Interactions of Dicarboxylic Porphyrins with Unilamellar Lipidic Vesicles: Drastic Effects of pH and Cholesterol on Kinetics†

Katerina Kuzelová and Daniel Brault*

Laboratoire de Biophysique, INSERM U.201, CNRS U.A. 481, Muséum National d'Histoire Naturelle, 43 rue Cuvier, 75231 Paris Cedex 05, France

Received February 21, 1995; Revised Manuscript Received May 22, 1995®

ABSTRACT: The effect of pH on the transfer of deuteroporphyrin from dimyristoylphosphatidylcholine (DMPC) unilamellar vesicles to human serum albumin is investigated using a stopped-flow with fluorescence detection. The kinetics of this process allows for the determination of the rate constants for the porphyrin exit from the outer vesicle layer to the bulk aqueous medium (k_{off}), and for its movement from the inner to the outer vesicle layer (k_{to}). Both k_{off} and k_{to} are found to strongly depend on the pH. The observed behavior can be described by classical titration curves and is most likely due to protonation equilibria involving the two carboxylic groups of the porphyrin. A pH increase accelerates the exit of the porphyrin. The reverse effect is observed for its movement through the bilayer. The presence of cholesterol in the DMPC bilayer also strongly affects the interactions of the porphyrin with the vesicles. The rate constant k_{to} is dramatically reduced by increasing the cholesterol content. An irregularity is noted around 10−20 mol % cholesterol. The results are discussed in relation to the preferential uptake of porphyrins by tumors, a basis of photodynamic therapy, and to possible pH-mediated relocalization of porphyrins among subcellular structures.

The retention of certain porphyrins by solid tumors, as compared to normal surrounding tissues, has long been recognized (Policard, 1924; Lipson et al., 1961). This retention coupled to the ability of these molecules to generate short-lived toxic species upon light irradiation (Spikes & Straight, 1990) forms the basis of a new therapeutic approach, photodynamic therapy. Following pioneering work by Dougherty et al. (1979), this therapy and laser technics have been developing jointly (for a general overview, see Kessel, 1990). Most of the current clinical treatments are carried out by using Photofrin, a preparation derived from a dicarboxylic porphyrin, hematoporphyrin. In fact, this preparation is a complex mixture made of more or less hydrophobic molecules including monomeric porphyrins as well as dimeric and oligomeric components (Dougherty, 1987). Free carboxylic chains are however retained in all of these structures.

There are still major unknowns regarding the mechanisms responsible for tumor selectivity and about the nature of the main targets at tumor or cellular levels. The selectivity for tumor cells has been suggested to arise from a favored low-density lipoprotein receptor-mediated pathway (Jori et al., 1984; Reyftmann et al., 1984). However, there is also large evidence for passive incorporation of porphyrins within cells (Dellinger et al., 1986; Böhmer & Morstyn, 1985). Studies on the kinetics and the reversibility of the porphyrin cellular uptake clearly show that passive incorporation is a biphasic process. Within a few tens of seconds, the porphyrin becomes loosely bound to the cytoplasmic membrane. A slower step involves migration to cytoplasm and internal membranes (Dellinger et al., 1986; Böhmer & Morstyn, 1985). The acidification of the tumor interstitial fluid is likely to take part in the selectivity of carboxylic porphyrins for tumor cells (Moan et al., 1980; Brault et al., 1986a,b; Brault, 1990; Pottier & Kennedy, 1990; Thomas & Girotti, 1989; Peng et al., 1991). Indeed, as well illustrated by studies on various amphiphiles including organic acids (Prestegard et al., 1979), fatty acids (Kamp & Hamilton, 1992, 1993; Doody et al., 1980), and bile acids (Cabral et al., 1987), the neutralization of the carboxylate groups of these molecules at lower pH accelerates the transbilayer movement considerably. The hydrophobic/hydrophilic balance and the charge of the molecules are also important determinants of porphyrin uptake and subcellular localization (Moan & Berg, 1992).

In a previous paper, the interactions of a dicarboxylic porphyrin, deuteroporphyrin, with dimyristoylphosphatidylcholine unilamellar vesicles, and the transfer of this porphyrin from the vesicles to albumin, were analyzed in detail from a kinetic point of view. In particular, we were able to identify the steps corresponding to the exit of the porphyrin from the outer leaflet of the bilayer and to its flip-flop between the two leaflets (Kuzelova & Brault, 1994). In the present paper, the effect of pH on these processes is investigated. Drastic changes in the exit and flip-flop rates with pH are demonstrated. In keeping with the above-mentioned studies, they are most likely related to the protonation state of the carboxylic chains of the porphyrin. In order to better mimic biological membranes, vesicles made of various mixtures of phospholipids and cholesterol are also studied. Although the flip-flop and exit rates are significantly reduced, the effect of pH is retained.

† This work was supported by a grant from the French government to one of us (K.K.).

® Abstract published in *Advance ACS Abstracts,* August 15, 1995.

0006-2960/95/0434-11245$09.00/0

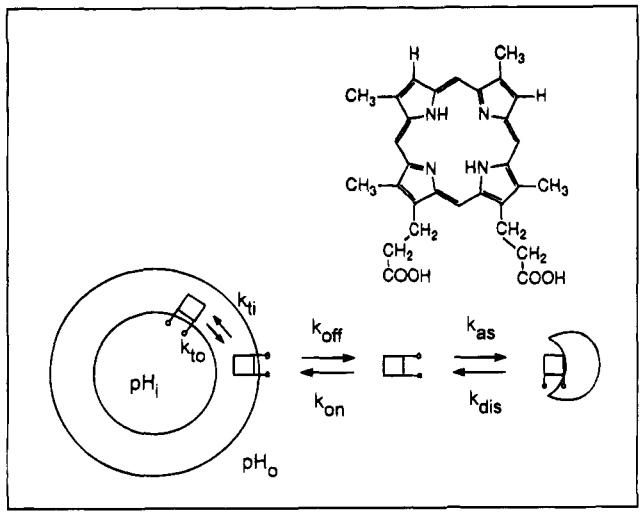


FIGURE 1: Structure of deuteroporphyrin (neutral form) and scheme for its transfer from vesicles to albumin.

These results are discussed with regard to the known effect of pH on the incorporation of porphyrins by cells (Moan et al., 1980; Böhmer & Morstyn, 1985), to the possible role of the acidification of the interstitial liquid in tumors as a selectivity factor, and to possible pH-dependent partitioning of porphyrins among subcellular structures. These results also have obvious relevance to the problem of heme biosynthesis, which involves translocation of porphyrins across mitochondrial membranes (Dailey, 1990).

MATERIALS AND METHODS

Measurements. Most of the kinetic measurements were performed with the aide of a Durrum-Gibson stopped-flow apparatus (Palo Alto, CA) equipped with fluorescence detection. Emission spectra and slower kinetics were recorded using a SPEX spectrofluorimeter (Edison, NJ). The experimental arrangements, as well as the procedure used to fit the signals by mono- or biexponential curves, were previously described in detail (Kuzelova & Brault, 1994).

Chemicals. Dimyristoylphosphatidylcholine (DMPC)[1] and lipid-free human serum albumin (HSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Cholesterol (chol) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Deuteroporphyrin (DP), the structure of which is shown in Figure 1, was prepared in our laboratory as described elsewhere (Brault et al., 1986b). Its purity, as checked by HPLC (Dellinger & Brault, 1987), was determined to be greater than 99%. The experiments were performed using 0.15 M NaCl solutions buffered with Tris (20 mM) or phosphate (20 or 60 mM).

Sample Preparation. Small unilamellar vesicles made of either pure DMPC or DMPC/chol mixtures were prepared by extrusion through polycarbonate membrane filters with pores of 0.05 μm, as previously described (Kuzelova & Brault, 1994). Typical stopped-flow experiments involved the following procedure. An aliquot (6.7 μL) of a 6×10^{-4} M DP solution in tetrahydrofuran was added to the vesicle preparation (20 mL). Then, the solution was incubated at 32 °C for 20 min to allow incorporation and partition of the porphyrin between the two hemileaflets of the vesicles. As these processes were slow for DMPC/chol vesicles, the porphyrin was added, in this case, to the lipid mixture prior to the vesicle formation. HSA was directly dissolved in the buffer. Phosphate or Tris buffers were used, depending on the pH ranges investigated. The pH of all vesicle and albumin solutions was controlled and eventually readjusted to the desired values (±0.01 unit). This readjustment was necessary, especially for the solutions containing high albumin concentrations. The effect of temperature on the pH of the buffered solutions was also carefully taken into account using correction curves. This effect was more important with Tris buffer. In the present work, all the pH values are given for 32 °C (the temperature of all measurements). Note that in our previous work (Kuzelova & Brault, 1994), the current pH value of 7.4 was measured at room temperature, corresponding to a value of 7.2 at 32 °C. After mixing in the stopped-flow, the total lipid concentration (DMPC or DMPC + chol) and the porphyrin concentration were 2×10^{-4} M and 1×10^{-7} M, respectively.

The size distribution of vesicles was determined by quasi-elastic light scattering using a Coulter N4M analyzer (Hialeah, CA). In all samples, only one population of vesicles was found. A very small increase of the vesicle diameter with pH was noted. Values of 59, 60, 63, and 64 nm (±2 nm, limits for 95% of population) were obtained for pH 5.8, 6.6, 7.2, and 8.3, respectively. The influence of cholesterol was more pronounced. At pH 7.2, the diameters were 63 ± 2, 67 ± 3, 70 ± 2, 73 ± 3, 77 ± 3, and 86 ± 5 nm for vesicles containing 0%, 10%, 17%, 23%, 33%, and 53% of cholesterol (molar fraction), respectively.

Rationale and Theoretical Models. (A) Transfer of Porphyrins from Vesicles to Albumin: Standard Experiments. Kinetic models for the interaction of a porphyrin molecule with a vesicle and for the transfer of a porphyrin from a vesicle to albumin have been presented in detail in our previous work (Kuzelova & Brault, 1994). Although the two systems give, to some extent, complementary information, the transfer to albumin is particularly useful. It makes it possible to avoid problems related to the aggregation of porphyrins in aqueous solutions, a phenomenon which takes a greater importance in the pH range investigated below. Indeed, although the transfer of the porphyrin occurs via the aqueous phase, its concentration in water can be kept very low by using vesicle and albumin concentrations high enough to shift the equilibria in the desired direction. In these conditions, the transfer follows two-exponential kinetics. Related systems involving transfer of fatty acids to albumin were previously examined in details by Kamp and Hamilton (1992, 1993). The validity of the scheme in Figure 1 has been verified in our previous paper. This system will be used throughout the present study. We also established (Kuzelova & Brault, 1994) the expressions connecting the observed rate constants to the intrinsic rate constants of the system (see Figure 1), i.e., the constants for porphyrin binding to the outer vesicle layer (k_{on}), for exit from the outer layer (k_{off}), for flip-flop movement toward the inner (k_{ti}) and the outer (k_{to}) layer, for the association of the porphyrin to albumin (k_{as}), and for the dissociation (k_{dis}) from albumin. The observed rate constants for the porphyrin transfer from vesicles to albumin were thus predicted to be:

[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DP, deuteroporphyrin; HSA, human serum albumin; chol, cholesterol.

$$k_{1,2} = (\alpha + \beta + \gamma + \delta \pm [(\alpha + \beta + \gamma + \delta)^2 - 4(\alpha\gamma + \beta\delta + \alpha\delta)]^{1/2})/2 \quad (1)$$

where

$$\alpha = k_{to}$$

$$\beta = k_{ti}$$

$$\gamma = \frac{k_{off}k_{as}[HSA]}{k_{on}[DMPC] + k_{as}[HSA]}$$

$$\delta = \frac{k_{dis}k_{on}[DMPC]}{k_{on}[DMPC] + k_{as}[HSA]}$$

We have shown previously (Kuzelova & Brault, 1994; Vever-Bizet & Brault, 1993) that the constant k_{on} is diffusion controlled and depends on neither the structure nor the ionization state of the porphyrin. Besides, the two rate constants for the passage of the porphyrin through the bilayer (k_{to}, k_{ti}) were found to be approximately the same (Kuzelova & Brault, 1994).

In the present work, we investigate the effects of the membrane composition and of the charge of the porphyrin molecule on its exit from the vesicles (k_{off}) and on its flip-flop (k_{to}, k_{ti}). These rate constants can be easily derived from the kinetics of porphyrin transfer from vesicles to albumin. For each pH value and vesicle composition, a set of measurements is performed using several albumin concentrations and a fixed vesicle concentration. The observed rate constants are fitted among the whole range of albumin concentrations using relation 1 to determine the pertinent intrinsic rate constants. It is also interesting to consider limiting conditions which illustrate some features of the system and provide first estimates of rate constants.

When the albumin concentration is low compared to that of vesicles ([DMPC] ≫ [HSA] and $\delta \gg \gamma$), γ and $\alpha\gamma$ can be neglected with regard to δ and $\alpha\delta$, respectively. Then, eq 1 reduces to:

$$k_{12} = \frac{(\alpha + \beta + \delta) \pm [(\alpha + \beta + \delta)^2 - 4\delta(a + \beta)]^{1/2}}{2} = \frac{(\alpha + \beta + \delta) \pm [(\alpha + \beta - \delta)^2]^{1/2}}{2} = \frac{\alpha + \beta + \delta \pm |\alpha + \beta - \delta|}{2}$$

The two observed rate constants, k_1 and k_2, reach the values

$$\alpha + \beta = k_{to} + k_{ti} \quad (2)$$

and

$$\delta = k_{dis} \quad (3)$$

The highest value corresponds to the faster phase (k_1) and the smallest value to the slower one (k_2).

At high albumin concentration ([HSA] ≫ [DMPC] and $\gamma \gg \delta$), eq 1 reduces to:

$$k_{1,2} = \frac{(\alpha + \beta + \gamma) \pm [(\alpha + \beta + \gamma)^2 - 4\alpha\gamma]^{1/2}}{2}$$

The development and rearrangement of the terms under the root lead to:

$$k_{1,2} = \frac{(\alpha + \beta + \gamma) \pm [(\gamma - \alpha)^2 + 2\beta(\alpha + \gamma) + \beta^2]^{1/2}}{2} = \frac{(\alpha + \beta + \gamma) \pm [(\beta - \alpha + \gamma)^2 + 4\alpha\beta]^{1/2}}{2}$$

The latter relation can be further simplified according to the relative values of the exit and flip-flop rate constants. When the flip-flop is slow (k_{to}, $k_{ti} \ll k_{off}$), the second-order term $\alpha\beta$ can be neglected, which yields:

$$k_{1,2} = \frac{\alpha + \beta + \gamma \pm |\beta - \alpha + \gamma|}{2}$$

$$k_1 = \gamma + \beta = k_{off} + k_{ti} \quad (4)$$

$$k_2 = \alpha = k_{to} \quad (5)$$

On the other hand, if the flip-flop is faster ($k_{to} \approx k_{ti} \gg k_{off}$), we have:

$$k_{1,2} = \frac{(\alpha + \beta + \gamma) \pm (\alpha + \beta)}{2}$$

$$k_1 = \alpha + \beta + \frac{\gamma}{2} = k_{to} + k_{ti} + \frac{k_{off}}{2} \quad (6)$$

$$k_2 = \frac{\gamma}{2} = \frac{k_{off}}{2} \quad (7)$$

The derivation of the rate constants of interest is illustrated in Figure 2. At pH 7.56, the flip-flop is much slower than the dissociation rate constants k_{off} and k_{dis}. The observed rate constant for the fast phase of the kinetics (k_1) is shown to vary from k_{dis} to $k_{off} + k_{ti}$ as a function of the albumin concentration according to relations 3 and 4 (see Figure 2a). It is worth noting that the shape of the curve depends on the ratio k_{as}/k_{on}. As predicted by relation 5, the rate constant of the slow phase yields the value of k_{to} by extrapolation to the higher albumin concentration (Figure 2b). The excellent precision on the value of k_{to} which is obtained in these transfer experiments is well illustrated in Figure 2b.

At low pH values, the rate of the flip-flop exceeds that of the exit from the outer vesicle layer which becomes the rate-limiting step ($k_{to} > k_{off}$). It can be predicted by numerical simulation (not shown) that the amplitude of the fast phase becomes negligible. The observed kinetics are practically monoexponential (Figure 2c). As predicted by relations 3 and 7, the observed rate constant of this single phase varies between k_{dis} and $k_{off}/2$ for the lowest and highest albumin concentrations, respectively.

(B) Transfer of Porphyrins from Vesicles to Albumin: pH Jump Experiments. As outlined above, at low pH, the flip-flop rate constant cannot be derived from experiments on the transfer of the porphyrin from vesicles to albumin. Direct measurements made by mixing vesicle solutions with the porphyrin dissolved in aqueous solutions were also precluded by porphyrin aggregation. Following previous studies (Eastman et al., 1991; Kamp & Hamilton, 1993), we therefore designed a protocol, hereafter named "pH-jump experiments", to determine the rate constant k_{to} in this pH range. In these experiments, the pH of the albumin solution was

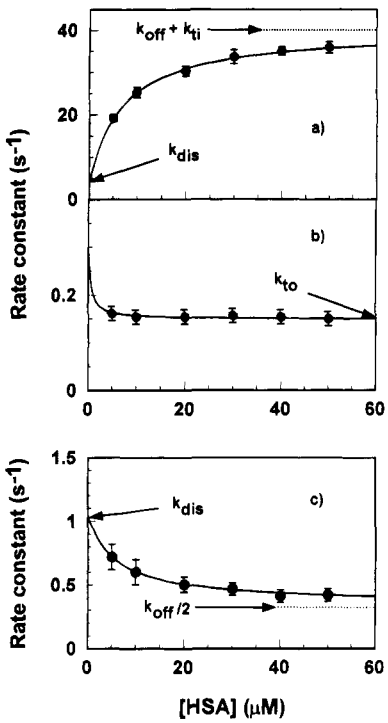
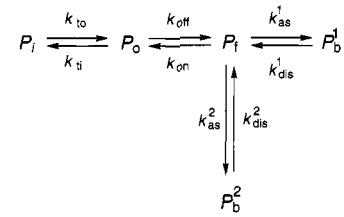


FIGURE 2: Observed rate constants of the transfer of deuteroporphyrin from DMPC vesicles to albumin: dependence on albumin concentration. Temperature: 32 °C. Each point corresponds to the mean and standard deviation calculated from 10−20 rate constant determinations. DMPC concentration was 2×10^{-4} M. (a) Fast phase at pH 7.56, Tris buffer; (b) slow phase at pH 7.56, Tris buffer; (c) single phase at pH 5.77, phosphate buffer. The full lines are theoretical curves calculated using eq 1 with the following values: (a) and (b) $k_{on} = 1.73 \times 10^6$ M^{-1} s^{-1}, $k_{off} = 40$ s^{-1}, $k_{as} = 5.9 \times 10^7$ M^{-1} s^{-1}, $k_{dis} = 3.5$ s^{-1}, $k_{to} = k_{ti} = 0.15$ s^{-1}. (c) $k_{on} = 1.73 \times 10^6$ M^{-1} s^{-1}, $k_{off} = 0.65$ s^{-1}, $k_{as} = 6.75 \times 10^7$ M^{-1} s^{-1}, $k_{dis} = 1.05$ s^{-1}, $k_{to} = k_{ti} = 5.3$ s^{-1}.

adjusted to a higher value than that of the vesicle solution. Upon mixing in the stopped-flow apparatus, the pH of the bulk aqueous solution (pH_o) reached a value intermediate between the initial pH's of the albumin and vesicle solutions. In the pH range investigated, this pH change took place within the mixing time (Benson, 1960). Thus, after mixing, the pH values were different outside (pH_o) and inside (pH_i) of the vesicles, the latter one being equal to the initial pH of the vesicle solution (see Figure 1). As discussed later, the exit of the porphyrin accelerated when the pH of the bulk aqueous solution was increased. By a convenient choice of the pH of the solutions before mixing, it was therefore possible to quickly remove porphyrin molecules from the outer layer and to make rate-limiting the movement of the porphyrin from the inner leaflet to the outer one. Consequently, biphasic kinetics were observed. The rate constants k_{off} and k_{to} were determined as usual from extrapolations at the highest albumin concentrations, but in this case they corresponded to the values pH_o and pH_i, respectively. Obviously, this protocol was valid only if the diffusion of protons through the bilayer was slow enough. This point was checked as described in the Results section.

(C) Porphyrin Binding Sites on Albumin. It was assumed in our previous paper (Kuzelova & Brault, 1994), as well as in the above analysis, that albumin presents only one strong binding site for porphyrins. This hypothesis, which was well sustained by experimental evidence at neutral pH (Rotenberg et al., 1987), might not be valid at lower pH as suggested by Reddi et al. (1981). Therefore, we have also examined a kinetic model based on the existence of two binding sites, according to the following scheme:



where P_i, P_o, P_f, P^1_b and P^2_b are the concentrations of the porphyrin located in the inner vesicle hemileaflet, in the outer vesicle hemileaflet, in the bulk aqueous solution, or bound to the first or the second albumin site, respectively. The kinetic description of this model (not shown) leads to a cubic equation predicting three observable rate constants.

As a matter of fact, a third, very slow kinetic phase was observed especially when the porphyrin transfer was investigated at low pH and for low albumin concentrations. The association and dissociation rate constants involving this second site were estimated by numerical simulation. It is important to note that the presence of this additional very slow phase did not modify the value of the intrinsic rate constants obtained using relation 1 by considering the first two phases.

RESULTS

Influence of pH. As previously shown (Kuzelova & Brault, 1994), the fluorescence spectra of deuteroporphyrin are sensitive enough to the environment to easily monitor the transfer of the porphyrin from the vesicles to albumin. The fluorescence spectra of the porphyrin incorporated in the vesicles have been shown to be the same in a large pH range (Brault et al., 1986b, 1993) and characteristic of a hydrophobic environment. This strongly suggested that the molecule remained oriented in each hemileaflet with its hydrophobic core buried in the lipidic phase and its carboxylic chains, either ionized or not, interacting with the polar heads of the phospholipids (Kuzelova & Brault, 1994; Brault, 1990). The fluorescence spectra of the porphyrin bound to albumin was also fairly independent of pH between 5.8 and 8.3 (not shown).

(A) Standard Experiments. These experiments involved solutions prepared at the same pH. Upon mixing of albumin with DMPC vesicles preloaded with porphyrin, a decay of fluorescence was observed when the excitation wavelength was set at 389 nm. Examples are given in Figure 3 for several pH's at 32 °C. As long as the pH was greater than about 6.7, two kinetic phases were clearly distinguished. The intrinsic rate constant for the exit of the porphyrin from the vesicles (k_{off}) and that for its movement from the inner leaflet to the outer one (k_{to}) were obtained from the dependence of the observed rate constants for the fast and slow phases on the albumin concentration using relation 1. An example is given in Figure 2a,b. In keeping with our previous paper (Kuzelova & Brault, 1994), k_{off} was calculated assuming that the values of the constants k_{ti} and k_{to} were similar. In any instance, as the flip-flop was slow compared to the exit, this assumption had little consequence in this pH range.

(B) pH-jump Experiments. As the first phase of the transfer kinetics slowed down and the second one accelerated

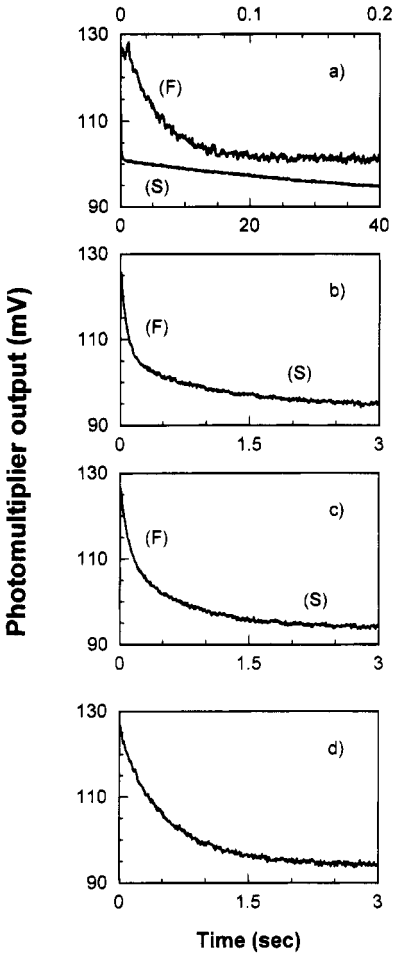


FIGURE 3: Fluorescence intensity changes recorded upon mixing albumin and DMPC vesicles preloaded with deuteroporphyrin. Temperature: 32 °C, excitation wavelength: 389 nm. Final concentrations after mixing: [DMPC] = 2×10^{-4} M, [DP] = 1×10^{-7} M, [HSA] = 5×10^{-5} M. (F) fast phase, (S) slow phase. (a) pH 7.8, fast phase: upper time scale, slow phase: lower time scale, (b) pH 7.0, (c) pH 6.8, (d) pH 6.4.

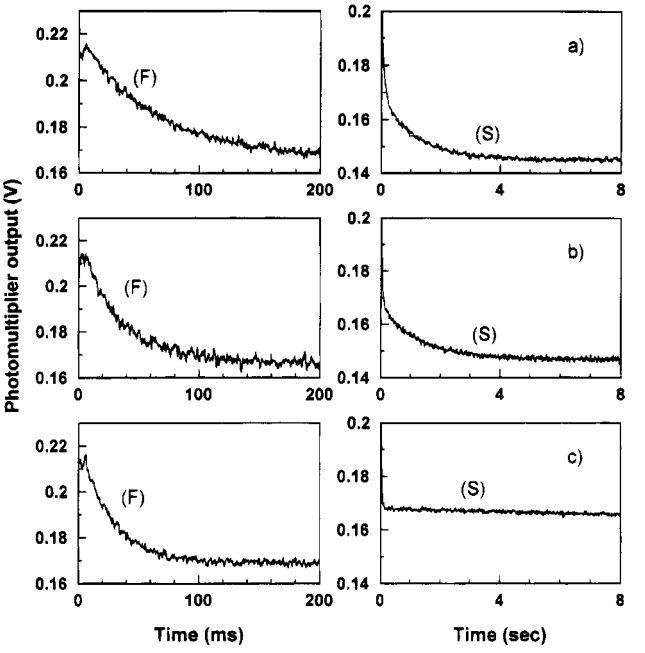


FIGURE 4: "pH-jump" control experiment. Fluorescence intensity changes recorded upon mixing DMPC vesicles preloaded with deuteroporphyrin and albumin. The pH was as follows: (a) 7.04 both in vesicle and albumin solutions (20 mM phosphate buffer); (b) 7.04 (20 mM phosphate buffer) in vesicle solution and 8.47 (60 mM phosphate buffer) in albumin solution, resulting in pH 7.7 outside of vesicles after mixing; (c) 7.7 in both vesicle and albumin solutions (20 mM phosphate buffer). (F) fast phase, (S) slow phase. Final concentrations after mixing: [DMPC] = 2×10^{-4} M, [DP] = 1×10^{-7} M, [HSA] = 5×10^{-5} M. Temperature: 32 °C, excitation wavelength: 389 nm. Note that the fast phase in (b) is the same as in (c), whereas the slow phase in (b) is the same as in (a).

with decreasing pH, it was no longer possible to distinguish between them below pH 6.7. Therefore, pH-jump experiments were designed as detailed in the experimental section. They consisted of mixing the porphyrin preloaded vesicles solution and the albumin solution which were prepared at different pH. This made it possible to create a pH difference between the bulk aqueous solution and the internal aqueous volume of the vesicles. In these conditions, the value of k_{to} was governed by the pH of the internal aqueous volume and that of k_{off} by the pH of the bulk solution. To be valid, this method requires that leakage of protons through the bilayer is slow enough. Although this assumption was supported by literature data (Kamp & Hamilton, 1992, 1993), it was checked again in our system by comparing the results obtained by the pH-jump method to those obtained by the standard method in a pH region where biphasic transfer was still observed. The porphyrin loaded vesicle solution was prepared at pH 7.04 (phosphate buffer, 20 mM). The pH of the albumin solution was 8.47 (phosphate buffer, 60 mM) which resulted in a pH of 7.7 in the bulk solution after mixing. Examples of the observed traces are given in Figure 4. The k_{off} and k_{to} values derived from the analysis of these biphasic kinetics corresponded exactly to those determined by standard experiments at pH 7.7 and at pH 7.04, respectively. Similar control experiments were also performed with pH-jump in the opposite way. The pH values were adjusted to 8.24 and 6.28 (20 mM Tris buffer) in the vesicle and albumin solutions, respectively. The pH after mixing was 7.71. Once again, the k_{off} value thus obtained was in excellent agreement with that found in conventional experiments at this pH.

The validity of the pH-jump method being established, it was used to determine the rate constants from pH 6.7 down to pH 5.8. For example (Figure 5), in order to determine k_{to} at pH 6.5, the pH was adjusted to 6.5 in the vesicle solution and to 8.5 in the albumin solution. The pH of the 1:1 mixture of the two solutions was measured to be 7.05. Thus, after mixing in the stopped-flow apparatus, the pH of bulk aqueous phase rapidly changed to 7.05 whereas the original value (6.5) was maintained in the vesicles. Consequently, the exit rate of porphyrin from the outer layer was accelerated and a well-defined second phase (related to the flip-flop) was observed (Figure 5b). The rate constants were then derived as described above.

Below pH 6.4, the kinetics appeared fairly monoexponential in standard experiments, and no significant improvement of the quality of the fit (see Kuzelova & Brault, 1994) was obtained by assuming biexponential decay. Having determined the k_{to} value from pH-jump experiments, we incorporated it as a constant in relation 1. Then, the value of k_{off} was determined by numerical simulations assuming that the observed monoexponential decay corresponded to the slower phase (k_2). Further simulations using the values

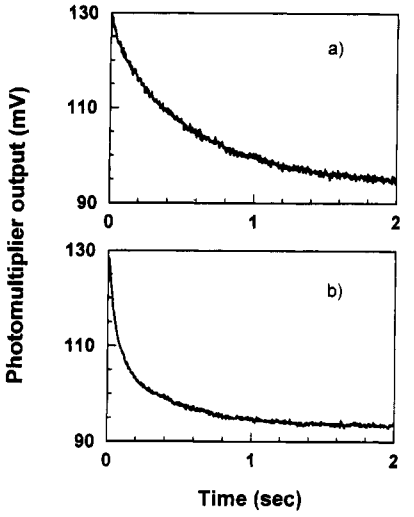


FIGURE 5: Example of a "pH-jump" experiment (b) as compared to a standard experiment (a). Fluorescence intensity changes recorded upon mixing DMPC vesicles preloaded with deuteroporphyrin (pH 6.5) and albumin. The pH of the albumin solution was (a) 6.5, (b) 8.5. The external pH after mixing was 7.05 in (b). Other conditions were the same as in Figure 4.

of k_{to} and k_{off} thus obtained did show that the relative amplitude of the faster component was only about 10% at pH 6.4. The amplitude of this component was still reduced at lower pH. In the most acidic conditions investigated, the exit of the porphyrin became rate-limiting compared to its flip-flop, and the rate constant of the observed monoexponential decay extrapolated to $k_{off}/2$ at the highest albumin concentration (see relation 7 and Figure 2c).

In Figure 6 is shown the dependence on pH of the k_{off} and k_{to} values obtained by using the different methods explained above. Above pH 9, k_{to} was lower than 0.01 s^{-1}.

The rate constants for the interaction of the porphyrin with albumin were estimated from plots such as that shown in Figure 2a. The dissociation constant (k_{dis}) is given by the intercept of the plot (see Figure 2a,c). Although the precision of this value was quite poor, especially for the highest pH values, k_{dis} appeared to increase with pH between 5.8 and 7.2 (compare extrapolations of panels a and c, Figure 2). The shape of the curve is determined by the relative values of k_{on} and k_{as}. It remained unchanged for pH ranging between 5.8 and 7.5. In fact, it was shown previously (Kuzelova & Brault, 1994; Vever-Bizet & Brault, 1993) that the k_{on} value approached the diffusion limit and depended only slightly on the pH in this region. Above pH 7.5, the curvature of plots such as that shown in Figure 2a was less pronounced, indicating a progressive reduction of the ratio k_{as}/k_{on} with increasing pH. Independent measurements of k_{on} were performed above pH 7.5 by mixing aqueous solutions of the porphyrin with vesicles (see Kuzelova & Brault, 1994, for the protocol). The k_{on} value was found to decrease slightly, attaining about 1.2×10^6 M^{-1} s^{-1} (3.6×10^{10} Ves^{-1} s^{-1} when expressed per mole of vesicles) at pH 8.5.

An additional very slow phase was observed in the transfer kinetics to albumin, especially at low pH. Whatever the excitation wavelength was, these slow changes were always in the same direction as the faster ones. The amplitude of this third phase was found to decrease with increasing albumin concentration. Both its relative amplitude and its rate constant decreased when the pH was increased. At pH

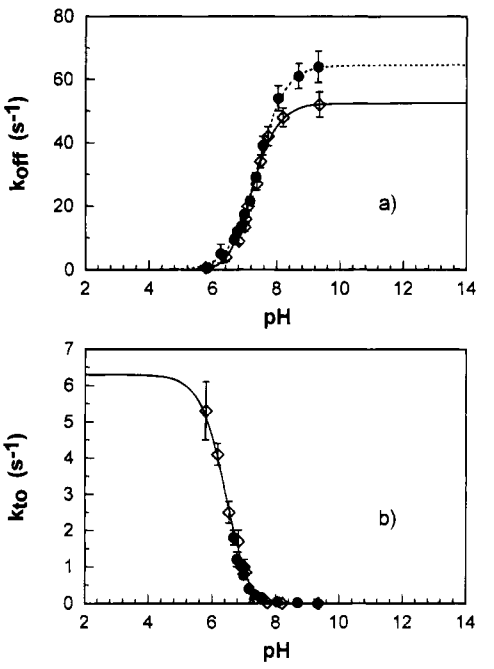


FIGURE 6: (a) The exit rate constant (k_{off}) of deuteroporphyrin from DMPC vesicles at 32 °C as a function of pH. (b) The flip-flop rate constant (k_{to}) of deuteroporphyrin from the inner to the outer layer of DMPC vesicles at 32 °C as a function of pH. The points below pH 6.7 were determined using the "pH-jump" method. (●) Tris buffer, (◇) phosphate buffer. Theoretical curves are calculated using the following: (—) $pK_1 = 7.2$, $pK_2 = 6.45$, $k_{off} = 0$, 0, and 52.5 s^{-1} for the neutral, monoanionic, and dianionic form, respectively, $k_{to} = 6.3$, 0, and 0 s^{-1} for the neutral, monoanionic, and dianionic form, respectively. (···) $pK_1 = 7.4$, $pK_2 = 6.75$, $k_{off} = 0$, 0, and 64.5 s^{-1} for the neutral, monoanionic, and dianionic form, respectively.

5.77, the rate constant of this third phase was around 0.05–0.2 s^{-1}, slightly increasing with the albumin concentration. Its relative amplitude decreased from about 45% of the total signal at the lowest albumin concentration (5×10^{-6} M) to about 20% at the highest one (5×10^{-5} M). At pH 7.15, the rate constant of the third phase was around 0.02–0.05 s^{-1}. Its contribution to the total signal was about 20% and less than 3% for albumin concentrations of 5×10^{-6} and 3×10^{-5} M, respectively. It was not observed at the highest albumin concentrations.

As numerical simulations suggested that such a behavior could be due to the existence of a second porphyrin binding site on albumin (see experimental section), we studied the inverse porphyrin transfer, i.e., from albumin toward the vesicles. In this case, when the fluorescence was excited at 389 nm, an increasing signal was observed. The third phase was still present. Moreover, for the same experimental conditions (i.e., identical pH and vesicle and albumin concentrations), the rate constant of this third phase was found to be identical to that determined in the direct transfer experiment.

Influence of Cholesterol. In our previous paper (Kuzelova & Brault, 1994), the effect of cholesterol was briefly described with the aim of providing further evidence for our scheme and for the assignment of the kinetic phases. As expected, at 32 °C, 50% mol/mol of cholesterol reduced the rate of porphyrin exit and seemed to obstruct its flip-flop. These effects are now investigated in more detail as a function of the cholesterol content and of the pH. The effects on k_{off} and k_{to} at 32 °C are shown in Figures 7 and 8. For

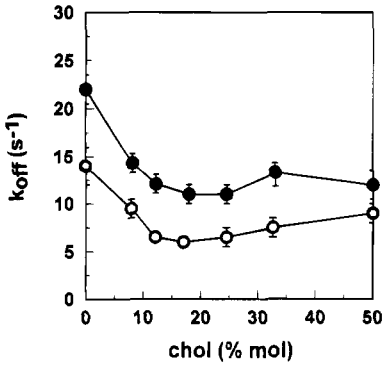


FIGURE 7: Influence of cholesterol (mol %) on the exit rate of deuteroporphyrin from DMPC/cholesterol vesicles. Temperature: 32 °C; pH: 7.15 (●), 6.9 (○).

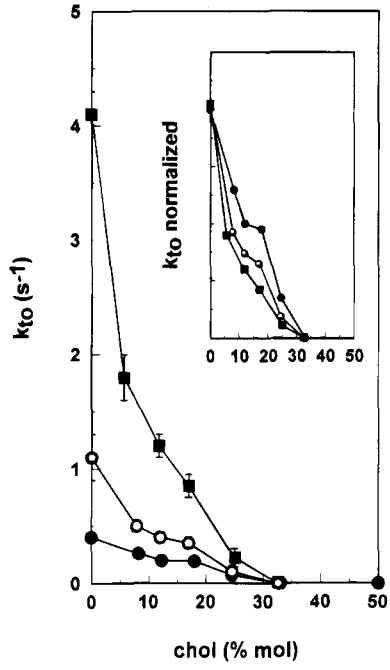


FIGURE 8: Influence of cholesterol (mol %) on the flip-flop rate of deuteroporphyrin in DMPC/cholesterol vesicles. Temperature: 32 °C; pH: 7.15 (●), 6.9 (○), 6.15 (■). The values at pH 6.15 were determined using the "pH-jump" method. Insert: Curves normalized to the same value at 0% chol.

mixtures of DMPC and cholesterol up to 17%, the kinetics were studied by using the stopped-flow apparatus. At 25% cholesterol, the second phase was slow enough to be observed even by using a standard spectrofluorimeter. At 33% cholesterol, the rate constant of the second phase was about 0.005−0.01 s^{-1}. At 50% cholesterol, a slow phase with a rate constant less than 0.001 s^{-1} was still seen at pH 6.15. At pH 7.15, the second phase was no longer observed up to 1 h. In this case, the shape of the emission spectra (see Figure 9) indicated that nearly half of the porphyrin remained in the vesicles after mixing with albumin. No change in the emission spectrum of the porphyrin−vesicles−albumin mixture was distinguished even after up to 3 h of incubation at 32 °C. When the incubation was prolonged until 22 h, a partial transfer of the porphyrin to albumin was suggested by the final fluorescence spectrum. However, a modification of the vesicles by albumin during this long period cannot be excluded. When the mixture was incubated at 50 °C, some changes were already observed after 30 min.

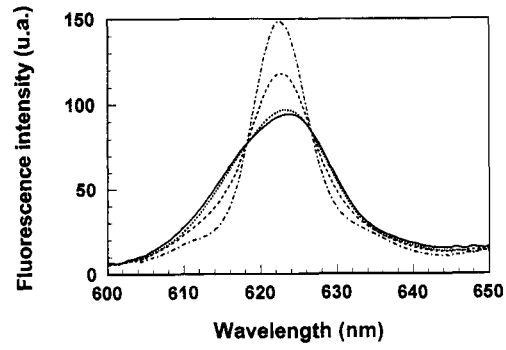


FIGURE 9: Emission spectra of deuteroporphyrin (1×10^{-7} M) at 32 °C, pH 7.15 (Tris buffer), in different environments. Excitation wavelength: 389 nm. (— • —) DMPC/chol vesicles. (- - -) Mixture of DMPC/chol vesicles and albumin: vesicles were prepared in the presence of deuteroporphyrin, and albumin was then added to the preparation. (• • •) Mixture of DMPC/chol vesicles and albumin: albumin was first mixed with the vesicle preparation, and deuteroporphyrin was added to the mixture. (—) HSA solution. Concentrations: [DMPC] = [chol] = 1×10^{-4} M, [HSA] = 5×10^{-5} M.

As a control, empty DMPC/chol (1:1 mol/mol) vesicles were first mixed with albumin, and then the porphyrin was added to the mixture. In this case, the emission spectrum indicated that nearly all porphyrin molecules were bound to albumin (see Figure 9).

DISCUSSION

As illustrated in Figure 6, the pH has a drastic effect on the kinetics of deuteroporphyrin interactions with DMPC vesicles. In the pH range investigated, this behavior is most likely due to protonation equilibria involving the two carboxylic groups of the porphyrin. Starting from the neutral form, as shown in Figure 1, successive deprotonations of the propionic chains lead to the monoanionic and dianionic forms. Corresponding pK's have been reported to be 5.4 ± 0.2 and 6.0 ± 0.2 in aqueous solutions (Brault et al., 1986b). As shown by previous steady-state partition experiments (Brault et al., 1986a,b; Brault, 1990), these pK's are shifted to higher values when the porphyrin is interacting with vesicles. Obviously, protonation−deprotonation steps at the interfaces between water and the phospholipid bilayer need to be considered to fully describe our system. Two limiting situations can be envisaged:

(i) The protonation and deprotonation are much faster than the interactions of the porphyrin with the lipidic bilayer. In this case, the porphyrin can be considered to be always at equilibrium with regard to its protonation status. Then, the system can be described in the same way as above, except that the intrinsic rate constants must be replaced by effective rate constants which are expected to depend on pH according to a classical titration curve.

(ii) The protonation and deprotonation are slow compared to the interactions with the bilayer. In this case, distinct populations of porphyrin molecules with different protonation status should coexist. At least a four-exponential signal should be observed instead of a biexponential one. The relative amplitudes of these four phases, but not their rate constants, would depend on the pH.

The pH dependence of the constants k_{off} and k_{to} strongly suggests that the first situation prevails. The pH-jump control experiments give definitive arguments. Indeed,

whether the porphyrin-loaded vesicle solutions are mixed with albumin solutions prepared at a greater or a lower pH, the observed exit rate constant (k_{off}) is characteristic of the actual pH_o after mixing. This means not only that the porphyrin incorporated in the outer vesicle hemileaflet undergoes rapid protonation−deprotonation equilibria, but also that the diffusion of protons at the water−phospholipids interface is fast enough. Rapid protonation−deprotonation equilibria were also assumed in related studies on fatty acids (Kamp & Hamilton, 1993).

As shown in Figure 6a,b, the dependence of k_{off} and k_{to} on pH can be fitted by classical titration curves of the form:

$$k = \frac{k(2-) + k(-)[10^{(pK_1-pH)}] + k(0)[10^{(pK_1+pK_2-2pH)}]}{1 + 10^{(pK_1-pH)} + 10^{(pK_1+pK_2-2pH)}}$$

where $k(0)$, $k(-)$, and $k(2-)$ stand for the rate constants (k_{off} or k_{to}) relative to the neutral, monoanionic, and dianionic porphyrin forms, respectively. Similar rate constants are obtained with Tris or phosphate buffers, except for the highest pH values. The exit of the dianionic form is somewhat faster when Tris buffer is used.

Several unknown parameters have to be optimized for each curve (pK_1, pK_2, and k_{off} or k_{to} for the various forms). Because plateaus are reached at low or high pH, the values of k_{to} and k_{off} can be estimated for the neutral and dianionic forms quite precisely: $k_{off}(2-) \approx 52\ s^{-1}$ (phosphate buffer) or 65 s^{-1} (Tris buffer), $k_{off}(0) \leq 0.5\ s^{-1}$, $k_{to}(2-) \leq 0.01\ s^{-1}$, and $k_{to}(0) \approx 6.5\ s^{-1}$ (phosphate buffer). On the other hand, there is a much greater uncertainty in the pK values and in the constants k_{to} and k_{off} characterizing the monoanionic form. Tentatively, we assume that pK values are the same whether the porphyrin carboxylic chains are located at the outer or the inner water−phospholipid interfaces of the vesicles; i.e., the same pK values are used to fit the curves describing the influence of pH on k_{off} or on k_{to}. Then, best fits are obtained with $pK_1 = 7.2 \pm 0.2$ and $pK_2 = 6.5 \pm 0.3$ when phosphate buffer is used (see curves in Figure 6). Similar apparent pK values have been reported for carboxylic groups of other organic molecules bound to lipidic bilayers (Ptak et al., 1980; Hamilton & Cistola, 1986; Ruf, 1987; Doody et al., 1980). Note that the apparent pK of the carboxylic groups of deuteroporphyrin are shifted by about 1.2 units toward higher values as compared to pK measured in aqueous solutions.

Large uncertainties remain on the values of k_{off} and k_{to} for the monoanionic form. Preliminary experiments (not shown) on the transfer of deuteroporphyrin monomethyl ester in which only one carboxylic group is free to ionize suggest that k_{off} for a monoanionic form is very low ($<1\ s^{-1}$). The theoretical fits given in Figure 6 were computed accordingly. It was also found necessary to suppose that the k_{to} value for the monoanionic form was small.

The present results are in line with various studies on ionizable amphiphiles that demonstrated a favored diffusion of the neutral forms across the phospholipid bilayer. Recently, Kamp and Hamilton (1993) described, both experimentally and theoretically, the flip-flop of a variety of acidic amphiphiles including fatty acids, fatty acids analogues, and bile acids, in egg phosphatidylcholine vesicles. The transfer across the bilayer was clearly related to the interfacial pK of the amphiphile. Similarly, Eastman et al. (1991) showed that the transport of phosphatidic acid through a bilayer involved the uncharged form. The transport of acidic amphiphiles was found to induce a pH gradient which was monitored using a pH-sensitive fluorescent probe entrapped inside the vesicles (Kamp & Hamilton, 1993). As a control, these authors followed the proton leakage after increasing the external pH. Equilibration of the pH between the entrapped water volume and the bulk aqueous solution was very slow, taking place within hours. Our present experiments to check the validity of the pH-jump protocol also show that, within the stopped-flow time scale, the proton leak is slow in the case of DMPC vesicles. Interestingly, asymmetric transbilayer distributions of phosphatidic acid or other ionizable amphiphiles can also be induced by transmembrane pH gradients (Hope & Cullis, 1987; Hope et al., 1989; Eastman et al., 1991).

Our results show further that pH also has an important effect on the desorption of the porphyrin from the bilayer. In keeping with previous work on fluorescent fatty acids (Doody et al., 1980), the exit of the negatively charged deprotonated form is accelerated.

The rate constants characterizing the porphyrin−albumin interactions are also found to depend to some extent on pH. The dissociation constant k_{dis} decreases with decreasing pH. In addition, the association rate constant, k_{as}, decreases above pH 7.5. This effect is likely due to changes in albumin conformation (Wilting et al., 1979) rather than to changes in the protonation state of the porphyrin. A decrease in the affinity constant, $K_A = k_{as}/k_{dis}$, above pH 7.5 was already reported for hematoporphyrin (Reddi et al., 1981).

The existence of a third very slow phase in the transfer kinetics presented above is fully consistent with the presence of a second porphyrin binding site on albumin. According to numerical simulations on experimental data, the dissociation rate constant k^2_{dis} for the second site is about 0.015 s^{-1} at pH 7.15. Assuming that the fluorescence properties of the porphyrin are the same at both albumin sites, the association rate constant for the second site can be estimated from the relative amplitude of the third phase, which yields a value of $k^2_{as} = 4.5 \times 10^5\ M^{-1}\ s^{-1}$. Since the main object of the present work was to investigate the interaction of porphyrins with lipidic bilayers, this slow phase was not investigated in detail, and the values of k^2_{as} and k^2_{dis} given here are only rough estimates. Nevertheless, some discrepancy was noted between the affinity constant K_A determined from steady-state measurements ($\approx 5 \times 10^7\ M^{-1}$ according to Rotenberg et al., 1987) and the ratio k_{as}/k_{dis} ($\approx 2 \times 10^7\ M^{-1}$) obtained from our previous kinetic experiments (Kuzelova & Brault, 1994). The existence of a second binding site characterized by very slow association and dissociation constants and by an affinity constant $k^2_{as}/k^2_{dis} = 3 \times 10^7\ M^{-1}$ would explain this discrepancy. At pH 5.77, values of $k^2_{as} \approx 5 \times 10^6\ M^{-1}\ s^{-1}$ and $k^2_{dis} \approx 0.07\ s^{-1}$ were deduced from the relative amplitude of the third phase and from its rate constant. The affinity constant of the second site would thus somewhat augment with decreasing pH. Secondary binding sites of albumin for hematoporphyrin are also unmasked at lower pH as shown by Reddi et al. (1981). Similar results were recently obtained in a related series of photosensitizers. Ambroz et al. (1994) investigated the binding of disulfonated aluminium phthalocyanine to albumin by means of time resolved fluorescence spectroscopy. At pH 7.4, the fluorescence decay was characterized by two lifetimes which

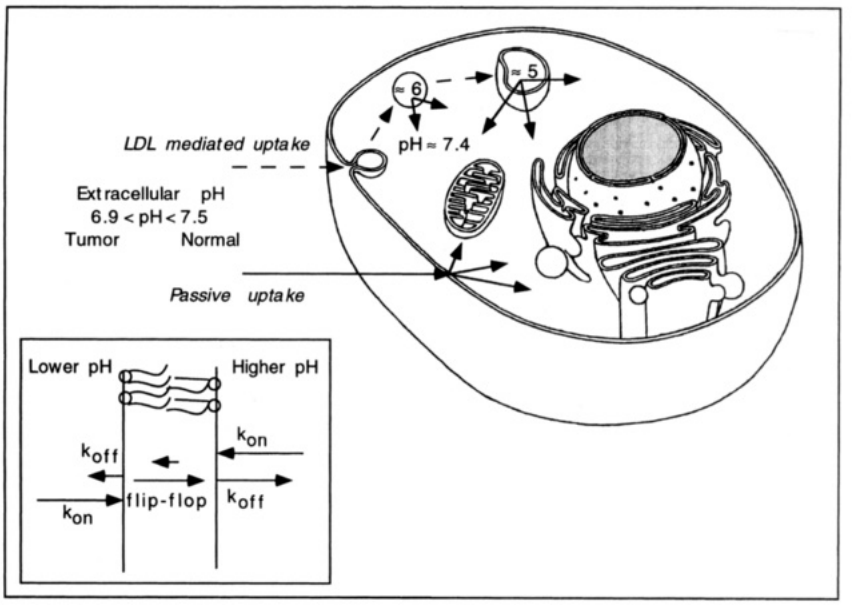


FIGURE 10: Scheme illustrating the passive uptake and the LDL hypothesis. The LDL endocytic pathway is indicated by dashed lines. The pH indicated in the organelles is lowered from endosomes (≈6) to lysosomes (≈5). Porphyrin diffusion is indicated by full lines. It accounts for leakage from the lysosomes (eventually from endosomes) or for passive uptake through the plasma membrane. The insert illustrates, by different arrow lengths, the kinetic effects arising from a pH difference between two aqueous compartments separated by a lipidic bilayer. The extracellular pH can vary from about 6.9 for tumor tissues to 7.5 for normal tissues.

might suggest the existence of two binding sites of comparable affinities.

It is important to note that, for the highest albumin concentrations, the transfer from vesicles to albumin is governed mainly by the rate constants relative to the vesicles (see Figure 2). Changes in the interactions of the porphyrin with albumin have no effect on the limit values ([HSA] → ∞) from which k_{off} and k_{to} are determined.

As shown in Figures 7 and 8, the presence of cholesterol in the lipid bilayer strongly affects the interactions of deuteroporphyrin with vesicles. The rate constant for the porphyrin flip-flop is dramatically reduced by increasing the cholesterol content. The decrease of k_{to} with the cholesterol content is not smooth, however. In the insert of Figure 8 the curves for the three different pH's are normalized to the same value at 0% cholesterol. This representation clearly shows that, at higher pH, the irregularity in the decrease of k_{to} around 10−20% cholesterol is more pronounced.

A possible explanation of this behavior might be found in the characteristics of the phase diagrams of phosphatidylcholine−cholesterol mixtures. A special phase state denoted liquid-ordered phase (Ipsen et al., 1987), β-phase (Vist & Davis, 1990), or liquid-gel phase (Huang et al., 1993) has been shown to characterize cholesterol-rich bilayers. In this state, the lipid chains are highly ordered, as in the gel phase, but their lateral mobility is high, comparable to that characterizing the liquid-crystal phase. Above the transition temperature of the pure lipids, liquid-crystal, β-phases, and coexistence of the two phases separated in macroscopic domains have been suggested at low, high, and intermediate cholesterol content, respectively (Ipsen et al., 1987; Vist & Davis, 1990). In the same line, Cruzeiro-Hansson et al. (1989) predicted fluctuations of cholesterol and lipid density leading to cluster structures in cholesterol−phosphatidylcholine bilayers. Similar effects are believed to occur in pure lipid bilayers near the phase transition temperature. Porphyrin molecules could diffuse more easily at the interface between domains with different lipid organization. It can be expected that this effect would be relatively more important for charged molecules, the flip-flop of which is much less favored. The curves shown in Figure 8 are smoother at the lowest pH, accordingly. It is worth noting that a drastic enhancement of deuteroporphyrin flip-flop rate was observed at the phase transition temperature in pure lipid bilayers (Kuzelova & Brault, 1994).

Besides the above-mentioned mechanisms, it is possible that cholesterol also modifies the apparent pK values. Indeed, small shifts in pK values, which can be illustrated in Figure 6b by a shift of the titration curve, would result in large changes in the flip-flop rate.

The rate of porphyrin exit from the bilayer, k_{off}, is also modified by the presence of cholesterol (Figure 7), although to a lesser extent than the flip-flop. It is progressively reduced to about one half at 15-20% cholesterol, and then little changes up to 50%. The increase of vesicle diameter with cholesterol content (see experimental section) might partly account for the observed changes (Kuzelova & Brault, 1994). In a related system, Cannon et al. (1984) reported a 2-fold reduction of the dissociation rate constant of hemin from phosphatidylcholine−dicetyl phosphate vesicles at room temperature.

Among the various hypotheses that have been put forward to explain the relative selectivity of porphyrins for tumor cells, two have received much attention. A low density lipoprotein (LDL) receptor-mediated pathway, favored in tumor cells, has been suggested to be preponderant in the case of lipophilic photosensitizers (Mazière et al., 1991). Indeed, lipoproteins are major carriers of hydrophobic porphyrins as shown by several groups (Jori et al., 1984; Reyftmann et al., 1984; De Smidt et al., 1993). Moreover, tumor cells possess an increased number of low density lipoprotein receptors. According to the LDL receptor-mediated hypothesis, the porphyrin should be driven through the endocytic pathway to the lysosomal compartment. In

fact, little or no evidence for lysosome labeling was found with hydrophobic photosensitizers such as protoporphyrin, Photofrin, or related compounds. On the contrary, localization in lysosomes was quite well demonstrated in the case of hydrophilic compounds (Moan & Berg, 1992). Experiments designed to check the LDL hypothesis by manipulating the number of LDL receptors on cells also led to controversial results (Biade et al., 1993; Kessel et al., 1993).

Alternatively, the low pH value of the interstitial fluid in tumors (Tannock & Rotin, 1989; Wike-Hooley et al., 1984) has been suggested to favor passive cellular incorporation of carboxylic porphyrins (Moan et al., 1980). This tumor peculiarity reflects the excretion of lactic acid by neoplastic cells which maintain high rates of glucose metabolism. Values of pH ranging from 6.4 to 7.4 have been reported for human tumors. On the other hand, the intracellular pH of the tumor cells remains virtually unaffected. This hypothesis gained support from studies on the effect of pH on the partition of porphyrins between lipidic vesicles and water (Brault et al., 1986a,b; Brault, 1990). Recent in vivo experiments with tumor bearing animals confirmed the importance of this pH effect (Thomas & Girotti, 1989; Peng et al., 1991). In those experiments an additional depletion of the pH of the tumor interstitial fluid was obtained by glucose administration. An increased uptake of hematoporphyrin derivative or Photofrin by the tumor, as well as an improved therapeutic effect, was clearly correlated to this pH drop.

Our results support the idea that kinetic effects related to the protonation state of the carboxylic chains underlie these phenomena. A pH difference between two aqueous compartments separated by a phospholipidic bilayer will drive molecules such as deuteroporphyrin from the lower to the higher pH. We can examine, for instance, the consequence of a difference of 0.5 unit, a value likely to be attained considering an intracellular pH of 7.4 and a pH of 6.9 for the tumor interstitial fluid. In our system, the exit rate of the porphyrin (k_{off}) at pH 6.9 is about 2 times smaller, and that for the movement across the bilayer (k_{to}) is 5 times larger than the respective values measured at pH 7.4. This kinetic effect is illustrated in the insert of Figure 10. In cholesterol-containing systems which better approach the properties of biological membranes, the kinetic effect of pH is maintained although the overall rates are reduced. Thus, the diffusion of this class of porphyrins through the plasmic membrane of tumor cells is expected to be accelerated when compared to normal cells which are surrounded by an interstitial fluid with a pH close to 7.5. On the other hand, the exit from tumor cells would be slower. After penetration in the cytoplasm, the porphyrin will diffuse and accumulate in various compartments such as mitochondria (Salet & Moreno, 1990), Golgi apparatus (Dellinger et al., 1988), or other subcellular membrane structures. This process is illustrated in Figure 10.

Alternatively, even if the LDL-mediated hypothesis holds, leakage of such porphyrins from lysosomes and even from endosomes is expected to be very fast. Indeed, the pH of these organelles is progressively lowered along the endocytic pathway to reach values as low as about 5 in lysosomes (Schmid, 1993). The curves shown in Figure 6 illustrate the tremendous effect which can be expected from such a pH difference. After leaking from the lysosomes, the porphyrin is expected to diffuse to other membrane structures as discussed above. It can be pointed out that the pH-jump experiments described above represent a simple model of those kinetic effects mediated by pH gradients.

ACKNOWLEDGMENT

We thank Dr. E. Fattal for measurements of vesicle sizes and Dr. G. Silver for her help in the preparation of the manuscript.

REFERENCES

Ambroz, M., MacRobert, A. J., Morgan, J., Rumbles, G., Foley, M. S. C., & Phillips, D. (1994) *J. Photochem. Photobiol. B: Biol. 22*, 105−117.

Benson, S. W. (1960) *The Foundation of Chemical Kinetics*, McGraw-Hill, New York.

Biade, S., Mazière, J. C., Mora, L., Santus, R., Mazière, C., Auclair, M., Morlière, P., & Dubertret, L. (1993) *Photochem. Photobiol. 57*, 371−375.

Böhmer, R. M., & Morstyn, G. (1985) *Cancer Res. 45*, 5328−5334.

Brault, D. (1990) *J. Photochem. Photobiol. B: Biol. 6*, 79−86.

Brault, D., Vever-Bizet, C., & Dellinger, M. (1986a) *Biochimie 68*, 913−921.

Brault, D., Vever-Bizet, C., & Le Doan, T. (1986b) *Biochim. Biophys. Acta 857*, 238−250.

Brault, D., Vever-Bizet, C., & Kuzelova, K. (1993) *J. Photochem. Photobiol. B: Biol. 20*, 191−195.

Cabral, D. J., Small, D. M., Lilly, H. S., & Hamilton, J. A. (1987) *Biochemistry 26*, 1801−1804.

Cannon, J. B., Kuo, F.-S., Pasternack, R. F., Wong, N. M., & Muller-Eberhard, U. (1984) *Biochemistry 23*, 3715−3721.

Cruzeiro-Hansson, L., Ipsen, J. H., & Mouritsen, O. G. (1989) *Biochim. Biophys. Acta 979*, 166−176.

Dailey, H. A. (1990) *Biosynthesis of Heme and Chlorophylls*, McGraw-Hill, New York.

Dellinger, M., & Brault, D. (1987) *J. Chromatogr. 422*, 73−84.

Dellinger, M., Vever-Bizet, C., Brault, D., Delgado, O., & Rosenfeld, C. (1986) *Photochem. Photobiol. 43*, 639−647.

Dellinger, M., Brault, D., & Vever-Bizet C. (1988) in *Photosensitisation* (Moreno, G., et al., Eds.) NATO ASI Series, Vol. H15, pp 387−390, Springer-Verlag, Berlin and Heidelberg.

De Smidt, P. C., Versluis, A. J., & Van Berkel, Th. J. C. (1993) *Biochemistry 32*, 2916−2922.

Doody, M. C., Pownall, H. J., Kao, Y. J., & Smith, L. C. (1980) *Biochemistry 19*, 108−116.

Dougherty, T. J. (1987) *Photochem. Photobiol. 46*, 569−573.

Dougherty, T. J., Lawrence, G., Kaufman, J. H., Boyle, D., Weishaupt, K. R., & Goldfarb, A. (1979) *J. Natl. Cancer Inst. 62*, 231−237.

Eastman, S. J., Hope, M. J., & Cullis, P. R. (1991) *Biochemistry 30*, 1740−1745.

Hamilton, J. A., & Cistola, D. P. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 82−86.

Hope, M. J., & Cullis, P. R. (1987) *J. Biol. Chem. 262*, 4360−4366.

Hope, M. J., Redelmeier, T. E., Wong, K. F., Rodrigueza, W., & Cullis, P. R. (1989) *Biochemistry 28*, 4181−4187.

Huang, T.-H., Lee, C. W. B., Das Gupta, S. K., Blume, A., & Griffin, R. G. (1993) *Biochemistry 32*, 13277−13287.

Ipsen, J. H., Karlström, G., Mouritsen, O. G., Wennerström, H., & Zuckermann, M. J. (1987) *Biochim. Biophys. Acta 905*, 162−172.

Jori, G., Beltramini, M., Reddi, E., Salvato, B., Pagnan, A., Ziron, L., Tomio, L., & Tsanov, T. (1984) *Cancer Lett. 24*, 291−297.

Kamp, F., & Hamilton, J. A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11367−11370.

Kamp, F., & Hamilton, J. A. (1993) *Biochemistry 32*, 11074−11086.

Kessel, D. (1990) *Photodynamic Therapy of Neoplastic Disease*, CRC Press, Boston.

Kessel, D., Garbo, G. M., & Hampton, J. (1993) *Photochem. Photobiol. 57*, 298−301.

Kuzelova, K., & Brault, D. (1994) *Biochemistry 33*, 9447−9459.

Lipson, R. L., Baldes, E. J., & Olsen, A. M. (1961) *J. Natl. Cancer Inst. 26*, 1−11.
Mazière, J. C., Morlière, P., & Santus, R. (1991) *J. Photochem. Photobiol. B: Biol. 8*, 351−360.
Moan, J., & Berg, K. (1992) *Photochem. Photobiol. 55*, 931−948.
Moan, J., Smedshammer, L., & Christensen, T. (1980) *Cancer Lett. 9*, 327−332.
Peng, Q., Moan, J., & Cheng, L.-S. (1991) *Cancer Lett. 58*, 29−35.
Policard, A. (1924) *C. R. Soc. Biol. 91*, 1423−1424.
Pottier, R., & Kennedy, J. C. (1990) *J. Photochem. Photobiol. B: Biol. 8*, 1−16.
Prestegard, J. H., Cramer, J. A., & Viscio, D. B. (1979) *Biophys. J. 26*, 575−584.
Ptak, M., Egret-Charlier, M., Sanson, A., & Boulossa, O. (1980) *Biochim. Biophys. Acta 600*, 387−397.
Reddi, E., Ricchelli, F., & Jori, G. (1981) *Int. J. Peptide Protein Res. 18*, 402−408.
Reyftmann, J. P., Morlière, P., Goldstein, S., Santus, R., Dubertret, L., & Lagrange, D. (1984) *Photochem. Photobiol. 40*, 721−729.
Rotenberg, M., Cohen, S., & Margalit, R. (1987) *Photochem. Photobiol. 46*, 689−693.
Ruf, H. (1987) *Biophys. Chem. 26*, 313−320.
Salet, C., & Moreno, G. (1990) *J. Photochem. Photobiol. B: Biol. 4*, 133−150.
Schmid, S. L. (1993) in *Subcellular Chemistry* (Bergeron, J. J. M., & Harris, J. R., Eds.) Vol. 19, pp 1−25, Plenum Press, New York and London.
Spikes, J. D., & Straight, R. C. (1990) in *Photodynamic Therapy of Neoplastic Disease* (Kessel, D., Ed.) Vol. I, pp 211−228, CRC Press, Boston.
Tannock, I. F., & Rotin, D. (1989) *Cancer Res. 49*, 4373−4384.
Thomas, J. P., & Girotti, A. W. (1989) *Photochem. Photobiol. 49*, 241−247.
Vever-Bizet, C., & Brault, D. (1993) *Biochim. Biophys. Acta 1153*, 170−174.
Vist, M. R., & Davis, J. H. (1990) *Biochemistry 29*, 451−464.
Wike-Hooley, J. L., Haveman, J., & Reinhold, H. S. (1984) *Radiother. Oncol. 2*, 343−366.
Wilting, J., Weideman, M. M., Roomer, A. C. J., & Perrin, J. H. (1979) *Biochim. Biophys. Acta 579*, 469−473.

BI9503864