5.8.2 Quantitative description.

Absorption.

The electrical field vector $\vec{E}$ and the transition dipole moment $\vec{\mu}_A$ define the probability P(θ,ϕ) for absorption:

$$P(\theta,\phi) = \left(\vec{\mu}_A \vec{E}\right)^2$$

The transition probability is therefore proportional to $\cos^2(\theta)$, if θ defines the angle between the electrical field vector and the transition dipole moment (see Figure 6.11.) The probability for a transition is therefore largest, if the transition dipole moment is oriented in the direction of the electrical field vector. The preferred excitation for small angles Θ is termed photoselection.

In addition to the probability of excitation, the absorption will also be determined by the number of molecules that are oriented between θ and $d\theta$ and between φ and $d\varphi$. An ensemble of statistically distributed molecules is described by

$$N(\varphi,\theta)d\theta d\varphi = \sin(\theta)d\theta d\varphi$$

The factor sin(θ) accounts for the reduced number of molecules with an orientation parallel to direction of polarization of the excitation beam as compared to the number of molecules that are oriented perpendicular to this direction.
The relative number Z(q,f) of excited molecules within the angles dθdϕ is therefore given by

$$Z(\theta,\varphi) = N(\theta,\varphi)P(\theta,\varphi)d\varphi d\theta$$

and

$$Z(\theta,\varphi) \sim \cos^2\theta \sin\theta d\theta d\varphi$$

Relative to the total number of excited molecules

$$N = \int_{\varphi=0}^{2\pi} \int_{\theta=0}^{\pi} Z(\theta,\varphi)d\theta d\varphi$$

the number of excited molecules within the angles $\theta \ldots \theta + d\theta$ and $\varphi \ldots \varphi + d\varphi$ is given by

$$W(\theta,\varphi)d\theta d\varphi = \frac{\cos^2\theta \sin\theta d\varphi}{\int_{\varphi=0}^{2\pi}\int_{\theta=0}^{\pi} Z(\theta,\varphi)d\theta d\varphi}$$

or after integration:

$$W(\theta,\varphi)d\theta d\varphi = \frac{2}{4\pi}\cos^2\theta \sin\theta d\theta d\varphi$$

The equation describes that the excitation by linearly polarized light leads to an anisotropic distribution of the excited molecules that is cylindrically symmetric to the direction of polarization of the incident radiation. The transition dipole moments of the excited molecules exhibited a preferred direction along the z-axis. Based on this anisotropic distribution of the excited molecules, the polarization of the emitted fluorescent light must be anisotropic.
It becomes further apparent that the absorption process of polarized light leads already to a depolarization of the emitted fluorescence.

Emission.

The plane of polarization of the emitted radiation of the molecule is determined by the orientation of the transition dipole moment of emission ($\vec{\mu}_E$). The depolarization, in addition to the contribution that is made by the absorption, contains two more contributions:

1. the relative orientation between the absorption and the emission transition dipole moments

2. molecular motions of the molecule within the lifetime of the excited state, which change the orientation of the transition dipole moment of emission that is given only by the geometry of the molecule.

If we consider a rigid isotropic sample, i.e. an orientation of the transition dipole moment of emission that is unchanged within the fluorescence lifetime of the excited state and we further assume that absorption and emission take place between the same electronic energy levels then the probability $P_z(\theta,\phi)$ for a polarization in the direction of the z-axis is proportional to $(\vec{\mu}_z\vec{e}_z)^2$ and therefore proportional to $\cos^2(\theta)$. The polarization of light in x-direction can be written as

$$P_x(\theta,\varphi) \sim (\vec{\mu}_x \cdot \vec{e}_x) \sim (\sin(\theta)\cos(\varphi))^2$$

$\vec{e}_x$ and $\vec{e}_z$ are the unit vectors for the respective directions in space.

The relative intensities $I_{||}$ and $I_{\perp}$ of the fluorescence radiation can be obtained by multiplication with the fraction W(θ, ϕ) of excited molecules and subsequent integration over the surface of the sphere:

$$I_{||} = \int_{\varphi=0}^{2\pi} \int_{\theta=0}^{\pi} \cos^2(\theta) \cdot W(\theta,\varphi) \cdot d\theta d\varphi = \frac{3}{5}$$

$$I_{\perp} = \int_{\varphi=0}^{2\pi} \int_{\theta=0}^{\pi} \sin^2(\theta)\cos^2(\varphi) \cdot W(\theta,\varphi) \cdot d\theta d\varphi = \frac{1}{5}$$

According to the assumption $(\vec{\mu}_a \,||\, \vec{\mu}_b)$ and rigid molecules, these equations give the maximum for the intensity parallel and the minimum for the intensity perpendicular to the z-axis. If the values are used to calculate the polarization P and the anisotropy A values of P=0.5 and A=0.4 are obtained for the maximum fluorescence polarization and the maximum fluorescence anisotropy of a sample of rigid molecules that are isotropically distributed in space.

For a given angle g between the transition dipole moments of absorption and emission, the fluorescence polarization can be written as:

$$P_0 = \frac{3\cos^2\gamma - 1}{\cos^2\gamma + 3}$$

or

$$A_0 = \frac{3\cos^2\gamma - 1}{5}$$

The index 0 means that the equations are only valid for molecules that do not reorient within the lifetime of the excited state. The general expression that takes the reorientation of the molecules into account is given by the Perrin equation:

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau_F}{\tau_C}\right)$$

and

$$\frac{1}{A} = \frac{1}{A_0\left(1 + 3\frac{\tau_F}{\tau_C}\right)}$$

τ_F is the fluorescence lifetime of the molecule and τ_C the rotational correlation time. The rotational correlation time is a characteristic quantity to describe rotational motions. It can be used to calculate the rotational diffusion coefficient:

$$\tau_C = \frac{1}{2D_{rot}}$$

The rotational diffusion constant of sphere-like molecules is given by

$$D_{rot} = \frac{k_B T}{V_h \eta}$$

k_B is the Boltzman constant, T the absolute temperature in K, and η the viscosity of the sample.

The Perrin equation is valid if it is assumed:

- molecules are spheres or there rotation has the same properties as the rotation of spheres with the volume V_h.
- the microviscosity in the direct vicinity of the molecule is the same as the overall viscosity of the sample.
- the rotational motion shall be unrestricted and statistic.
- the depolarization shall only be caused by differences in the transition dipole moments of absorption and emission and by Brownian molecular motions, but not by energy transfer, reabsorption or other effects.

The Perrin equation can be used to discuss the observed fluorescence polarization for different values of the fluorescence lifetime τ_f and the rotational correlation time t_c:

1. Is the rotational correlation time large compared to the fluorescence lifetime of the excited molecule, then there is no motion of the molecule within t_f and the polarization of the fluorescence emission is at maximum.

2. is the rotational correlation time much smaller compared to the fluorescence lifetime of the excited molecule, then the fluorophores are statistically reoriented compared to their orientation at the moment of excitation. The intensity of fluorescence parallel and perpendicular to the z-axis is then the same and polarization and anisotropy are zero.
If the orientation of the fluorophores changes within the fluorescence lifetime and the rotational correlation time and the fluorescence lifetime are of similar magnitude, then the distribution of the molecules remains anisotropic and the polarization will be characterized by values between 0 and 0.5 (the maximum value of polarization).

With the Perrin equation it is possible to obtain the rotational mobility of a fluorophore and the viscosity of the sample or the hydrated molecular volume of the fluorophore.

5.8.3 Static and time resolved fluorescence polarization.

In the determination of fluorescence polarization, two methods are distinguished, the time continuous fluorescence polarization and the time resolved fluorescence polarization. Continuous fluorescence polarization is observed by exciting the sample with light of constant intensity. Measuring the parallel and perpendicular components of the fluorescent light leads to average intensities:

$$I_{||} = \frac{1}{\tau_F}\int_0^\infty I_{||}(t)dt, \qquad I_{\perp} = \frac{1}{\tau_F}\int_0^\infty I_{\perp}(t)dt$$

The obtained polarization or anisotropy therefore also represents an average value.

In time-resolved fluorescence polarization the sample is excited with a short laser pulse and the time dependence of the intensities parallel and perpendicular to the z-axis is measured. The decay of both fluorescence intensities is exponential with time. Directly after the pulse, the anisotropy of the emission is given by the anisotropy of the absorption (photoselection). In contrast, molecules that are fluorescent at a later time will have performed a molecular rotation. The experimental determination of the polarization as a function of time after the light pulse therefore shows a decay from P_0 (A_0) to a final value which is determined by the fluorescence lifetime and the rotational correlation time. For spherical molecules this decay is exponential and the time after which the polarization or the anisotropy is only 1/e times as high as at time 0 corresponds to the rotational correlation time, τ_c, which can thus be determined experimentally.

5.9 Applications

5.9.1 Estimation of the lateral diffusion coefficient.

The formation of excimers is a diffusion controlled process. The rate of excimer formation of pyrene labelled lipids can be used to determine the diffusion coefficient in membranes parallel to the membrane surface (lateral diffusion). The ratio of the quantum yields of monomers and excimers can be expressed as

$$\frac{\Phi_E}{\Phi_M} = \frac{k_{fE}}{k_{fm}} \cdot k_a \cdot c \cdot \tau_E$$

The ratio of quantum yields is proportional to the intensity ratio I_E/I_M :

$$\frac{\Phi_E}{\Phi_M} \propto \frac{I_E}{I_M}$$

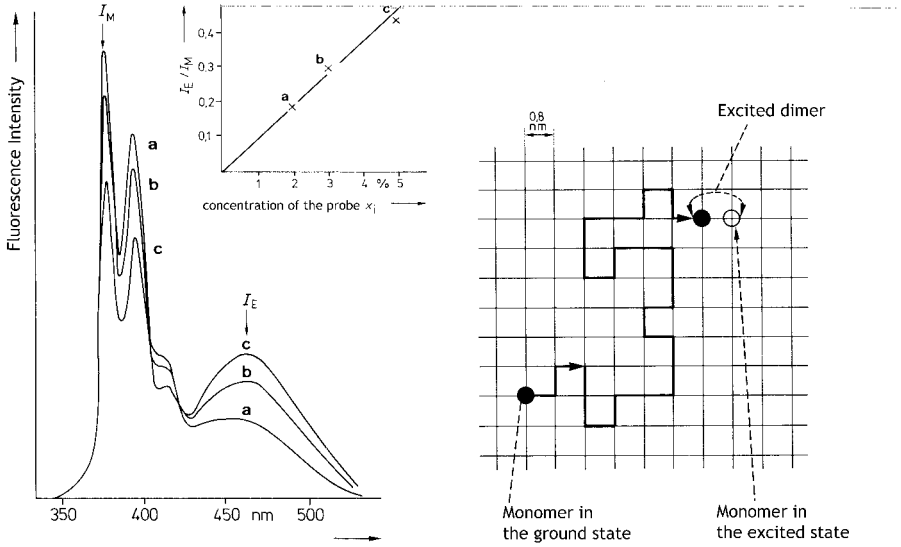
If the transition rates are k_{fE} and k_{fM} for the transitions of the excited monomer and the excited dimer to the ground state are known and if the lifetime τ_E of the excimer was determined, the intensities I_E and I_M can be used to determine the excimer formation rates $k_a \cdot c$. The value of $k_a \cdot c$ is dependent on the probability of collision between M and M* and can be related to the diffusion coefficient of monomers. For the diffusion in the lipid bilayer we consider a statistical random walk process. The lipid bilayer is regarded as an approximately two-dimensional grid. The association rate

$$k_a \cdot c = \nu_j \cdot n_s$$

is proportional to the jump frequency ν_j of the labeled molecules between different positions within the grid. The average number of jumps, which a monomer performs until an excimer is formed shall be n_s. With the Einstein-relation and the mean jump distance λ the diffusion coefficient is given by

$$D = \frac{1}{4}\nu_j \lambda^2$$

The diffusion coefficient is a quantitative means to describe the fluidity of the membrane. The lateral diffusion of lipids in the liquid-crystalline L_α-phase is described by diffusion coefficients in the range of 10^{-8} to 10^{-7} cm^2/s. This diffusion is slow compared to the diffusion of water molecules ($D=10^{-6}$ to 10^{-5} cm^2/s), which is based on the more complex molecular composition of the phospholipids, which consist of two long hydrophobic chains



Fluorescence spectra of pyrene decanoic acid in fluid dipalmitoyl phosphatidylcholine membranes. The labeled fatty acid was added in concentrations of 2, 3, and 5 mol-%. The increase of the intensity ratio of the fluorescence of the excimer over the fluorescence of the monomer can be used to determine the diffusion coefficient.

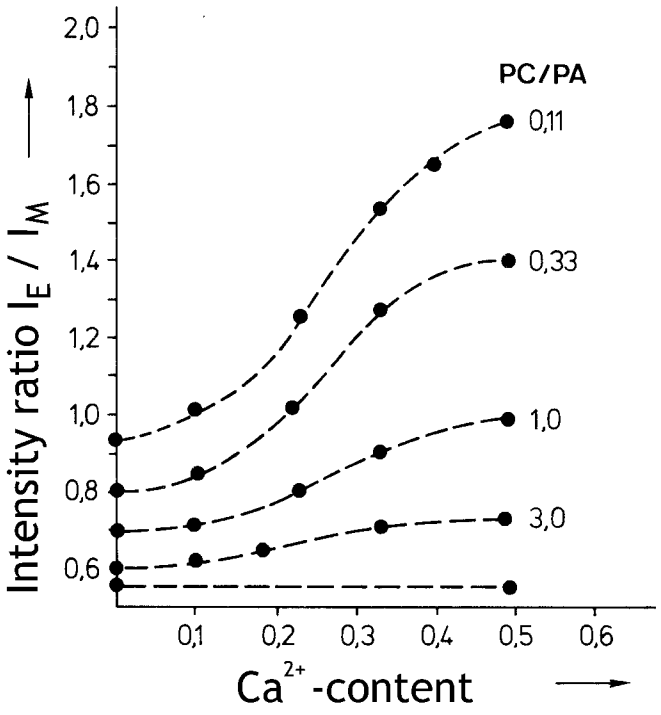
Schematic representation of a "random walk". The fluorescent probe migrates statistically from one site on the membrane to another. If a non excited and an excited pyrene label occupy two neighboring sites, an excimer can be formed.
(after Galla, H.J. et al., (1979), J. Membrane Biol. 48, 215)

Figure 5.12 Estimation of the lateral diffusion coefficient by excimer formation

that are covalently linked to glycerol and a headgroup. Below the phase transition temperature of the phospholipids, the diffusion coefficient of the lipids is even smaller, 10^{-11} to 10^{-12} cm^2/s. These diffusion coefficients below the phase transition temperature can no longer be determined by the excimer experiment, because the fluorescence lifetime of the excited pyrenes is too short at to allow for dimer formation in this diffusion controlled process.
Phase separation problems in lipid bilayers.

The equation

$$\frac{I_E}{I_M} \propto \frac{\Phi_E}{\Phi_M} = \frac{k_{fE}}{k_{fm}} \cdot k_a \cdot c \cdot \tau_E$$

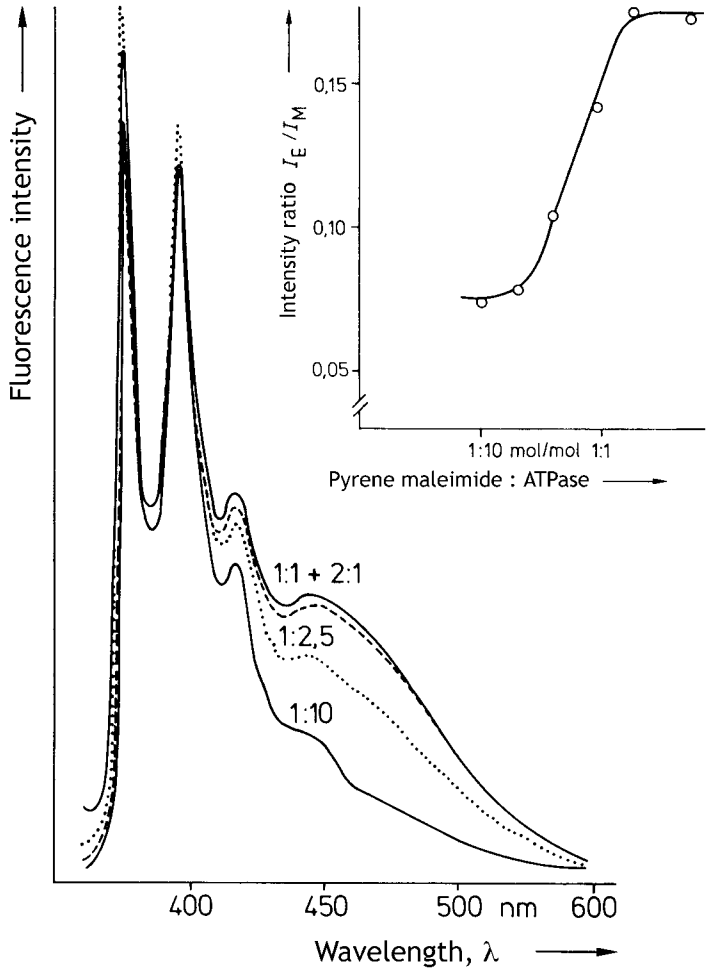


Ratio of the fluorescence intensities of excimers and monomers of pyrene decanoic acid in membranes composed of phosphatidylcholine and phosphatidic acid. The excimer formation increases at increased calcium concentrations. The reason for this increase is a calcium induced phase separation.
(after Galla, H.-J., Sackmann, E. (1975), J. Amer. Soc. 97, 4114)

Figure 5.13 Use of excimer formation to detect calcium induced phase separations in lipid bilayers.

shows that the intensity ratio of the fluorescence of the excimer over the fluorescence of the monomer depends on the concentration of the excimer forming label. For a diffusion controlled process, the intensity ratio increases linearly with the concentration, if the labels are statistically distributed. This is not the case if the probe partitions preferentially into a specific membrane area. A local increase in the concentration of the probe and thus an increased intensity ratio will be observed. In Figure 5.13 , this is shown on the example of a lipid mixture of negatively charged phosphatidic acid (PA) and zwitterionic phosphatidylcholine (PC). The temperature is chosen such that both lipids are in the liquid-crystalline phase in the absence of calcium. The calcium ions form a complex with phosphatidic acid which is now in the crystalline state. Both lipids are now inmiscable and fluid PC-domains are formed that are separated from rigid domains of CaPA. The extend of such a phase separation depends on the concentration of Ca. The excimer formation is facilitated in the fluid PC phase, but not in the crystalline

CaPA phase. In addition, the fluorescent probe becomes more soluble in the fluid PC phase. A local increase in concentration leads to an increased formation of excimers.



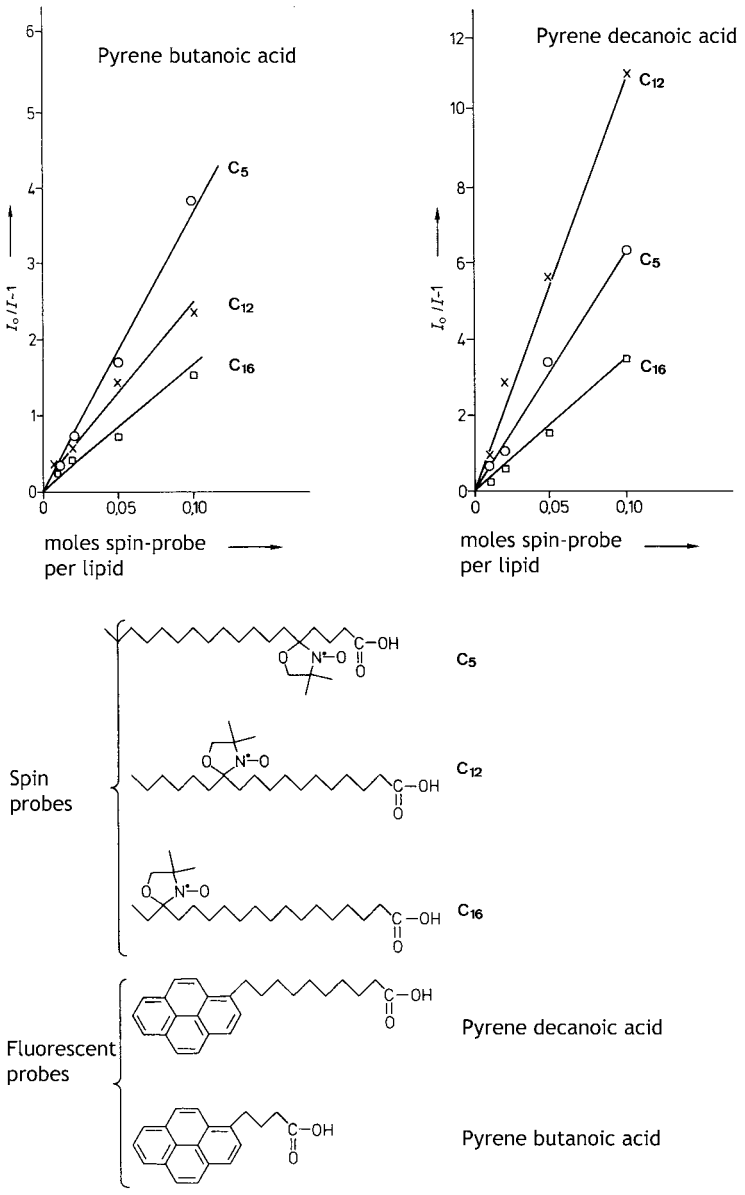
Emission spectra of pyrene labeled ATPase in lipid-bilayers. With increasing molar ratio of pyrene maleimide to ATPase, an excimer band can be observed. (after Lüdi, H., Hasselbach, W. (1983) Eur. J. Biochem. 130, 5)

Figure 5.14 Detection of oligomers of calcium ATP-ase by excimer formation.

5.9.2 Protein Association.

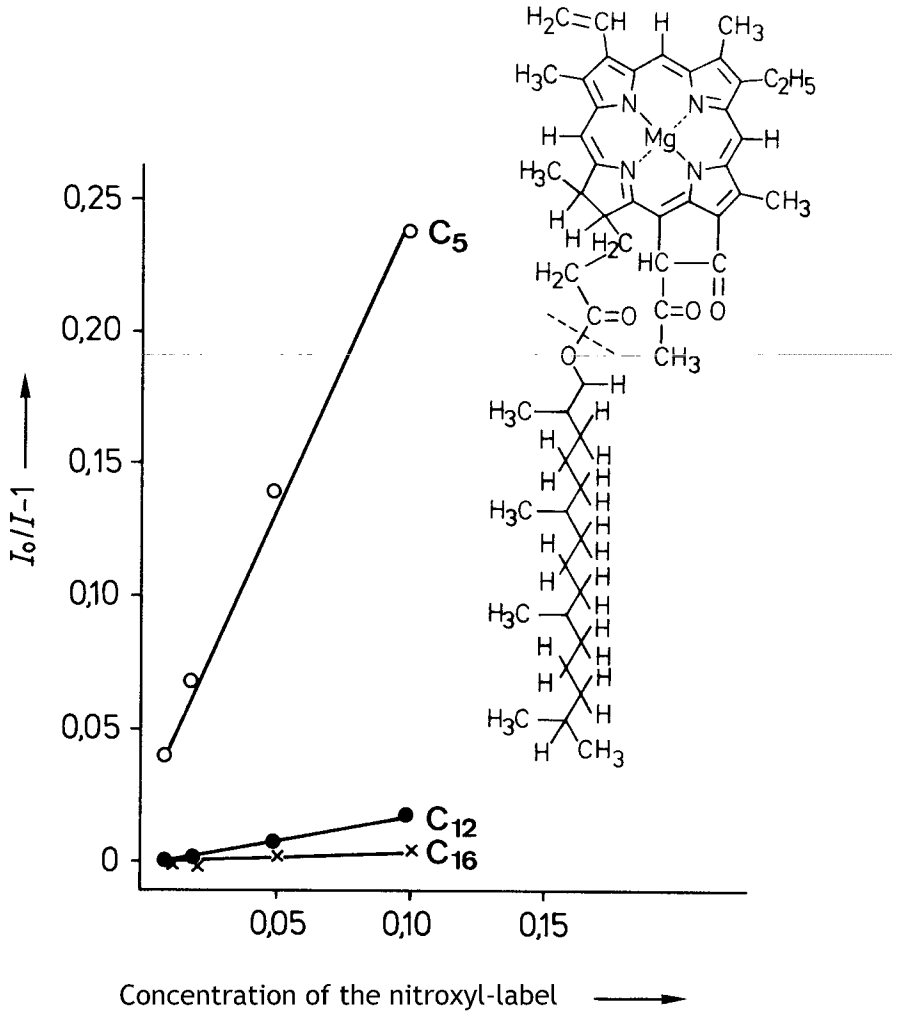
The formation of excimers is not limited to investigate lipid phase behavior. For example, it could be observed that the Ca^{2+} -dependent ATPase forms oligomers in membranes. The protein was covalently labeled by a pyrene maleimide. With increased concentration of the labeled protein, excimer formation was observed. A possible double labeling of the protein was excluded such that it had to be assumed that the protein forms oligomers in membranes.

Only a regular orientation of the protein would make the excimer formation possible.



Fluorescence quenching of pyrene butanoic acid and pyrene decanoic acid in lipid bilayers by different radicals. (after Luisetti, J. et al. (1979) Biochim. Biophys. Acta 552, 519).

Figure 5.15 Fluorescence quenching by different membrane bound quenchers.



Fluorescence quenching of chlorophyll A in lipid bilayers
(after Luisetti et al. (1979), Biochim. Biophys. Acta 552,519)

Figure 5.16 Fluorescence quenching of chlorophyll by spin-labeled lipids.

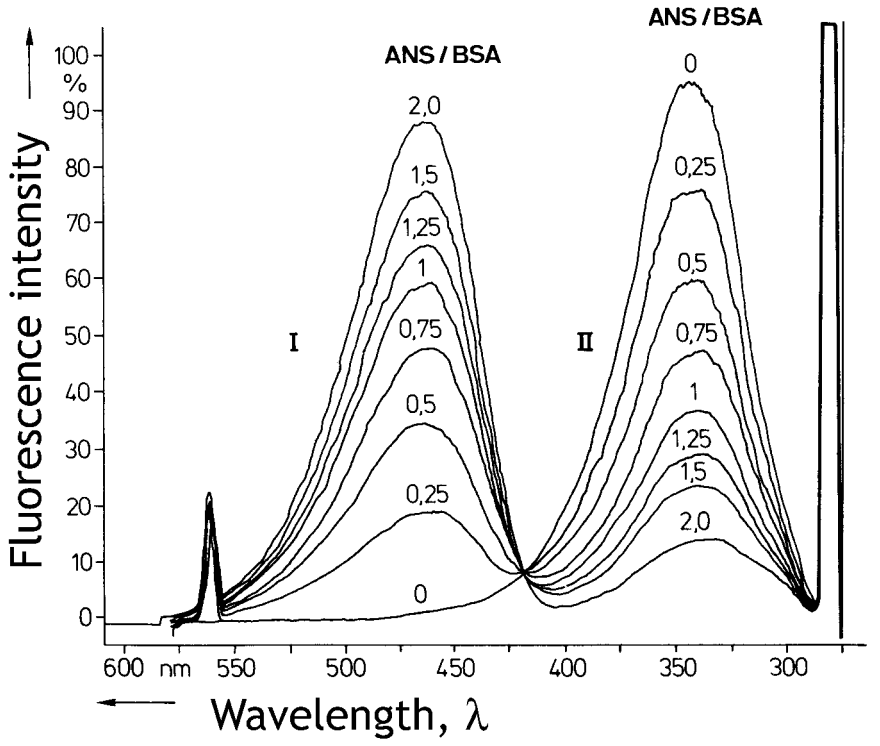
5.9.3 Localization of fluorophores in membranes.

The investigation of the efficiency of fluorescence quenching by radical probes made it possible to determine the position of a fluorophore in a lipid bilayer. Especially suitable are fatty acids of lipids with nitroxyl groups, which are attached at different carbon atoms along the acyl chain. Figure 5.15 shows the Stern-Volmer plots of the fluorescence quenching of the fluorophores pyrene butyric acid and pyrene decanoic acid by different radical quenchers. C5, C12, and C16 -doxyl labeled stearic acids were used. Fluorescence quenching of pyrene butyric acid is most effective with C5-doxyl stearic acid spin-label and less effective the deeper the quenching doxyl group is located in the membrane. In case of pyrene decanoic acid, the C12 doxyl stearic acid was found to be the most effective quencher. The position of the fluorophore can be determined from the known position of the nitroxyl group.

This method was subsequently used to determine the position of the porphyrine ring of chlorophyll in biomembranes (Figure 5.16).
The fluorescence quenchers used in that study were the same as the fluorescence quenchers that were used to detect the locations of pyrene butyric acid and pyrene decanoic acid. The strongest quencher is the C5-doxyl labeled stearic acid which is closest to the membrane surface, i.e. the apolar / polar interface. The quenching efficiencies of the C12 and C16 radical probes are significantly lower (C12) or not observed at all (C16). The results indicate that the porphyrin ring is located close to the polar headgroup region.

5.9.4 Distance determination by resonance energy transfer.

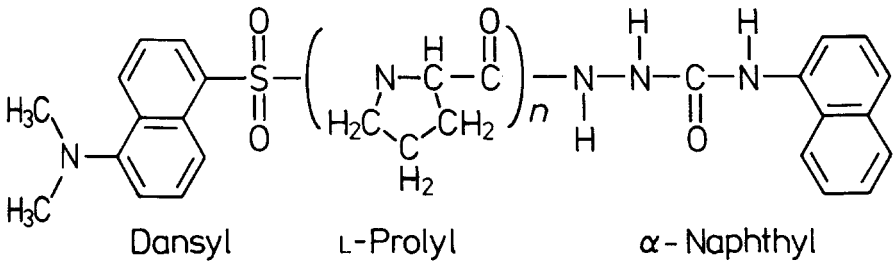
The fluorescent probe ANS binds to serum albumin and the fluorescence quantum yield is increased upon binding. The absorption can also be observed by fluorescence energy transfer (Figure 5.17). Serum Albumin has tryptophans which form donor acceptor pairs. When Trp is excited, the fluorescence of the tryptophan decreases with increasing ANS / tryptophan ratio. At the same time the fluorescence emission of the ANS increases. The energy transfer is maximal if all binding sites are occupied, at which ANS binds to the protein. The number of ANS binding sites can therefore be determined by measuring the energy transfer (fluorescence intensities of ANS and of protein) at different ratios of ANS to protein.

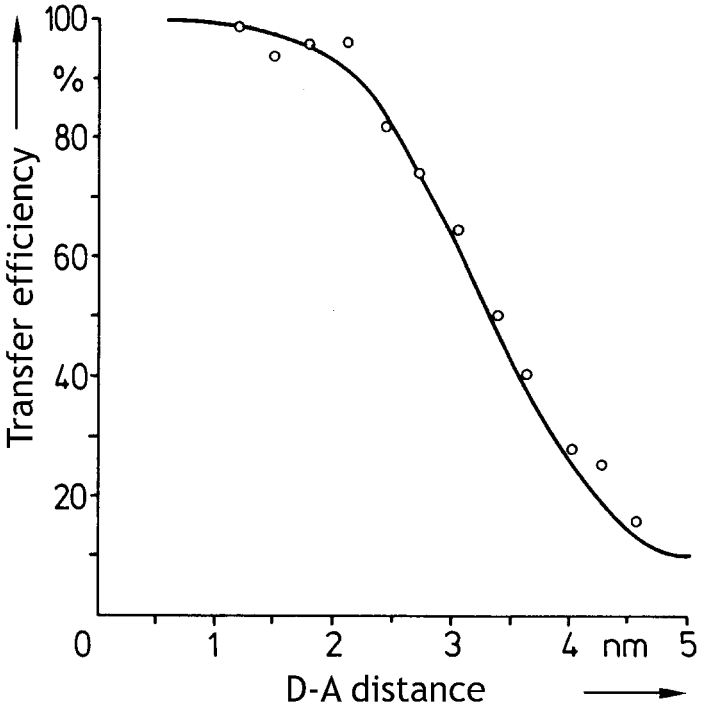


Energy transfer on the example of ANS bound to bovine serum albumin. ANS is excited at 340 nm, the wavelength at which trp fluorescence emission occurrs.

Figure 5.17 Energy transfer on the example of ANS bound to BSA

Based on the distance dependence, energy transfer is a suitable method to determine the separations between two fluorescent groups. The distance R_0 must be known and close to the distance that is to be estimated. Figure 5.18 demonstrates the establishment of a spectroscopic ruler based on the chromophores dansyl (acceptor) and naphtyl (donor).





Experimental test of the r^6-dependence. The donor D (α-naphtyl -group) and the acceptor A (dansyl-group) are separated by prolyl residues. The transfer efficiency decreases with increasing distance (number of prolyl residues).

Figure 5.18