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STUDIES OF HEMATOPORPHYRIN AND HEMATOPORPHYRIN DERIVATIVE EQUILIBRIA IN HETEROGENEOUS SYSTEMS

PORPHYRIN-LIPOSOME BINDING AND PORPHYRIN AQUEOUS DIMERIZATION

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Two processes of porphyrins in heterogeneous systems containing aqueous and membrane phases have been studied with hematoporphyrin and hematoporphyrin derivative: Dimerization equilibrium in the aqueous phases and porphyrin-membrane binding equilibrium using liposomes as models for biological membranes. The interrelationship of aqueous aggregations and membrane binding was probed and the porphyrin aggregation state in the membrane, at equilibrium, was assessed. Fluorimetric techniques were employed. The dimerization equilibrium constants, at neutral pH and 37°C were found to be $2.8 \cdot 10^5$ M^{-1} and $1.9 \cdot 10^6$ M^{-1} for hematoporphyrin and its derivative, respectively. Over a porphyrin concentration range going from monomer-dominant to dimer-dominant systems, we have found that only monomers are bound to the membrane. The respective monomer-liposome binding constants, found to be independent of the initial monomer/dimer distribution in the aqueous phase, were determined to be $1.6 \cdot 10^3$ M^{-1} and $4.1 \cdot 10^3$ M^{-1} at neutral pH and 37°C for hematoporphyrin and its derivative, respectively. The monomer-liposome interaction was found to perurb the initial monomer/dimer distribution in the aqueous phase, so that the monomers residing at equilibrium in the membrane originate from both monomers and dimers in the aqueous phase.

Introduction

Protoporphyrin IX and several of its derivatives, in particular hematoporphyrin derivative, are known to have potential roles in cancer treatment due to their following properties: (1) they have shown preferential affinity for several types of malignant tissues, which can be useful for tumor localization [1–8]; and (2) they have cytotoxic activity when membrane-embedded and irradiated by light in the visible range, thus potentially useful for tumor regression [1,2,9–20]. To perform in either role, the porphyrin has to make its way from the site of administration, usually in aqueous phase (for example, intravenous injection in clinical application [16,19,20] or directly into the medium in cell cultures [13–15,17–18]), to its site of action in the target cell, presumably in the membrane [12–14,21]. Obviously, any processes in which the porphyrin will be involved along its way, will affect its diagnostic and cytotoxic activities. Major among these processes are porphyrin aggregations in aqueous phases [22–24], porphyrin binding to serum proteins [25,26], porphyrin-membrane binding (including monomers and aggregates) [11,13,18,21,27] and porphyrin aggregations in the membrane phase (if at all). In fact, it has been argued that the major determinant of differences

in activity among porphyrins, in vivo, is in reaching the target and retention there [28].

In the present communication, we report on our molecular-level studies on several of these processes, namely aggregations in aqueous phases, membrane-binding, and the interrelationship between these two processes, for two porphyrins: Hematoporphyrin derivative and its parent compound, Hematoporphyrin IX.

In dealing with these two porphyrins, one has to be aware of their heterogeneity. Both hematoporphyrin IX and hematoporphyrin derivative preparations, commercial as well as laboratory-prepared excepting hematoporphyrin IX if used immediately after chromatographic separation, are each a mixture of several porphyrin species containing hematoporphyrin IX as the major fraction and other more hydrophobic porphyrins, the fraction of the latter higher in hematoporphyrin derivative than in hematoporphyrin IX [8,14,28–31]. Still, since the hematoporphyrin IX and hematoporphyrin derivative preparations, rather than the purified components, are commonly used in research and therapy (see for example, Refs 4–7,16–20), we have focused on the former at this stage of our studies.

Aggregations of porphyrins, such as protoporphyrin and its deutero, hemato and meso derivatives have been studied previously by Karns et al. [24] at alkaline pH and by Brown et al. [23] at neutral pH using absorption spectrospcopy. We have used fluorescence spectroscopy to determine the equilibrium constant of the first aggregation step, dimerization, and have reported our data for protoporphyrin and hematoporphyrin IX elsewhere [22].

In order to study the binding of porphyrins to membranes at the molecular level, we have started with a model system - large unilamellar vesicles (LUV). This type of liposome was chosen since they are a better model for cell membranes than sonicated unilamellar vesicles (SUV) which are too small, or multilamellar vesicles (MLV) which have too many lamella. There have been several studies of porphyrin-liposome systems, aimed at assessing the photodynamic activities of porphyrins in such model systems [8,27]. However, in those preparations whether the liposomes were of the multilamellar or the sonicated type, the porphyrins were included in the swelling solution and were therefore located in the lipid regions of the liposomes as well as in their inner aqueous phases. Obviously, such systems are not suitable for the purpose of studying the binding of porphyrins from an aqueous to a membrane phase.

Three specific issues were addressed in the present study: (a) the dimerization equilibria of hematoporphyrin IX and hematoporphyrin derivative, in aqueous solutions; (b) binding equilibria of hematoporphyrin IX and hematoporphyrin derivative to neutral fluid liposomes, over porphyrin concentration ranges spanning from monomer-dominent to dimer-dominant systems; (c) the aggregation states of hematoporphyrin IX and hematoporphyrin derivative in the membrane phase at equilibrium, and their relation to the porphyrin aggregation states in the aqueous phase.

Materials and Methods

I. Materials

Hematoporphyrin derivative was purchased from Porphyrin Products, Logan, UT, U.S.A., prepared according to Lipson et al. [10]. Egg phosphatidylcholine (type VII-E) and hematoporphyrin IX were purchased from Sigma Chemical Co. Triton X-100 was purchased from Serva Feinbiochemica, Heidelberg. All other reagents were of analytical grade.

Phosphate-buffered saline was prepared from 3.3 mM KH_2PO_4 3.3 mM Na_2HPO_4/4.7 mM KCl and 143 mM NaCl, adjusted to pH 7.2.

Fluorescence spectra were recorded on a Perkin-Elmer spectrofluorometer model MPF-48B. Absorption spectra were recorded on a Cary 118 spectrophotometer.

II. Preparation and characterization of the liposomes

Large unilamellar vesicles of phosphatidylcholine were prepared according to the procedure of Szoka et al. [32]. The liposome preparation was passed through a polycarbonate filter with a pore size of 0.6 μm and only the filtrate was collected. This set an upper limit to the size of liposome used in the binding experiments. Quantitative determination of the lipid was carried out by a colorimetric method according to Ref. 33.

III. Dimerization equilibrium of porphyrins

For the preparation of hematoporphyrin IX and hematoporphyrin derivative solutions, and the determination of hematoporphyrin derivative dimerization equilibrium constants in aqueous solutions using fluorescence data, we have used previously detailed procedures [22]. In this type of experiments, as well as in those described in the following section, the hematoporphyrin IX and hematoporphyrin derivative preparations behaved each as far as the sensitivity of our experimental methods could detect as a single species. For aqueous and organic phases, respectively, the excitation peaks for both porphyrins were at 394 nm and 397 nm, emission peaks for hematoporphyrin IX were at 615 nm and 625 nm, and emission peaks for hematoporphyrin derivative were at 617 nm and 627 nm.

IV. Procedure of porphyrin-liposome binding experiments

For each binding experiment a series of reaction mixtures were prepared. All mixtures, buffered by phosphate buffered saline, contained a constant porphyrin concentration (in the 10^{-8}–10^{-6} M range) and increasing concentrations of liposomes ranging from 0.1 to 1.7 mg/ml lipid. Doing it the other way, i.e. holding the liposome concentration constant and varying the concentrations of the porphyrin from one reaction mixture to the other is not suitable for the present systems, since each porphyrin concentration would have a different monomer/dimer distribution. The reaction mixtures were incubated for 2 h at 37°C. The liposome-associated porphyrin was separated from unreacted aqueous porphyrin by ultracentrifugation for 1 h, at 225 000 × g at 25°C. For a quantitative determination of the liposome-bound (in the pellet) and aqueous (in the supernatant) porphyrin, Triton X-100 was added to aliquots from both fractions (for each reaction mixture) to a final detergent concentration of 0.6%. The fluorescence emission spectra of each sample was recorded, and its porphyrin concentration was determined from a calibration curve of known porphyrin concentrations in 0.6% Triton X-100. We have verified that one cycle of centrifugation was sufficient for quantitative separation of the unreacted (aqueous) porphyrin, from the liposome-bound material.

Results

I. Dimerization equilibrium constants of hematoporphyrin IX and hematoporphyrin derivative

Within the porphyrin concentration range of 10^{-8}–10^{-6} M we have found fluorescence intensity of aqueous solutions of hematoporphyrin derivative to increase with increasing total porphyrin concentration in a nonlinear saturating pattern. Typical behavior is illustrated in Fig. 1a. This indicates a decrease in the fraction of the fluorescent species with increasing porphyrin concentration. In organic and in detergent solutions we found the peak emmision to be shifted to the red with an increase in intensity compared to aqueous solution. In contrast to the latter solvent, the intensity in the organic-type phases was found to increase linearly with increasing porphyrin concentrations, within the said concentration range, as illustrated by a typical example presented in Fig. 1b. The behavior observed in the organic and detergent systems indicates that in these systems the porphyrin is fully monomeric [22].

Data of the type illustrated in Fig. 1a can be processed to yield the magnitude of the dimeriza-

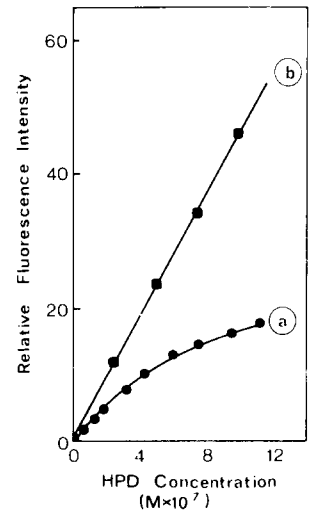

Fig. 1. Increase in peak fluorescence intensity of hematoporphyrin derivative, with increasing porphyrin concentration, in aqueous and detergent media. (a) Hematoporphyrin derivative in phosphate buffered saline, at 25°C. Excitation was at 394 nm, emission peak at 617 nm. (b) Hematoporhyrin derivative in 0.6% Triton X-100, at 25°C. Excitation was at 397 nm, emission peak at 627 nm.

TABLE I

DIMERIZATION EQUILIBRIA CONSTANTS OF PORPHYRINS AT NEUTRAL pH

Porphyrin	K_D (M^{-1})	
	25°C [a]	37°C [b]
Hematoporphyrin IX	$4\ (\pm 0.4)\cdot 10^5$	$2.8(\pm 0.4)\cdot 10^5$
Hematoporpyrin derivative	$2.3(\pm 0.7)\cdot 10^6$	$1.9(\pm 0.9)\cdot 10^6$
Protoporphyrin IX	$3\ (\pm 0.4)\cdot 10^7$	–

[a] Data reproduced from Ref. 22.
[b] This work.

tion equilibrium constant, according to the following equation:

$$[T]/F = 2K_D k^2 F + k \qquad (1)$$

where [T] is the total porphyrin concentration expressed as monomers, F is the fluorescence intensity at a given wavelength, k an apparent coefficient relating the fluorescence intensity at a given wavelength to the concentration of the fluorescent species (i.e. $F = [M]/k$, the monomer being the only fluorescent porphyrin species in the present systems [22]) and K_D is the dimerization equilibrium constant.

The magnitudes of K_D obtained for hematoporphyrin derivative and hematoporphyrin IX, from experiments similar to those illustrated in Fig. 1a, are listed in Table I.

II. Porphyrin-liposome binding

In the present study we have concentrated on the binding of hematoporphyrin IX and hematoporphyrin derivative monomers and dimers to the liposomes. To stay within the conditions where the dominant aggregation state is dimers and where the fraction of aggregates higher than dimers is negligible, concentration range 10^{-7}–10^{-5} M for hematoporphyrin IX and 10^{-8}–10^{-6} M for hematoporphyrin derivative were chosen. Our studies encompassed systems in which the monomer fraction was 85–90%, up to systems in which the dimeric fraction was 60% of the total porphyrin present. The limit at the lower end was set by the extremely low porphyrin concentrations (and therefore small fluorescence signal) that would be required to achieve systems with a monomer fraction higher than 90: We did not go beyond 60% dimers, to avoid systems in which aggregates higher than dimer could no longer be neglected.

We have recorded the fluorescence spectra of each reaction mixture at three stages. These will be described by following the typical example illustrated in Fig. 2 for hematoporphyrin derivative: First, we recorded the emission spectra of the reaction mixture at the end of the incubation period (i.e., at equilibrium). This spectra should give a qualitative indication that the reaction has taken place, and that at least some of the porphyrin, initially in the aqueous phase, has entered the lipid regions of the liposome and is now in an 'organic' phase. The observed peak intensity

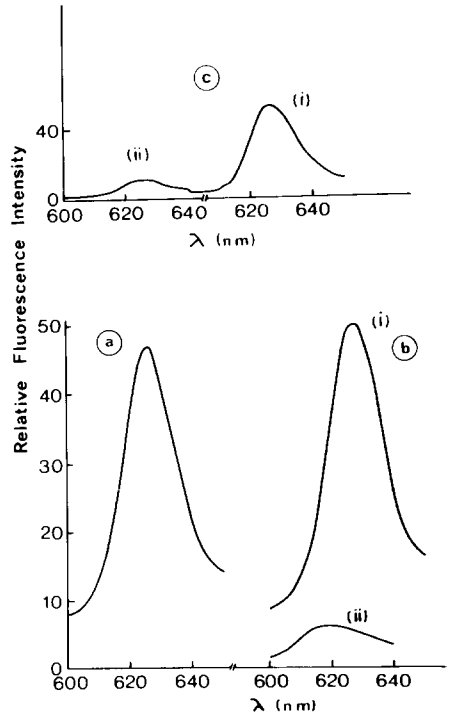

Fig. 2. Fluorescence emission spectra of hematoporphyrin derivative-liposome reaction mixture at different stages. Composition of initial reaction mixture: $2\cdot 10^{-7}$ M hematoporphyrin derivative and $1.33\cdot 10^{-3}$ M lipid. Excitation was at 397 nm. (a) Total reaction mixture, at the end of the incubation period (see Methods for further details). (b) Reaction mixture, after separation by ultracentrifugation. (i) Pellet, suspended in phosphate buffered saline to volume of original reaction mixture. (ii) Supernatant. (c) (i) Pellet (sample bi) after detergent treatment. (ii) Supernatant (sample bii) after detergent treatment.

should be between those for pure aqueous and for pure organic phases (i.e. between 617 and 627 nm). As shown in Fig. 2a, the data bear out this expectation, giving an observed peak at 625 nm.

Next, the liposomes containing bound porphyrin (presumably) were separated from excess unreacted porphyrin by centrifugation, as detailed under Methods. Typical emission spectra of the pellet (suspended in buffer) and of the supernatant, are also illustrated in Fig. 2b (i and ii). Indeed, the data show the pellet fraction to contain porphyrin in a hydrophobic medium, therefore assumed to be liposome-associated. Washing the pellet in buffer, then re-centrifuging, did not result in loss of porphyrin from this fraction. Thus the porphyrin seems to be tightly-bound rather than loosly-associated.

For a quantitative determination of the porphyrin in the pellet and in the supernatant, aliquots were dissolved in Triton X-100, as detailed under Methods. Under these conditions all the porphyrin should be in the monomeric state. This has been affirmed by others [14,31] and in the present study by the linearity of the calibration curves (recall Fig. 1). Typical emission spectra for the pellet and supernatant after detergent treatment are also illustrated in Fig. 2c (i and ii). Data similar to those illustrated in Fig. 2 were recorded for each of the reaction mixtures, in a given binding experiment. The concentration of liposome-bound porphyrin was calculated, from the detergent-treated samples, as also detailed under Methods.

Typical data of such binding experiments, for hematoporphyrin IX and hematoporphyrin derivative, from systems having various monomer/dimer initial distributions, are illustrated in Fig. 3, showing the increase of liposome-bound porphyrin with increasing lipid concentrations. For all cases, saturation was observed with a hyperbolic pattern, indicative of a binding process with only one type of binding site within the range of ligand and macromolecule concentrations studied.

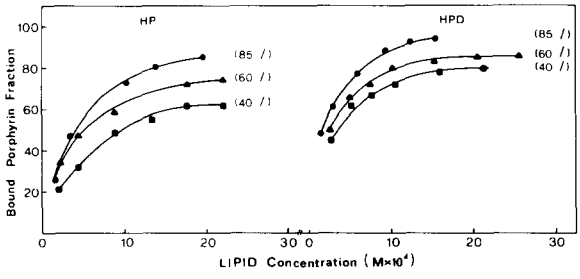

Fig. 3. The increase in the fraction of liposome-bound porphyrin (of the total porphyrin in the reaction mixture) with increasing lipid concentrations at 37°C, buffered by phosphate buffered saline. Numbers in parenthesis are the monomer fractions in the aqueous phase, prior to addition of the liposomes.

III. Aggregation state of the membrane-bound porphyrin

In order to probe the aggregation state of the porphyrin in the membrane at equilibrium, we have compared the fluorescence emission spectra of the porphyrin in the pellet before and after the triton treatment (recall Figs. 2b and 2c). If the porphyrin in the liposome is an equilibrium mixture of monomers and aggregates (presumably dimers) then the detergent treatment should result in an increase in the fluorescent intensity of the sample, since it transforms all the membrane-bound porphyrin to the monomeric state. If, on the other hand, all the membrane-bound porphyrin is already in the monomeric state, the detergent treatment should not effect a significant increase in the fluorescence intensity. The latter was found to be the case, as shown by the data listed in Table II.

Discussion

I. Monomer-dimer equilibria in aqueous phases

We have previously shown through fluorimetric

TABLE II

EFFECTS OF DETERGENT TREATMENT ON THE PEAK FLUORESCENCE INTENSITY OF LIPOSOME-BOUND PORPHYRIN

HPD, hematoporphyrin derivative. HP, hematoporphyrin IX.

Initial aggregation state in aqueous phase (% monomers)	Ratio of post-triton to pre-triton peak fluorescence intensity	
	HPD	HP
85	0.85	1.00
60	1.00	1.16
40	1.20	1.08

studies [22], and others have shown through absorption spectroscopy studies [23,24] that among porphyrin IX molecules derivatized at the vinyl residues, protoporphyrin is the most highly aggregated and hematoporphyrin IX is the least. Hematoporphyrin derivative has not been studied previously, and the present data show it to fall between protoporphyrin and hematoporphyrin IX (recall Table I). The increase in the magnitude of the dimerization constants, from $4 \cdot 10^5$ for hematoporphyrin IX to $2.3 \cdot 10^6$ for hematoporphyrin derivative (at 25°C) agrees with the experimental observations that hematoporphyrin derivative has a higher content of hydrophobic species than hematoporphyrin IX [8,28–31], and that the dimerization is due to favorable hydrophobic interactions between the monomers [1,2,22,24].

Having at hand the magnitudes for K_D, makes it possible to determine the state of aggregation (i.e. quantitative monomer/dimer distribution) of a given porphyrin solution within the concentration ranges of 10^{-9}–10^{-7} M for protoporphyrin, 10^{-8}–10^{-6} M for hematoporphyrin derivative and 10^{-7}–10^{-5} M for hematoporphyrin IX. We have made use of data of this type, assessing the binding of porphyrins to membranes over a range of monomer/dimer distributions, as will be shown in following sections of this discussion.

II. Porphyrin aggregation states in the liposomes at equilibrium

One of the major findings of the present study is that at equilibrium, only monomers were found in the liposomal membranes, even though the aqueous phase of this heterogeneous system has both monomers and dimers (recall section III of Results).

This finding leads immediately to the following question: Where do the monomers embedded in the membrane come from? If the monomer-liposome binding process does not perturb the initial monomer/dimer distribution in the aqueous phase to an extent that would be measurable experimentally in our system, then the porphyrin in the membrane can only come from the original monomer concentration in the aqueous phase. Furthermore, that concentration would set an upper limit to the concentration of liposome-bound monomers at saturation. If, on the other hand, the original monomer/dimer distribution is sufficiently perturbed to be measurable, then that limit does not apply.

The typical data illustrated in Fig. 3 unambiguously shows that the lat6ter is the case, i.e. significant perturbation does occur. For example, in hematoporphyrin derivative systems having initial monomer fractions of 85%, 60% and 40% (in the aqueous phase), the respective fractions of liposome-bound monomers at saturation are seen to be 95%, 85% and 80%. For hematoporphyrin IX, it is 60% and 40% vs. 75% and 60%, respectively.

Thus, the data clearly indicate an involvement of the dimers, ending as monomers, in the membrane binding. Whether this involvement is direct or indirect, will be assessed in the following section.

III. Porphyrin-liposome binding equilibrium in systems varying in their initial state of porphyrin aggregation

Any scheme introduced to represent the porphyrin-liposome binding processes we have studied must account for the findings discussed in the previous section, i.e. the presence of monomers only in the membrane phase, originating from both monomers and dimers in the aqueous solution. Furthermore, at least two equilibria have to be taken into account–dimerization in the aqueous phase and monomer-liposome binding.

Assuming that the involvement of the dimer is indirect–through dissociation into monomers (the latter being the only species binding to the liposomes with a stoichiometry of 1:1)–these two equilibria suffice and the following simple scheme can represent the process:

$$2M \overset{K_D}{\rightleftharpoons} D; \qquad K_D = [D]/[M]^2 \tag{2}$$

$$M + L \overset{K_{BM}}{\rightleftharpoons} ML; \qquad K_{BM} = [ML]/[M][L] \tag{3}$$

The free and porphyrin-bound liposomes are denoted as L and ML respectively. M and D respectively denote the monomer and dimer. K_{BM} is the equilibrium binding constant of the porphyrin monomers of the liposomes– the parameter we are seeking. K_D has been defined previously (see Eqn. 1).

By denoting the concentration of ML as $[T_b]$ (the total concentration of liposome-bound porphyrin expressed per monomer), and by expressing the dimer concentration through Eqn. 2 while taking into account conservation of matter, the following expression is obtained:

$$2K_D[M]^2+[M]-([T]-[T_b])=0 \quad (4)$$

Eqn. 4 yields an expression for [M], the concentration of the free monomers in the aqueous solution. This can then be introduced into Eqn. 3 to obtain the magnitude of K_{BM} from the experimentally measured quantities [T], $[T_b]$ and [L], and the independently determined magnitude of K_D, giving the following:

$$K_{BM}=\frac{[T_b]}{\left(\frac{-1+\sqrt{1+8K_D([T]-[T_b])}}{4K_D}\right)[L]} \quad (5)$$

Binding data of the type illustrated in Fig. 3 have been processed according to Eqn. 5. The magnitudes of K_{BM}, for hematoporphyrin IX and hematoporphyrin derivative, obtained from experimental data of systems ranging from monomer dominant to dimer-dominant, are listed in Table III.

The most striking finding to emerge from this data is the single constant magnitude of K_{BM} for a given porphyrin, regardless of the state of aggregation in the aqueous phase: $4.1 \cdot 10^3$ (mol lipid/liter)$^{-1}$ for hematoporphyrin derivative and $1.6 \cdot 10^3$ (mol lipid/liter)$^{-1}$ for hematoporphyrin IX. These magnitudes, showing hematoporphyrin derivative to be more tightly bound than hematoporphyrin IX, are similar to the pattern observed for the dimerization constants (recall Table I). This finding agrees with our hypothesis which assumes that when hematoporphyrin derivative or hematoporphyrin IX monomers and dimers in aqueous solution encounter membrane-enclosed particles (namely liposomes), then only the monomers bind to the liposomes. The consequent decrease in the free monomer concentration in the aqueous phase causes the dimers to dissociate, thus contributing 'fresh' monomers to the monomer pool in the aqueous phase. When the system comes to rest, equilibria must exist between the free monomers and dimers in the aqueous solution and the bound monomers in the liposomes. This equilibria is defined by two constants, one for porphyrin dimerization in the aqueous phase and the other for porphyrin monomer-liposome binding.

As already discussed in previous sections, under the experimental conditions of the present study, each preparation behaved as a single species. We do not mean this to say that each preparation was, in fact, a single species, but rather that it did not undergo significant changes in composition in the course of the present experiments. This might not be the case for long incubation periods, of the order used in some clinical protocols, as the time-interval between porphyrin administration and photoactivation (48 h and more, see for example Refs. 5,6,16,19,20). Under such experimental conditions, it is quite conceivable that significant changes in composition will be detected (i.e. 'fractionation' between aqueous and membrane phases, including inner aqueous phases of the membrane-enclosed particles [28–31]). This issue will be pursued, not only with liposomes composed of phosphatidylcholine only, but with other lipids as well.

On the other hand, we argue that the data we gathered, although limited to dimers in terms of porphyrin aggregates and their effects on membrane binding, is relevant to hematoporphyrin IX and hematoporphyrin derivative administration in vivo. The total doses administered are such that

TABLE III

EQUILIBRIUM BINDING CONSTANTS OF PORPHYRIN MONOMERS TO PC LIPOSOMES (LUV), AT NEUTRAL pH, 37°C

HPD, hematoporphyrin derivative. HP, hematoporphyrin IX. n is the number of experiments.

Initial aggregation state in aqueous phase (% monomers)	K_{BM} (M^{-1})	
	HPD	HP
85	$(4.2 \pm 0.8) \cdot 10^3$ ($n=13$)	$(1.8 \pm 0.3) \cdot 10^3$ ($n=10$)
60	$(4.1 \pm 0.7) \cdot 10^3$ ($n=15$)	$(1.4 \pm 0.3) \cdot 10^3$ ($n=24$)
40	$(4.0 \pm 0.8) \cdot 10^3$ ($n=12$)	$(1.7 \pm 0.3) \cdot 10^3$ ($n=13$)

aggregates higher than dimers would be expected [6,7,16–20]. However, the scavenging action of serum proteins [15,26] reduces the remaining free aqueous porphyrin, pushing towards the dominance of dimers as the porphyrin aggregates present in vivo.

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