Biochimica et Biophysica Acta, 367 (1974) 326–337

BBA 76767

STUDIES ON THE INCORPORATION OF FLUORESCENT PIGMENTS INTO BILAYER MEMBRANES

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(Received May 20th, 1974)

SUMMARY

1. A technique has been developed which is appropriate for quantitative fluorescence analysis of pigments in artificial lipid-bilayer membranes. The technique is based upon taking a monochromatic picture of the point where a parallel light beam crosses the membrane. The density of the negative can be used as a standard for the fluorescence intensity. A calibration curve is obtained by substituting the membrane with an optical microcell filled with solutions of the examined pigment. In a modified version the photographic film is replaced by a sensitive photodiode. Thus a continuous observation is possible. The absolute sensitivity of the technique is given.

2. The incorporation of rubrene, magnesium octaethylporphyrin and of three amphiphilic flavins, substituted with long hydrocarbon chains into bilayer membranes has been investigated. If identical concentrations of these pigments are added to the membrane-forming solution, vast differences in the membrane concentrations are found. The amount of incorporated pigment also depends on the kind of lipid as well as the lipid preparation. The experiments with the amphiphilic flavins indicate that the hydrophilic flavin nucleus is situated in the membrane/water interface, whereas the hydrophobic hydrocarbon chain sticks into the lipophilic interior of the bilayer.

INTRODUCTION

Artificial bimolecular lipid films play an important role as models of biological membranes. Their relatively simple structure allows insights into molecular processes which are relevant to the much more complicated natural membranes. By the adsorption and incorporation of various substances into artificial lecithin bilayers, parts of the biological systems can be imitated and insight into the intrinsic properties of membranes can be obtained [1–11].

If membranes are formed according to Müller and Rudin (sometimes called the brush technique) [12] the constituents of the membrane have to be dissolved in alkane. This solution is brushed into a hole of a Teflon frame and the bimolecular state develops spontaneously. In general the composition of the membrane-forming solution and the composition of the membrane itself will be different. Some previous

results suggest a separation of different molecules during this process [14, 6]. In this paper quantitative results are reported concerning such separation processes. Recently Müller and Montal reported a new technique for forming bilayer membranes which is less subject to separation and composition problems [15]. Bilayers are formed by connecting two monolayer films. These membranes are free of alkane solvent and the membrane-forming process is likely to exclude separation of the admixed molecules [16, 17].

In order to investigate the composition, structure and fluidity of natural and artificial membranes, fluorescence label probes are incorporated [18–26]. By employing appropriate compounds, changes in spectra, in degree of polarisation [21, 22] and in quantum yield give information on the location and mobility of the probe [23], on relaxation processes [24] and phase transitions [25, 26] (for review see ref. 18). In the present paper a new class of fluorescent molecules is used for incorporation: flavin molecules are rendered lipid-soluble by substitution with long hydrocarbon chains.

Two aspects led to the synthesis of the amphiphilic flavins and to their incorporation into bilayer membranes. Firstly, the spectral property of the flavin nucleus shows a distinct fluorescence at about 520 nm which is sensitive to the polarity of the solvent and thus, these compounds are suitable as fluorescence labels for membrane studies. The second aspect is to investigate anisotropic chemical reactions of membrane-bound flavins either in plane bilayer membranes or in liposome preparations. Such systems, in which the flavin nucleus is embedded in lecithins, could serve as models for enzymatic reactions because two essential reaction conditions are imitated: anisotropy and sterical selection.

EXPERIMENTAL

Measuring method

The experimental set-up of the technique employed is shown in Fig. 1. The parallel light beam of a CW-Ar-laser passes some neutral density filters to reduce the light intensity and enters a black box made of plywood. Then the beam crosses a quadrangular glass cuvette with a plane Teflon septum for the membranes placed

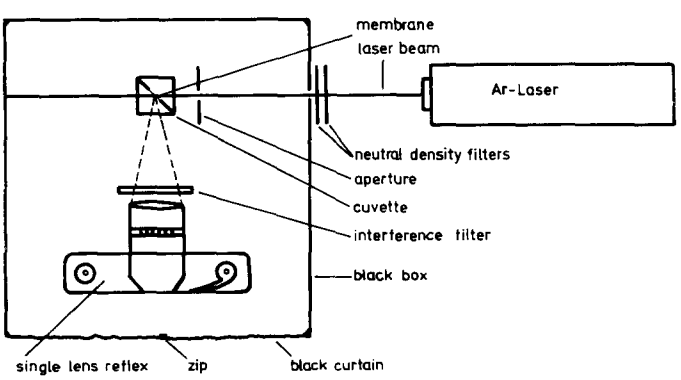


Fig. 1. Experimental arrangement for fluorescence photography. Different apparatus components are represented in different scales.

at an angle of 45° to the incident beam. Perpendicular to the beam a single lens reflex camera is mounted with a narrow-band interference filter (Balzers, Filtraflex B40) in front of the lens.

The laser, model 252 CW Argon with a mirror for wavelength selection, was manufactured by Cryophysics. In all experiments a single line of 457.9 nm was used. The light was plain polarized. In most of the reported experiments, the plane of the *E*-vector was oriented perpendicular to the plane which results from the laser beam and the direction of observation. The output power of the laser line is 0.05 W and the beam diameter is 1.7 mm. The camera was a Zeiss Icarex 35 S with a Tessar 2.8/50-mm lens. The lens was focussed at the nearest possible distance. In addition, an extension ring and an ancillary lens were used to give a picture scale of about 1 : 1. The film was an Ilford FP 135 black and white panchromatic safety film with a sensitivity of 125 ASA. Its sensitivity is almost independent of the wavelength in the region between 450 and 650 nm.

In order to analyse the exposed and developed pictures, they were framed and projected. The differences between the exposed and the unexposed parts of the film were measured with a photodiode.

In a modified technique the film is replaced by a blackened metal plate with a bore in its centre. In this hole a sensitive Silicon photodiode (Motorola MRD 500) is mounted. On illumination the diode generates a photovoltage which is measured with a Keithley electrometer model 610 B. With an input impedance of 10^{14} Ω there is a maximal sensitivity correlated to a response time of about 30 s. Normally an input resistor of 10^{10} Ω was chosen, which gives a 10-fold smaller sensitivity but a response time of 3 s. The photovoltage of the diode was recorded on a strip chart recorder.

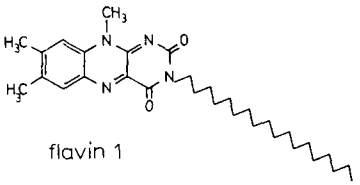
The membranes were formed in the usual way by brushing the membrane-forming solution into a circular hole (8 mm in diameter) in a Teflon frame submerged in an aqueous KCl solution 10^{-1} M. The bimolecular state of the membrane is reached within 30–45 min. The "blackness" of the films was observed independently through a telescope. From this observation small lenses of bulk liquid in the bilayer are estimated to have diameters less than 0.01 mm. The composition of the membrane-forming solutions was always 5 mM phosphoglycerides in *n*-decane plus various amounts of pigments. Into this solution 10 % (v/v) *n*-butanol is added in order to increase the membrane stability. The quoted pigment concentrations are related to decane.

Liposomes were prepared according to Batzri and Korn [27]. A solution of 30 mM lecithin in ethanol, containing various amounts of pigments, is rapidly injected into an aqueous solution of 10^{-1} M KCl to give a final ethanol concentration of 2.5 % in water.

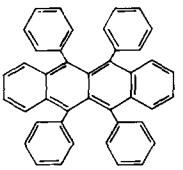
Materials

Dioleoyllecithin and phosphatidylserine 1 were obtained from Supelco and were used without further purification. Phosphatidylserine 2 was purchased from Koch-Light Laboratories and purified by column chromatography. Inositol was obtained from General Biochem and purified by column chromatography. The different preparations of dierucoyllecithin 1 and 2 were synthesized by K. Janko in the laboratory of Professor P. Läuger. Both preparations have been shown to be identical

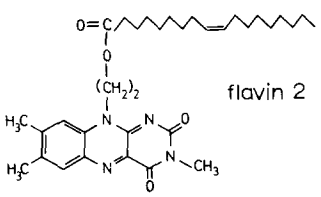
by NMR and thin-layer chromatography. K. Janko also prepared egg-lecithin and egg-serine according to Singleton [28]. All phosphoglycerides, except those from Supelco, gave a single spot on the thin-layer chromatogram.



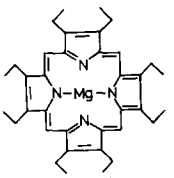
flavin 1



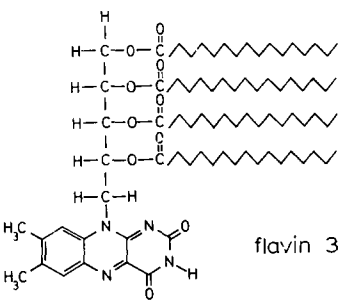
rubrene



flavin 2



Magnesium octaethylporphyrin



flavin 3

3-octadecyl-lumiflavin (flavin 1) and 3,7,8-trimethyl-10-[2-(octadec-(9c)-enoyl-oxy)-ethyl]-isoalloxazine (flavin 2) were synthesized by Dr W. Knappe. Both flavins were finally purified by column chromatography on neutral aluminia and gave the correct elemental analysis for C, H and N. Flavin 2 turns out to be slightly soluble in water whereas flavin 1 is completely insoluble. Riboflavin tetrapalmitate (Flavin 3) was synthesized according to Yagi [29]. The substance was chromatographically purified three times.

5,6,11,12-tetraphenylnaphtacen (rubrene) was purchased from the Aldrich Chemical Company. It is insoluble in water. The maximum fluorescence lies at 550 nm and is hardly influenced by changing the polarity of the solvent.

Magnesium octaethylporphyrin was a gift from Dr Fuhrhop. In diluted solutions the maximum fluorescence is at a wavelength of 584 nm which shifts about 2 nm when solvents of different polarity are used. The fluorescence intensity varies within 25 % on changing the solvent. Magnesium octaethylporphyrin is also water insoluble.

Calibration

Calibration was carried out with a flat optical microcell of 0.01-cm thickness filled with solutions of the compounds being investigated. The microcell was adjusted

in the same manner as the membrane. The mirror of the reflex camera allows a control so that no disturbing light reflexes are seen by the photodiode. By multiplying the concentration of the calibration solutions c_c by the thickness of the microcell d an area concentration c_a is obtained:

$$c_a = c_c \cdot d \tag{1}$$

Fig. 2a shows a calibration curve of 3-methyl-lumiflavin in H_2O resulting from the photographic method. A density quantity S is defined as the logarithm of the ratio of the light intensities at the exposed I_s and an unexposed spot I_0:

$$S = \log \frac{I_s}{I_0} \tag{2}$$

The curve shows linear behaviour at low concentrations and saturation of density towards high area concentrations. This relative decrease is not due to fluorescence quenching but is a property of the photographic film. The observed characteristic is well known from film photometry.

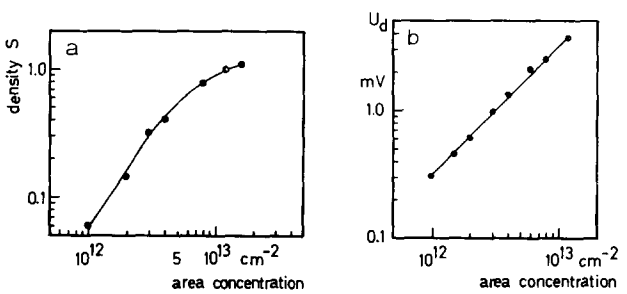


Fig. 2. (a) Photographic film density S as a function of lumiflavin concentration in a flat microcell at a wavelength of 520 nm. By multiplying the volume concentration of the calibration solution with the thickness of the glass the given area concentration is found. (b) Photovoltage of a photodiode which replaces the photographic film in the same dependence on lumiflavin concentration.

The calibration curve obtained with the photodiode is quite different (Fig. 2b). In the most interesting part of the flavin area concentration from 10^{12} to 10^{13} molecules/cm^2 the figure shows a linear characteristic in photovoltage. The noise of the photovoltage is about 10 %. It is due to instabilities of the laser. The photovoltage is a linear function of area concentration as well as of the light intensity under all conditions used. The calibration solutions were chosen so that no fluorescence quenching occurred.

The sensitivity of both detecting systems is nearly the same when the photodiode is shunted with 10^{10} Ω. The absolute sensitivity at 514 nm was about 10^5 quanta/cm^2 per s entering the front lens. A 10-fold higher sensitivity is reached when the diode works on open circuit.

RESULTS

Fig. 3a shows a photograph taken from a lecithin bilayer membrane containing 0.2 mM flavin 1 in the membrane-forming solution. The black crossing point of the

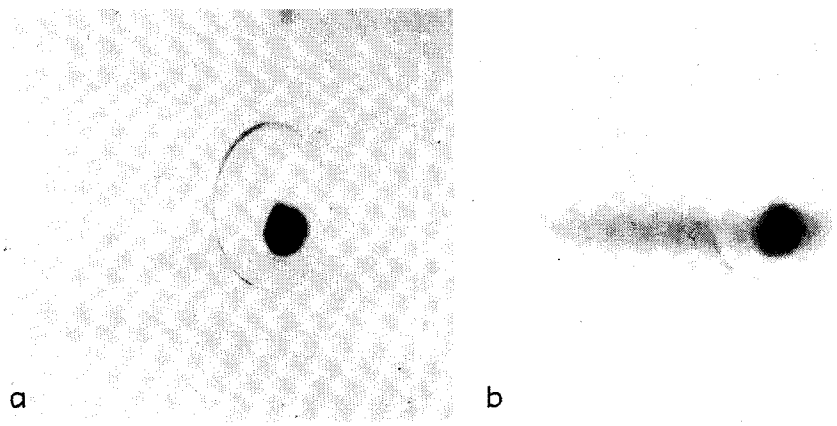


Fig. 3. (a) Picture of a dierucoyllecithin 1 membrane containing 0.2 mM flavin 1 in the membrane-forming solution taken at a wavelength of 520 nm. The exposure time was 10 s and the light intensity was reduced to 5%. (b) Picture of a dierucoyllecithin 1 membrane in an aqueous flavin 2 solution ($9 \cdot 10^{-8}$ M) taken at a wavelength of 520 nm. The exposure time was 10 s and the light intensity was reduced to 5 %.

laser beam with the membrane is surrounded by the oval torus which is irradiated by stray light. It can be seen that distinct separation of the membrane signal from the disturbing illumination of the torus is achieved. By using the calibration curve (Fig. 2a) the film density yields an area concentration of flavin 1 in the membrane of $9 \cdot 10^{12}$ molecules/cm^2. Fig. 3b shows a membrane with flavin 2 which had been added to the aqueous phase ($9 \cdot 10^{-8}$ M). In contrast to Fig. 3a, fluorescence of the aqueous phase along the laser beam is observed indicating the water-dissolved pigment. The membrane-bound flavin 2 causes the higher film density in the membrane centre. From the difference of these densities the area concentration of flavin 2 in the membrane is calculated as 10^{13} molecules/cm^2. The same results are found when the photodiode is used as the detecting system. Because of its easier operation and higher accuracy the diode was used throughout the further experiments.

The fluorescence time courses are similar to those reported in the literature [6, 30]. Immediately after membrane formation the fluorescence signal is high because of the relative thickness of the membrane. When the bimolecular region crosses the light beam the signal decreases to a steady-state value which shows a noise of about 20 %. When the membrane is destroyed mechanically the signal drops to zero. Presumably the high noise is caused by macroscopic wobbling of the membrane where parts of the illuminated membrane move in and out of the measuring volume. Small lenses of bulk liquid can be excluded as responsible for membrane noise because their diameter is less than 1/100 of the illuminated part of the membrane.

Using different narrow-band interference filters, the fluorescence spectra of membrane-bound compounds can be obtained. Membranes containing flavin 1 or flavin 2 show fluorescence spectra with a maximum at 520 nm indicating a polar micro-environment of the flavin nucleous in the bilayer membrane.

The incorporation of the compounds flavin 1, flavin 2, flavin 3 and magnesium octaethylporphyrin is shown in Fig. 4. Up to an apparent membrane concentration of about 10^{13} molecules/cm^2, the incorporation is nearly a linear function of the con-

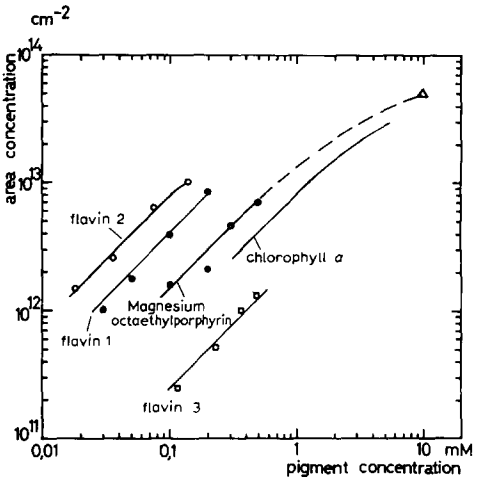


Fig. 4. Area concentration c_m of different fluorescent probes in bilayer membranes formed from dierucoyllecithin 1 as a function of their concentration in the membrane-forming solution. The data points are fitted by straight lines drawn at an angle of 45°. The chlorophyll *a* curve has been reproduced from the literature [6]. The flavin curves were measured using a 520-nm narrow-band interference filter, whereas the magnesium octaethylporphyrin curve was measured using a broad band interference filter (Balzers K-5).

centration in the membrane-forming solution c_s. The linear dependence of flavin 1 fluorescence from the concentration c_s (up to $c_s = 0.2$ mM) has already been found in preliminary experiments by W. G. Pohl (unpublished) using a fluorescence apparatus described earlier [31]. At higher concentrations fluorescence quenching begins and the employed method of area-concentration determination fails. The straight lines of Fig. 4 are drawn at an angle of 45°. With flavin 3 the area concentration cannot exceed $1.3 \cdot 10^{12}$ molecules/cm^2 because of the inhibition of membrane formation. Already 0.3 mM flavin 3 in the membrane-forming solution doubles the time required to reach the bimolecular state.

Flavin 2 is slightly soluble in water. When dissolved in the membrane-forming solution only, diffusion into the surrounding water phase is observed until equilibrium is reached within one to two hours. A similar result has been obtained in preliminary experiments by W. G. Pohl (unpublished). If on the other hand flavin 2 is dissolved in the aqueous phase only ($5 \cdot 10^{-8}$ M) equilibrium is reached as soon as the bilayer is formed. An area concentration of $6 \cdot 10^{12}$ molecules/cm^2 was then calculated for this experiment.

Polarization of the fluorescence light was observed with flavin 1 as well as with flavin 2. Incorporation of the amphiphilic flavins does not influence the electrical membrane resistance.

Rubrene in the membrane-forming solution yields a high-fluorescence signal which drops to zero when the membrane becomes bimolecular, i.e. practically no incorporation can be seen. An upper limit of area concentration of $2 \cdot 10^{11}$ molecules/cm^2 of rubrene can be given at a concentration of 0.5 mM in the membrane-forming solution. The rate of membrane formation is not influenced by rubrene.

A linear relationship between the magnesium octaethylporphyrin concentra-

tion of the membrane-forming solution and the area concentration of the membrane is observed up to $6 \cdot 10^{12}$ molecules/cm^2. At higher concentrations fluorescence quenching takes place. This is indicated by the smooth line. Because of the high molar extinction coefficient of magnesium octaethylporphyrin ($\varepsilon = 4.8 \cdot 10^5$ at 410 nm) an absorption spectrum of one single bilayer could be measured with a sensitive spectrophotometer (Aminco DW-2). 11 mM porphyrin in the membrane-forming solution leads to an area concentration of $5 \cdot 10^{13}$ molecules/cm^2. This is indicated by the open triangle in Fig. 4. It should be noted that with membranes containing magnesium octaethylporphyrin, large deviations of measuring points ($\pm 30\,\%$) are found even within the same membrane or in membranes containing equal amounts of pigment.

For comparison, the incorporation of chlorophyll *a* into bilayer membranes of dioleoyllecithin, measured by absorption [6] is included in Fig. 4.

From the linear portions of the curves an enrichment factor A for the individual compounds can be evaluated:

$$A = \frac{c_m/d}{c_s} \tag{3}$$

where d is the membrane thickness, c_m the area concentration in the bilayer and c_s the pigment concentration in the membrane-forming solution. A is a function of the pigment as well as the lecithin. All results of Fig. 4 were obtained with dierucoyllecithin 1 (except for chlorophyll *a*). Differences in A, therefore, reflect differences in the incorporation of the pigments. The membrane thickness d is chosen to be 70 Å. The enrichment factors for the different pigments are listed in Table I.

TABLE I

ENRICHMENT FACTORS A OF SOME FLUORESCENT PROBES

The value A for dioleoyllecithin was calculated by using an area concentration of $3.2 \cdot 10^{14}$ molecules/cm^2.

Pigment	Enrichment factor A	Lecithin
Flavin 1	98	Dierucoyllecithin 1
Flavin 2	190	Dierucoyllecithin 1
Flavin 3	6	Dierucoyllecithin 1
Rubrene	<1	Dierucoyllecithin 1
Chlorophyll *a*	19	Dioleoyllecithin
Dioleoyllecithin	≈ 152	—

Flavin 1 was used to study the influence of the lipid on the incorporations. Table II summarizes the enrichment factors of flavin 1 in different phosphoglycerides, calculated in the same way as those of Table I. The factors vary from 29 in phosphatidyl serine 2 to 105 in dioleoyllecithin. Even different preparations of identical phosphoglycerides differ considerably from each other. Only identical lecithin preparations yield equal enrichment factors.

TABLE II

ENRICHMENT FACTORS A OF FLAVIN 1 IN DIFFERENT PHOSPHOGLYCERIDES

Dierucoyllecithin 1 and dierucoyllecithin 2 are different preparations. Phosphatidylserine 1 was obtained from Supelco and phosphatidylserine 2 from Koch-Light Laboratories (see Materials).

Phosphoglyceride	Enrichment factor *A*
Dioleoyllecithin	105
Dierucoyllecithin 1	98
Dierucoyllecithin 2	43
Egg lecithin	43
Phosphatidylserine 1	88
Phosphatidylserine 2	29
Egg serine	58
Phosphatidyl inositol	38

DISCUSSION

Fluorescent pigment molecules in lecithin bilayer membranes are estimated quantitatively and characterized by two photographic techniques. In both techniques the membrane is focussed by a single lens reflex system. The photographic objective leads to a high resolutions which allows distinction of the membrane, the torus and laser beam. Two different detecting systems are used, a photographic film and a photodiode. Quantification is based on the comparison of the membrane fluorescence signal with the fluorescence of isotropic calibration solutions containing the same chromophore.

A critical point in determining the pigment concentration in bilayers with fluorescence intensity measurements is the uncertainty which arises from the non-isotropic orientation of the pigment molecules in the membrane. The molar absorption coefficient as well as the molar fluorescence intensity are different in the isotropic and non-isotropic state. For example, Steinemann et al. [32] reported a factor of 1.2 in bilayers containing chlorophyll *a* in order to correlate the isotropic absorption cross-section with that in the membrane. As in most cases the geometrical arrangement of the electronic transition moments is unknown, an exact determination of concentration cannot be obtained either by fluorescence or by absorption measurements.

Spectral shifts and changes in the quantum yield of fluorescence have also to be discussed when the calibration solution is compared with the membrane. In aqueous solutions as well as in the membrane-bound state, all flavins mentioned in this paper show the same fluorescence maximum and quantum yield. The latter is demonstrated by liposome preparations containing the amphiphilic flavins. Therefore, with respect to this point, no correction for flavin measurements is necessary. Since the fluorescence maximum of flavins shifts to shorter wavelengths (down to 500 nm) in solvents with lower dielectric constant and the quantum yield rises at the same time, it can be stated that the flavin nuclei of the membrane-bound amphiphilic flavins are in a hydrophilic environment. Therefore, an arrangement similar to that found for chlorophyll is assumed [32, 34]. The hydrophilic flavin nucleus and the porphyrin ring, respectively, are situated in the polar interface region of the bilayer or jut into the aqueous phase while the hydrophobic hydrocarbon chains are anchored in the

apolar interior of the membrane. If the flavin nucleus is located between the polar groups of the lecithin, it is likely that the chromophore is surrounded by water molecules, since water molecules are assumed to be located between the lecithin dipoles to lower the mutual repulsion [33].

At low concentrations the fluorescence of magnesium octaethylporphyrin shows little difference in spectral shift and quantum yield when the polarity of the solvent is changed. Since the fluorescence intensity of the membrane-bound porphyrin is measured with a broad band interference filter no correction is necessary.

Enrichment factors have been introduced to describe concentration changes during membrane formation. As in some cases the concentration changes exceed by far the errors in the calculation of the pigment concentration, it has to be concluded that separation occurs. The membrane formation seems to be a sensitive process in which the separation of different molecules can take place. During the membrane-forming process the solvent decane is displaced by the lipids and the pigments. This is indicated by enrichment factors greater than 1. The incorporation seems to depend on the number of fatty acid chains, the flexibility and the amphiphilic character of the compounds.

In flavin 2 the flavin nucleus and the hydrocarbon chain are connected by an ester group which renders the molecule more flexible than the corresponding C–N–bond in flavin 1. In addition, flavin 2 contains a double bond in the hydrocarbon chain, whereas flavin 1 has a saturated chain. A better adaption of flavin 2 to the lecithin molecule is, therefore, possible. The result is a better incorporation of flavin 2 than of flavin 1. The four hydrocarbon chains of flavin 3 are oriented in all directions when the chains are in the all-trans configuration. They represent a bulky structure with strong sterical hindrance. Thus it is easy to imagine that this molecule can prevent the parallel arrangement of the lecithin molecules. This is in keeping with the inhibition of membrane formation at low concentrations.

At high pigment concentrations in the membrane-forming solution the concentration in the bilayer appears not to be proportional to the observed fluorescence. This is caused by fluorescence quenching effects. By the formation of flavin dimers the fluorescence maximum shifts by about 27 nm to longer wavelength [35]. But at high flavin 1 or flavin 2 concentrations in the membrane no shift is observed. Appreciable amounts of dimers can, therefore, be excluded and quenching by energy transfer seems to prevail. For flavin 1 and flavin 2, quenching begins at an area concentration of 10^{13} molecules/cm^2 which corresponds to a mean distance between the molecules of 45 Å in one membrane interface. The quenching of magnesium octaethylporphyrin, however, begins at an area concentration of $6 \cdot 10^{12}$ molecules/cm^2 corresponding to a mean distance of 58 Å. It is interesting to note that for chlorophyll *a* fluorescence quenching begins at the same value of $6 \cdot 10^{12}$ molecules/cm^2 [6].

Because of the solubility of flavin 2 in water a partition coefficient can be calculated from the concentration in the aqueous phase c and the area concentration in the membrane c_m:

$$\beta = \frac{c_m}{c} \qquad [cm] \tag{4}$$

Using the concentrations given above, a value for flavin 2 of $\beta = 0.2$ cm is obtained. This value is ten times larger than the one found for the hydrophobic ions dipicryl-

amine and tetraphenylborate, which are also accumulated in the membrane interface [3]. This is not surprising since flavin 2 is a neutral molecule and, in contrast to ions, no repulsive electrostatic forces are active. Recently Zingsheim and Haydon [36] determined the area concentration of the water-soluble fluorescence probe 1-anilino-8-naphthalene sulfonate (ANS) bound to a bilayer membrane formed from glycerol monooleate. They found an interfacial concentration of 10^{13} molecules/cm^2 when ANS was dissolved $3.1 \cdot 10^{-5}$ M in 0.1 M KCl. These values result in a partition coefficient $\beta = 5.3 \cdot 10^{-4}$ cm which is approximately 400 times smaller than that of flavin 2. Despite this poor binding, ANS is an appropriate fluorescence probe because the quantum efficiency increases strongly in the membrane-bound state. Therefore, the fluorescence signal originating from the membrane is more intensive than that originating from the water phase. This holds for ANS as well as for flavin 2.

ACKNOWLEDGEMENT

The author wishes to thank Mr L. Carls for excellent technical assistance, Professor P. Läuger and Dr G. Pohl for helpful discussions, Professor P. Läuger for supplying lecithins and Professor P. Hemmerich for his interest in this work. This work was financially supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 138.

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