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Surface Potential and Energy-Coupling in Bioenergy-Conserving Membrane Systems*

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Abstract

In order to understand the localization of dyes and the nature of their responses in membranes and particularly in those involved in energy-conservation processes, the influence of micelles of neutral and ionic surfactants on the pK_a of solubilized fluorophoric (umbelliferone) and chromophoric (bromthymol blue and methyl red) indicator dyes is studied. It is shown that the pK_a of the indicator adsorbed onto micelles shifted towards the acid extreme with cationic micelles, to the alkaline side with anionic micelles while it was not significantly modified by the neutral ones. Maximal displacements were observed with Methyl Red where the difference in pK_a between anionic and cationic micelles was as large as 3 pH units. Phospholipid liquid crystals (Liposomes) of phosphatidylcholine with and without adsorbed long-chain ions introduced in order to confer to it a net surface charge induced displacements of the pK_a of UBF analogous to those detected in the presence of detergent micelles. It was demonstrated that UBF can monitor reversal of charge phenomena such as that obtained by the interaction of phosphatidylcholine + dicetyl phosphate liposomes (anionic colloid) with poly-L-lysine (cationic colloid). The partition of the indicator dyes between micellar and aqueous phases was determined by gel filtration revealing the *quasi* exclusive presence of the dyes in the micellar phase. Fluorescence polarization measurement of solubilized UBF in either ionic micelles or submitochondrial particles indicate that the dye tumbling rate is as rapid as in pure water suggesting that the dye is mobile in an interfacial environment where it can experience modifications due to changes in surface potential. The use of UBF as a probe of respiration-dependent energy-linked reactions in submito-

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chondrial particles is presented. The available data on the use of indicator dyes in mitochondrial, chloroplast and bacterial chromatophore membranes is reevaluated, on the basis of the evidence of the extreme sensitivity of these probes to surface charge. The implications of these results and considerations are discussed in terms of the importance of the surface potential in the primary event of the energy-coupling process in oxidative and photosynthetic phosphorylation.

Introduction

The primary event in the energy-coupling process in oxidative and photosynthetic phosphorylations still remains an open question. (For a recent review see Slater, 1971 [1].) The realization of the importance of the membrane, enlightened by the postulation of the chemiosmotic hypothesis of energy-coupling [2-3] and by the stringent association of membrane structure in energy-conserving systems, has established that the membrane does not just function as an inert frame where the energy-conserving machinery is located, but plays a major role in the mechanistics of these biological processes. Considerable effort has been directed towards the elucidation of the relationship between transmembrane potential gradients and energy-conservation, and though most of the evidence appears to substantiate this proposal, the nature of the primary event and its involvement in the generation of the electrochemical potential remains unsolved [4-7].

In the majority of the studies performed using dyes to monitor so-called "bulk" pH changes, it has not been established whether the dye is indeed incapable of becoming bound to the membrane. Clearly, any rational interpretation of results depends on the actual location of the dye. In the present study we have found that probes such as umbelliferone, bromthymol blue and methyl red have high partition coefficients towards detergent micelles and phospholipid liposomes. At the concentrations used they would therefore monitor surface pH changes reflecting the distribution of protons induced by the surface potential. Our evidence indicates that in the membrane studies hitherto published, the reported pH changes are the surface pH values reflecting probably surface charges, the surface potential being responsible for the observed results.

The present paper will first deal with the use of solubilized indicator dyes to determine the surface potential and pH of detergent micelles as originally suggested by Hartley [8] and Hartley and Roe [9] and further elaborated by Mukerjee and Banerjee [10]. This will be followed by an extension of the method to phospholipid dispersions (liposomes) (cf. Bangham [11]) where it is applied to monitor reversal of charge phenomena. The partitioning of the dyes between micellar and aqueous phases is determined by gel filtration. Fluorescence polarization of the

dye is used to obtain information about its motional freedom in the interface (surface). These results will be used as a basis for the interpretation of the energy linked changes of these dyes in mitochondrial as well as chloroplasts and bacterial chromatophore membranes and a general discussion will be presented in terms of the current hypotheses of energy coupling in oxidative and photosynthetic phosphorylation.

Materials and Methods

Fluorescence measurements were performed in a Farrand spectrofluorometer (Farrand Optical Co. Inc., New York) and absorption measurements in a Cary Model 15 recording spectrophotometer (Applied Physics Corporation, Monrova, California) at the wavelengths indicated in Figure and Table legends where the composition of the reaction mixtures is also indicated. For the pH calibrations a radiometer Model 22 pH meter (Radiometer-Copenhagen) with expanded scale attachment was used.

Fluorescence polarization was measured with an instrument built according to the design of Weber and Bablouzian [12].

Submitochondrial particles derived by sonic disruption of beef-heart mitochondria were prepared as described by Fessenden and Racker [13] and Lee and Ernster [14].

Protein was determined by the biuret method [15]. Phospholipid liquid crystals (liposomes) [11] were prepared by suspension of the phospholipid in water (usually 10 mg/ml) followed by a 15 min sonication period in a Branson sonifier Model B-12 (Branson Sonic Power Co., Connecticut, U.S.A.) at the maximum power output allowable for the microtip. A cylindrical, aluminium vessel with a volume of 5.0 ml was used. During the sonication, the temperature was maintained at 4°C.

The Sephadex G-25 chromatography was performed on columns 1.8 cm inner diameter with a bed height of about 22 cm. The excluded (V_o) and included (V_i) volumes were determined by the volumes of elution of Dextran blue 2000 (M.W. $\sim 2 \times 10^{6}$) (determining optical density at 630 nM) and 2-4-dinitrophenol (determining absorbance at 420 nm), respectively according to Fish, Mann and Tanford [16]; representative elution patterns are illustrated in Fig. 5.

All experiments presented were performed at a temperature of 25° ± 1°C.

Sodium dodecyl sulfate, and the alkyl (dodecyl, tetradecyl and hexadecyl) trimethyl ammonium bromides were high purity lots obtained from British Drug Houses Ltd., England. Triton X-100, octadecylamine, dicetyl phosphate and poly-L-lysine (M.W. 127500)

were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Chromatographically pure egg phosphatidylcholine, sheep brain phosphatidylethanolamine and phosphatidyl-L-serine were purchased from General Biochemicals (Ohio, U.S.A.) and the dyes used in this work were obtained from the indicated sources: umbelliferone (7-hydroxycoumarin), (Hopkin and Williams Ltd., England); bromthymol blue (3,3-dibromothymol-sulfonphtalein) (Eastman, Rochester, N.Y.); methyl red (K & K Laboratories Inc., Plainview, N.Y.); Sephadex G-25, medium grade, was obtained from Pharmacia, Uppsala, Sweden. All chemicals were of the highest purity commercially available. Glass redistilled water was used throughout.

Results

Influence of Ionic and Neutral Surfactant Micelles on the Apparent Dissociation Constant (pK_a) of Solubilized Indicators Dyes

The uncorrected excitation spectra of UBF* with constant emission at 450 nm (the wavelength of maximum emission) in water and in the presence of micelles† of anionic (sodium dodecyl sulfate-SDS-) and cationic (dodecyl trimethyl ammonium bromide-DTABr) surfactants are presented in Fig. 1A. The three spectra were performed at a bulk pH of the aqueous phase of 7.4. It is illustrated that while the spectrum of UBF in SDS presents two peaks, one at 370 nm corresponding to the ionized form and a second one at approximately 335 nm associated to the unionized form of the indicator, in DATBr it only presents one highly fluorescent maximum (please note scale calibrations) at approximately 390 nm; an intermediate situation is obtained in water. This experiment is repeated at several pH's in the range of 3-12 and the relative fluorescence intensity corresponding to the ionized form is used in the construction of Fig. 1B. The curves present the typical sigmoid form characteristic of weak acid indicators and from them the apparent dissociation constant, pK_a, is computed at the pH corresponding to 50% of the maximum fluorescence intensity. It should be noted that while the pK_a of UBF in water is 7.75 or in the presence of micelles of the neutral surfactant Triton X-100 (pK_a = 7.8) (Table I), it is shifted to the

* Abbreviations used: BTB, bromthymol blue, 3-3,dibromothymol-sulfonphtalein; UBF-, umbelliferone, 7-hydroxycoumarin; MR-, methyl red, *p'*-dimethyl amino azobenzene-*o*-carboxylic acid; CTABr, DTABr, and TTABr, cetyl (hexadecyl), dodecyl and tetradecyl trimethyl-ammonium bromides, respectively; SDS, sodium dodecyl sulfate.

† The critical micelle concentrations (CMC) of the detergents used in this study are: 8, 0.1, 1.0, and 15 mM for SDS, Triton X-100, CTABr and DTABr respectively (these values are in agreement with previous reports, Emerson and Holtzer [17]; Chávez [18]; Shinoda, *et al.* [19]).

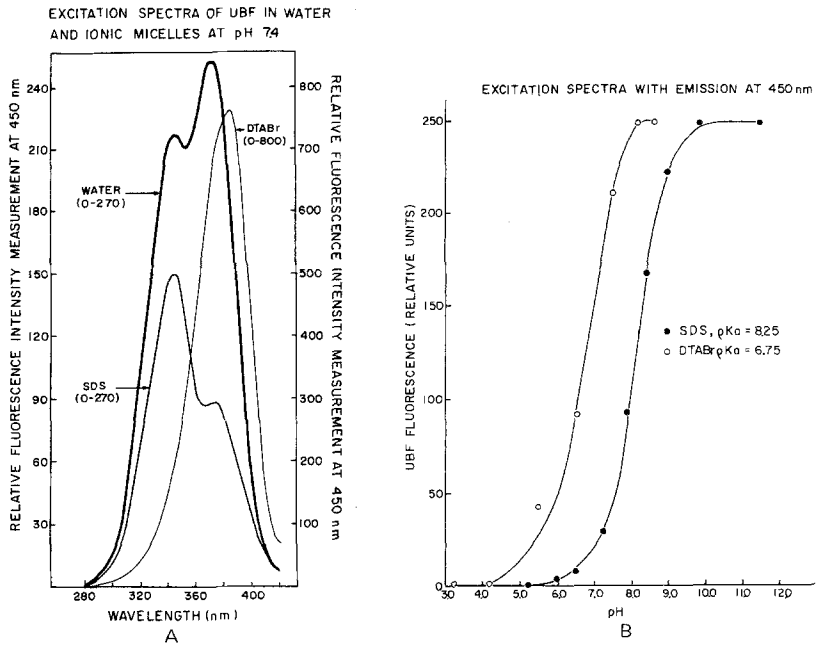

Figure 1. Influence of ionic micelles on the ionization properties of umbelliferone. A: Excitation spectra of UBF with emission constant at 450 nm in aqueous and ionic micellar solutions of SDS and DTABr. Reaction mixture: 10 μM UBF, 6 mM tris-Cl, pH 7.6 and when indicated 24 mM SDS and 24 mM DTABr. Final volume = 3.0 ml. Temperature = 25 ± 1°C. B: pH titration of UBF in the presence of SDS and DTABr micelles. Excitation spectra with emission at 450 nm were performed at each pH and the maximum fluorescence at 370 nm for SDS and 390 nm for DTABr were used to construct the figure. The experimental conditions are the same as those of Fig. 1A.

TABLE I. Influence of surfactant micelles and phospholipid liquid crystals on the ionization of umbelliferone

Non-aqueous phase	pK_a		
	Cationic	Neutral	Anionic
Surfactant micelles	DTABr 6.85	Triton X-100 7.8	SDS 8.25
Phospholipid Liquid crystals	Phosphatidyl-choline + octadecylamine 7.5	Phosphatidyl-choline 7.75	Phosphatidyl-choline + dicetylphosphate 8.0

Excitation spectra were recorded maintaining the emission constant at 450 nm. The experimental conditions are the same as those given for Figs. 1 and 4. The concentration of Triton X-100 was 5 mM and of UBF was 10 μM and 1 μM for the system containing micelles and phospholipid liquid crystals, respectively.

acid side (pK_a = 6.75) when adsorbed onto cationic micelles and to the alkaline extreme (pK_a = 8.25) in the presence of anionic micelles.

In Fig. 2A are illustrated the absorption spectra of BTB in water, anionic SDS micelles and cationic DTABr micelles at a bulk pH of the aqueous phase of 7.0. The peaks at 618 nm and 420 nm are associated with the ionized and unionized forms of the dye respectively. This experiment was repeated at several pH's in the range of 4.0-12.0 and the absorbance at 618 nm measured and plotted in Fig. 2B as a function of pH. It is illustrated that the pK_a of BTB in water is approximately 7.18 and shifts towards the acid extreme when adsorbed onto DTABr micelles giving a pK_a = 6.45 and towards the alkaline side in the presence of SDS micelles (pK_a = 8.35).

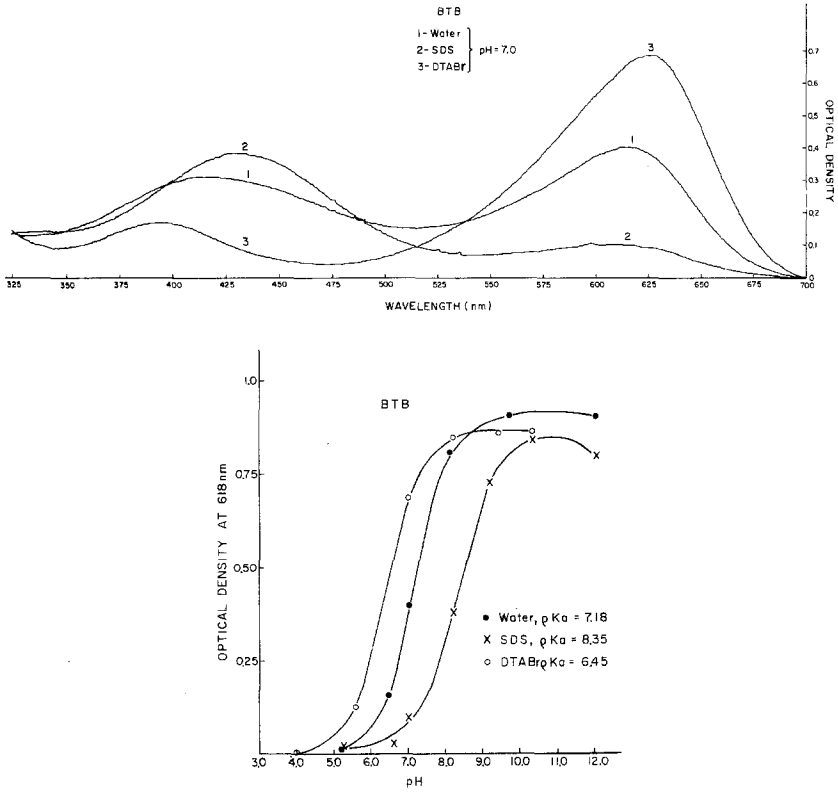

Figure 2. Influence of ionic micelles on the ionization properties of bromthymol blue. A: Absorption spectra of BTB in aqueous and micellar solutions of SDS and DTABr. Reaction mixture: 16.7 μM BTB, 1.67 mM tris-Cl, pH 7.0 and when indicated 24 mM SDS and 24 mM DTABr. Final volume 3.0 ml. Temperature 25 ± 1°C. B: pH titration of BTB in water and in the presence of SDS and DTABr micelles. Experimental conditions as those for Fig. 2A.

Analogous observations have been obtained with methyl red and are illustrated in Fig. 3A, B. In Fig. 3A the absorption spectra of MR in water, SDS and DTABr micelles all at a bulk pH of the aqueous phase of 4.2 are presented. The absorbance at 520 nm, corresponding to the ionized form of MR was measured between pH 1.5 and 8.5 and presented as a function of pH in Fig. 3B. It is readily apparent that the neutral micelles of Triton X-100 do not significantly modify the pK_a of MR (pK_a = 5.2) with respect to that characteristic of the aqueous phase (pK_a = 4.95). In contrast to this, large deviations of the pK_a of MR are observed when adsorbed onto ionic micelles; the difference in pK_a ($\triangle pK_a$) between neutral and anionic micelles being of 1.45 and between neutral and cationic micelles of 1.5 (Table II).

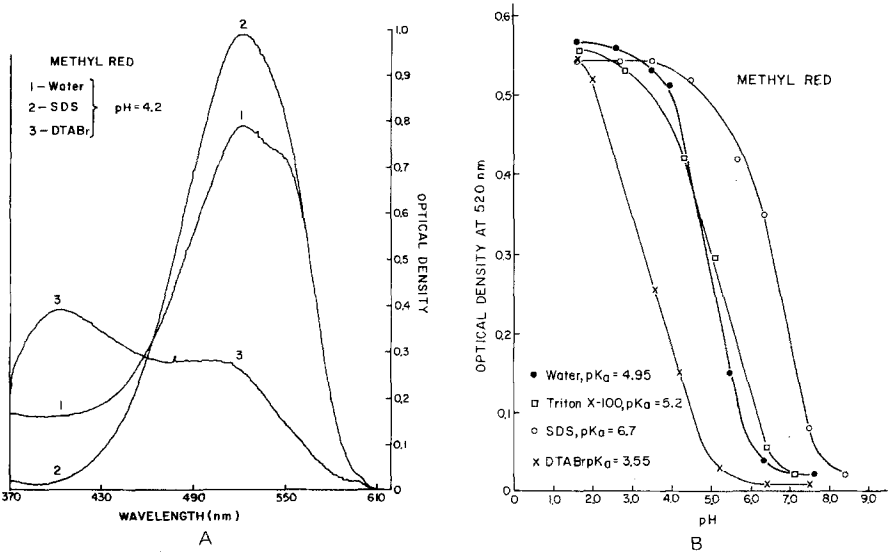

Figure 3. Influence of ionic micelles on the ionization properties of methyl red. A: Absorption spectra of MR in aqueous and micellar solutions of SDS and DTABr. Reaction mixture: 33.3 μM MR, and when indicated, 24 mM SDS, 24 mM DTABr. In all cases the pH = 4.2. Final volume: 3.0 ml. Temperature 25 ± 1°C. B: pH titration of MR in water, and in the presence of non-ionic (Triton X-100) and ionic micelles (SDS and DTABr). Reaction mixture: 16.7 μM MR, and when indicated 24 mM SDS, 24 mM DTABr, 5 mM Triton X-100. Final volume 3.0 ml. Temperature 25 ± 1°C.

Influence of Ionic and Neutral Phospholipid Smectic Mesophases on the Apparent Dissociation Constant of Umbelliferone

In Fig. 3A, the pH titrations of UBF in the presence of neutral, anionic and cationic phosphatidyl-choline liquid crystals are illustrated. The computed pK_a's are presented in Table I in comparison to those

TABLE II. Influence of ionic and non-ionic surfactants on the ionization of methyl red[a]

Surfactant	pK_a	pK_a difference between neutral and ionic surfactant	Surface potential difference between neutral and ionic surfactant[b] (mV)
None (water)	4.95	–	–
24 mM $DTAB_r^+$	3.675	1.5	+89
24 mM SDS	6.625	1.45	−86
5 mM Triton X-100 5.175	5.175	–	–

[a] These tabulated data are obtained from Fig. 3B.
[b] Ψ values calculated according Eq. 3.

obtained when adsorbed onto surfactant micelles. The pK_a deviations detected in the presence of phospholipid dispersions are qualitatively analogous to those obtained with surfactant micelles though significantly smaller in extent. It is worth noting that a fluorescence enhancement of 10- to 20-fold was detected in lipid relative to detergent micellar systems. To assess the potentiality of UBF to monitor surface charge dependent phenomena, the reversal of charge obtained by the interaction of colloid anions with colloid cations to form complex coacervates (dicomplexes) [20] was selected, and the results are illustrated in Fig. 4B. The starting material consists of anionic liposomes composed phosphatidylcholine and dicetylphosphate to which increasing concentrations of poly-L-lysine are added. The pK_a of UBF in the presence of anionic liposomes is approximately 8.0, and becomes 7.55 when the molar concentration of polylysine is about 1/1000 that of dicetyl phosphate. No further modification of the pK_a of UBF is obtained by larger increments in polylysine concentration until above 1 : 1 w/w dicetyl-phosphate/polylysine visible precipitation of the system occurs. It is of interest to note that the pK_a obtained after addition of 100 μg of polylysine ($pK_a = 7.75$) is analogous to that of phosphatidylcholine (neutral colloid) while that measured at 200 μg ($pK_a = 7.55$) is equivalent to that of phosphatidylcholine plus octadecylamine (cationic colloid). It is very significant that Kimelberg and Papahadjopoulos [21] have reported the effectiveness of polylysine in reducing the electrophoretic mobility of anionic phosphatidylserine liposomes. These results are highly indicative of the surface potential sensitivity of solubilized dyes of the type herein described.

Partitioning of Indicator Dyes between Micellar and Aqueous Phases

A fundamental question that has been raised in the mechanistic evaluation of the energy-linked changes of BTB and other dyes in

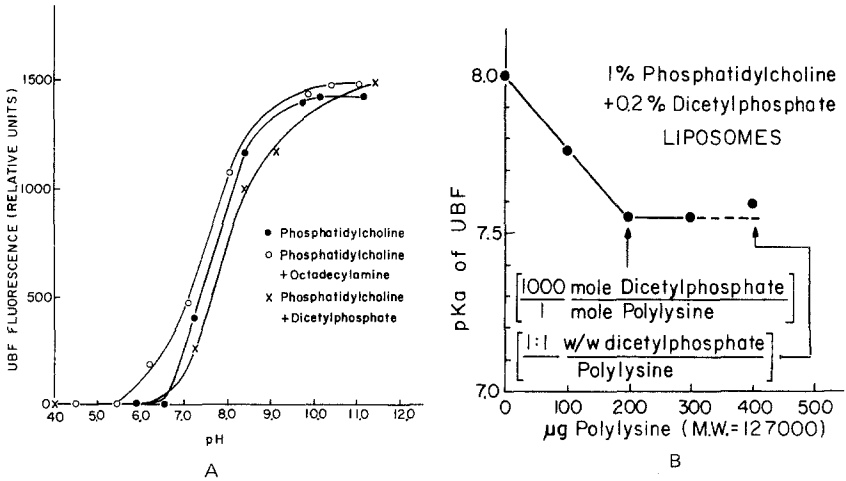

Figure 4. Influence of neutral and charged phosphatidylcholine liquid crystals on the ionization properties of umbelliferone. A: pH titration of UBF in the presence of phosphatidylcholine liquid crystals. Excitation spectra with emission at 450 nm were performed at each pH and the maximum fluorescence at 370 nm used to construct the figure. Reaction mixture: 1 μM UBF, 1 mM tris-Cl, and 0.2 ml of phosphatidylcholine liposomes (1%) (w/w) or mixed phosphatidylcholine (1%) (w/w) + dicetylphosphate (0.2%) (w/w) or + octadecylamine (0.2%) (w/w) as indicated, all in a final volume of 3.0 ml. Temperature 25 ± 1°C. B: Effect of polylysine on the pK_a of UBF adsorbed onto ionic phospatidylcholine + dicetylphosphate liposomes. Reaction mixture: 1 μM UBF, 1 mM tris-Cl, 0.2 ml of phosphatidylcholine (1%) + dicetylphosphate (0.2%) liposomes and increasing concentration of polylysine as indicated. Final volume 3.0 ml. Temperature 25 ± 1°C.

energy-transducing membranes is that of the different partition and hence mobility of these probes in distinct metabolic states (cf. [22]) (Jackson and Crofts [23]). To answer this question we have determined the partition coefficient (K) of UBF and MR between micellar and aqueous phases according to the gel filtration method of Herries, Bishop and Richards [24]. The rationale behind the method is that the cross-linked dextran gel Sephadex G-25 will exclude from the interior of the particles solutes larger than approximately 5000 in molecular weight. Surfactant micelles have molecular weights well above this figure, for example CTABr = 61,700 and SDS = 25,600 [19], and thus would be expected to be excluded from the gel. Inside the gel particles, the detergent should be as monomer units. According to Herries *et al.* [24], "If the solvent for a Sephadex column consists of an aqueous detergent solution at a concentration well above the CMC, the movement of an additional low molecular weight solute added as a sample will depend upon the partition coefficient of this latter material between the micellar and aqueous phases."

The elution pattern of such a column is shown in Fig. 5A. The absorbance scale refers to the volume at which dextran blue (630 nm) and 2-4-dinitrophenol (420 nm) are eluted in order to determine V_o and V_i respectively as shown in upper portion of the figure. In the lower portion of the figure are presented three UBF fluorescence peaks corresponding from left to right to the elution patterns of UBF carried out with solutions of 10 mM, 7 mM, 5 mM, and 1 mM CTABr and distilled water respectively using columns equilibrated with the elution solution. The pattern shows that while above the CMC of CTABr the UBF fluorescence peak aappears at the V_o position, it appears at the $V_o + V_i$ position in the absence of detergent or when the solvent contained CTABr below or at the CMC. Hence, above the CMC the change in elution volume depends on the detergent concentration. On the basis of this evidence and with the use of the plot of Fig. 5B and the equations derived by Herries *et al.* [24] one arrives to a value of the partition coefficient (K) of UBF between micellar and aqueous phases of 2301, indicating that in the presence of micelles all the dye is solubilized.

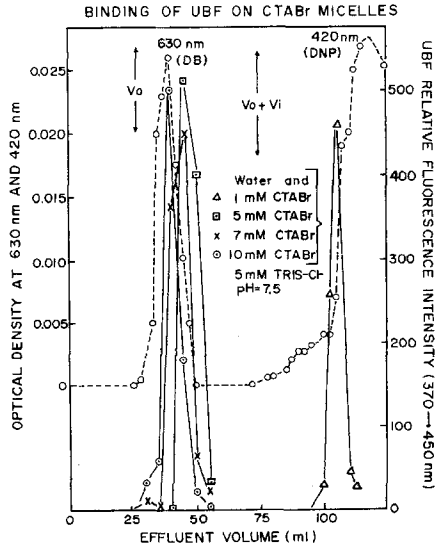

Figure 5A. Elution diagrams of UBF on a column of Sephadex G-25 in the presence of CTABr. The gel bed was 22 cm high and 1.8 cm in diameter. The left-hand ordinate gives the absorbance of dextran blue 2000 and 2-4 DNP at 630 nm and 420 nm, respectively. The right-hand ordinate indicates the UBF fluorescence intensity in arbitrary units. The abscissa is the same for all four patterns. The column parameters V_o and V_i are shown by the arrows. The concentration of UBF was 10^{-6} M in all cases. The columns were equilibrated with the indicated elution solution.

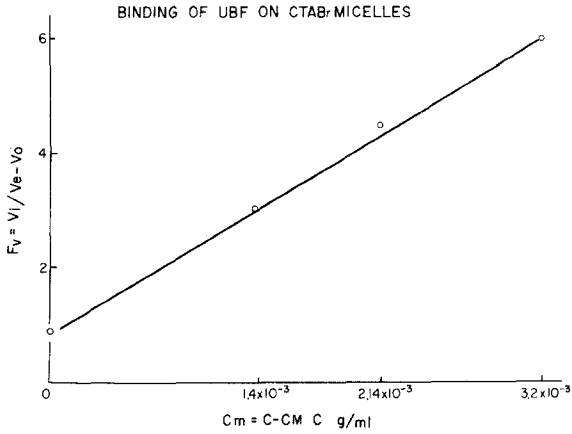

Figure 5B. Elution of UBF on a column of Sephadex G-25 in the presence of CTABr. The column parameters and elution volumes are taken from Fig. 5A and plotted according to the following equation (cf. [24]):

$$\frac{V_i}{V_e - V_o} = f_v = \frac{\bar{v}(K-1)}{k'K_D} Cm + \frac{1}{k'K_D}$$

where V_e = effluent volume; $\bar{v}$ = partial specific volume of a detergent molecule in a micelle; K = the partition coefficient of solute between micellar and aqueous phases; k = constant of proportionality between solute adsorbed per unit volume of gel matrix and equilibrium concentration of monomer solute in liquid; K_D = ratio of solute concentration in imbibed liquid to concentration in non-micellar portion of external liquid; C = total concentration of detergent in eluting liquid in g/ml; Cm = C – CMC = concentration of micelles in starting solution in g/ml. Combination of slope and intercept term permits both K and the product $k'K_D$ to be determined.

A value of the distribution coefficient of bromophenol purple between micelles and bulk of 3×10^5 has been derived by Mukerjee and Banerjee [10].

In Figs. 6 and 7 similar experiments using MR as the solubilized dye are illustrated. When CTABr cationic micelles are used a K value of 2458 is obtained (Fig. 6A B) in agreement with that found for UBF. However, when Triton-X-100 non-ionic micelles are utilized a K value of 476 is calculated, indicating a marked dependence of the dye partition on the nature of the surface charge.

Fluorescence Polarization of UBF in Micellar, Membranes and Aqueous Phases

In order to get information about the motional freedom of UBF, the degree of fluorescence depolarization (P) was measured when solubilized in micelles or membranes.

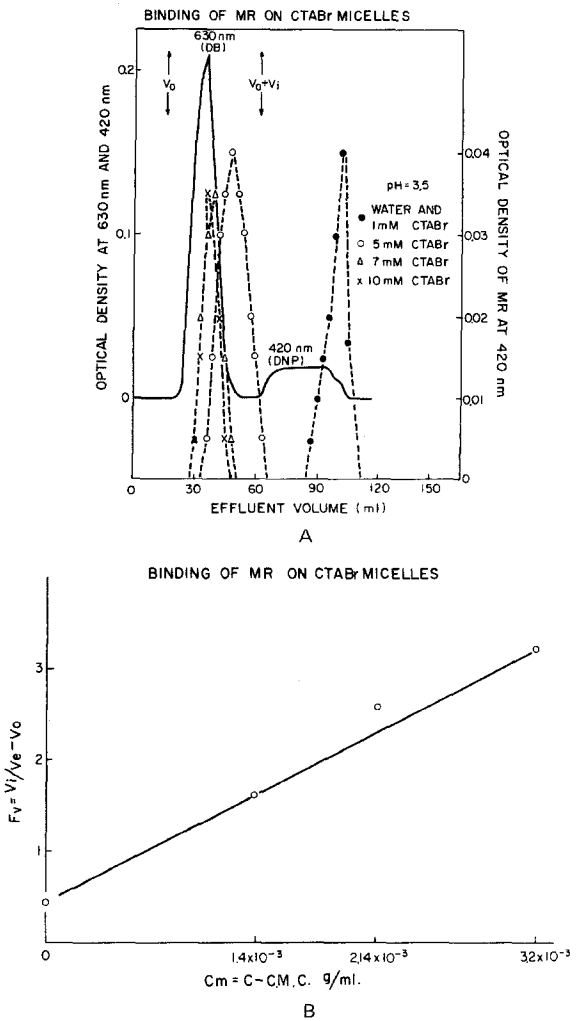

Figure 6A and B. Elution of MR on a column of Sephadex G-25 in the presence of CTABr. The concentration of MR was 10^{-6} M in all cases. The data are plotted as described in Fig. 5 legend.

In Table IV comparative results obtained with UBF and ANS (1-8-anilinonaphthalene-sulfonate), a dye known to fluoresce only when solubilized in apolar environments [25-26], are presented. It should be noted that while negligible changes in P were obtained when UBF was in the presence of either ionic micelles or submitochondrial particles as compared to that observed in water, marked increments of P were

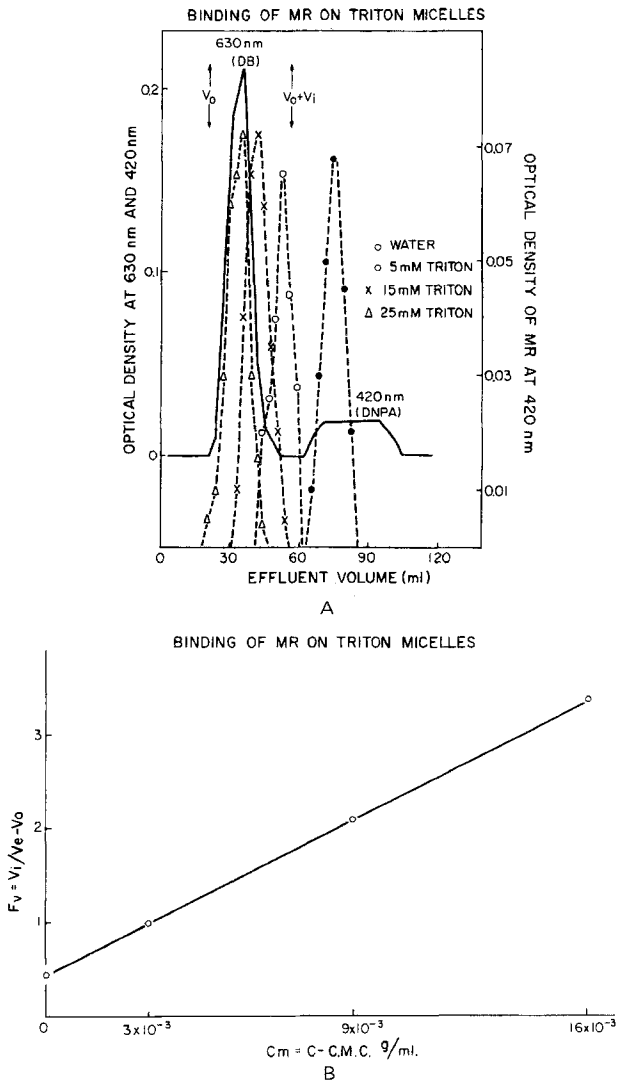

Figure 7A and B. Elution of MR on a column of Sephadex G-25 in the presence of Triton-X-100. The concentration of MR was 10^{-6} M in all cases. The data are plotted as indicated in Fig. 5 legend.

detected with ANS under analogous conditions. This fact indicates that a critical distinction between solubilized dyes should be made, i.e., dyes which are bound and partially immobilized (e.g. ANS) and those bound but still preserve essentially the same motional freedom they exhibit in

water (e.g. UBF). It is important to note that both UBF and ANS give large P values when in the presence of BSA [25]. These results indicate that the low UBF fluorescence polarization values observed in the micelles and membranes are not due to a long lifetime of the excited state of this dye. In the absence of actual measurements of τ, the lifetime of the excited state for UBF, no actual rotational relaxation rates can as yet be calculated. However since there is only a very small change in quantum yield of the probe in all the systems tested, the difference in P can only indicate a very high rotational freedom in the micelles and membranes.

TABLE III. Comparison of Zeta (ζ)-potential (microelectrophoresis) and Ψ calculated from the pK_a of UBF (according to Eq. 3)[a] of phosphatidylcholine (neutral and bearing net charge) liquid crystals

Potential	Lecithin + octadecylamine	Lecithin	Lecithin + dicethyl phosphate
(mV)	(cationic)	(Zwitterionic)	(anionic)
Zeta (ζ)[b]	+20	0	−20
Ψ	+14.8	0	−14.8
Ψ			(+Polylysine) + 14.8

[a] These tabulated data are obtained from Fig. 4A and B and calculated according to Eq. 3.

[b] Data from Papahadjopoulos and Watkins [23].

TABLE IV. Degree of fluorescence polarization of solubilized dyes in aqueous, micellar and membranous phases

Medium	p(%) UBF[a]	ANS[b]
0.25 M Sucrose 0.1 M Tris acetate, pH 7.4	0.82	—
A particles (1.6 mg/ml) in 0.25 M sucrose 0.1 M tris-acetate, pH 7.4	1.02	20.4
0.1 M CTABr 0.005 M tris-acetate, pH 7.4	0.85	6.9
0.51% BSA	37.5	30.0

[a] UBF concentration = 10^{-6} M

[b] ANS concentration = 10^{-5} M
Temperature = 25.8°C.

Comparison of UBF aand BTB as Probes of the Electron-transfer-Dependent Energy-linked Reactions in Submitochondrial Particles

The changes in fluorescence and absorbance of UBF and BTB respectively upon initiation of electron-transfer reactions in submitochondrial particles are illustrated in Fig. 8. The particles have been

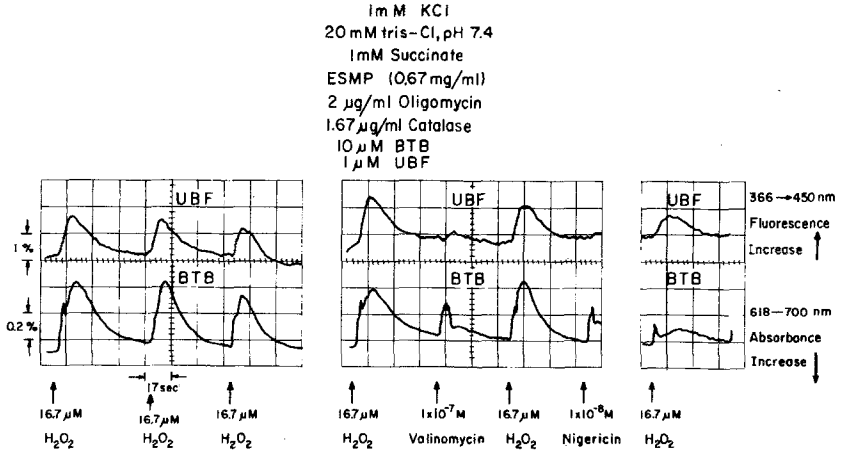

Figure. 8. Comparison of umbelliferone and BTB as probes of electron-transfer-dependent energy-linked reactions in submitochondrial particles. Reaction mixture: 1 mM KCl, 20 mM tris-Cl, pH 7.4, 1 mM succinate, submitochondrial particles (0.67 mg/ml), 2 μg/ml oligomycin, 1.67 μg/ml catalase, 10 μM BTB, 1 μM UBF. Final volume, 1.0 ml. Temperature 25 ± 1° C. This experiment was performed at the Johnson Research Foundation, University of Pennsylvania, using the combination dual wavelength spectrophotometer and fluorimeter, as described by Azzi, Chance, Radda and Lee [40]. We are grateful to Professor Britton Chance for enabling us to present this experiment.

rendered anaerobic by prior addition of 1 mM succinate and are supplemented with catalase; hence, addition of 16.7 μM H_2O_2 which is converted to oxygen, results in an increase in UBF fluorescence simultaneous to a decrease in BTB absorbance, both exhibiting a half-time of rise of approximately 4 sec and reaching a peak after about 12 sec of the H_2O_2 addition; thereafter both signals return to the original base line with a half-time of approximately 10-15 sec. This oxidation-reduction cycle is repeated three more times, when 1×10^{-7} M valinomycin is added; this ionophore does not significantly modify the subsequent aerobiosis-anaerobiosis cycle. Thereafter, 1×10^{-8} M nigericin is introduced resulting in a reduction of the UBF signal by about 50% and of the BTB signal by approximately 70% as would be expected from the uncoupling effect exerted by the

valinomycin-nigericin couple in the presence of K^+ (cf. [4]). These preliminary observations indicate that UBF is responding to some energy-linked functions of submitochondrial particles.

Discussion

The results of this study illustrate the profound modifications of the ionization properties of indicator dyes solubilized at an ionic interface. The apparent indicator dissociation constants are calculated in terms of the overall (H^+), i.e. a pH titration. However, the (H^+), at a surface differs from that in bulk because of the electrical potential difference of the surface with respect to the bulk, which determines a Boltzmann distribution of H^+ in the interfacial region. Accordingly, the pK_a of the indicator is subject to the influence of the surface charge and is given by the following equation [9]:

$$pK_s = pK_b - \frac{\epsilon\psi}{2.3\,kT} \tag{1}$$

where pK_s = is the pK of the indicator at the surface, in the solubilized state, pK_b the indicator's pK in the bulk aqueous phase, ψ, the surface potential (for a thorough discussion on surface potential see Davies and Rideal [27]), ϵ, the electronic charge, T, the absolute temperature, k, the Boltzmann constant. At 25°, this equation becomes:

$$pK_s = pK_b - \psi/59.16 \tag{2}$$

This relation indicates that only when $\psi = 0$, $pK_s = pK$, implying that the surface and bulk pH values are the same; that for a positively charged interface, the bulk pK_b value is lower than pK_s and that for a negatively charged surface the reverse is true. The indicators utilized in this study follow this relation, i.e. the pK_a values were always lower in positively charged interfaces and higher when adsorbed onto negatively charged surfaces.

Being aware of the fact that the surface potential will influence the distribution of all ions, not only H^+, and despite the limitations of the double layer theory (cf. [27]), it is worth using such a conceptual approach not with the aim of obtaining high precision values but as an approximate estimation of the magnitude of surface potential necessary to account for the observed pK_a shifts.

Rearrangement of Eq. (2) results in the following expression:

$$59.16\,(pK_s - pK_b) = -\psi \tag{3}$$

Table II presents the difference in pK_a values of MR between neutral (non-ionic) and cationic and anionic detergent micelles. Substituting these pK_a values in Eq. 3 results in a difference in surface potential, ψ,

of +89 mV between neutral and cationic micelles (more positive for DTABr) and of −86 mV between neutral and anionic micelles (more negative for SDS).

Hartley and Roe [9] suggested that Eq. (2) could be expressed in terms of the zeta potential (i.e. the electric potential at the slipping plane between the fixed and flowing liquid; cf. [27]), as follows:

$$pK_s = pK_b - \zeta/59.16 \tag{4}$$

A comparison of ζ values of phosphàtidylcholine (neutral, positively and negatively charged) liquid crystals as calculated with Eq. (4) with those derived from the microelectrophoresis data obtained by Papahadjopoulos and Watkins [28] is illustrated in Table III. As can be noted, there is a reasonable agreement between the ζ values calculated from Eq. (4) and the ζ-potential measurements. Moreover, it is worth noting the significant reversal of potential (from −14.8 to 14.8 mV, i.e. $\triangle\zeta$ = 29.6 mV) induced by the interaction of poly-L-lysine with anionic phosphatidylcholine liposomes.

If the values obtained by using Eq. (4) are assumed to reflect ζ-potential values, and the relationship between the surface potential (i.e. the potential difference at the plane of the ionizable groups), and the ζ-potential, is accepted to be in general $\psi \cong 2\zeta$ (cf. [27]), then the calculated values from the pK_a of MR give ψ values for SDS micelles of −172 mV (when the bulk concentration of salt is less than 0.01 M) which are in agreement with the calculated values from electrokinetic potentials of −178 mV (ζ potential −92.3), presented by Davies and Rideal [27], and of −200 mV calculated by Mukerjee and Ray [29].

A surprising result of these studies is the finding that even though the umbelliferone is shown to partition towards the micelles and liposomes, as evidenced by its reflecting the surface charge and from the actual determination of partition coefficients, its fluorescence emission is nearly completely depolarized either in the presence of micelles, liposomes or submitochondrial particles.

In contrast, ANS fluorescence emission shows a polarization of fluorescence of 6.9% for micelles (value of 16% was published by Flanagan and Ainsworth [30]) and in submitochondrial particles it was 20.4% (a value of 19% was published by Brocklehurst, Freedman, Hancock and Radda [31]). As shown by Weber [25] a low value for P is related to a fast rotational relaxation rate of the molecule or alternatively to a long lifetime of the excited state. The high polarization values for umbelliferone in the presence of serum albumin, which are higher than those of ANS, indicate that the lifetime of the excited state is equivalent or even shorter than that of ANS. Thus the evidence is that umbelliferone is partitioning to the amphipathic interfaces studied but is still capable of rotational movements nearly equal to those of the dye in water.

Taking into account:

1. The high magnitude ($>10^3$) of the UBF partition coefficient between micellar and aqueous phases.
2. The absence of UBF immobilization when solubilized in micelles or membranes as judged by fluorescence polarization, and
3. The sensitivity of UBF to changes in surface potential and the close quantitative agreement between the calculated ψ values from pK_a measurements and the measured ζ-potential by microelectrophoresis, it is tempting to suggest that UBF exhibits a space-averaged location where despite being solubilized in an environment susceptible to experience marked fluctuations in surface charge, it preserves the high motional freedon observed in water.

To the best of our knowledge, this is the second case of a dye-interface interaction where the dye is bound and still highly mobile Waggoner, Griffiths and Christensen [32] reported such a behavior of the 2,4-dinitrophenyl hydrazone of 2,2,6,6,tetramethyl-4-piperidone nitroxide-free radical when solubilized in micellar phases and proposed a new model of dye solubilization "as a dynamic association with an aggregate of SDS molecules in which the probe preserves a random spatial orientation and experiences a relatively polar time-averaged environment". Furthermore, they stress: "Perhaps the most interesting aspect of this study is the emphasis on the dynamic nature of solubilization. The ESR results, and to some extent the NMR data, serve to illustrate that the static models for the incorporation of foreign molecules in micelles have limited significance."

It is plausible that the differences in $\triangle pK_a$ determined with the dyes studied reflect varying degrees of penetration into the charged region, MR being located closest to the charge region while BTB and especially umbelliferone being present towards the outer micellar region.

Since MR can experience electrochromic effects, that is, electric field induced changes in its absorption spectrum [33] an actual change of the intrinsic pK_a of MR cannot be discarded.

It clearly emerges from the above discussion that solubilized indicator dyes are subject to the influence of the surface charge existent at interfaces and respond directly to it by changes in their ionization behavior.

An additional consideration is worth noting in assessing the nature of dye responses in energy-conserving membrane systems, that of the importance of the direct interaction of the electric field present in the membrane with the dye. This type of interaction, known as electrochromism [34] has been well studied by the physical chemists (cf. [35]) but no significant attention to it has been payed by the membrane biologists (cf. [36]).

Thus, one can no longer ignore the surface potential when interpreting

the energy-linked changes of indicator dyes in mitochondrial, chloroplasts and bacterial chromatophore membranes. Furthermore, it is tempting to reinterpret all the available evidence on the use of these dyes in energy-transducing membranes on the basis of the observations hereby reported and suggest the participation of the surface potential in the primary event of the energy-coupling process. It is not difficult to envisage the generation of a surface potential by a transmembrane electron-transfer reaction. It is worth mentioning in this respect the data of Chance, Crofts, Nishimura and Price [37] and Chance, McCray and Bunkenburg [38] on the kinetic sequence of redox reactions and color changes of solubilized bromcresol purple in Chromatium chromatophores (we have measured a $\triangle pK_a$ of solubilized bromcresol purple between cationic CTABr and anionic SDS micelles of 2.25). They report that while the half-time response of the dye to a 20 nsec flash is 400 μsec the response of other identifiable electron transport events is 1-2 orders of magnitude faster. Grunhagen and Witt [39] have studied UBF solubilized in chloroplasts and report a half-time of rise of UBF fluorescence after 2×10^{-5} sec short flashes (using the repetitive pulse technique) of 20-30 msec. This difference between bromcresol purple and UBF may again be indicative of the different degree of penetration into the charged surface, UBF being more superficial than bromcresol purple. The electron-transfer-dependent change in surface charge would lead to the installation of a membrane potential with the consequent redistribution of ions according to conventional electrochemical principles (cf. [3]). This scheme (see also [7, 38, 39]), that considers the surface potential as an intermediate between the primary redox reactions and the transmembrane events, incorporates the most attractive features of the current hypotheses of energy-coupling in oxidative and photosynthetic phosphorylations.

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