Migration and Partitioning of Pyrene and Perylene between Lipid Vesicles in Aqueous Solution Studied with a Fluorescence Stopped-Flow Technique

Mats Almgren

Contribution from the Department of Physical Chemistry, Chalmers University of Technology and the University of Gothenburg, S-412 96 Gothenburg, Sweden. *Received June 3, 1980*

Abstract: A fluorescence stopped-flow technique is described in which excitation energy transfer of the Förster type between pyrene and perylene is utilized to monitor the migration of these aromatic molecules between lipid vesicles in aqueous solution. It is shown that the migration occurs via the aqueous phase, the rate being controlled by the exit rate of the solubilized molecules from the vesicles. The exit rate, and hence the migration rate, is diffusion controlled and is simply related to the size of the vesicles and the partition coefficient of the molecules between the lipid bilayer and the aqueous phase. The exit rate constants at 18 °C were obtained as $3 \pm 0.5\ s^{-1}$ for perylene and $77 \pm 6\ s^{-1}$ for pyrene from soybean lecithin vesicles containing 10% dicetyl phosphate in 2 mM Tris buffer and 8 vol % of ethanol. Similar results were obtained with egg lecithin and dimyristoylphosphatidylcholine vesicles, whereas no relaxation process was detected in pure dicetyl phosphate vesicles.

Introduction

Although the phenomenon of solubilization of hydrophobic molecules in amphiphilic structures of various kinds—micelles, membranes, etc.—has been known and utilized for a long time,[1] it was only recently that the first systematic study of the dynamics of the solubilization process was published.[2] It was then shown that the rate of association and dissociation of aromatic molecules with/from ionic micelles was close to diffusion controlled.

The results for the rates of the exit and entrance processes in small structures like micelles may not be taken as representative for the rates of these processes in larger structures. In a previous paper[3] the excimer emission of pyrene was utilized to monitor the migration of this molecule between lipid vesicles in stopped-flow experiments. It was shown that the migration process was controlled by the exit rate of pyrene from vesicles into the aqueous phase. In the present contribution it will be shown that the Förster type excitation energy transfer between pyrene and perylene[4] may be advantageously utilized to monitor the migration of both these molecules between vesicles. The migration process will be characterized, and the diffusion-controlled character of the exit and entrance processes will be accounted for by a phenomenological model of the dynamic partitioning of the aromatic molecules between the lipid bilayer and the aqueous solution.

The main body of this work deals with vesicles composed of soybean lecithin with some dicetyl phosphate added to give a net negative charge. Similar studies of the transfer of fluoroescent molecules between vesicles of dipalmitoyl- or dimyristoylphosphatidylcholine were published recently. Sengupta et al.[5] and Galla et al.[6] studied the migration of pyrene, pyrenedecanoic acid, and a pyrene–lecithin derivative, whereas Doody et al.[7] used pyrenenonanoic acid. Kano et al.[8] measured the migration rates of alkylated alloxazines and isoalloxazines by means of excitation energy transfer. Most of these results indicate very slow transfer processes. Even for pyrene the relaxation times were several seconds.[5,6] Some complementary experiments were performed by using vesicles from dimyristoylphosphatidylcholine (DMPC), egg lecithin, or pure dicetyl phosphate (DCP) in order to allow a direct comparison with some of the earlier results.

Experimental Section

An Aminco–Morrow stopped-flow apparatus was adapted for fluorescence measurements as described earlier.[3] The signal from the photomultiplier was fed to a Biomation 850 digital waveform recorder, via a differential amplifier which permitted the substraction of the DC signal. The digital data in the 2048 channels of the waveform recorder (8 bits resolution) were transferred to a Commodore 3016 computer and further processed by the addition of 8–32 succesive channels. Normally a signal averaging of 4–12 consecutive shots was performed in the computer.

The resulting data were fitted to kinetic laws with a constant and one or two exponential terms by means of a nonlinear least-squares procedure (utilizing a Marquardt algoritm;[9] the procedure was adapted to the particular fitting function by deriving analytical expressions for all sums involving only the fitting function or its derivatives; in this way the computation time was reduced drastically).

Vesicles were formed from Epicuron 200 soybean lecithin (Lucas–Mayer) to which normally 10 mol % of dicetyl phosphate (Sigma) had been added to give the vesicles a net negative charge. Aliquots of 0.80 mL of a 25 mM ethanol solution of the lecithin (on the basis of an assumed average molecular weight of 750) were added by injection[10] with a syringe into 10 mL of vigourously stirred water with 2 mM Tris buffer (pH 7.3). The resulting solutions were only slightly turbid. At higher buffer concentration and/or lower net charge much more turbid solutions resulted. Vesicles prepared by sonication–ultracentrifugation from the same starting material at a buffer concentration of 20 mM Tris have been shown to be quite monodisperse with a radius of 320 ± 40 Å.[11]

Pyrene was added to the vesicle solutions by injecting small aliquots of a 10 mM solution of pyrene in ethanol. The solubility of perylene in ethanol is too low to make this method tractable. Perylene was therefore introduced into the lecithin–ethanol solution prior to the formation of the vesicles.

Principles of the Method. Consider the mixing of equal volumes of two vesicle solutions, with equal vesicle concentrations but different concentrations of the solubilized molecules. Assume that the mean number per vesicle of one of the molecules, e.g., perylene, is $\bar{n}_{Pe}$ in the mixed solution. Denote the corresponding mean number pertaining to the vesicles from solution 1 by $\bar{n}_{1Pe}(t)$ and from solution 2 by $\bar{n}_{2Pe}(t)$.

(1) (a) M. E. L. McBain and E. Hutchinson, "Solubilization and Related Phenomena", Academic Press, New York, 1955. (b) J. H. Fendler and E. J. Fendler, "Catalysis in Micellar and Macromolecular Systems", Academic Press, New York, 1975. (c) J. H. Fendler and A. Romero, *Life Sci.*, **20**, 1109, 1977; (d) J. H. Fendler, *Acc. Chem. Res.*, **13**, 7 (1980).

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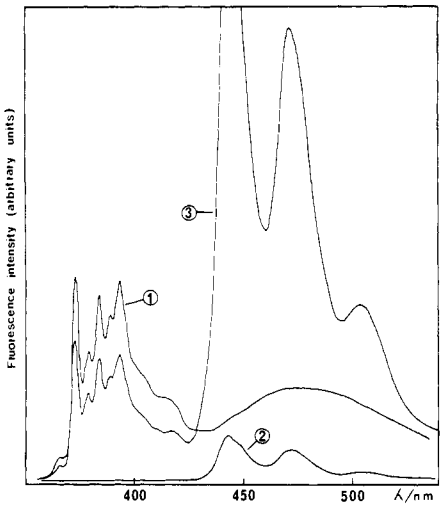

Figure 1. Fluorescence spectra of perylene and pyrene in vesicles. The vesicles were composed of 1.8 mM soybean lecithin and 0.2 mM dicetyl phosphate in 2 mM tris buffer. Curve 1 represents fluorescence from pyrene, 25 μM, curve 2 represents perylene, 10 μM, and curve 3 represents both pyrene and perylene at these concentrations. The excitation wavelength was 335 nm.

Evidently, $\bar{n}_{1Pe}(t) = \bar{n}_{Pe} + \Delta n_{Pe}(t)$ and $\bar{n}_{2Pe} = \bar{n}_{Pe} - \Delta n_{Pe}(t)$. It was shown previously[3] that if the migration process occurs via the aqueous phase, then the migration rate is controlled by the exit rate k^- of the molecule from the vesicle and follows a first-order kinetic law

$$\Delta n(t) = \Delta n(0) \exp(-k^- t) \quad (1)$$

The fluorescence intensity from a set of equal vesicles containing n_{Py} absorbing molecules each will be proportional to n_{Py}. The intensity depends also on the microconcentration of the energy acceptor, perylene, in the lipid bilayer in a way that may be expressed by a function $f(n_{Pe})$.

$$I_e = n_{Py} f(n_{Pe}) \quad (2)$$

In the measurements the actual mean number of solubilized molecules per vesicle was large, of the order of 100. Under these conditions the mean numbers $\bar{n}_{Pe}$ and $\bar{n}_{Py}$ may be used in eq 2.[3] This is still so even if the vesicle size varies, since the important quantity for the observed fluorescence intensity is the microconcentration in the bilayer.

The functional form of the fluorescence intensity dependence on $\bar{n}_{Pe}$ is accessible experimentally by measurement of the intensity at constant pyrene concentration and varying perylene concentration. The empirical results may be given as a power series

$$f(\bar{n}_{Pe}) = A_0 + A_1\bar{n}_{Pe} + A_2\bar{n}^2_{Pe} + A_3\bar{n}^3_{Pe} + \ldots \quad (3)$$

At time t the observed signal is the sum of the contributions from vesicles from solutions 1 and 2

$$S(t) \propto \bar{n}_{1Py} f(\bar{n}_{1Pe}) + \bar{n}_{2Py} f(\bar{n}_{2Pe}) \quad (4)$$

Subtracting the signal at equilibrium and introducing eq 3 and eq 1 for $\Delta\bar{n}_{Pe}$ and Δn_{Py}, eq 4 transforms into eq 5.

$$S(t) \propto \Delta n_{Pe}(0)\Delta n_{Py}(0) \times (2A_1 + 4A_2\bar{n}_{Pe} + 6A_3\bar{n}^2_{Pe}) \exp[-(k_{Pe}^- + k_{Py}^-)t] + \Delta n^2_{Pe}(0)(2A_2\bar{n}_{Py} + 6A_3\bar{n}_{Pe}\bar{n}_{Py}) \exp(-2k_{Pe}^- t) + \Delta n_{Py}(0)\Delta n^3_{Pe}(0)2A_3 \exp[-(3k_{Pe}^- + k_{Py}^-)t] \quad (5)$$

Normally the third-order term in the power expansion of $f(n)$ in eq 3 will be quite small (or it will be possible to choose experimental conditions so that it becomes small). The effect of this term on the signal in eq 5 is then mainly small corrections of the amplitudes of the two exponential terms that depend on A_1 and A_2. The third exponential term will have a low amplitude, and in the present case where $k_{Py}^- \gg k_{Pe}^-$ the time constant will be close to that of the first exponential term. Neglecting third-order terms there remains

$$\Delta S(t) \propto (2A_1 + 4A_2\bar{n}_{Pe})\Delta n_{Pe}(0)\Delta n_{Py}(0) \exp[-(k_{Py}^- + k_{Pe}^-)t] + 2A_2\bar{n}_{Py}\Delta n^2_{Pe}(0) \exp(-2k_{Pe}^- t) \quad (6)$$

Results

Soybean Lecithin Vesicles. Fluorescence spectra of pyrene (curve 1), perylene (curve 2), and both pyrene and perylene (curve 3) in vesicles at representative concentrations are shown in Figure 1. The dramatic sensitization of the perylene fluorescence by pyrene is obvious. The fluorescence intensity at 470 nm on excitation at 335 nm of vesicle solutions with constant pyrene concentration and varying perylene concentration is presented in Figure 2 and provides a representation of $f(\bar{n}_{Pe})$. If $f(\bar{n}_{Pe})$ is at most a second order polynomial in $\bar{n}_{Pe}$, then

$$(I - I_0)/[\mathrm{Pe}] \propto (A_1 + A_2[\mathrm{Pe}]) \quad (7)$$

where I_0 is the fluorescence intensity without added perylene. The data are represented also in this way in Figure 2 and are seen to give a good linear plot with negative slope.

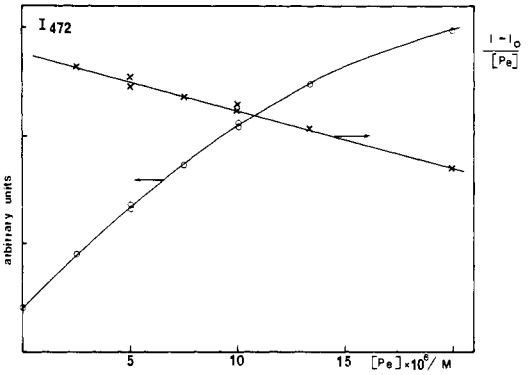

Figure 2. Fluorescence intensity at 470 nm on 335-nm excitation from vesicle solutions containing 25 μM pyrene and varying concentrations of perylene (O). × represents the data plotted according to eq 7.

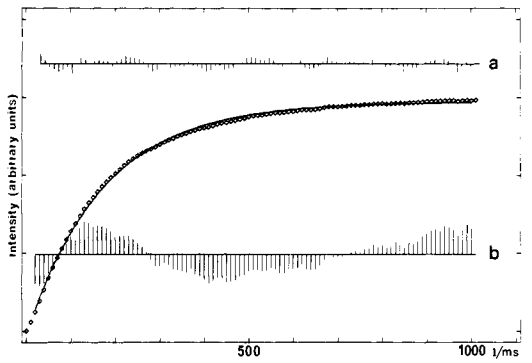

Figure 3. Relaxation signal from stopped-flow experiment where vesicles with pyrene and perylene were mixed with vesicles with pyrene. Solution composition as in Table I. The full-drawn curve represents a least-squares fit to the experimental data (◊). The histograms represents the deviations between data and model for fitting functions with two-exponential terms (a) and one-exponential term (b).

The stopped-flow experiments may be carried out under four principally different initial conditions: (i) Vesicles with pyrene and perylene are mixed with vesicles with only pyrene. (ii) Vesicles with pyrene and perylene are mixed with vesicles with only perylene. (iii) Vesicles with pyrene are mixed with vesicles with perylene. (iv) Vesicles with perylene and pyrene are mixed with empty vesicles.

(i) Pe,Py,Ves + Py,Ves. The fluorescence intensity will increase as the perylene molecules become distributed over all vesicles. Equation 6 predicts a single exponential term since $\Delta n_{Py} = 0$.

The experimental results in Figure 3 are in accord with this prediction, even though the difference plot shows a systematic deviation from a single exponential law. Two exponential terms (plus, as always, a constant) give, of course, a much better fit with a more random distribution of deviations. This deviation from exponentiality will be discussed more fully below. The probable explanation is that it is due to a broad vesicle size distribution. The rate constant obtained from a single exponential fit will be used as a representative mean value.

(ii) Pe,Py,Ves + Pe,Ves. No change of the fluorescence intensity will result from the migration of pyrene in this case since the excitation energy-transfer efficiency is determined by the

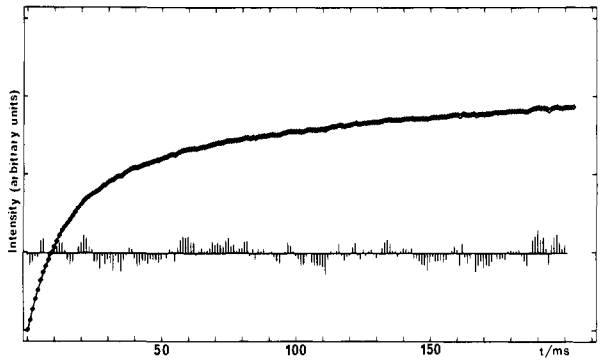

Figure 4. Relaxation signal from stopped-flow experiment of type iii: mixing of vesicles with pyrene and vesicles with perylene. The histogram represents the deviation between data and a model with two-exponential terms.

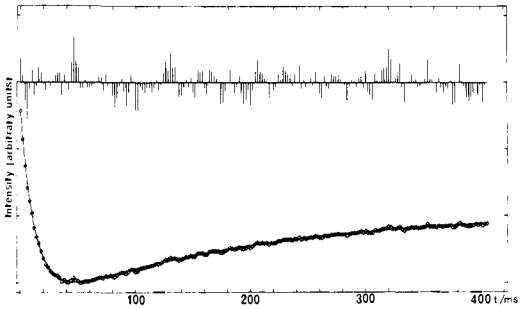

Figure 5. Relaxation signal from type iv stopped-flow experiments: vesicles with both perylene and pyrene mixed with empty vesicles.

perylene concentration. The prediction was fully borne out by the experimental results.

(iii) Py,Ves + Pe,Ves. The results from case i and previous measurements on the migration of pyrene[3] show that pyrene migrates at least 1 order of magnitude faster than perylene. Qualitatively, the following is expected

$$\text{Py,Ves} + \text{Pe,Ves} \xrightarrow{\text{fast}} \text{Py,Ves} + \text{Pe,Py,Ves} \xrightarrow{\text{slow}} \text{Pe,Py,Ves}$$

The fast initial migration of pyrene should be accompanied by a drastic increase in fluorescence intensity. A further increase is expected during the slow step (which is identical with the process in case i).

The experimental results, Figure 4, show the expected behavior. The rate law of eq 6 predicts in this case two exponential terms with amplitudes of equal sign.

(iv) Py,Pe,Ves + Ves. In this case the initially high fluorescence intensity will be expected to decrease rapidly as pyrene migrates out from the vesicles containing perylene and then increase again slowly during the migration of perylene into the vesicles which now contain pyrene. Equation 6 predicts a two exponential law with the same time constants as in case iii but now with amplitudes of opposite sign. The results are in full agreement with the prediction (Figure 5).

The qualitative features are thus entirely satisfactory. Values of the rate parameters determined from several experiments on different samples are collected in Table I. The range of variation reported reflects mainly differences between different samples. In case iv the slow process was studied separately in experiments with a long time base. In these experiments it was evident that the slow process showed the same type of deviation from exponentiality as in the case i experiments. The rate parameter $2k_{Pe}^-$ = 5.5 s^{-1} from the one exponential fit was typically split into 8 ± 3 s^{-1} and 3.5 ± 2.5 s^{-1}. A consequence of this nonexponentiality is that the values of the rate parameter determined in case iii experiments which were all done on a comparatively short time scale tended to be too high: the early part of the slow process was favored. Conversely, the separate study of the slow process in type iv experiments stresses the long time behavior and gives too low a value.

Table I. Rate Parameters for Pyrene and Perylene Migration in Aqueous Lipid Vesicles at 18 °C[a]

start solutns	$2k_{Pe}^-$/ s^{-1}	$k_{Pe}^- + k_{Py}^-$/ s^{-1}	$2k_{Py}^-$/ s^{-1}
Pe,Py,Ves + Py,Ves (i)	6.5 ± 0.5		
Pe,Ves + Py,Ves (iii)	9.0 ± 1.3	74 ± 4	
Pe,Py,Ves + Ves (iv)	5.0 ± 1.0	86.5 ± 5	
Py,Ves + Ves			140 ± 5

[a] The vesicle composition was 1.8 mM soybean lecithin and 0.2 mM dicetyl phosphate in 2 mM Tris buffer of pH 7.3. The total concentrations of pyrene and perylene were 25 and 10 μM, respectively.

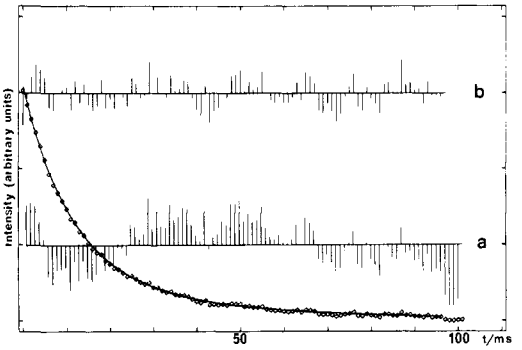

Figure 6. Relaxation signal from stopped-flow study of pyrene migration at 10 °C. Vesicles (1.8 mM lecithin, 0.2 mM dicetyl phosphate in 2 mM Tris buffer) containing pyrene (50 μM) mixed with empty vesicles. The deviation plots represent deviations from one-exponential term model ($2k^-$ = 72 s^{-1}) (a) and two-exponential term model (93 and 30 s^{-1}) (b).

With these difficulties in mind, the results in Table I show on the whole good agreement between the different cases. From the results a value k_{Pe}^- = 3 ± 0.5 s^{-1} may be deduced, and for the fast process k_{Py}^- = 77 ± 6 s^{-1}.

A redetermination of the exit rate constant for pyrene under the present experimental conditions, using the excimer formation technique,[3] gave k_{Py}^- = 70 ± 3 s^{-1}, in good agreement with the value from the fast process.

The reason for the nonexponentiality observed for the perylene migration process is not fully established. It could indicate that perylene might be solubilized in kinetically different sites in the vesicles. However, considering the expected dependence of k^- on the size of the vesicles (mentioned in ref 3 and more fully discussed below), it seems more likely that the explanation is a wide distribution of vesicle sizes. The corresponding effect should then also show up for the migration of pyrene. In separate experiments the migration of pyrene was studied at 10 °C (the process is too fast at 18 °C to give the necessary precision in the data) with excimer fluorescence monitoring. Figure 6 shows the results from one and two exponential fits to such data. The rate parameter from the one-exponential fit, 72 ± 1 s^{-1}, is seen to split into 93 ± 5 and 30 ± 9 s^{-1} on a two-exponential fit. The behavior is very similar to that observed for perylene.

Other Vesicle Systems. In order to compare the results of the present study with those for the transfer of pyrene,[5,6] pyrene decanoic acid,[5,6] or pyrenenonanoic acid[7] between vesicles of dipalmitoyl- or dimyristoylphosphatidylcholine, some complementary experiments were performed with various vesicle systems by using the excimer fluorescence technique.

Egg Lecithin (Sigma Type III-E). The vesicles were prepared as before by injection. Measurements on vesicles in 2 mM Tris buffer with pyrene added both after and before the vesicle preparation gave similar results. The relaxation process was somewhat slower than for pyrene in soybean lecithin vesicles, $2k_{Py}^-$ = 37 ± 3 s^{-1} at 15 °C, and showed the same type of nonexponentiality. The relaxation process was faster for vesicles prepared in 0.15 M NaCl, without buffer, $2k_{Py}^-$ = 56 ± 3 s^{-1}. This preparation was also less turbid. Extended (3 h) sonication of these solutions using a bath-type sonicator, with air and pyrene present, did not change the relaxation process significantly.

Dimyristoylphosphatidylcholine (Sigma, Synthetic 98%). The temperature dependence of the relaxation time was investigated for a vesicle solution containing 1 mM DMPC, 4% by volume ethanol, and 7.5 μM pyrene (no buffer). The relaxation curves were similar to those for the natural lecithins. The results were $2k_{Py}^- = 123$ s^{-1} (35 °C), 97 (30 °C), 75 (27 °C), 64 (25 °C), 45 (20 °C), 40 (15 °C), and 37 s^{-1} (10 °C). The rate constant increases faster with temperature above the transition point (close to 25 °C) than below it. Measurements with perylene–pyrene (end concentrations 7.5 μM of each, type iv conditions) gave $k_{Py}^- + k_{Pe}^- = 36$, 53, and 83 s^{-1} at 18, 26, and 31.5 °C, respectively, and at the same temperatures $2k_{Pe}^- = 2.0$, 3.3, and 6.7 s^{-1}.

Dicetyl Phosphate. No relaxation process was detected for molecularily dissolved pyrene in pure dicetyl phosphate vesicles. This is understood as a consequence of the unusual behavior of pyrene in these vesicles.

(i) The solubility of pyrene was much lower than in other vesicles. The presence of microcrystals of pyrene was revealed in the fluorescence emission and excitation spectra. In the emission spectrum a peak appeared at about 10-nm shorter wavelength than the normal excimer emission and with a more pointed appearance. This quite weak emission was readily detected on excitation at 370 nm where the absorption of molecular pyrene is very weak. The solubility at 25 °C seemed to be close to 15 μM in a 1.25 mM DCP solution (8% ethanol), corresponding to a partitioning coefficient of about 1.5×10^4, or about a factor of 4 smaller than that for pyrene between heptane and water with 8% ethanol. Equation 18 then predicts a relaxation time too short to be resolved.

(ii) Even well below the saturation limit (solutions with a molar ratio pyrene/DCP down to 1:500 were investigated) the fluorescence emission and excitation spectra showed that pyrene was present in two different environments in the vesicles. The relative amounts of pyrene in these two environments depended on the pyrene concentration. The emission favored by high pyrene concentrations showed a higher excimer/monomer ratio but was weaker than the low concentration form, so that the excimer emission intensity was actually less. This resulted in a solution with 0.5 mM of DCP and 5 μM pyrene giving about the same excimer emission intensity as one with the same concentration of pyrene and twice as much DCP. The signal in the stopped-flow experiments will then be very weak.

At concentrations of pyrene above the saturation limit in DCP a complex relaxation pattern was observed, which reached into the region of tens of seconds.

Thus, it was not possible to reproduce the long relaxation times for pyrene in DMPC reported earlier.[5,6] Only vesicle solutions containing microcrystals of pyrene gave relaxation processes in the range of seconds.

Discussion

The discussion will focus upon the results for soybean lecithin vesicles. Results for other vesicle systems will be used only in comparisons with earlier studies.

Exchange on Vesicle Collisions. One of the reasons for studying the migration of perylene in addition to the migration of pyrene was the anticipation that this much less water-soluble molecule would provide a more sensitive test of whether exchange upon vesicle collisions is important. Without going into any details of such a migration process it seems safe to predict that the mechanism implies a dependence on the vesicle concentration. Case iv type experiments were carried out both with normal vesicle solutions and with solutions that had been diluted with 6 parts of 2 mM Tris buffer containing 8 vol % of ethanol. No change of the rate parameters of either process could be detected (Table II).

Since the vesicles in this case were highly charged (10% dicetyl phosphate) and the ionic strength quite low (2 mM Tris), a corresponding dilution experiment was carried out by using vesicles with only 2% dicetyl phosphate, at 20 mM Tris concentration. The vesicles were now larger, giving a turbid solution. There was still only a slight change in the rate parameters on dilution.

Table II. Dilution Experiments at 18 °C[a]

vesicle preparatn	$2k_{Pe}^-$/s^{-1} undil	$2k_{Pe}^-$/s^{-1} diltd	$k_{Pe}^- + k_{Py}$/s^{-1} undil	$k_{Pe}^- + k_{Py}$/s^{-1} diltd
2.0 mM lec, 0.2 mM DCP, 2 mM Tris (I)	5.0 ± 1	5.8 ± 1	86.5 ± 5	81.5 ± 2.5
2.0 mM lec, 0.04 mM DCP, 20 mM Tris (II)	2.1 ± 0.7	2.0 ± 0.4	36.5 ± 1	32.5 ± 1.5
ratio I/II	2.4	2.9	2.4	2.5

[a] Rate parameters from type iv experiments using two different vesicle preparations, at lecithin concentration 2 mM and diluted with buffer to 0.13 mM. In the undiluted samples the pyrene concentration was 25 μM and the perylene concentration 10 μM.

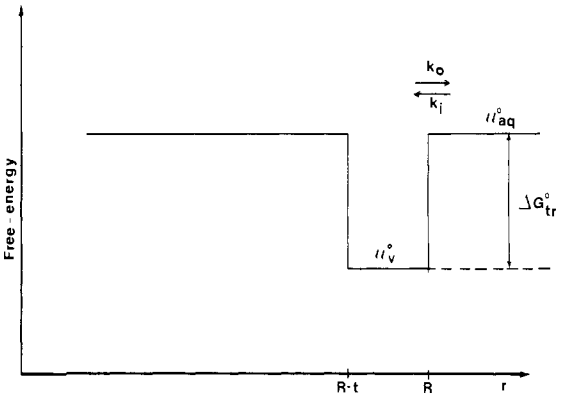

Figure 7. The vesicle lipid bilayer as a free-energy well for hydrophobic solutes. For further explanations see text.

Diffusion-Controlled Dynamics of Partitioning. Before discussing the phenomenological model of partitioning of the aromatic molecules between the lipid bilayer and the aqueous solution (Figure 7) similar to one proposed for solubilization in micelles,[2] it is in place to consider the ambigous nature of certain parameters that enter the description. What is experimentally accessible is the (mean) number of molecules, $\bar{n}$, that become associated with the vesicle (or micelle or other small structure), and a distribution constant K_D which relates $\bar{n}$ to the concentration of the species in the aqueous phase and which further is the ratio of the two well-defined rate constants k^+ and k^-

$$K_D = \bar{n}/[P] = k^+/k^- \tag{8}$$

$$\mathrm{Ves,P}_x \underset{k^+}{\overset{xk^-}{\rightleftarrows}} \mathrm{Ves,P}_{x-1} + \mathrm{P} \tag{9}$$

For obtaining a measure of the standard free energy of transfer[12] of P from the aqueous phase to the vesicles, a molar volume V must be assigned to the part of the structure which is available to the solute. This is an ambigous step, not only because the size of the structure usually is poorly defined but also because the solute may be very heterogenously distributed between different parts of the structure. The latter will not have any important kinetic

(12) In this paper as earlier in ref 2 the standard free energy of transfer is based on the molarity concentration scale. The reason for this choice rather than the more popular mole fraction scale was originally simply the fact that a solubility in a given volume of a hydrocarbon solvent is about the same, irrespective of the size of the solvent molecules. The mole fraction scale would also introduce unnecessary complications in the kinetic theory. Since then Ben-Naim's[13] lucid and penetrating discussion of the concept of standard free energy of transfer has come to my attention and has further substantiated the correctness of this choice. Ben-Naim also introduced the useful concept of a *generalized* standard free energy of transfer, equal to the difference in *pseudo-chemical potential* of the solute in the two phases. The standard free energy of transfer used here is such a generalized quantity, which goes over into the conventional standard free energy of transfer in the limit of very low concentration in both phases. For a full discussion of these concepts see ref 13.

(13) (a) A. Ben-Naim, *J. Phys. Chem.*, **82**, 792 (1978). (b) A. Ben-Naim, "Hydrophobic Interactions", Plenum Press, New York, 1980.

consequences as long as all solubilization sites are kinetically equivalent on the time scale of the processes studied. This is expected to be the case for the partitioning of the aromatic molecules of the present study; however, for polar molecules a difference between those associated with the inner and outer surfaces would certainly be expected.

Having defined a molar volume of the structure, a mean microconcentration of the solute in the structure is obtained, and from this a partitioning coefficient, K_p, and a (generalized) standard free energy of transfer[12]

$$c_v = \bar{n}/\bar{V} \tag{10}$$

$$K_p = c_v/[P]_{aq} = K_D/\bar{V} \tag{11}$$

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

The parameters c_v, K_p, and ΔG°_{tr} are thus all subject to the ambiguity connected with $\bar{V}$. A further ambiguity is associated with the rate constants k_o and k_i, the probability per time unit that a molecule just inside (outside) the outer radius will jump out (in). The meaning of "just inside" and "just outside" is not well-defined. The distance between these positions will be denoted λ. A short consideration shows that the products λk_o and λk_i are well-defined quantities. It is also clear that the ratio $k_i/k_o = K_p$.

Consider now a situation where the concentration of P inside the vesicle is kept at zero, and the concentration far from the vesicle is $[P]_{aq}$. The inward flow of P is given by eq 13, where

$$J_+ = 4\pi DR([P]_{aq} - c_+) = 4\pi R^2 \lambda k_i c_+ \tag{13}$$

c_+ is the concentration of P just outside the vesicle surface. Eliminating c_+ with the last equality the rate constant for the entrance process is obtained as in eq 14. The exit rate constant

$$k^+ = \frac{N_A J_+}{[P]_{aq}} = 4\pi DRN_A(R\lambda k_i/D)/(R\lambda k_i/D + 1) \tag{14}$$

is similarily obtained from a consideration of an outward flow process with concentration c_v inside the vesicles and zero far from the vesicles.

$$J_- = (k_o c_v - k_i c_+)4\pi R^2 \lambda = 4\pi RDc_+ \tag{15}$$

$$k^- = \frac{N_A J_-}{c_v \bar{V}} = \frac{4\pi RDN_A(R\lambda k_i/D)}{VK_p(1 + R\lambda k_i/D)} \tag{16}$$

Equations 14 and 16 provide expressions for the entrance and exit rate constants that are quite general. Diffusion-controlled kinetics ensues if $R\lambda k_i \gg D$, when the expressions simplify to

$$k^+ = 4\pi RDN_A \tag{17}$$

$$k^- = 4\pi RDN_A/\bar{V}K_p \tag{18}$$

For similar systems that only differ in size k_i would be about constant. The processes would therefore become more nearly diffusion controlled with increasing size. Since it was found that the solubilization of aromatic hydrocarbons was close to diffusion controlled already in micelles,[2] diffusion-controlled kinetics would be expected also in the case of the much bigger vesicles.

In these derivations it has been tacitly assumed that the vesicles in the solution are well separated so that the presence of other vesicles in the vicinity of a central vesicle will not disturb the diffusion process. Noyes[14] discussed this and other limitations to the diffusion treatment of chemical reactions. For a diffusion process in a system with spherical symmetry the steady-state concentration course is given by $c(r) = c(\infty)(1 - R/r)$. A perturbation of less than 10% requires that the average distance to neighboring vesicles be greater than 20 R. In the typical situation of the present work the average distance between the vesicles was only about 12 R. However, in the stopped-flow experiments a given vesicle was surrounded by both "empty" and "filled" vesicles, and the effects of these will to some extent cancel. At very high vesicle concentrations an increased exchange rate would be expected due to the decreased separation distance, even without any vesicle collisions. The dilution experiments reported in Table II and in the previous paper[3] do give an indication of a slight increase in the migration rate at the higher concentrations that may be due to such an effect (vesicle collisions would be expected to affect the low migration rate of perylene more strongly than that of pyrene).

The Effect of Size on the Exit Rate. Equation 18 predicts an interesting and potentially useful dependence of k^- on the size of the structure. For unilamellar vesicles the volume available for the hydrophobic molecules is expected to increase as R^2. It then follows that k^- is proportional to $1/R$. For other particles like microemulsion particles, multilamellar liposomes, or living organisms a $1/R^2$ dependence may be more appropriate. A quantitative study of this size dependence is underway.

The difference in size dependence between eq 16 and 18 provides a means of deciding the degree of diffusion control. There is already strong evidence that the process is diffusion controlled under the prevailing conditions: (i) a comparison with k^- for the exit of pyrene from micelles shows the expected change by about a factor of 10;[2,3] (ii) qualitatively, there is always a decrease in k^- for both pyrene and perylene in parallel with an increase in the vesicle size, as evidenced by increasing turbidity of the solution; (iii) in the experiment reported in Table II with bigger vesicles k^- decreased by the same factor for both pyrene and perylene from the values pertaining to the smaller vesicles.

The Partition Coefficient K_p. The partition coefficient is a measure of the partitioning of the solute between the lipid bilayer and the surrounding aqueous phase. A change in this factor does well to explain the observed effect of ethanol on the exit rate constants. (It was erroneously stated in the previous communication[3] that the addition of 8 vol % of ethanol had no effect on k^-. The error was caused by some unfortunate circumstances.)

The effect of ethanol was assessed by determining the exit rate constant of pyrene by the excimer fluorescence method.[3] A sample prepared in the usual way by injection to produce an aqueous solution with 2 mM of lipids and 8 vol % of ethanol was diluted sixfold both with 2 mM aqueous Tris buffer and with 2 mM Tris buffer to which 8 vol % of ethanol had been added. The resulting ratio of $k_{Py}^-(8\%)/k_{Py}^-(1.3\%)$ was obtained as 1.6.

The addition of ethanol to the solutions changes the diffusion coefficient. From data in the literature the ratio $D(1.3\%)/D(8\%)$ was estimated as 1.23.[15]

The effect of the alcohol on the partition coefficient is expected to be mainly due to a change of the solubility in the aqueous phase, or of μ_{aq}^0. The alcohol will also be present in the vesicle bilayer. However, the solubility of both pyrene and perylene is about as large (within a factor of 2) in ethanol as in a hydrocarbon solvent, whereas the solubilities in water are several orders of magnitude lower. The solubility of pyrene in water containing about 1 and 8 vol % of ethanol was determined by measuring the fluorescence intensities from a series of solutions prepared by injection of microlitre aliquots of a pyrene in ethanol solution into water and 8% ethanol–water. Below the saturation limits the fluorescence intensities increased in proportion to the pyrene concentration. Above the saturation limit the intensities were constant. The intersection of the straight lines from these two regions gives the saturation point, $0.65 \pm 0.05 \times 10^{-6}$ M in water with about 1% of ethanol and $1.27 \pm 0.05 \times 10^{-6}$ M in water with 8 vol % of ethanol.

From these effects of ethanol on diffusion coefficient and solubility eq 18 predicts

$$k_{Py}^-(8\%)/k_{Py}^-(1\%) = 1.58$$

in excellent agreement with the experimental value.

For vesicles of known size the partition coefficient may be estimated from measured values of the exit rate constant. The

(14) R. M. Noyes in "Progress in Reaction Kinetics", Vol. 1, G. Porter, Ed., Pergamon Press, London, England, 1961.

(15) "CRC Handbook of Chemistry and Physics", 52nd ed., CRC Press, Cleveland, Ohio, 1971.

mean size of the vesicles in the preparations used to get the data of Table I may be estimated as follows. For well-defined vesicles with a radius of 32-nm values of k_{Py}^- at different temperatures were determined previously.[3] From these results a value of $k_{Py}^- = 35\ s^{-1}$ at 18 °C may be interpolated. From the measured k_{Py}^- of Table I, correcting for the ethanol effect and assuming that the $1/R$ dependence of eq 18 applies, a vesicle radius of 25 nm is obtained.

With the assumption of a double-layer thickness of 3 nm[16] the volume of the vesicles is obtained as about 20000 $(nm)^3$. Since the volume of the two C_{16} chains of a lecithin molecule is about 1 $(nm)^3$,[12] the aggregation number is about 20000.

With these values for the vesicle dimensions, an assumed diffusion coefficient of $10^{-5}\ cm^2\ s^{-1}$, and the measured k^- the partition coefficients are obtained as $K_p(Py) = 3.4 \times 10^5$ and $K_p(Pe) = 80 \times 10^5$. These values may be compared with the partition coefficients for the aromatic molecules between a hydrocarbon phase and water. For pyrene the solubility in a hydrocarbon[2] is about 0.072 M and in water is 6×10^{-7} M,[17] giving a partition coefficient of 1.2×10^5, somewhat lower than that for the vesicles. For perylene the solubility in heptane was estimated as 0.6×10^{-3} M, while that in water has been given as 1.6×10^{-9} M.[18] The partition coefficient is then only 3.7×10^5 or a factor of 20 smaller than that suggested by the exit rate for the vesicles.

This observation indicates that the solubility of perylene in the vesicle bilayer is much greater than in heptane, which was also found by direct observation. The highest perylene/lipid ratio obtained in any of the preparations was estimated by absorption measurements as close to 0.01, corresponding to a microconcentration of 0.017 M in the lipid bilayer (this ratio was not exceeded even when higher concentrations were aimed at and may be close to the saturation limit).[19] The partitioning coefficient based on 0.017 M as the solubility in the bilayer becomes $K_p(Pe) = 100 \times 10^5$, in much better agreement with the estimate from the exit rate.

A possible explanation of the remarkable solubilizing power of the vesicles in the case of perylene could be that the bilayer actually contained aggregates of perylene. However, there was no indication from either absorption or fluorescence spectra of aggregation. Furthermore, the aggregation would imply a concentration dependence on the partitioning coefficient and also k^-. This was tested experimentally as follows.

Case i experiments were performed under the same conditions as in Table I with end concentration of perylene of both 10 and 2.5 μM. The results were in perfect agreement. At the high perylene concentration a single exponential fit gave $2k_{Pe}^- = 5.75 \pm 0.50\ s^{-1}$, which split into 8.7 and 3.2 s^{-1} on two-component analysis. The corresponding values at low perylene concentration were $2k_{Pe}^- = 5.79 \pm 0.55\ s^{-1}$, splitting into 8.8 and 3.3 s^{-1}. Thus, there is no indication of an aggregation process occurring in the vesicles. (Incidentally, such a process would have provided an alternative explanation to the nonexponentiality of the results.)

A second, more thought-provoking explanation of the high solubility of perylene in the vesicle bilayer would be that the insertion of perylene relaxes some packing constraints of the bilayer so that the total system gains free energy although the perylene concentration is above that of normal solubility in a hydrocarbon phase. Such a stabilization of the bilayer would be a quite general effect, determined mainly by the size and form of the hydrophobic molecule. However, it would be relatively unimportant for the solubility of a molecule which, like pyrene, already has a high solubility in a normal hydrocarbon. This effect would have a number of consequences on the molecular level, e.g., regarding the location and orientation of the solutes. At present, this explanation is a mere speculation. The phenomenon is worth further study.

Conclusions and Comparison with Earlier Investigations

(i) The transfer mechanism is a migration through the aqueous phase. The rate of the process is then determined by the exit rate of the solute from the vesicles.

(ii) In the case that diffusion away from the vesicle surface sets the limit to the exit rate constant the simple eq 18 relates the rate constant to the diffusion coefficient of the solute in the aqueous phase, the size of the vesicle, and the partitioning coefficient of the solute between the vesicle bilayer and the aqueous phase. The results are in agreement with this equation, which suggests that the transfer process is diffusion limited.

All investigators[5-8] agree on the first point: there is no observable dependence of the transfer times on the vesicle concentration. The mechanism of the transfer process is a migration through the aqueous phase. However, Doody et al.[8] conclude from their results that the rate-limiting step is the passage over a free-energy barrier just outside the surface of the bilayer created by the "bound-water structure" in this layer. Their main argument for this conclusion was that the free-energy change associated with the formation of the kinetic transition state, as estimated with absolute rate theory ($k^- = kT/h \exp[-\Delta G^{\ddagger}/RT]$), was greater than the standard free energy of transfer from the bilayer to the aqueous phase, $\Delta G^\circ = RT \ln (x_{ves}/x_{aq})$. There is reason to question the validity of this comparison, both regarding the use of absolute rate theory for the diffusion of the pyrene nonanoic acid out from the bilayer,[20] and the comparison of a ΔG based on a mole fraction ratio with one calculated from the rate constant.

Instead of pursuing this line of thought we will use the partitioning data of Doody et al. to calculate a partitioning coefficient as defined in eq 12. The value thus obtained for the ionized form of PNA is close to the value for pyrene in the present work. Equation 18 would then predict a value of the exit rate constant of the same magnitude as for pyrene or 1 order of magnitude larger than that actually found. However, there is a trend in the partitioning data, most remarkable for the acidic form of the probe but considerable also for the anion: at 15 °C the partioning coefficient changes by a factor of 4 on a change of the concentration in the aqueous phase by a factor of 14. The trend is probably an indication on association in the aqueous phase, which was shown by Smith and Tanford[22] to be of critical importance for the partitioning of long-chain alkyl carboxylic acids between heptane and water. It is thus quite probable that the partitioning coefficient of PNA is appreciably bigger than that found by Doody et al.

Kano et al.[8] utilized singlet excitation energy transfer between alkylated alloxazines and isoalloxazines to study the migration of these molecules between vesicles in type iv experiments. Since no data on the partitioning are available, it is not possible to conclude whether their long relaxation times are in accord with eq 18.

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