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Effects of Membrane Physical Parameters on Hematoporphyrin-Derivative Binding to Liposomes: A Spectroscopic Study

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Summary. Physical parameters of membrane bilayers were studied for their effect on the binding of hematoporphyrin derivative (Hpd), which is used as a sensitizer in photodynamic therapy of cancerous tissues. The purpose of this study was to clarify which parameters were relevant, under physiological conditions, to the selectivity of Hpd binding to cancer cells. Fluorescence spectroscopy was used to measure the relative partitioning of the dye between the lipid and aqueous media. Increasing the microviscosity of the liposomes' membranes by various bilayer additives results in a strong reduction of Hpd binding, to an extent independent of the specific additive. The effect of temperature near the physiological value as well as the effect of cross membrane potential are small. Surface potential does not affect the binding constant, indicating that the binding species does not carry a net electric charge.

Key Words hematoporphyrin derivative · dye-liposome interaction · fluorescence polarization · membrane order and microviscosity · membrane electric potentials

Introduction

Hematoporphyrin derivative (Hpd)[1] has been used in recent years as a photosensitizing agent in photodynamic therapy of cancerous cells *in vitro* and *in vivo* (Dougherty et al., 1978). The two properties of Hpd that enable its use in this therapeutic mode are its selective accumulation by cancerous cells in the body (Rasmussen-Taxdall, Ward & Figge, 1955; Gomer & Dougherty, 1979) and the photo-sensitization of cell damage caused by either the formation of highly reactive singlet oxygen or by collisional energy transfer from the excited porphyrin molecules (Grossweiner, Patel & Grossweiner, 1982). We have recently shown (Malik, Gozhansky & Nitzan, 1982; Nitzan, Gozhansky & Malik, 1983; Ehrenberg, Malik & Nitzan, 1985) that the binding, or solubilization, of Hpd at the cytoplasmic membrane of bacteria, evidenced by a characteristic spectral shift of the main fluorescence band from 613 to 631 nm, is a prerequisite for the photodynamic killing of the bacteria by Hpd. It was also shown that protoporphyrin, another photosensitizer of human cancer cells, tends to accumulate and cause damage mainly to the cytoplasmic membrane (Malik & Djaldetti, 1980). It is therefore reasonable to anticipate that membranal factors that modulate Hpd binding to the membrane contribute to the preferred binding and retention in malignant cells.

In this study we examined the effect of a few membranal physical parameters on the binding of Hpd to lipid vesicles. Among these parameters were the microviscosity of the membrane, which was altered by adding various lipids to the vesicles' preparation or by the effect of temperature, and changes in the membrane surface charge density and cross-membrane potential difference. The strong effects on Hpd binding were monitored by the characteristic fluorescence bands of membrane-bound and aqueously-dissolved Hpd.

Materials and Methods

Chemicals

Hematoporphyrin derivative (Hpd) was prepared by the method of Dougherty et al. (1978). Hematoporphyrin (Sigma) was treated by glacial acetic acid/sulphuric acid (19 : 1 vol/vol). The product was brought into solution in 1 N NaOH and then the pH was adjusted to 7.0. This stock solution, containing a mixture of acetylation and dehydration products (Clezy et al., 1980) was used as is. Egg phosphatidylcholine, cholesterol, dimyristoyl- and dipalmitoylphosphatidylcholine, dicetylphosphate (DCP) and 1,6-diphenyl-1, 3,5-hexatriene (DPH) were purchased from Sigma.

[1] *Abbreviations:* Hpd, hematoporphyrin derivative; DPH, 1,6-diphenyl-1,3,5-hexatriene; DCP, dicetylphosphate; DMPC, dimyristoyl-phosphatidylcholine; DPPC, dipalmitoyl-phosphatidylcholine.

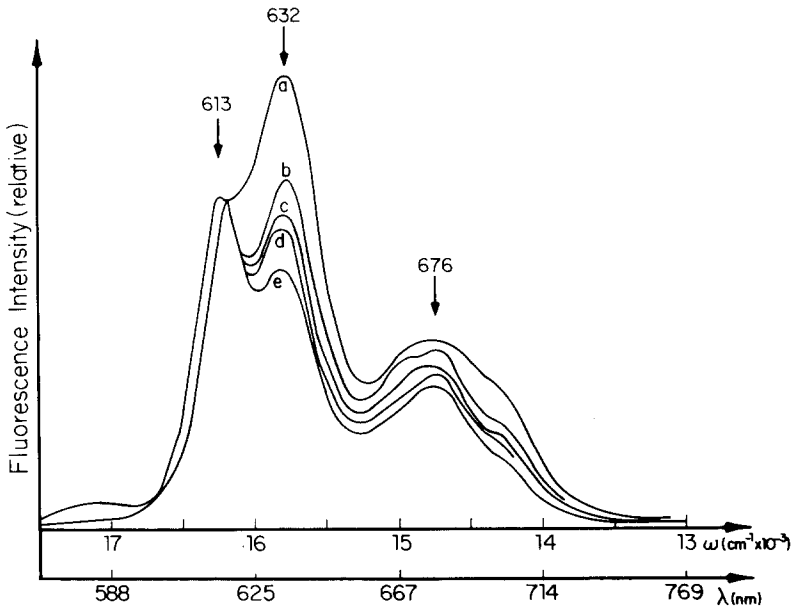

Fig. 1. Fluorescence spectra of Hpd in suspensions of lecithin vesicles, with cholesterol content of 0% (*a*), 10% (*b*), 20% (*c*), 50% (*d*), 70% (*e*). Total lipid concentration: 0.07 mg/ml. Hpd concentration: 10 μg/ml · λ_{exc} = 395 nm. Spectra were measured at 20°C. Spectra were normalized at 613 nm

Table. Effect of additives in lecithin liposomes on the membranal polarization and on the partitioning of Hpd between the lipid and aqueous media[a]

	Cholesterol			DCP			DPPC			DMPC		
	P	r_∞	F_L/F_W	P	r_∞	F_L/F_W	P	r_∞	F_L/F_W	P	r_∞	F_L/F_W
0	0.148	0.048	1.000	0.135	0.040	1.000	0.133	0.038	1.000	0.110	0.026	1.000
10	0.185	0.075	0.430	0.155	0.052	0.752	0.164	0.059	0.744	0.156	0.053	0.802
20	0.237	0.121	0.384	0.188	0.077	0.690	0.200	0.087	0.659	0.152	0.050	0.740
30	0.258	0.142	0.424	0.247	0.131	0.300	0.195	0.083	0.652	0.164	0.059	0.730
40							0.230	0.114	0.631	0.199	0.086	0.494
50	0.291	0.176	0.324									
70	0.347	0.238	0.254									

[a] P is the measured fluorescence polarization. r_∞ is the limiting anisotropy of DPH calculated by the empirical equation of Van der Meer et al. (1986). F_L/F_W is the ratio between the integrated fluorescence intensities of the lipidic and water-solubilized Hpd, normalized to that obtained with liposomes of pure lecithin.

Preparation of Liposomes

Lipid vesicles were prepared by evaporating the solvent from the solution of phosphatidylcholine in chloroform/methanol and redissolving in ethylether and adding here, when necessary, a desired lipid additive in ether or chloroform solutions. The ether was evaporated thoroughly, and water or buffer was added to form a total lipid content of 1 mg/ml. The sample was vortexed for about 3 min and then sonicated in a round bath sonicator (Laboratory Products, Hicksville, N.Y.) to clarity and was used as stock solution. Large unilamellar vesicles were prepared by freezing the above-described liposomes at liquid nitrogen temperature, letting them thaw at room temperature and then resonicating for 30 sec (Pick, 1981). These large vesicles could be pelleted at 300,000 × g for the study of Hpd binding.

Cross-membrane potential was generated by preparing a liposome suspension of 5 mg/ml in 0.2 M NaCl or KCl and diluting into 0.2 M KCl or NaCl, respectively. Addition of valinomycin (Sigma) to a final concentration of 10^{-7} M induces K^+ diffusion potential, and gramicidin, 10^{-6} M (Sigma) is used to abolish this potential by causing opposite Na^+ diffusion. Usually, the liposomes that were prepared for this purpose also contained ~10% cholesterol, to diminish the leakiness of the liposomes to the ions present in the suspension (Sims et al., 1974).

Fluorescence Measurements

Fluorescence spectra were taken in a set-up composed of Hg or Xe light source (Oriel), Spex 1403 monochromator at 0.5 nm resolution or better, RCA C31034-02A-cooled photomultiplier and photon-counting electronics interfaced to an Apple II microcomputer. The computer also controlled the monochromator drive (Meiri et al., 1983) and was used to resolve the bands in the fluorescence spectra, by a parameter-fitting program (Ruckdeschel, 1981).

Steady-state fluorescence polarization was measured with liposome samples that were incubated for ~½ hour with DPH at a 1 μM concentration. DPH fluorescence was excited at 365 nm through a UV polarizer (Polaroid HNPB-UV), and the emission was measured at 430 nm through a rotatable analyzer, which was followed by a depolarizer to avoid polarization effects at the monochromator's gratings. These effects as well as scattering from the sample were checked and corrected for (Litman & Barenholtz, 1982). Absorption spectra were measured on a Perkin-Elmer Lambda-9 spectrophotometer.

Results and Discussion

Effect of Cholesterol, DMPC and DPPC on Hpd Binding to Liposomes

The fluorescence spectra of Hpd in suspensions of lipid vesicles containing varying concentrations of cholesterol are shown in Fig. 1. A few emission bands can be seen in the spectra. The fluorescence of aqueously dissolved Hpd appears at 613 nm and the fluorescence of Hpd bound to the lipid vesicles shows up at 632 nm (Srivastava, Anand & Carper, 1973; Andreoni et al., 1982; Ehrenberg et al., 1985), and this red shift reflects the better stabilization of the Hpd-excited state by lipid solvation. This shift is also observed in Hpd fluorescence spectra in common organic solvents of low polarity such as ethanol, methanol, dioxan, etc. (Kessel & Rossi, 1982). A third emission band, seen at 676 nm, was assigned to a transition from a different excited electronic state(Srivastava et al., 1973). However, this assignment is still questionable, especially because this long wavelength fluorescence band of the lipid-bound Hpd shifts down to 667 nm when excited at 458 nm instead of the 395-nm excitation employed to obtain the spectra of Fig. 1. It also does not seem likely that this emission band is a vibrational splitting, because the gap between the fluorescence bands varies by hundreds of cm^{-1} upon changing the solvent or excitation wavelength.

The most prominent result that can be observed in Fig. 1 is the decrease in the fluorescence intensity at 632 nm relative to that at 613 nm upon increasing the cholesterol content of the liposomes. This change is indicative of the fact that at increased cholesterol concentrations less Hpd is bound to the liposomes. The Table summarizes the ratios of the fluorescence bands of membrane bound and aqueous Hpd at varying contents of cholesterol, as well as the steady-state fluorescence polarization of DPH in these membranes. The absolute values of the polarization of the mixed phosphatidylcholine/cholesterol vesicles are very close to those obtained before (Shinitzky & Inbar, 1976).

The measured steady-state fluorescence polarization of DPH is related to two different physical properties of the lipid bilayer-probe system. One is the restricted motion of the probe in the membrane, in the nanosecond time scale, which is caused by the membrane viscosity, and the other is the anisotropic distribution of the probe in the membrane bilayer. These dynamic and static parameters, respectively, can be delineated by fluorescence polarization decay measurements (Heyn, 1979; Jahnig, 1979; Lakowicz, 1983). Based on the vast body of data on DPH fluorescence polarization in various phospholipid membranes, empirical equations were derived for both the "true," dynamic microviscosity (Van Blitterswijk, Van Hoeven & Van der Meer, 1981; Shinitzky, 1984; Van der Meer, 1984), as well as for the limiting anisotropy r_∞, which is reached after a long time in decay experiments (Van der Meer, Van Hoeven & Van Blitterswijk, 1986). The latter is related to the static order parameter, or the packing of the membrane lipids. The Table also includes the values of r_∞ for the various membrane samples that were calculated by the empirical formula of Van der Meer et al. (1986).

A similar observation of decreased solubilization of an external molecule in the lipid bilayer, upon increasing the cholesterol content in the membrane, was observed with the fluorescent dye merocyanine 540 (Williamson, Mattocks & Schlegel, 1983). In that study it was concluded that this effect is predominant when the vesicle bilayers are in the fluid phase and the inclusion of cholesterol induces closer packing of the lipid molecules. There is additional evidence that the partitioning of low molecular weight solutes such as glucose (Chen, Lund & Richardson, 1971), alkanes (Miller, Hammond & Porter, 1977), ANS (Shinitzky, 1979), or alcohols (Zavoico, Chandler & Kutchai, 1985) in lipid bilayers decreases with an increase in the measured fluorescence polarization. In our study, the bilayer packing effect on the partitioning was monitored and correlated quantitatively as an increased membrane polarization.

Another study (Suwa, Kimura & Schaap, 1978) has shown that at temperatures above the gel-fluid phase transition, an increased cholesterol content in the membrane caused some decrease in the yield of cholesterol oxidation products, formed by photosensitization with regular hematoporphyrin. This finding could not be used, in our opinion, as a direct proof of the effect of cholesterol on hematoporphyrin binding to the membrane, but rather could result from the dilution of the cholesterol photodestruction when it is present at high concentration, even if the amount of membrane-bound hematoporphyrin remained the same. In our study, however, the fluorescence measurement is a direct indication of the

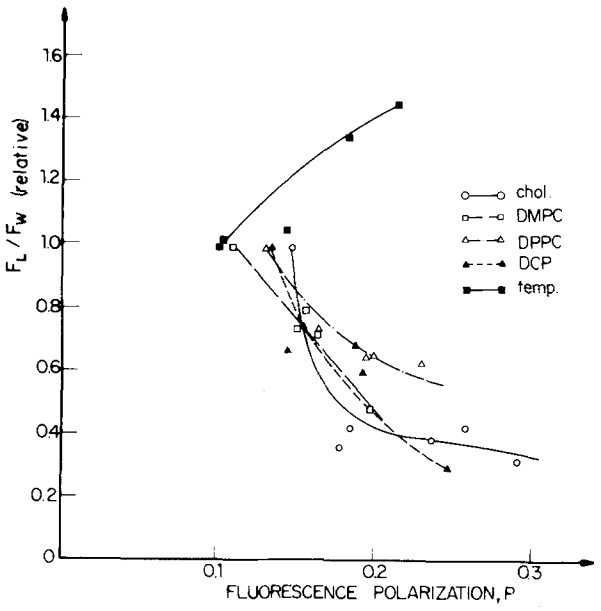

Fig. 2. The ratio between the measured integrated fluorescence intensities of lipid-bound, (F_L, band centered at 632 nm) and aqueous Hpd (F_W, centered at 613 nm), as a function of the fluorescence polarization, which was altered by the indicated lecithin additives (at 20°C) and by temperature variation

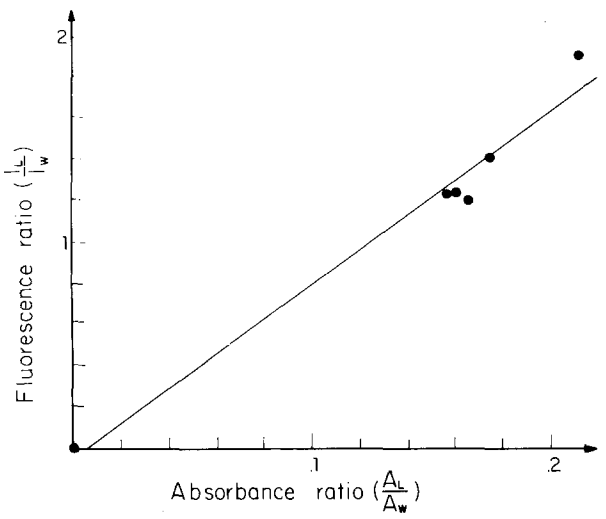

Fig. 3. Ratio of fluorescence intensities of Hpd in the lipid (F_L) and aqueous media (F_W) for a set of liposome preparations with 0 to 40% DPPC *vs.* the corresponding ratio of peak absorbancies of Hpd in the two media after they were separated by ultracentrifugation. The liposome precipitates were dissolved in methanol. The straight line is a least squares fit. Hpd concentration: 12.5 μg/ml.

incorporation of the tumor localizing Hpd to the vesicles' membranes.

We have also checked the effect of the addition of specific phosphatidylcholines to the membranes. As can be seen in the Table and Fig. 2, the addition of DMPC and DPPC increases the polarization of DPH in the bilayer and at the same time decreases the extent of Hpd binding to the membrane. From the monotonous change in both the polarization and Hpd binding upon increasing the relative content of the added lipids, it is clear that no phase separation occurs and that the membranes are in their fluid phase at all the component ratios employed here.

It is important to notice in Fig. 2 that the dependence of the membrane-Hpd binding, on the observed DPH polarization, is practically independent of the membrane components. Thus the packing of the membrane bilayer, which is reflected in the polarization, is the parameter that is important in determining the extent of Hpd binding. This is an important result, which may turn out to be at least part of the reason for the selective, or enhanced, binding of Hpd to malignant cells, because of the lowered microviscosity and packing of the cytoplasmic membranes of some cancerous cells (Van Blitterswijk, 1984).

In our discussion above, it is inherently implied that the change in the relative fluorescence intensities, upon increasing the content of a lipid additive to the membrane, is indicative of a change in the extent of binding of the Hpd to the membrane, and it does not reflect a mere change in the fluorescence quantum yield of the bound Hpd due to the altered lipid composition. We checked this by separating the vesicles from the aqueous solvent by ultracentrifugation, dissolving the precipitate in methanol and measuring the absorbances of these solutions as well as the aqueous supernatants. The plot of relative fluorescence intensities *vs.* relative absorbances for the different preparations of varying DPPC content (Fig. 3) indicates no change of fluorescence quantum yield, but rather a dependence of fluorescence intensity on Hpd binding to the membrane. From the slope in Fig. 3, and from our observation that the light absorbance at 395 nm of Hpd in tetrahydrofuran is 1.2 times higher than in methanol, we can calculate that the fluorescence quantum yield of membrane-bound Hpd is 10.15 ± 0.5 times higher than in water, assuming that tetrahydrofuran provides a medium similar to that felt by membrane-bound Hpd. The position of the fluorescence band of the bound Hpd is indeed indicative of a low-polarity medium with a dielectric constant smaller than 10 (Ehrenberg et al., 1985; Kessel & Rossi, 1982). It should be noted, however, that Hpd probably does not immerse itself fully into the apolar, chain region of the bilayer. This is based on the simple fact that the solubility of Hpd in alkane sol-

vents, like hexane or octane as well as in very apolar solvents like benzene or chloroform, is extremely small. The binding must resemble somewhat the way amphiphiles are immersed in lipid bilayers.

The temperature effect on Hpd binding was measured with pure lecithin liposomes. It can be seen in Fig. 2 that as the temperature is lowered, the membrane polarization increases as well as the relative amount of bound Hpd. To separate the pure, thermodynamic effect of the temperature on the binding from the indirect influence through the change in the membrane order and viscosity, we divided the measured lipidic/aqueous fluorescence intensity ratios at the different temperatures by the temperature-independent effects observed with the lipid additives at the same polarization values. The net thermodynamic effect of the temperature is shown in Fig. 4. The standard change in the Gibbs free energy of Hpd transfer from aqueous to lecithin environment is in the range of -1.7 kcal · mole^{-1}, based on the effect of DPPC on the polarization, to -2.4 kcal · mole^{-1}, using the cholesterol results.

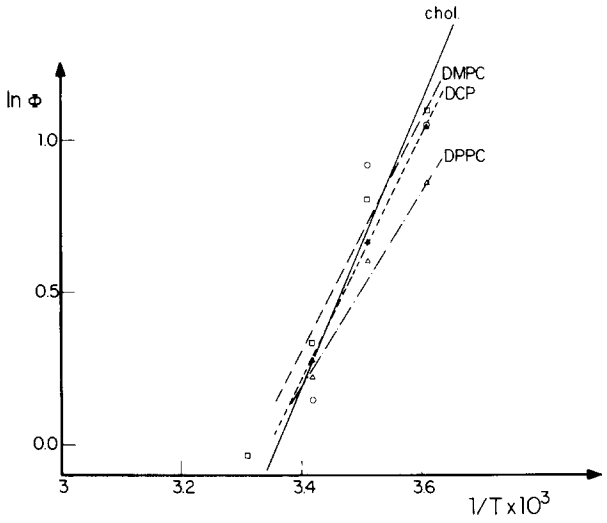

Fig. 4. Plots of the equation $\Delta G^o = -RT \ln\phi$ for the net temperature dependence of the ratio of fluorescence bands of Hpd in the lipid and water environments (ϕ). The effect of microviscosity was eliminated using the Hpd binding/viscosity relationship observed with cholesterol, DMPC, DPPC and DCP. λ_{exc} = 395 nm

Effect of Surface Potential

Under the drastic conditions employed to produce Hpd, a mixture of acetylation, dehydration (Clezy et al., 1980) and possibly etherification and polymerization products (Dougherty, Potter & Weishaupt, 1984) are formed, and this mixture is usually used in studies and clinical treatment. At pH = 6.5, the core nitrogens are mainly doubly protonated, i.e., neutral, and side chain carboxylic groups are anionic. We were interested in finding out whether the species that binds to the membrane is a neutral or charged molecule. To answer this question we imposed a membrane surface potential by incorporating in the bilayer the negatively charged lipid dicetylphosphate and monitored its effect on Hpd binding through the fluorescence spectrum. The electric potential difference across the diffuse layer between the membrane's surface and the solvent bulk, ψ_s, can be calculated by the Gouy-Chapman equation (McLaughlin, 1977; Ehrenberg, 1986)

$$\sinh\left(\frac{ze\psi}{2kT}\right) = A\sigma C^{-1/2} \qquad (1)$$

where σ is the surface charge density; C is the concentration of an electrolyte of charge z; e, k and T have their usual meanings; and A is a constant equal to 136.2 M$^{1/2}$ · Å^2 at 20°C. Since the area occupied by a phospholipid molecule in a bilayer is ~60 Å^2 (Levine & Wilkins, 1971), the surface potential can be calculated at any electrolyte concentration. For instance, at 5% DCP in the bilayer and 1 mM KCl in the solution, $\sigma = 3.3 \times 10^{-3}$ charges · Å^{-2} and $\psi = -170$ mV, and at 30% DCP, $\psi = -195$ mV. Under such potentials, the concentration of ions has a Boltzmann distribution along an axis perpendicular to the membrane surface. For the above-mentioned examples the concentration of a univalent anion near the surface is higher than the bulk concentration by a factor of 840 and 2250, respectively. These numbers could be lower if there is ion binding to the membrane. We should thus observe a strong dependence of Hpd binding on the surface charge density, i.e., on DCP concentration in the membrane. However, the results, which are included in Fig. 2, show that the binding is governed by the order and microviscosity of the membranes and is very similar to the variation caused by the other additives shown in the figure. We can thus conclude that the species that binds to the membrane is a noncharged or zwitterionic Hpd derivative. This result has, of course, an important significance on the considerations that have to be taken in devising specific derivatives of Hpd in future syntheses.

Effect of Cross-Membrane Potential

The cross-membrane, Nernst, potential difference is a parameter that plays an important role in cellu-

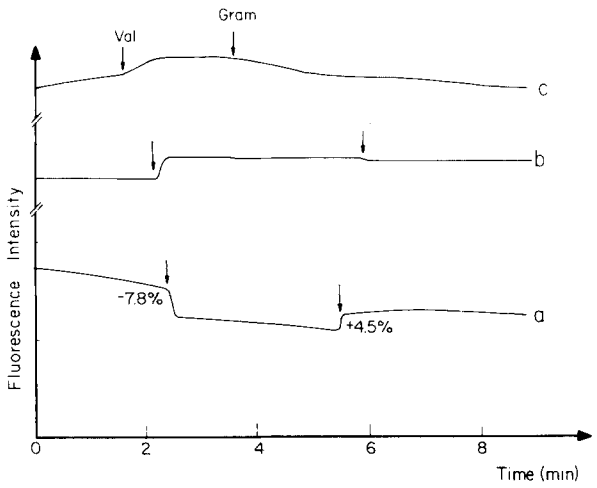

Fig. 5. The response of Hpd fluorescence to valinomycin-induced, K^+ diffusion potential. Lecithin vesicles containing 10% cholesterol were suspended to a final concentration of 0.1 mg/ml. Valinomycin (10^{-7} M) and gramicidin (10^{-6} M) were added from concentrated ethanol stocks. (*a*) The vesicles were sonicated in 0.2 M KCl and diluted 45-fold into 0.2 M NaCl. The fluorescence was measured at 632 nm. (*b*) As in *a*, the fluorescence was measured at 613 nm. (*c*) Vesicles in 0.2 M NaCl were diluted into 0.2 M KCl and the fluorescence was measured at 632 nm

lar activity and regulation. We checked its effect on Hpd binding to liposomes by generating valinomycin-induced, K^+ diffusion potential on these liposomes. As can be seen in Fig. 5, the fluorescence intensities of the membrane-bound and aqueously dispersed Hpd exhibit changes in opposite directions when the diffusion potential is created. The relative decrease of the lipid Hpd is 7.8% per 98 mV potential difference, negative inside. The mechanism of this response could be electrochromic coupling between the membrane electric field and the chromophoric transition dipole (Loew, 1982), which does not involve any redistribution of the dye between the phases, or a variety of other mechanisms (Waggoner & Grinvald, 1977; Waggoner, 1979) that arise from redistribution, aggregation or penetration of the dye in the lipid phase. We are now pursuing the mechanism of this potentiometric response. In any event, it is obvious that physiological membrane potentials do not modulate Hpd binding to lipid membranes to a significant extent.

In summary, the effect of membrane order and microviscosity is clearly an important parameter influencing the distribution constant of Hpd into lipid bilayers and is important in affecting the photodynamic effect in human cells as well (Salzberg et al., 1985). Electric field parameters are found to be of small importance and probably have little physiological relevance.

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