcompensated for by the greater effect of the corresponding pK's of EDTA.

In contrast to the *isotropic* system, we observed a basically different pattern for the pH dependence of the *anisotropic* system (AFl 3/egg-lecithin vesicles; Fig. 12). The efficiency of photoreduction is constant between pH 1 to 6, but decreases in a sigmoidal pattern above pH = 6. This probably reflects a flavin-photo-pK close to 8 [12]. The only known pK of flavin in the ground state that is close to this is 8.4 for the blue radical FlH˙ [2] and, therefore, its involvement cannot be excluded. On the other hand, our previous work [34, 41] demonstrates that pK's known from *isotropic* flavin chemistry are usually increased by several units upon membrane binding. On this basis, the pK of the N1-hydrogen of the flavin of 6.3 as indicated by the arrow in Fig. 12 could well be shifted towards 8 by the specific membranous microenvironment. A survey including all relevant pK values of flavin has been given in the review by Bruice [2].

Schreiner et al. [43] and Schreiner and Kramer [42] demonstrated that the pK of $^3(HFl_{ox}^+)^*$ is as high as 5, compared to zero in the ground state. Based on a well-known relation introduced by Brønstad, the dependence of photoreduction on the ionic strength observed for the *isotropic* system reflects the involvement of a charged species in the rate-limiting step. This, however, is not the case for the *anisotropic* system, since the reaction is completely independent of a high ionic concentration present (curves are not shown). In conclusion, our present data are not sufficient to unequivocally attribute a particular pK to the observed pH dependence under *anisotropic* conditions.

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