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Effects of pH on the binding of hematoporphyrin derivative to monolayer and bilayer membranes

M.R. Rich, G. Ferraro and S.S. Brody

Biophysics Laboratory, Department of Biology, New York University, New York, NY (USA)

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The effects of pH on the binding of hematoporphyrin derivative (HpD) to monolayer and bilayer membranes have been studied. Absorption spectra of HpD bound to phosphatidylcholine (PC) liposomes indicate that there is greater binding of HpD to lipid films at acidic, tumorcidal pH conditions than at normal tissue pH. These results were found to correlate with surface pressure measurements of monolayer films formed under similar conditions. Surface potential measurements in conjunction with surface pressure measurements from monolayer films suggest that at low pH (i.e. ≤ 6.6) porphyrin intercalates within the lipid film to reach relatively high concentrations, while at higher pH (i.e. ≥ 7.4) the porphyrin preferably adsorbs to the lipid film at the monolayer/water interface.

Introduction

The therapeutic effects of photoradiation therapy are based in part on the selective uptake and retention of the aggregated fraction of HpD by tumor tissue. While the underlying mechanism for this uptake has not yet been fully elucidated, it appears to be related to a number of specific properties of the tumor, namely, poor lymphatic drainage, presence of macrophages that take up aggregated materials, pinocytosis, an elevated concentration of lipoprotein receptors and low tumor pH [1]. Studies in tumors have shown that the interstitial fluid pH ranges from 5.8 to 7.2 whereas the distribution for normal tissue has been found to be between 7.1 and 7.4 [2,3].

While the effects of pH on the ionization of drugs and hence their binding and transport across the normal cell membrane have been well-documented [4], the role of pH in HpD uptake by tumors has only recently been established. It has been proposed by several investigators that under low pH, haematoporphyrin and many of its derivatives get protonated, thus more lipophilic, resulting in an increase in cellular uptake [5–8]. *In vitro* studies using cell cultures have demonstrated increased uptake of HpD at low pH [9,10].

The significance of pH effects on porphyrin uptake are reflected in recent *in vivo* studies. Thomas et al. [11] have demonstrated that sarcoma rat tumors made hyperglycemic exhibited decreased pH and increased ability to accumulate HpD; photoradiation of these tumors resulted in a greater cell kill. Recently, Peng et al. [12] demonstrated that mice bearing a human melanoma LOX resulted in significant increased uptake of Photofrin II in the tumor after administration of glucose inject. Nordihyroguaiaretic acid, an inhibitor of gylcolysis seem to abolish the effect.

In order to gain greater insight into the role that pH plays in HpD uptake by tumors, the binding of HpD to phosphatidylcholine bilayers and monolayers in a pH range of 5.9–8.0 has been studied. To this end, surface pressure (π) and surface potential (ΔV) measurements for monolayer films in conjunction with spectroscopy of liposomes have been used.

Materials and Methods

Hematoporphyrin derivative (from Porphyrin Products, Logan, UT) and phosphatidylcholine (Avanti Polar Lipids, Birmingham, AL) were used without any further purification.

Monolayer studies were carried out in a Wilhelmy plate film balance, housed in a light free chamber. Lipid films were formed to 20 dyne/cm on an aqueous

Abbreviations: Hp, hematoporphyrin; HpD, hematoporphyrin derivative; HvD, hydroxyethyl vinyldeuteroporphyrin; PP, protoporphyrin.

Correspondence: M.R. Rich, Biophysics Laboratory, Department of Biology, New York University, 1009 Main Building, Washington Square, New York, NY 10003, USA.

subphase consisting of 10 mM KCl and 10 mM potassium phosphate buffer (pH 5.9–8.0). After the film stabilized HpD was injected from under the barrier. The solution was prepared by dissolving HpD in a 0.1 M solution of NaOH and slowly bringing the solution to the desired pH by addition of aliquots of 1 M HCl. The final concentration of HpD used was such to bring the subphase to 25 μg/ml. A magnetic stirrer located in the subphase, used to facilitate rapid dispersion of the pigment, had no effect on the stability of the film. A Perkin-Elmer autobalance (AM-2) and sand blasted platinum blade was used to measure surface tension. The sensitivity of the balance permitted measurement of the film pressure, π, with a precision better than 0.1 dyne/cm. The area of the film was kept constant as a change in π was recorded. Surface potential measurements were made using an Am^{241} electrode placed 0.5 cm above the surface of the film, a Ag-AgCl electrode in the subphase, and an electrometer (Kiethley, Model 610). Surface potential was recorded as a change in lipid monolayer surface potential ($\Delta\Delta V$). Changes in surface potential and surface pressure were concurrently recorded onto a Hewlett Packard strip chart recorder.

Liposomes were prepared by dissolving 10 mg of phosphatidylcholine in chloroform. After evaporation under nitrogen, the lipid was dispersed by vortexing for 10 min. The lipid mixture was allowed to swell for 1 h at 5 °C before sonicating using a Bronson Sonicator set at 3 for 20 min. The buffer used was the same as that for monolayer studies. The liposomes were centrifuged at 5000 ×. The supernatant containing the liposomes was then mixed with a solution of HpD to attain a final concentration of 0.2 mg/ml lipid and 90 μg/ml of HpD. The mixture was incubated in the dark for 1 h and then passed through a Sephadex G-50 column to remove unbound porphyrin. Binding of HpD to liposomes were assayed using a Cary-14 spectrophotometer.

Results and Discussion

Pure lipid monolayer films formed from PC at an air/water interface yielded reproducible films. The area/molecule and surface potential, ΔV, at 20 dyne/cm were found to be 85 Å^2 and 375 mV, respectively, in agreement with those in the literature [13]. No significant changes were observed in either surface pressure or surface potential for PC films in the pH range of 5.9–8.0. This was expected since PC is insensitive to pH over the range 4–9 [14].

Fig. 1a shows the change in π as a function of time after injection of HpD into the subphase. After a lag time of several minutes, a pH-dependent increase in surface pressure was observed. As the pH of the subphase was reduced, both $\Delta\pi/t$ as well as the final

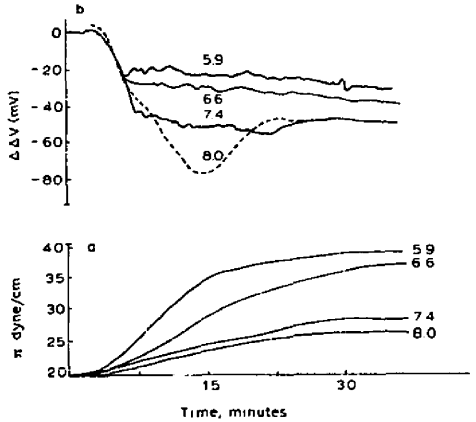

Fig. 1. (a) Change in surface pressure (π) as a function of time. The subphase consisted of 10 mM KCl and 10 mM phosphate buffer. (b) Change in surface potential ($\Delta\Delta V$) as function of time over pH range 5.9–8.0. Surface potential measurements were recorded concurrently with surface pressure.

saturation value of π increased. Final values for π at pH 5.9, 6.6, 7.4 and 8.0 were 39, 36, 27, 25 dyne/cm, respectively. High values for π at low pH indicate that components of HpD are intercalating within the lipid film resulting in a decrease in film surface tension as the total area of the film is held constant; surface pressure, π is generally considered to be equal to the reduction of the pure lipid surface tension, γ, by the film, i.e., $\pi = -\Delta\gamma$.

HpD is known to be composed of several components among them hematoporphyrin (Hp), protoporphyrin (PP), hydroxyethyl vinyldeuteroporphyrin (HvD), and an aggregated fraction believed to be the active component of HpD [15]. If we assume the area/molecule of a typical porphyrin (monomer) in a compressed state to be 70–85 Å^2 over the pH range used here [16], then using an area/molecule for phosphatidylcholine of 85 Å^2 reveals a relatively high porphyrin to lipid ratio at equilibrium $\approx 1:1$ at pH 5.9 compared with $\approx 0.25:1$ at pH 7.4. The high ratios of porphyrin to lipid at low pH found in monolayers are unlikely to occur in cell membranes. They do however reflect the relative driving force of HpD into the lipid core that is associated with different ionic species.

Fig. 1b shows the change in surface potential over time. At higher pH values (i.e. ≥ 7.4) $\Delta\Delta V$ approaches -50 mV at equilibrium, while at low pH (5.9) a significantly lower $\Delta\Delta V$ of -20 mV is observed. The high value for $\Delta\Delta V$ at higher pH in conjunction with a relatively small change in surface pressure, suggests that ionic forms of HpD, while not intercalated within the monolayer film, contribute substantially to $\Delta\Delta V$. Surface potential, ΔV, is related to the dipole moment by $\Delta V = 4\pi n\mu_T$ where n represents the number of

dipoles per unit area (cm^2) and μ_T represents the total dipole contribution from water/monolayer and monolayer/air interfaces Since the individual dipole fields superpose additively [17], a change in surface potential may be the result of an additional dipole component, or to a change in lipid dipole component. Both conditions require ionic forms of HpD situated at the water/monolayer interface. It therefore appears that while HpD at higher pH values may not be intercalated in high concentrations within the lipid film, porphyrins may very likely be adsorbed at the water/monolayer interface.

It is interesting to note that under all pH conditions binding of HpD to monolayers is complete after 30 min. Moan et al. [18] found a time dependence of HpD in tumors whereby the amounts of Hp and HvD did not bind significantly after 1 h while that of PP increased by about 100% from 1 h to 8 h. Thomas et al. [11] showed continued uptake of aggregated fractions at pH 6.4. This suggests that *in vivo*, rapid uptake (turnover in the cell membrane) of HpD aggregated fractions may occur, or that binding of HpD to serum protein limits the rate of uptake.

Fig. 2 shows the relative absorbance of liposomes incubated with HpD after removal of unbound HpD. The maximum peak slightly above 400 nm are indicative of porphyrin monomers bound to lipid [19]. The broadened peak for HpD bound to liposomes at pH 6.0 is similar to the pH sensitive 440 nm band observed in solutions of hematoporphrin IX, which may be the result of bound dimers and/or aggregate fractions [20]. Relative absorbance changes of bound HpD as a function of pH were found to correlate well with surface pressure changes from monolayer films under similar conditions; decreasing pH in both cases substantially increased binding of HpD to monolayers and bilayers. The non-linear (sigmoidal) relationship between π and pH as well as relative absorbance and pH (Figs. 3a and 3b), is representative of pH dependence on protonation of HpD components and hence their hydrophobicity [21] and dipole moment.

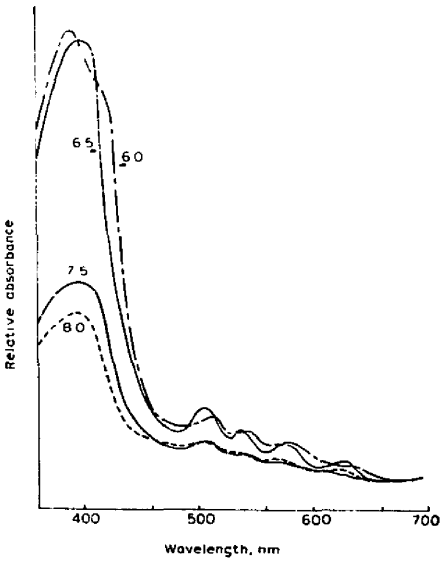

Fig. 2. The effect of pH on binding of HpD to phosphatidylcholine liposomes. Relative absorbance was measured after 1 hour incubation of HpD with liposomes, followed by column exclusion of unbound HpD. The bathing solution consisted of 10 mM KCl and 10 mM phosphate buffer.

In conclusion, analysis of HpD–lipid interactions using absorbance studies in conjunction with monolayer studies suggests that at low, tumorcidal pH, hydrophobic fractions of HpD, possibly aggregate in nature, intercalate within the bilayer. These results are in good agreement with data previously published by other workers [21,22]. The driving force at low pH is rather substantial, forcing high concentrations of aggregated fractions into the bilayer. At higher pH, HpD may

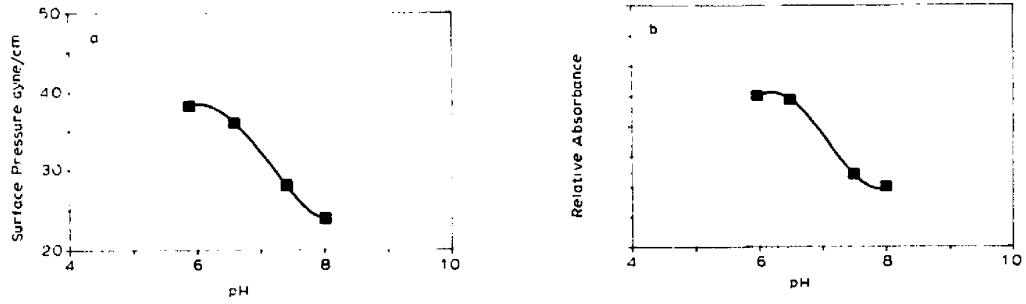

Fig. 3. (a) Surface pressure π (dyne/cm) as a function of pH. (b) Relative absorbance as a function of pH. Conditions are the same as in Fig. 1.

intercalate at low concentrations within the bilayer, with additional fractions being adsorbed at the monolayer interface. Through various cellular mechanism these fractions are taken up within the cell allowing further solubization of HpD into the membrane.

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