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Porphyrin-membrane interactions: binding or partition?

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Porphyrins are photodynamic drugs employed in an experimental tumor-treatment modality in which cell membranes are one of the primary drug-action sites. To gain insight into the nature of the interaction of these drugs with those primary sites we have studied the affinity of porphyrins to the lipid moieties of biological membranes, at the molecular level. The association of porphyrins to large unilamellar liposomes, modeling the lipid regions of biological membranes was studied (at equilibrium) for deuteroporphyrin IX and protoporphyrin IX, at neutral pH and 37 °C, taking into account porphyrin aggregation. Two thermodynamic approaches were investigated: (i) Simple partition equilibria between the external aqueous phase and the lipid bilayer, for drug monomers and dimers. (ii) Binding equilibria of drug monomers and dimers to the lipid bilayer. Using two types of experimental design and processing the data according to the expectations of both approaches, three different models for the binding (differing in the participation assigned to the dimer) were considered. Our major findings are: (a) The data clearly do not fit with the expectations for simple partition equilibria, nor with binding models assuming direct participation of the dimers. (b) The data fit well with a binding process, in which the membrane binds the porphyrin monomers only, with the dimers participating indirectly through the aqueous dimerization equilibrium. (c) At 37 °C and neutral pH, for liposomes composed of phosphatidylcholine/cholesterol at molar ratios of 3:2, we found for both investigated species a binding constant of $2.3 \cdot 10^4$ M^{-1}. (d) For each species the binding constant is independent of the initial and final states of drug aggregation in the aqueous phase.

Introduction

Porphyrins, investigated for tumor treatment such as protoporphyrin IX and its derivatives [1–7] have high affinities for biological membranes [8–12], especially for the lipid moieties of the bilayers as shown by studies with liposomes [9,10,12,13].

The interaction of a relatively small molecule (such as these porphyrins) with membranes has been generally approached as a binding process. However, the ability of the porphyrin molecule to interact with the lipid matrix itself without any specific membrane component (such as a protein) serving as a receptor, points to an alternative approach: Treating the porphyrin-membrane association as a partition process, the small molecule distributed, at equilibrium, between the external aqueous phase and the lipid moieties of the membrane-enclosed particles.

In view of the involvement of the porphyrin-membrane association in the therapeutic activities of these molecules, we found it desirable to have

Abbreviations: LUV, large unilamellar vesicles; PC, phosphatidylcholine.

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the ability to define and describe this association in quantitative thermodynamic terms, testing binding vs. partition. Having this ability should give added molecular insight into the activities of porphyrins in biological systems as well as have practical applications, for the treatment itself.

In the present work we have used liposomes to model the lipid matrix of biological membranes, measuring the association of deutero- and protoporphyrin IX to the liposomes, at equilibrium. The deuteroporphyrin IX data obtained were processed according to both approaches, with the dual aim of understanding the association of porphyrins with membranes, at the molecular level, and assessing which of the two approaches fits the experimental observations better. Following this, the protoporphyrin IX data were processed according to the best-fitting approach.

As in previous studies [10,14], we focussed first on deuteroporphyrin since it represents the core structure common to all porphyrin IX species employed in tumor therapy.

Materials and Methods

Materials

Deuteroporphyrin was purchased from Porphyrin Products (Logan, Utah). Protoporphyrin and lipids were purchased from Sigma Chemical Co. All other materials were of analytical grade.

Fluorescence spectra were recorded on a Perkin-Elmer spectrofluorimeter model MPF-44B.

Methods

Porphyrin stock solutions were prepared and handled as previously described [9,13–15]. Large unilamellar liposomes of the reverse-phase evaporation type were prepared according to Ref. 16, from phosphatidylcholine and cholesterol at the final molar ratios of 3 : 2.

Two experimental designs were used:

(I) Porphyrin concentration held constant within the range of 0.08–0.8 μM (initial monomer fraction of 50–90%). Lipid concentration varied over the 0.01–0.1 mM range. The desired initial monomer/dimer distribution was obtained by controlling the total porphyrin concentration and using the previously determined dimerization constant [14].

(II) Lipid concentration held constant at 50 μM. Porphyrin concentration varied over the 0.1–1.0 μM range.

In all experiments the liposome-porphyrin reaction mixtures, in phosphate-buffered saline at pH 7.2, were incubated in the dark for 2 h at 37 °C. Separation of the liposomal fraction was by ultracentrifugation at 225 000 $\times$ g. Porphyrin concentrations in the pellet (suspended in the phosphate buffer) and in the supernatant were determined fluorometrically, adding Triton X-100 to a final concentration of 0.6% in order to dissolve the liposomes and monomerize the porphyrin in the system (see Refs. 9, 10 and 13 for additional details). Excitations were at 397 and 405 nm and peak emissions were at 622 and 635 nm for deuteroporphyrin IX and protoporphyrin IX, respectively.

Theoretical expectations

Several models and quantitative expressions for the binding or partitioning of porphyrin into liposome systems will be considered below. The models and expressions are restricted to the range of porphyrin systems we have studied, namely to those in which the presence of aggregates of higher order than dimers is negligible [11,14]. In addition, we have made use of a key experimental observation – at equilibrium we found all liposome-associated porphyrin to be in the monomeric state regardless of the initial and final monomer-dimer distributions in the external aqueous phase. This experimental observation will be presented in a later section of this report.

In all expressions we have introduced the term [Tb], which is an experimentally-measurable quantity of the total concentration of liposome-associated porphyrin (at equilibrium) expressed per units of monomers. As will be shown, the specific liposome-porphyrin species contributing to [Tb] will differ according to the specifics of the model.

I. Binding equilibria

The following three models, differing in the nature of the dimer involvement, are proposed.

Ia. Indirect involvement of dimers

In this case only the monomers bind to the

liposomes and the participation of the dimers is indirect, through their dissociation into monomers. Two simple equilibria suffice to describe the system [9,10,13]:

$$2\,M \overset{K_D}{\rightleftharpoons} D \tag{1}$$

$$M + L \overset{K_{ML}}{\rightleftharpoons} ML \tag{2}$$

where K_D and K_{ML} represent the dimerization and monomer-liposome binding equilibrium constants, respectively; M, D, L, and ML denote the monomer, the dimer, the liposomes and the porphyrin-liposome species, respectively.

For this system the measurable quantity [Tb] is simply:

$$[Tb] = [ML] \tag{3}$$

[ ] denoting, in general, molar concentration.

Simple manipulation of Eqns. 1–3, taking conservation of matter into consideration, will yield an explicit expression for the desired parameter K_{ML}:

$$K_{ML} = \frac{[Tb]}{[M][L]} = \frac{[Tb]}{([L]/4K_D)\left(-1+\sqrt{(1+8K_D([T]-[Tb]))}\right)} \tag{4}$$

where [T] is the total porphyrin concentration (expressed per units of monomers) in the system.

Ib. Direct involvement of dimers – competitive binding

In this case both monomers and dimers bind to the liposome, competing on the same liposomal site with the liposome-bound dimer undergoing dissociation (in situ). In addition to the processes expressed in Eqns. 1 and 2 above, the following are also required for complete description of the system:

$$D + L \overset{K_{DL}}{\rightleftharpoons} DL \tag{5}$$

$$M_2L \overset{K'_D}{\rightleftharpoons} DL \tag{6}$$

where K_{DL} and K'_D are the dimer-liposome equilibrium binding constant and the dimerization equilibrium constant at the liposomal site, respectively.

[Tb] is this case corresponds to:

$$[Tb] = [ML] + 2[M_2L] \tag{7}$$

Taking together Eqns. 1, 2, 5–7 and conservation of matter will give:

$$\frac{[Tb]}{[M][L]} = K_{ML} + A[M] \tag{8}$$

$$A = 2K_DK_{DL}/K'_D \tag{9}$$

Ic. Direct dimer involvement – noncompetitive binding

In this case both monomers and dimers bind to the liposome, but the sites are different, the binding therefore noncompetitive. For independent binding and for dimerization in the membrane dimer site being independent of the state of occupation at the monomer site, the following equilibria together with Eqn. 6 can describe the system:

$$\begin{array}{l} M \overset{K_{ML}}{\rightleftharpoons} ML + D \underset{K_{ML}}{\overset{K_{DL}}{\rightleftharpoons}} \\ L + \\ D \overset{K_{DL}}{\rightleftharpoons} DL + M \end{array} MDL \tag{10}$$

$$M_3L \overset{K'_D}{\rightleftharpoons} MDL \tag{11}$$

[Tb] for these systems is given by:

$$[Tb] = [ML] + 2[M_2L] + 3[M_3L] \tag{12}$$

Taking Eqns. 6, 10 and 11 together and rearranging, will give:

$$\frac{[Tb]}{[M][L]} = K_{ML} + A[M] + B[M]^2 \tag{13}$$

where A is the same combination of constants given in Eqn. 9 and B is given by:

$$B = 3K_DK_{DL}K_{ML}/K'_D \tag{14}$$

II. Partition equilibria

In dealing with partition processes in which one of the phases is a microparticle such as a mem-

brane-enclosed biological or artificial system and the other a larger phase such as the external aqueous phase one should be aware that the interfaces constitute a nonnegligible fraction of the smaller phase. Thus, the region into which the molecule is assumed to partition has to be specified. In the present case we have limited our considerations to partition of the porphyrin into the lipid regions beyond the interfaces. Regions in which the relationship $(\partial\mu^0)/\partial x) = 0$ (μ^0 is the standard chemical potential) can be assumed to hold for the partitioning molecule, indicating this molecule is truely dissolved in the medium. We will refer to this as simple partition equilibria.

We did not go into the issue of porphyrin partitioning into the interface region since in our opinion this process cannot be sufficiently distinguished from binding: In the interface the standard chemical potential of the porphyrin would vary with the molecule's location. Also, the porphyrin would not be truely dissolved, but limited to a narrow space, not much different from a binding site.

For the distribution of porphyrin between the external aqueous phase and the lipid regions of the liposomes, the expressions obtained for monomer and dimer species, at equilibrium, can take the following forms.

IIa. Partition of monomers

$$\frac{[M]_l}{[M]_w} = \exp\left(\frac{(\mu_M^0)_w - (\mu_M^0)_l}{RT} + \frac{z_M F}{RT}(\psi_w - \psi_l)\right) \tag{15}$$

where $[M]_w$ and $[M]_l$ are the concentrations of the deuteroporphyrin monomers in the aqueous and in the liposomal phases, respectively; $(\mu_M^0)_w$ and $(\mu_M^0)_l$ are the standard chemical potential of M, ψ_w and ψ_l are the electrical potentials in the aqueous and lipid phases, respectively.

For constant experimental conditions in terms of total porphyrin and electrolyte concentrations, temperature and the nature of the two phases, the right-hand side of Eqn. 15 corresponds to an apparent partition coefficient (denoted $(K_{MP})_{app}$) which should be independent of the total lipid level in this heterogeneous system. Therefore:

$$(K_{MP})_{app} = \frac{[M]_l}{[M]_w} = \frac{[Tb]}{[M]_w} \tag{16}$$

IIb. Partition of dimers

For the dimers, considering those partitioning into the lipid undergo monomerization (as the experimental data show), the partition process, at equilibria, can be described by the following expressions:

$$\frac{[M]_l^2}{[D]_w} = \frac{1}{K_D'} \exp\left(\frac{(\mu_D^0)_w - (\mu_D^0)_l}{RT} + \frac{z_D F}{RT}(\psi_w - \psi_l)\right) \tag{17}$$

$$(K_{DP})_{app} = \frac{[M]_l^2}{[D]_w} = \frac{[Tb]^2}{[D]_w} \tag{18}$$

where all notations are as defined previously and the subscript D indicates that the property or factor specified are those of the dimer.

Results and Discussion

I. Deuteroporphyrin IX-lipid association and the aggregation state of the liposomal porphyrin

Carrying out experiments according to the first experimental design, we found the fraction of bound deuteroporphyrin IX to increase with the increase in lipid concentration, as exemplified by the data illustrated in Fig. 1 (a and b) for initial monomer levels of 90% and 50%, respectively.

To assess the aggregation state of the liposomally-bound porphyrin, we compared the fluorescence emission of a sample of intact liposomes

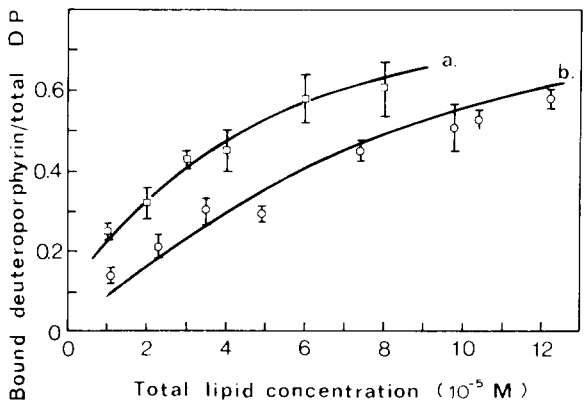


Fig. 1. The increase in liposome-associated porphyrin with the increase in lipid concentration, for systems with initial aqueous monomer fractions of 0.91 (curve a) and 0.60 (curve b), respectively. The points are experimental, the curves introduced in order to illustrate the trends. Ordinate: The fraction of liposome-associated porphyrin. Abscissa: The total lipid concentration in the system.

suspended in buffer, to a similar (or the same sample dissolved by detergent (see Methods for details). The latter treatment releases and monomerizes all bound porphyrin [9,10,13]. If some of the liposome-bound porphyrin were in the non-fluorescent dimeric state, the fluorescence intensity should increase from the pre to the post-detergent treatment, and be dependent on the initial aggregation state. If, on the other hand, all bound porphyrin is already in the monomeric state, the detergent treatment should not result in any significant increase in fluorescence. As illustrated by the data listed in Table I, the latter was the case. Increasing the dimer fraction (per mole monomers) from 10% to 50% had virtually no effect on the post- to pre-detergent ratios.

II. Partition equilibria

If the porphyrin-liposome association is a simple partition process, fitting with the expectations of Eqns. 15 and 18, then the partition coefficient should be a constant, independent (under similar experimental conditions) of the following: (i) The total drug level in the system. (ii) The relative magnitudes of the two phases. A test of the latter is illustrated in Fig. 2: Data of the type illustrated in Fig. 1 were processed according to Eqns. 15 and 18 (left-hand sides), then the partition coefficients obtained were plotted vs. the increasing lipid concentrations. Since all reaction mixtures were of equal volumes, an increase in the lipid concentration represents an increase in the liposome phase at the expense of the external aqueous phase, thus changing the ratio of the magnitudes of the two phases.

TABLE I

THE EFFECT OF DETERGENT TREATMENT ON PEAK FLUORESCENT INTENSITY (EMISSION) OF LIPOSOME-BOUND DEUTEROPORPHYRIN, FOR INCREASING DIMER CONTENT

Increases are normalized to starting stage of 6% dimers.

Increase in initial dimer fraction	Increase in fluorescence ratio (post- to pre-detergent)
1.8	0.98
2.6	1.04
3.4	1.11
3.8	1.06

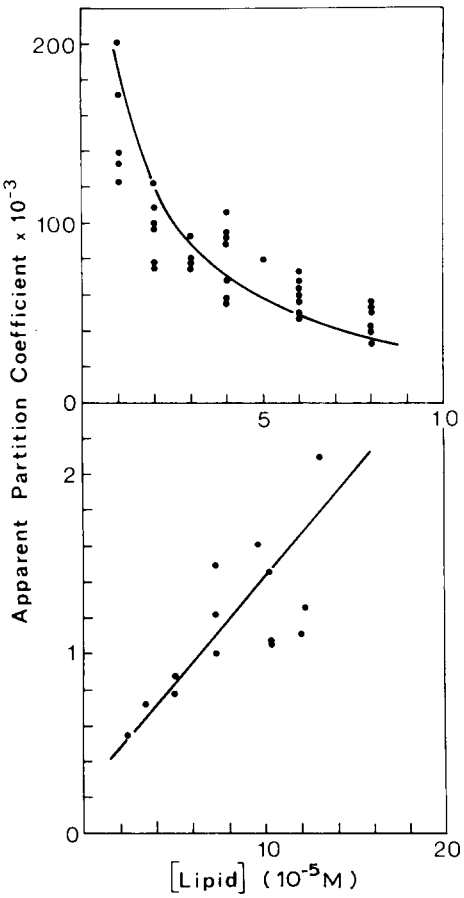


Fig. 2. The experimentally observed relationship between the apparent partition coefficients of deuteroporphyrin monomers (upper section) and dimers (lower section) and the relative sizes of the external aqueous phase and the lipid bilayers. Points are experimental data, the curves drawn in order to illustrate the trends. Ordinate: Apparent partition coefficients, obtained from the experimental data according to Eqns. 15 and 18 (see text for additional details). Abscissa: The increase in the weight of the lipid bilayer phase, expressed per mole lipid.

The data exemplifying the partition of monomers is taken from highly monomeric porphyrin solutions (% monomers = 90) and that for dimers taken from systems in which half of the porphyrin is already in the dimeric state. As clearly seen, for both monomers and dimers, the results are contrary to the expectations. Instead of a single value, independent of the lipid concentrations, the magnitude of the monomers' partition coefficient decreases and that of the dimers increases, with the increase in lipid concentration. Thus, neither the

interactions of monomers nor those of the dimers with the lipid moieties of biological membranes fit with the expectations of simple partition equilibria.

III. Binding equilibria

For each initial monomer level, data of the type illustrated in Fig. 1 were processed to yield K_{ML} – the monomer-liposome binding constant according to first binding model (see Eqns. 1–4, above). The results obtained, listed in Table 2, show the most significant finding for these experiments: The data yield the same magnitude for the binding constant, corresponding to $(2.3 \pm 0.7) \cdot 10^4$ M^{-1} regardless of the initial monomer/dimer distribution. Thus the data fit, unambiguously, with the model found to fit other porphyrins also [9,10,13] in which only the monomers bind to the liposomes and the dimers are involved indirectly, through their dissociation into monomers.

In order to add support to the fit of the model discussed above and to test whether this is the only model which fits the data, we carried out experiments according to the second design. If these data were to fit the models of direct dimer involvement, then plotting [Tb]/([M][L]) vs. [M] should yield either a linear line with a slope > 0 for the competitive model (Eqn. 9) or a nonlinear curve for the non-competitive model (Eqn. 3). Typical data, plotted in Fig. 3, clearly illustrate that neither is the case. A linear relationship is obtained, but with a slope of zero, thus ruling out the expectations for both the competitive and the noncompetitive binding models. In fact, the dependence obtained indicates that both the *A* and *B* terms in Eqns. 9 and 13 have zero values, reducing the said equations to Eqn. 4. Moreover, the magnitude of the binding constant (K_{ML}) obtained from the data illustrated in Fig. 2 (the intercept) is $(2.3 \pm 0.7) \cdot 10^4$ M^{-1}, in good agreement with the magnitude obtained from the experiments of the first experimental design (recall Table II).

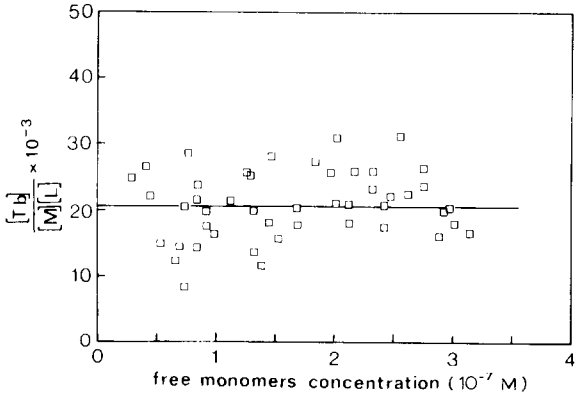


Fig. 3. A test of the liposome-porphyrin association data according to the competitive and noncompetitive binding models for direct dimer involvement. The points are the experimental data plotted according to Eqn. 9 in the text. The solid line is the results of linear regression. Ordinate: The ratio (at equilibrium) of liposome-bound porphyrin to the product of free monomer and lipid. Abscissa: The concentration of free monomers (at equilibrium).

IV. Protoporphyrin IX-liposome binding

The association of protoporphyrin IX to liposomes was studied according to the two experimental designs detailed under Methods above. As with deuteroporphyrin IX, we studied the association at initial aggregation states ranging from 90 to 50% monomers, setting the desired states by manipulating porphyrin concentration as detailed in Table III.

Applying the conclusions from the analysis of the deuteroporphyrin IX data, we have processed the protoporphyrin IX according to Eqn. 4. Over the entire range of aggregation states studied, as shown in Table III, the data yielded the same binding constant which is also similar to that

TABLE II

EQUILIBRIUM BINDING CONSTANTS OF DEUTEROPORPHYRIN IX MONOMERS TO LIPOSOMES (LUV, PC/CHOLESTEROL, 3:2) AT 37°C AND NEUTRAL pH

n is the number of experimental determinations.

Initial aggregation state in aqueous phase (% monomers)	K_{ML} (10^{-4} M^{-1}) (mean ± S.D.)
50–60	2.3 ± 0.5 (*n* = 17)
60–70	2.5 ± 0.7 (*n* = 16)
70–80	2.1 ± 0.7 (*n* = 13)
80–90	2.2 ± 0.8 (*n* = 14)
95	2.6 ± 0.7 (*n* = 20)
Combined [a]	2.3 ± 0.7

[a] Combined indicates pooling together all determinations, regardless of aggregation state.

TABLE III

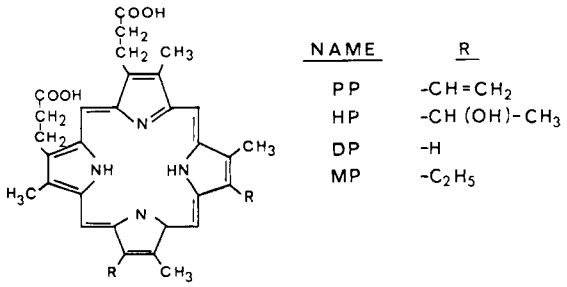
EQUILIBRIUM BINDING CONSTANTS OF PROTOPORPHYRIN IX MONOMERS TO LIPOSOMES (LUV, PC/CHOLESTEROL, 3:2) AT 37°C AND NEUTRAL pH

PP, protoporphyrin IX.

Experimental design	Concentrations		Initial aggregation state (% monomers)	K_{ML} (10^{-4} M^{-1})
	lipid (μM)	total PP (μM)		
I	65–390	0.30	56	1.8 ± 0.6
I	22–130	0.40	50	2.1 ± 1
II	100	0.05 ± 0.50	47–83	2.5 ± 0.9
Combined				2.3 ± 0.9

determined for deuteroporphyrin IX, $(2.3 \pm 0.9) \cdot 10^4$ M^{-1}.

As can be seen in Fig. 4 deutero- and protoporphyrin IX differ, structurally, at two side chain positions only: The biologically-occurring protoporphyrin IX has vinyl residues where the deuteroporphyrin IX derivative has hydrogen residues only. Probing other reactivities of proto- and deuteroporphyrin IX, we have found quantitative differences between the two, for example in the dimerization constants [14]. Yet, we have determined the same magnitude of porphyrin-liposome binding constants for them. Thus, it seems that at least for the present liposome system (i.e. type and lipid composition) and experimental conditions (such as pH, temperature and state of aggregation) the interaction with the membrane is not sensitive to the peripheral structural differences of deutero- vs. protoporphyrin IX.



NAME	R
PP	$-CH=CH_2$
HP	$-CH(OH)-CH_3$
DP	$-H$
MP	$-C_2H_5$

Fig. 4. Formula structures of proto- and deuteroporphyrin IX, respectively, PP and DP. HP, hematoporphyrin. MP, mesoporphyrin.

V. Conclusions

Using two experimental designs, which alternate the lipid and the porphyrin in terms of constant and variant components, testing partition vs. binding and, within binding, testing three different models, our findings allow the following conclusions:

(1) Binding rather than partition is the better thermodynamic approach for the quantitative treatment of the association of porphyrin with the lipid moieties of biological membranes (modeled by liposomes).

(2) Among porphyrin monomers and dimers only the former bind to the membrane, the dimers participating indirectly by their ability to dissociate into monomers. For both deuteroporphyrin IX and protoporphyrin IX, at neutral pH and 37°C, the binding constant of the monomers to liposomes composed of phosphatidyl-choline and cholesterol (molar ratios of 3:2) was determined to be $(2.3 \pm 0.7) \cdot 10^4$ M.

(3) Since both deutero- and protoporphyrin IX have the same magnitude for the porphyrin-liposome binding constant, it seems that the tetrapyrrole core and all constant side-chain substituents carry the major responsibility for the porphyrin-liposome affinity. As to the variant side chains, in the present case they are seen to have no effect. We suggest this observation is in agreement with the binding approach: In the process of binding specific moieties of the binding molecule are expected to interact with specific components at the site, while other parts of the binding molecule can have little or no interactions. The latter leading to little or no effect of such moieties on the binding. On the other hand, in a partition process the whole molecule is expected to solubilize in the organic phase, in this case the hydrocarbon interior of the lipid bilayer. We suggest such a process would be influenced by all moieties of the partitioning molecule.

Yet, taking into account that both the hydrogens and the vinyls are two-dimensional residues and neither have polar residues within them, the possibility of an effective role for porphyrins with other variant side chains cannot be excluded. There could be cases of steric and/or electrostatic interferences, with either negative or positive contribu-

tions to the overall porphyrin-membrane affinity. An example for a negative contribution, leading to decrease in the affinity can be seen for hematoporphyrin and for hematoporphyrin derivative [9,10,13].

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References

1 Dougherty, T.J., Kaufman, J., Goldfarb, A., Weishaupt, K., Boyle, D. and Mittleman, A. (1978) Cancer Res. 38, 2628–2635
2 Ito, T. (1983) Studia Biophys. 94, 1–6
3 Kessel, D. (ed.) (1986) Methods in Porphyrin Photosensitization, Plenum Press, New York
4 Kessel, D. and Dougherty, T.J. (eds.) (1983) Porphyrin Photosensitization, Plenum Press, New York
5 Kinsey, J., Cortese, D., Moses, H., Ryan, R. and Branym, E. (1981) Cancer Res. 41, 5020–5026
6 Kohn, K. and Kessel, D. (1979) Biochem. Pharmacol. 28, 2465–2470
7 Spikes, J. (1975) Ann. N.Y. Acad. Sci. 244, 395–399
8 Kessel, D. and Rossi, E. (1982) Photochem. Photobiol. 35, 37–41
9 Margalit, R. and Cohen, S. (1983) Biochim. Biophys. Acta 736, 163–170
10 Margalit, R., Cohen, S. and Rotenberg, M. (1986) in Methods in Porphyrin Photosensitization (Kessel, D., ed.), pp. 303–311, Plenum Press, New York
11 Moan, J. and Sommer, S. (1983) Cancer Lett. 21, 167–175
12 Spikes, J., Matis, W. and Rogers, M. (1983) Studia Biophys. 94, 19–24
13 Cohen, S. and Margalit, R. (1985) Biochim. Biophys. Acta 813, 307–312
14 Margalit, R. and Rotenberg, M. (1984) Biochem. J. 219, 445–450
15 Margalit, R., Shaklai, N. and Cohen, S. (1983) Biochem. J. 209, 547–553
16 Szoka, F. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. USA 75, 4194