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PROTOLYTIC BEHAVIOR OF INDICATOR DYE IN THE MODEL MEMBRANE SYSTEMS. I. DYE PARTITIONING INTO LIPID PHASE

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Protolytic and partition equilibria of indicator dyes in the model lipid systems have been analyzed. A methodological approach has been developed allowing the partition coefficients of the protonated and deprotonated dye forms to be derived from spectrophotometric measurements. The most effective ways of employing the indicator dyes for monitoring the changes in the lipid bilayer properties have been suggested.
KEY WORDS: protolytic equilibria, indicator dye, partition coefficient, lipid bilayer

The vast majority of functionally significant membrane processes including molecular recognition, adsorption, fusion phenomena, ion transport, formation of multienzyme complexes are controlled by chemical composition and physical properties of the interfacial region of a lipid bilayer. This is a heterogeneous, dynamic, highly anisotropic region containing phosphorylcholine moiety, glycerol backbone, carbonyls, upper methylene groups of acyl chains and hydrational water [1, 2]. Molecular organization and physicochemical characteristics of lipid-water interface are influenced by a variety of factors, particularly, by the membrane interactions of proteins [3], ions [4], pharmacological agents [5]. To address this issue a number of powerful physical techniques have been employed, including NMR [6, 7], ESR [8, 9], Raman, infrared and fluorescent spectroscopy [10, 11, 12]. Of interest in this regard is also a method of pK_a-probes based on examining the protolytic equilibria of pH-indicator dyes [13, 14]. The dye partitioning between the aqueous and lipid phases is accompanied by the shift of its apparent ionization constant (pK_a). This shift stems from the differences in partition coefficients of the protonated and deprotonated dye forms [13, 15]. In turn, the dye partition coefficients are determined by a number of factors including surface and dipole electrostatic potentials, interfacial hydration, membrane molecular packing, etc. [16]. Therefore, variations of this parameter may be indicative of the changes in the structure and physicochemical properties of the lipid-water interface. The dye partitioning into lipid phase can be quantitatively characterized by examining pK_a dependencies on lipid concentration [15]. However, the employed approach has a serious drawback associated with the necessity of varying pH in rather wide limits (up to 4 pH units) to ensure reliable pK_a estimation. This may give rise to undesirable pH – dependent changes in the structure of lipid bilayer, and conformation of protein molecules resulting in modification of the interactions between membrane constituents. In the present study we made an attempt to evaluate the possibility of circumventing this problem. Our main goal was to choose most effective ways of employing the indicator dyes for monitoring the changes in the lipid bilayer properties and to work out an optimal methodology for acquisition and quantitative interpretation of experimental data.

THEORY

The thermodynamic acid-base equilibrium constant of indicator dye in a buffer solution is given by [15]:

$$K_a^w = \frac{a_{H^+} a_{In}}{a_{HIn}} \cong \frac{F_{H^+} F_{In}^o}{F_{HIn}^o} \qquad (1)$$

where a_{H^+}, a_{In}, a_{HIn} are the activities of the protons, deprotonated (In) and protonated (HIn) dye forms, respectively; $F_{H^+}, F_{In}^o, F_{HIn}^o$ are the concentrations (mol dm^{-3}) of the corresponding species. Denoting the total dye concentration by D_o one obtains:

$$D_0 = F_{In}^o + F_{HIn}^o \;; \qquad F_{In}^o = \frac{D_o}{1 + \frac{F_{H^+}}{K_a^w}} \qquad (2)$$

In a suspension of lipid vesicles the above protolytic equilibrium is shifted due to the dye distribution between aqueous (*w*) and lipid (*L*) phases so that D_o can be written as:

$$D_0 = F_{In} + F_{HIn} + B_{In}^L + B_{HIn}^L \tag{3}$$

This process is quantitatively described in terms of partition coefficients defined as:

$$P_{HIn}^L = \frac{n_{HIn}^L v_w}{n_{HIn}^w v_L} = \frac{B_{HIn}^L v_w}{F_{HIn} v_L}; \quad P_{In}^L = \frac{n_{In}^L v_w}{n_{In}^w v_L} = \frac{B_{In}^L v_w}{F_{In} v_L} \tag{4}$$

where *n* is the number of moles of different dye species in aqueous and lipid phases, v_w, v_L are the volumes of these phases given by: $v_L = N_A C_L V_L$; $v_w = v_t - v_L$, N_A is Avogadro's number, V_L is the mean volume of lipid molecule, v_t is the total volume of the system ($v_t = 1\,\mathrm{dm}^3$). In the range of lipid concentrations commonly employed v_L is much less than v_t, i.e. $v_w \approx v_t$.

Given that

$$F_{HIn}^w = \frac{F_{In} F_{H^+}}{K_a^w}; \qquad B_{In}^L = \frac{F_{In} P_{In}^L v_L}{v_w}; \quad B_{HIn}^L = \frac{F_{HIn} P_{HIn}^L v_L}{v_w} = \frac{F_{In} F_{H^+} P_{HIn}^L v_L}{K_a^w v_w} \tag{5}$$

Thus, Eq. (3) can be transformed to:

$$D_0 = F_{In} + \frac{F_{In} F_{H^+}}{K_a^w} + \frac{F_{In} P_{In}^L v_L}{v_w} + \frac{F_{In} F_{H^+} P_{HIn}^L v_L}{K_a^w v_w} = F_{In}\left(1 + \frac{F_{H^+}}{K_a^w} + \frac{P_{In}^L v_L}{v_w} + \frac{P_{HIn}^L F_{H^+} v_L}{K_a^w v_w}\right) \tag{6}$$

The process of dye partitioning into lipid phase can be examined through monitoring the absorbance changes of the In or HIn species. In the case where the absorbance measured in buffer solution (A_o) or liposomal suspension (A_L) is determined by both deprotonated and protonated dye forms the following relationships hold:

$$A_o = \varepsilon_f^{In} F_{In}^o + \varepsilon_f^{HIn} F_{HIn}^o; \qquad A_L = \varepsilon_f^{In} F_{In} + \varepsilon_{In}^L B_{In}^L + \varepsilon_f^{HIn} F_{HIn} + \varepsilon_{HIn}^L B_{HIn}^L \tag{7}$$

where $\varepsilon_f^{In}, \varepsilon_{In}^L, \varepsilon_f^{HIn}, \varepsilon_{HIn}^L$ are the extinction coefficients of the free ($\varepsilon_f^{In}, \varepsilon_f^{HIn}$) and bound ($\varepsilon_{In}^L, \varepsilon_{HIn}^L$) In and HIn species, respectively. By combining the Eqs. (2) - (7) the difference between the dye absorbances in a buffer and liposomal suspension can be written as:

$$\Delta A_L = A_o - A_L$$

$$\Delta A_L = \frac{D_o\left(\varepsilon_f^{In} - \varepsilon_f^{HIn}\right)\left(P_{In}^L \frac{v_L}{v_w} + P_{HIn}^L \frac{v_L F_{H^+}}{K_a^w}\right) - D_o \frac{v_L}{v_w}\left(1 + \frac{F_{H^+}}{K_a^w}\right)\left(\varepsilon_{In}^L P_{In}^L + \varepsilon_{HIn}^L P_{HIn}^L \frac{F_{H^+}}{K_a^w}\right)}{\left(1 + \frac{F_{H^+}}{K_a^w}\right)\left(1 + \frac{F_{H^+}}{K_a^w} + P_{In}^L \frac{v_L}{v_w} + P_{HIn}^L \frac{F_{H^+}}{K_a^w}\frac{v_L}{v_w}\right)} \tag{8}$$

here $F_{H^+} = 10^{-pH}$.

RESULTS AND DISCUSSION

The theory predicts that the absorbance changes resulting from the dye membrane partitioning are determined by the parameters depending on the dye microenvironment in the lipid phase (P_{In}^L, P_{HIn}^L, ε_{In}^L, ε_{HIn}^L) and experimental variables (pH and C_L). Fig. 1 and Fig.2 show how the variations in P_{In}^L, P_{HIn}^L and ε_{In}^L, ε_{HIn}^L manifest themselves in the behavior of ΔA_L (pH) plots. Fig. 1 represents the ΔA_L (pH) dependencies calculated from Eq. (8) assuming that $\varepsilon_{HIn}^L = \varepsilon_f^{HIn} = 0$, $\varepsilon_f^{In} = 2\times10^4$ $\mathrm{M^{-1}cm^{-1}}$. Importantly, the condition $\varepsilon_{HIn}^L = \varepsilon_f^{HIn} = 0$ can be satisfied by measuring ΔA_L at the wavelength (λ_{In}) where contribution of deprotonated form to the dye absorbance proves to be negligibly small. It appeared that ΔA_L attains positive values and has a clear maximum if $P_{HIn}^L > P_{In}^L$ (Fig. 1, A). Note that lipid vesicles employed to mimic the properties of biological membranes are usually negatively charged. This gives rise to proton accumulation near the lipid-water interface [16, 17]. Thus, the assumption that $\varepsilon_{In}^L \le \varepsilon_f^{In}$ seems reasonable because membrane binding of In form is likely to result in its conversion to HIn form whose extinction coefficient at the wavelength λ_{In} is less than ε_f^{In}. In the case where $P_{HIn}^L < P_{In}^L$ the sign of ΔA_L at a given pH depends on the difference

between ε_{In}^{L} and ε_{f}^{In} (Fig. 1, B). However, this difference has a slight effect on ΔA_L when P_{HIn}^{L} is more than 100-fold greater than P_{In}^{L} (Fig. 1, C).

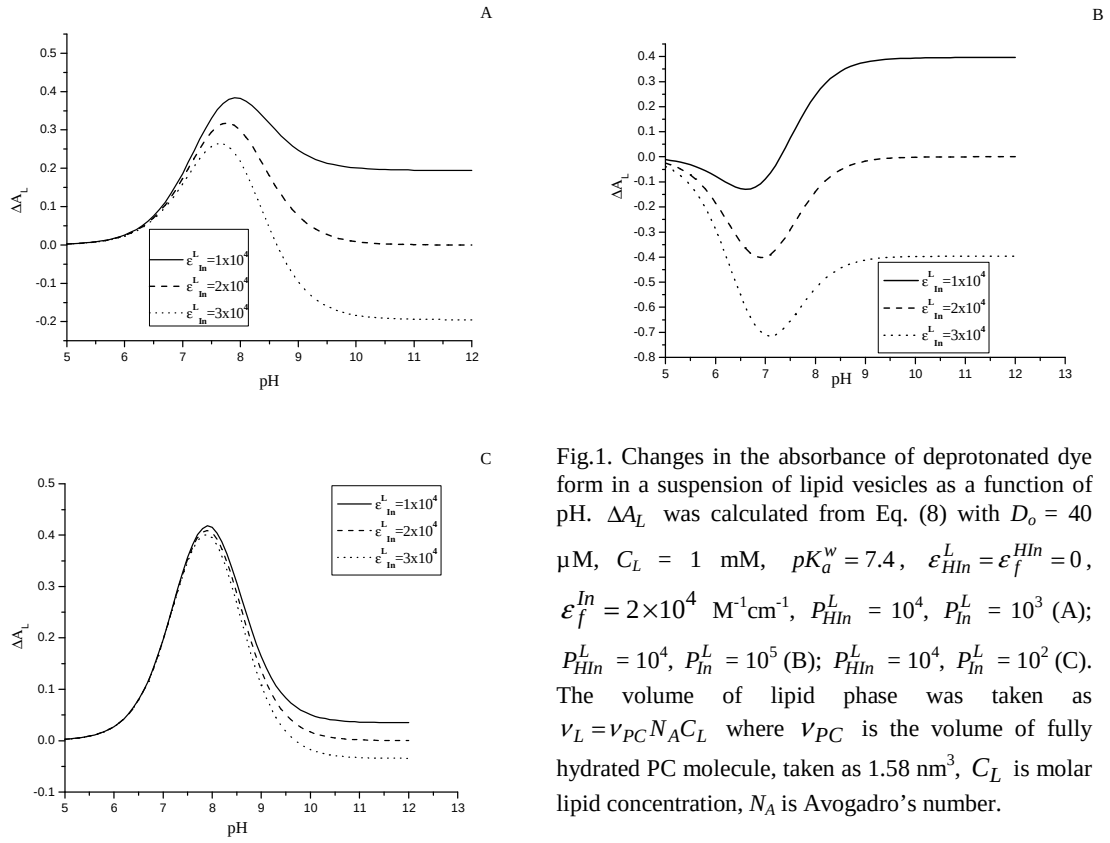

Fig.1. Changes in the absorbance of deprotonated dye form in a suspension of lipid vesicles as a function of pH. ΔA_L was calculated from Eq. (8) with D_o = 40 μM, C_L = 1 mM, $pK_a^w = 7.4$, $\varepsilon_{HIn}^{L} = \varepsilon_{f}^{HIn} = 0$, $\varepsilon_{f}^{In} = 2\times10^4$ M^{-1}cm^{-1}, P_{HIn}^{L} = 10^4, P_{In}^{L} = 10^3 (A); P_{HIn}^{L} = 10^4, P_{In}^{L} = 10^5 (B); P_{HIn}^{L} = 10^4, P_{In}^{L} = 10^2 (C). The volume of lipid phase was taken as $\nu_L = \nu_{PC} N_A C_L$ where ν_{PC} is the volume of fully hydrated PC molecule, taken as 1.58 nm^3, C_L is molar lipid concentration, N_A is Avogadro's number.

Fig. 2 represents the ΔA_L(pH) dependency in the case when $\varepsilon_{In}^{L} = \varepsilon_{f}^{In} = 0$, $\varepsilon_{f}^{HIn} = 2\times10^4$ M^{-1}cm^{-1} at a certain wavelength λ_{HIn} where the dye absorbance is determined by the protonated form. In this case ΔA_L has a sigmoid shape without clear maximum for various combinations of P_{HIn}^{L} and P_{In}^{L}.

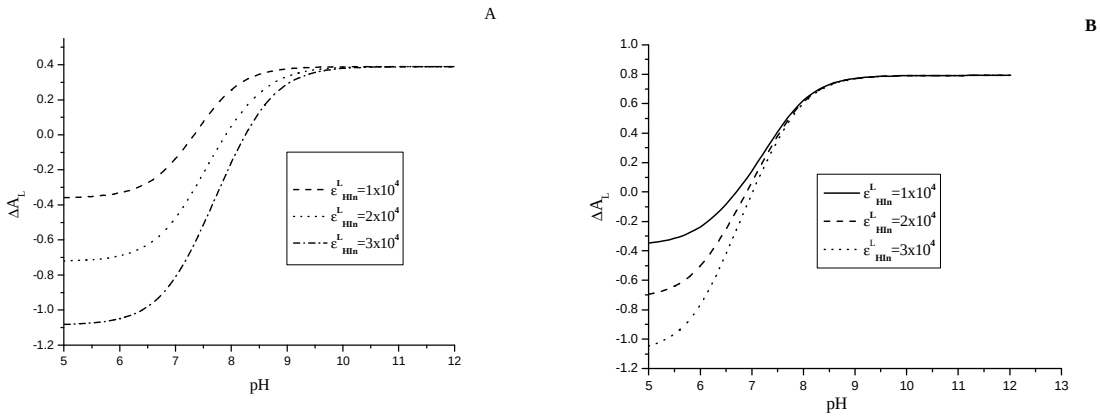

Fig.2. Changes in the absorbance of protonated dye form in a suspension of lipid vesicles as a function of pH. $\varepsilon_{In}^{L} = \varepsilon_{f}^{In} = 0$, $\varepsilon_{f}^{HIn} = 2\times10^4$ M^{-1}cm^{-1}, P_{HIn}^{L} = 10^4, P_{In}^{L} = 10^3 (A); P_{HIn}^{L} = 10^4, P_{In}^{L} = 10^5 (B).

Fig.3 plots pH_{max} as a function of partition coefficients. It appeared that pH_{max} value weakly depends on ε_{In}^{L}. The above simulated data can be used for rough estimation of initial values of P_{HIn}^{L}, P_{In}^{L} and ε_{In}^{L} appropriate for further fitting of the above model to the experimental data. For instance, if pH_{max} experimentally

observed for a given lipid concentration is around 7.6, one possible combination of partition and extinction coefficients is $P^L_{HIn} \approx 10^{4.6}, P^L_{In} \approx 10^4 (\varepsilon^L_{In} = 2\times10^4)$.

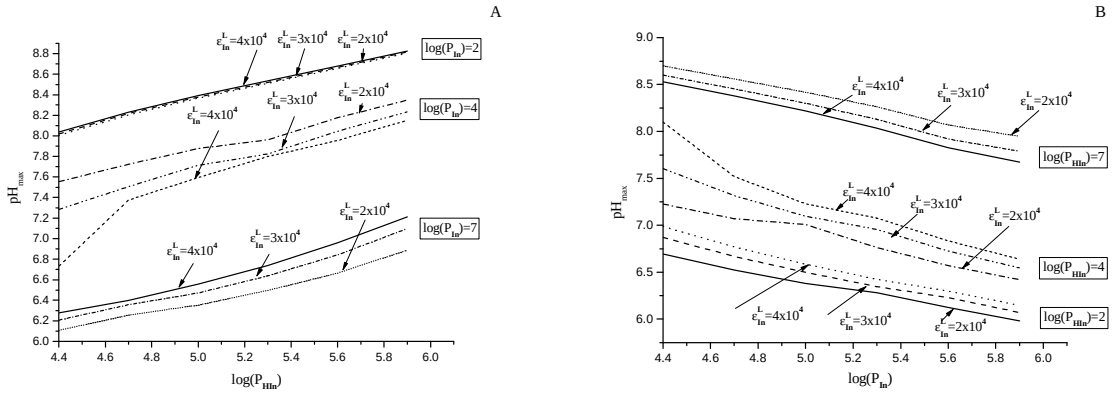

Fig. 3. pH_{max} dependencies on the partition coefficients of protonated (A) and deprotonated (B) dye forms.

Next, it was of interest to ascertain what pH region ensures most accurate experimental estimation of P^L_{HIn} and P^L_{In}. Clearly, in this region $\Delta P_{HIn,In}$ is coupled with the largest $\Delta\Delta A_L$, i.e. P^L_{HIn} and P^L_{In} derivatives of ΔA_L take up their maximum or minimum at a certain pH (pH_{ext}) (Fig. 4). Note that pH_{ext} proves to be virtually independent of the extinction coefficients (Fig. 4).

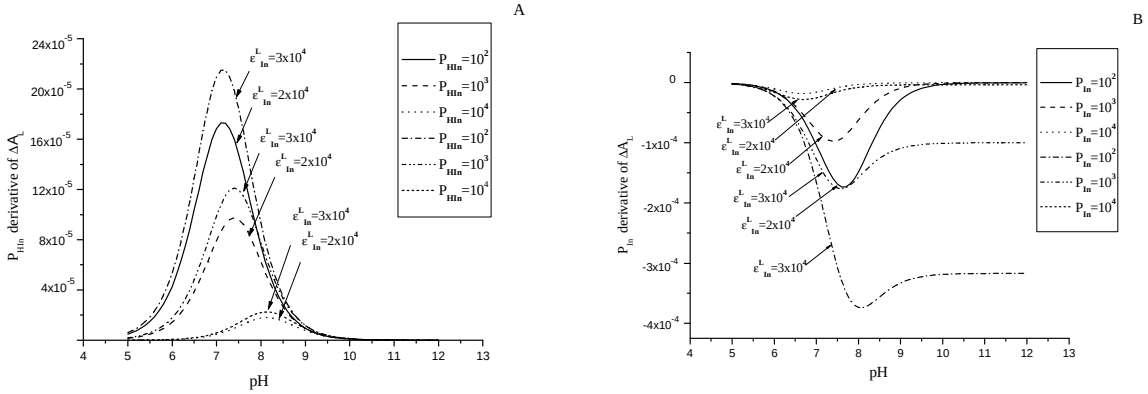

Fig. 4. P^L_{HIn} and P^L_{In} derivatives of ΔA_L: P^L_{In} =10^3 (A), P^L_{HIn} =10^3 (B).

Estimating the deviations of pH_{ext} from pH_{max} $|\Delta pH|$ ($|\Delta pH| = |pH_{max} - pH_{ext}|$) as a function of the partition coefficients showed that within the range of partition coefficients typical for the majority of indicator dyes ($2 \le P_{HIn,In} \le 6$) the difference between pH_{ext} and pH_{max} does not exceed 2 pH units. This implies that pH values falling in the range $pH_{max} \pm 1$ are preferable for ΔA_L measurements. Importantly, pH_{max} is determined not only by the dye or membrane intrinsic properties, but also on the lipid concentration (Fig. 5). Hence, by varying C_L one can shift the maximum of ΔA_L (pH) dependency towards desirable pH region where perturbations of lipid or protein structure are expected to be minimal. On the other hand, as indicated above, ΔA_L (C_L) dependencies as such or combined with ΔA_L (pH) plots can be used for the estimation of partition coefficients. In this case it is important to extend the range of employed lipid concentrations to a region where ΔA_L (C_L) plots become non-linear and approach a plateau (Fig. 6).

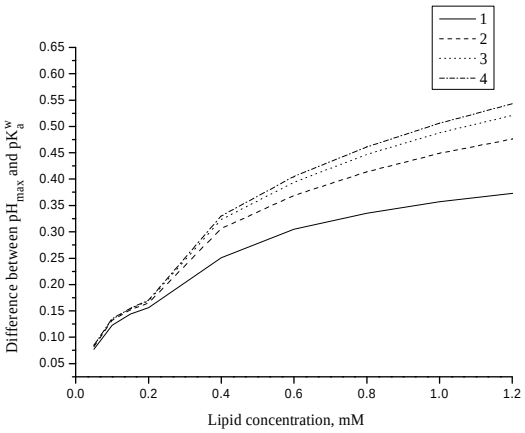

Fig. 5. Difference between pH_{max} and pK_a^w. $P_{HIn}^L = 10^4$, $P_{In}^L = 10^3$ (1), $P_{In}^L = 10^{2.5}$ (2), $P_{In}^L = 10^2$ (3), $P_{In}^L = 10$ (4).

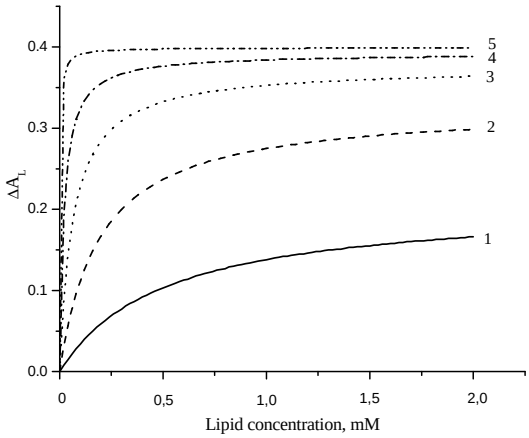

Fig. 6. Changes in the absorbance of deprotonated dye form ($\varepsilon_f^{HIn} = 0$, $\varepsilon_{HIn}^L = 0$) in the suspension of lipid vesicles as a function of lipid concentration: $P_{In}^L = 10^3$, $P_{HIn}^L = 10^4$: 3.5(1), 4(2), 4.5(3), 5(4), 6(5); $\varepsilon_{In}^L = 2\times10^4$ $M^{-1}cm^{-1}$.

CONCLUSIONS

To summarize, the principal features of indicator dye partitioning into model membrane systems and some guides for better experimental design can be outlined as follows. (i) The change in the absorbance of a given dye form on going from a buffer solution to a suspension of lipid vesicles attains its maximum value at a certain pH (pH_{max}). (ii) The pH_{max} value depends on the partition coefficients of the HIn and In dye species and lipid concentration, being slightly sensitive to the changes in the dye extinction coefficient resulting from chromophore transfer into lipid environment. (iii) The partition coefficients of different dye forms in the lipid system can be derived in three ways involving fitting of the data sets ΔA_L (C_L), ΔA_L (pH) or ΔA_L (pH, C_L). The accuracy of recovering these parameters is highest at pH values close to pH_{max}. The proposed methodological approach offers an advantage of minimizing the pH-induced perturbation of the lipid and protein membrane constituents and relative simplicity of recovering the dye partition coefficients.

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