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Localization of dipyridamole molecules in ionic micelles: effect of micelle and drug charges

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Abstract

The localization of the coronary vasodilator dipyridamole (DIP) in cationic cetyltrimethylammonium chloride (CTAC), anionic sodium dodecylsulfate (SDS) and zwitterionic *N*-hexadecyl-*N,N*-dimethyl-3-ammonio-1-propanesulfonate and lysophosphatidylcholine (HPS and LPC) micelles was investigated using fluorescence quenching by quenchers with known localization in the micelle (TEMPO and 5-doxyl and 12-doxyl stearic acids). The use of fluorescence quenching jointly with fluorescence and ^{1}H-NMR spectral measurements shows that DIP molecules in both protonated and nonprotonated forms are localized in micelles near the region which separates their polar and nonpolar parts, the polarizable heteroaromatic cycle of DIP being close to the polar part and the nonpolar substituents penetrating the hydrophobic interior of the micelle. The electrostatic interaction between the protonated DIP molecules and micelle charges either moves DIP into the micelle interior (for cationic and zwitterionic micelles) or draws it closer to the micelle surface (for anionic ones). Our results could be relevant to the mechanism of DIP action since many data indicate the interaction of the drug with cell membranes. The ability of DIP to localize near the membrane surface with the substituents immersed into a hydrophobic moiety could be essential for the drug interaction with P-glycoprotein, which is responsible for mediation of the effects of several antitumour drugs.

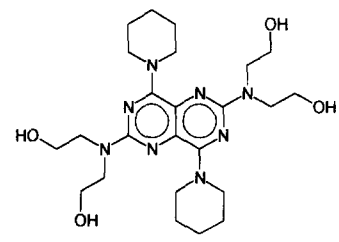
Keywords: Vasodilator; Dipyridamole; Fluorescence quenching; Micelle; Drug localization

1. Introduction

Dipyridamole (DIP), 2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido-[5,4-d]pyrimidine, well known for a number of years as a coronary vasodilator [1,2], recently has been also recognized as a coactivator of a number of antitumour compounds [3–7]. Studies of multidrug resistant cells have shown that DIP reduces the drug resistance of the cells due to its inhibition of the function of P-glycoprotein, which is an efflux pump of lipophilic substances localized in cell membranes [5,6]. These data suggest that the DIP molecules should also be localized in the cell membrane. The same conclusion can be drawn from the data on inhibition of lipid peroxidation by DIP [8], which is associated with scavenging of oxygen free radicals by the drug.

The biological activity of DIP and its derivatives (DIPD) depends upon the nature of the substituents in different positions of the molecule [9–11]. This effect is certainly related to the influence of these substituents on the binding of the DIP derivatives to microheterogeneous biological structures (membranes, macromolecules), as it was shown for bovine serum albumin [12]. However, existing explanations of the relative activity of DIP and DIPD [11] do not take into account their localization in the membrane (at the membrane surface or in a hydrophobic moiety deep inside the membrane). This is an important issue since it is related to the mechanism of drug action, especially to the problem of mediation of this action through either binding of the drug to membrane proteins or modifications promoted in the lipid structure.

The structure of DIP includes tertiary nitrogen atoms in the aliphatic substituents, diethanolamine and piperidine groups, and in the aromatic pyrimido-pyrimidine nucleus



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responsible for the transition of the DIP molecules in the physiological pH range from protonated to nonprotonated (charged and noncharged) form (pK_a 5.8 in homogeneous aqueous solution) [13,14]. The nitrogen protonation site has not yet been definitely determined, probably due to involvement of all nitrogens in the π-conjugation of the molecule. In conjugated system, a binding of a single hydrogen promotes effective deexcitation decreasing sharply the fluorescence emission and affecting drastically the optical absorption. Owing to ionizable headgroups of lipids, the surface of biological membranes often possess a net charge. Therefore, the binding properties and localization of these two forms of the DIP molecule, protonated and neutral, may differ considerably.

The present work was aimed at the determination of the localization of DIP in ionic micelles, and its dependence on the charges of DIP and micelle. Micelles are a simplified model of the membranes, since they do not posses a bilayer structure and have the spherical form. However, the presence of polar and hydrophobic regions in their structure allows one to study the affinity of small molecules to membrane systems. Micelles are often used as a model of biological membranes [15–19]. DIP solutions have the long wavelength absorption band in the spectral range 350–450 nm and the fluorescence emission at 450–550 nm allowing the use of fluorescence spectroscopy to study DIP interaction with biological and model systems in solutions [12,20]. Fluorescence quenching by quenchers with known position in the membranes or in the micelles is used to determine the localization of the fluorophores [21,22].

We use the quenching of DIP fluorescence in cationic cetyltrimethylammonium chloride (CTAC), anionic sodium dodecylsulfate (SDS) and zwitterionic lysophosphatidylcholine (LPC) and *N*-hexadecyl-*N,N*-dimethyl-3-ammonio-1-propanesulfonate (HPS) micelles by 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO) and spin labeled stearic acids with the nitroxide groups at the fifth and twelfth carbon atoms of the chain (5-DSA and 12-DSA, respectively). Comparison of the results of fluorescence and NMR spectral analyses is also performed in order to assess the localization of the drug in the membrane model system.

2. Materials and methods

DIP, LPC and HPS were purchased from Sigma, SDS was from Bio-Rad, CTAC was from Herga and recrystallized twice from methanol-acetone 1:4 and dried under vacuum. The quenchers, 5-DSA, 12-DSA and TEMPO, and the solvents D_2O and $CDCl_3$ were purchased from Aldrich. All other reagents were analytical grade and used without further purification. Distilled deionized water was used as solvent for aqueous solutions.

In fluorescence measurements, the DIP concentration in acetate (pH 2.0 and pH 5.0), phosphate (pH 7.0) and borate (pH 9.0) buffers was $(0.5–1.0) \cdot 10^{-5}$ M so that the solution optical density was less than 0.1 at $\lambda_{exc} = 415$ nm. The concentration of micelles in all cases was sufficient for the total binding of DIP [23] ([CTAC] = 0.076 M, [LPC] = 0.0125 M, [HPS] = 0.040 M, [SDS] = 0.100 M). In quenching experiments, small aliquots of 10 mM solutions of nitroxide radicals in ethanol were added to the fluorescence cuvette, and the fluorescence intensity at maximum emission wavelength was monitored. The determination of association constants of DIP to micelles was performed in fluorimetric titrations at fixed concentration of the drug and with additions of small aliquots of concentrated detergent solutions directly to the fluorescence cuvette. Data were analyzed as described in [20,23].

In NMR measurements the DIP concentration was in the range $(1–5) \cdot 10^{-3}$ M in D_2O, $CDCl_3$, and aqueous solutions of SDS and CTAC, and $5 \cdot 10^{-5}$ M in CCl_4 (saturated solution). The pH value was adjusted by the addition of HCl or NaOH. The millimolar concentration of DIP in D_2O was reached at acid pH (pH 2.0), and after the addition of micelles the pH was raised to 7.0 (CTAC) or 5.0 and 9.0 (SDS).

Fluorescence measurements were performed on a Jasco FP-777 spectrofluorometer, the absorption was monitored on a Hitachi U-2000 spectrophotometer, the fluorescence

Table 1
DIP fluorescence lifetimes (τ), bimolecular quenching constants ($k_q \cdot 10^{-11}$) association constants (K_a) and Stern-Volmer constants (K_{SV})

pH	Quencher	CTAC 0.076 M		HPS 0.04 M		LPC 0.0125 M		SDS 0.1 M		EtOH
		2.0	7.0	2.0	7.0	2.0	7.0	5.0	9.0	
τ(ns)		11.2	17.2	6.2	16.7	2.2	17.2	2.7	17.1	12.8
$K_a(M^{-1})$		11	3170	25	1600	750	7500	> 3000	1800	
$k_q(M^{-1}\ s^{-1})$	TEMPO	0.23	0.19	0.17	0.20	2.2	0.27	0.62	0.14	0.05
	5-DSA	0.30	0.25	0.37	0.34	2.5	0.53	1.00	0.20	0.06
	12-DSA	1.35	0.33	1.53	0.68	7.1	0.91	–	0.43	0.06
K_{SV} (M^{-1})	TEMPO	260	310	108	330	475	460	167	236	69
	5-DSA	340	350	221	550	542	913	522	329	73
	12-DSA	1520	545	946	1138	1553	1558	–	702	72
η		4.5	1.8	4.3	2.1	2.9	1.7	–	2.1	–

lifetimes (τ) were measured using a nanosecond lifetime system from PTI, model LS-1, ^{1}H-NMR spectra were run on a Bruker AC-200 spectrometer. The pH values were measured with a Corning 130 pH meter equipped with a glass Ag/AgCl semimicro combination electrode. All measurements were made at 295 K.

3. Results and discussion

The maximum positions and the band shapes of the absorption and fluorescence spectra of DIP in homogeneous solutions change with the protonation of the DIP molecules [13]. The quantum yield of fluorescence (ϕ_{fl}), which is about 100% for the nonprotonated form in homo- and heterogeneous solutions, is reduced by a factor of 6–10 upon the protonation [13,23]. The fluorescence lifetime of the protonated DIP molecules is less than that of the neutral ones (see Table 1). The fluorescence decay kinetics of DIP both in homogeneous solutions and in micelles is the sum of two exponentials. In the present work, we used the average τ values calculated according to the procedure described elsewhere [14].

The pK_a value of DIP changes due to the binding of DIP molecules by micelles, and the value and sign of this change, ΔpK_a, depends on the micelle type [23]. We measured the DIP fluorescence quenching at two different pH values for every type of micelles used (see Table 1) so that DIP molecules were either fully protonated or nonprotonated. Quenching by 12-DSA, 5-DSA and TEMPO was measured at both pH values for all types of micelles. Fluorescence was excited at $\lambda_{exc} = 415$ nm, the emission was monitored at $\lambda_{em} = 485$ nm. Stern-Volmer plots for the quenching of DIP in CTAC micelles at pH 7.0 are presented in Fig. 1. The effect of different positional isomers of stearic acid nitroxides is evidenced. Stern-Volmer plots were nicely fitted to straight lines. The values of the Stern-Volmer quenching constants (K_{SV}) are given in Table 1.

The values of bimolecular quenching constants (k_q) were calculated as $k_q = K_{sv}/\tau$, and are also shown in Table 1. In homogeneous ethanolic solutions, k_q is the same for all three quenchers and has a value close to the diffusion limit of k_q for dynamic quenching. Similar effect was obtained for TEMPO and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) in water for both protonated and nonprotonated DIP forms. In the presence of micelles, these values are much higher than those in homogeneous solutions. In previous experiments we have shown that the association constant (K_b) of TEMPO with all types of micelles was higher than 3000 M^{-1} [20]. For more hydrophobic 5-DSA and 12-DSA, even higher values of K_b may be expected. Thus, at micelle concentrations used the quenchers were totally bound.

NMR experiments have shown that the paramagnetic fragment of 5-DSA molecule is localized near the polar

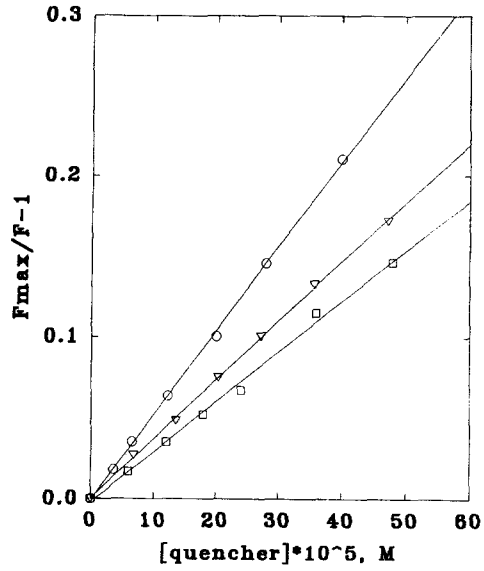

Fig. 1. Stern-Volmer plots for the DIP fluorescence quenching by nitroxide radicals in CTAC (80 mM) solutions in phosphate buffer at pH 7.0, (○—○) 12-DSA, (▽—▽) 5-DSA, (□—□) TEMPO. Excitation at 415 nm, emission at 485 nm.

part of the micelle, whereas the paramagnetic fragment of 12-DSA is situated in the nonpolar internal part of the micelle [24]. Due to the high flexibility and dynamic nature of the micelle the probe is not at a precisely fixed position. It is reasonable to assume that in average the paramagnetic fragment of 12-DSA lies deeper by 7 methylene links (6 Å) into the nonpolar part of the micelle as compared to 5-DSA. More hydrophilic TEMPO does not penetrate into the micelle interior. High k_q values show that the DIP molecules should also be localized within the micelles. This is in agreement with the values of the association constants (see Table 1) and with the absence of DIP fluorescence quenching by iodide in the presence of micelles [23].

High k_q values might be explained by several reasons:

(1) Formation of a complex between the DIP molecules in the ground state and the quencher. However, we did not observe changes in the absorption and fluorescence DIP spectra indicating the complex formation, and the k_q values in the homogeneous solutions do not exceed the diffusion limit. Thus, we have no reason to admit the complex formation.

(2) The K_{sv} values were calculated from the average concentrations of quenchers in solutions. The local quencher concentrations in the micelles are many times higher, since the total volume of micelles (V_{mic}) is less than the solution volume (V_{sol}). Therefore, in order to obtain real K_{sv} values, the K_{sv} values in Table 1 should be divided by the ratio of local and average concentrations of quencher:

$$V_{sol}/V_{mic} = [\text{quencher}]_{local}/[\text{quencher}]_{average} = n;$$

$$K_{sv,real} = K_{sv}/n;\ k_{q,real} = k_q/n;\ n \gg 1$$

and we have the usual dynamic quenching in the liquid internal part of the micelle. However, in this case the k_q value would be the same for different radicals and in different solvents, in contrast with the experimental results (Table 1).

(3) DIP molecules are localized in a certain region of the micelle and we have the static quenching of the DIP fluorescence by the radicals. This assumption is strongly supported by the following observations.

The DIP fluorescence spectra in all types of micelles are nearly the same as in the polar ethanolic solution and differ from that in nonpolar cyclohexane (Fig. 2). The DIP solution fluorescence spectra are due to the $\pi \leftarrow \pi^*$ transition of π-electrons in the heteroaromatic cycle [13]. The similarity of the DIP fluorescence spectra in ethanolic solutions to those in micelles shows that the polarizable heteroaromatic cycle of the DIP molecule has a polar environment, i.e., it is located close to the external polar part of the micelle. This is in agreement with the results showing preferential localization of some aromatic compounds near the polar headgroup region of the micelles [25]. DIP solubilization in nonpolar solvents (cyclohexane, hexane, CCl_4) is very low, while in polar ones (ethanol, chloroform) it is quite high, being in the millimolar range. This also shows the preference of DIP to the polar environment.

Comparison of the ^{1}H-NMR spectra of DIP in two polar solvents ($CDCl_3$ and D_2O at pH 2.1), nonpolar CCl_4, in CTAC (at pH 2.0 and pH 8.6) and SDS (at pH 5.0 and pH 9.0) in D_2O solutions is quite informative. The whole ^{1}H-NMR spectrum of DIP in $CDCl_3$ is shown in Fig. 3. The protons of the CH_2 groups which are close to the heteroatoms (nitrogen and oxygen atoms in the ethanolamines and nitrogen atoms in the piperidines) give their signals between 3.6 and 4.2 ppm. The other CH_2 groups of the piperidine rings give a signal at about 1.8 ppm, and will not be considered further. The signal from the OH groups of the ethanolamine substituents is observed at about 4.4–4.5 ppm. In aqueous solutions, or in

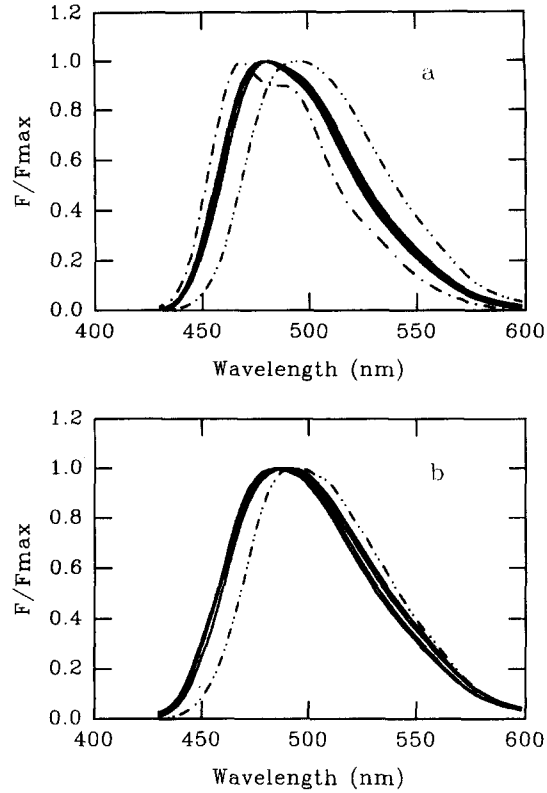

Fig. 2. DIP fluorescence spectra in phosphate buffer at pH 7.0 (a) and in acetate buffer at pH 2.0 (b) for aqueous solutions, and in cyclohexane. (– · –) in cyclohexane, (– · · –) in water, (—) in CTAC, HPS, LPC, SDS and ethanol. Excitation at 415 nm. Concentrations of detergents as in Materials and methods.

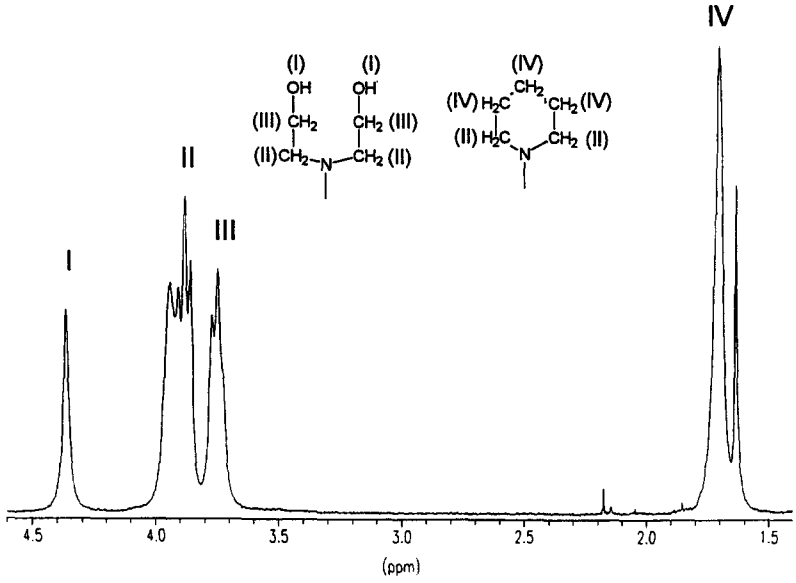

Fig. 3. 200 MHz ^{1}H-NMR spectrum of DIP in $CDCl_3$.

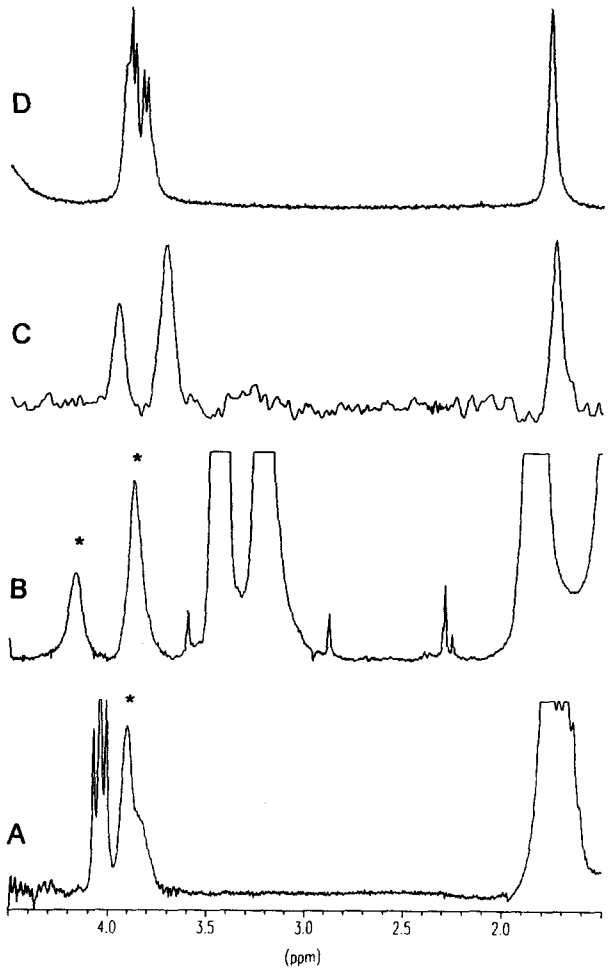

Fig. 4. 200 MHz ^{1}H-NMR spectra of DIP in different solutions: (A) in 0.02 M SDS (pH 5.0), (B) in 0.075 M CTAC (pH 8.6), (C) in CCl_4, (D) in D_2O (pH 2.0). The DIP resonances in charts A and B are marked by asterisk.

$CDCl_3$ and CCl_4 containing considerable amount of water as impurity, the hydroxyl resonance is broadened and practically disappears due to exchange with water.

In polar media ($CDCl_3$, D_2O) three peaks at 3.6–4.2 ppm are observed: two multiplets linked by spin-spin coupling and, hence, belonging to the CH_2 groups of the ethanolamines, and a broad singlet belonging to the CH_2 of the piperidine and overlapping the low-field ethanolamine peak (Fig. 3 and Fig. 4).

In non-polar CCl_4 two ethanolamine peaks collapsed into a broad nonresolved envelope, and the piperidine peak was well separated from it (Fig. 4). The piperidine peak moved from about 3.9 ppm in $CDCl_3$ to 4.0 ppm in CCl_4. The NMR spectra in CTAC at both pH values, 2.0 and 8.6, are very similar to that in CCl_4 (Fig. 4). It means that the substituents in the DIP molecule in both protonated and nonprotonated forms are localized in the nonpolar internal part of the CTAC micelle. However, in SDS at pH 5.0 the piperidine peak shifts to 3.95 ppm (very close to the ethanolamine envelope), and the whole pattern is very similar to that observed in water except for the unresolved spin coupling due to immobilization of the drug (Fig. 4). It means, evidently, that for the protonated DIP form the substituents displace from the nonpolar to more polar surrounding or, alternatively, that in SDS micelles the access of water molecules to the interior of the micelle is greater.

Taken together, fluorescence and NMR data lead to the conclusion that DIP molecules are localized in the micelle at the 'border' of the polar and nonpolar regions so that the heteroaromatic cycle is placed close to this border and the substituents are located in the nonpolar part. In the case of SDS, the attraction between negative charges of SDS and positive charges of the protonated DIP molecules maintains the drug closer to the micelle surface.

Since both the quencher and the DIP molecules are located in micelles at the border of polar and nonpolar regions, it is reasonable to assume that the quenching of DIP fluorescence in the presence of saturating concentration of micelles is mainly static. The values of k_q well above the diffusion limit also give evidence for static quenching. Studies on the mechanism of quenching of several fluorophores by nitroxide radicals in aqueous solution have evidenced that the quenching is neither dynamic nor entirely static, and is due to an electron exchange deexcitation pathway [26].

In the case of static quenching, the fluorescence intensity depends on the quencher concentration according to the expression

$$F_0/F = \exp(V[\text{quencher}])$$

where V (M^{-1}) is the quenching sphere of action.

For low levels of quenching (F_0/F is close to 1) it is possible to write

$$F_0/F = 1 + V[\text{quencher}] \quad (1)$$

The V value obtained from our data equals exactly the Stern-Volmer constant, K_{sv}, obtained above and presented in Table 1. For the neutral DIP form in all types of micelles, the maximum K_{sv} value was obtained for 12-DSA, and the minimum one for TEMPO, the K_{sv} value for 5-DSA being intermediate. The K_{sv} of the protonated DIP molecules in positively charged (CTAC) and zwitterionic (HPS, LPC) micelles changes in a manner similar to that for the nonprotonated DIP molecules. However, K_{sv} for 12-DSA in this case increases as compared to 5-DSA. The ratio $K_{sv}(12\text{-DSA})/K_{sv}(5\text{-DSA}) = \eta$ is much higher than that for the nonprotonated DIP form (Table 1). The η value is minimum for the LPC micelles, which have a negative charge near the 'border' separating the polar and nonpolar parts of the micelle. For the negatively charged SDS micelles, the K_{sv} for 12-DSA is very low (non measurable), and the maximum K_{sv} is observed for 5-DSA (Table 1).

From our fluorescence quenching data we conclude that neutral DIP molecules are localized in the micelle between the paramagnetic centers of 12-DSA and 5-DSA, closer to

12-DSA (see Table 1) and this localization does not depend on the micelle charge. Upon DIP protonation, the electrostatic repulsion shifts the DIP molecules from the positively charged surface of CTAC and positively charged internal 'border' between polar and nonpolar parts of HPS, displacing them away from the paramagnetic center of 5-DSA and nearer to 12-DSA. In LPC this 'border' is negatively charged. The equilibrium between the attraction of the DIP molecules to this 'border' and their repulsion from the positively charged surface of the micelle reduces the influence of the DIP charge in this case. For SDS, which has a negatively charged external surface, the electrostatic attraction shifts the DIP molecules nearer to the 5-DSA paramagnetic center. This conclusion is strongly supported by ^{1}H-NMR and fluorescence spectral data. Since the DIP molecule has linear dimensions about 7 Å, i.e., comparable with the distance between nitroxide fragments in 5-and 12-DSA, our pattern of localization should be considered as an average one.

The DIP molecules have a high preference for the micelle interior. Even in the presence of CTAC at low pH, when the DIP solubilisation is quite large (0.015 M), and a repulsion between the protonated DIP molecules and the positively charged micelle surface reduces considerably the association constant (by a factor of over 100, see Table 1), the DIP molecules are localized in the micelle interior. This is quite different from the behaviour of the local anaesthetic dibucaine, which in its protonated form tends to be localized in the outer part of the micelle where its accessibility to iodide is increased, while that for nitroxyl stearic acids is significantly reduced [19]. It seems that an increase in DIP solubility due to protonation is not sufficient to overcome rather high hydrophobicity of the molecule. This result might be quite relevant to the pharmacological action of DIP. It has been recently suggested that its effect could be linked to a P-glycoprotein found in multidrug resistant cells [5,6]. The ability of the substituents in the DIP molecule to be located in a nonpolar environment could be crucial for the biological activity which is modulated by the P-glycoprotein. It could also explain the high sensitivity of the biological activity of DIP derivatives both to the structure and position of the substituents in the molecule.

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