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Binding of dipyridamole to phospholipid vesicles: a fluorescence study

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Abstract

Binding and localization of the vasodilator and antitumor drug coactivator dipyridamole (DIP) and one of its derivatives, RA25, to phospholipid vesicles of DMPC (dimyristoylphosphatidylcholine) and DPPC (dipalmitoylphosphatidylcholine) was studied using fluorescence spectroscopy as well as quenching of fluorescence. The analysis of fluorescence data indicates that neutral dipyridamole binds to the phospholipids in their liquid crystalline phase with an association constant of 950 M^{-1} and 1150 M^{-1} to DMPC and DPPC, respectively. Protonation of DIP leads to a 3-fold reduction of the association constant. For the gel phospholipid phase, the binding is smaller (a factor of 2), independently of pH, suggesting that the more flexible lipid packing in the liquid crystalline phase facilitates the binding of the drug. The association constant of RA25 neutral form is considerably lower than for DIP, being around 295 M^{-1}. Fluorescence quenching with nitroxides TEMPO and stearic acid doxyl derivatives suggests the localization of DIP to be closer to the 5th carbon of alkyl chain. The quenching effect of 5-DSA below the lipid phase transition suggests that a strong static quenching may be operative. The quenching effect of 16-DSA is almost as great as that for 5-DSA below the phase transition, being even higher above the phase transition. This effect is probably due to the *trans*-gauche isomerization of the stearic acid nitroxide, making the encounter of its paramagnetic fragment with the DIP chromophore possible. Our data are consistent with DIP location close to the bilayer surface in the border of hydrophobic-polar heads interface which is similar to the data in micellar systems. In the case of RA25, the drug is in the outer part of the head group interface being much exposed to the aqueous phase and being significantly less accessible to the membrane nitroxide quenchers. © 1997 Elsevier Science B.V.

Keywords: Fluorescence; Vasodilator drug; Dipyridamole; Phospholipid; Drug interaction; Fluorescence quenching; Drug binding; Drug localization

1. Introduction

Biological membranes are extremely complex structures that require models of much less complexity to investigate different aspects of essential bilayer properties and functions [1]. The structural framework of the membrane is formed by a lipid bilayer: two parallel monolayers with their headgroups on the outside surfaces and their tails pointing inwards [2].

Lipid vesicles have been used as membrane model

Abbreviations: DIP, dipyridamole; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; DSA, doxyl stearic acid; LPC, lysophosphatidylcholine; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; HPS, *N*-hexadecyl-*N*,*N*-dimethyl-3-ammonium-1-propanesulfonate; SDS, sodium dodecylsulfate; ESR, electron spin resonance

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systems for analysing protein–lipid or drug–membrane interactions [3]. Vesicles are becoming important systems to study various physicochemical properties or biological phenomena and they may be used as carriers for drug delivery [4,5]. Similar to the micellar systems, vesicles have the ability to solubilize compounds that may be localized in different regions of the bilayer: (a) in the hydrophobic core; (b) at the headgroups region; or (c) in the inner or outer aqueous phases [6,7].

Physical properties of vesicles have been investigated in some detail considering the molecular organization of the alkyl chain in the hydrophobic core of the bilayer [1]. These systems may experience a phase transition with a characteristic temperature (T_c), which reflects the degree of the chain packing in the bilayer [6,8,9]. The changes in the phospholipid phase state upon their transition are believed to affect membrane function by changing the structure and/or the activity of membrane-associated protein [10]. Recently, much work has been done on the perturbation of the membrane lipid ordering by association of foreign molecules, particularly drugs [11–13]. If these compounds possess hydrophobic groups that facilitate and promote the binding to biological membranes, this may affect the fluidity of the bilayer altering the phase transition. The drug may introduce a charge into the membrane leading to a change in its function [14]. The site of the drug location within the lipid moiety is of importance since it relates to the problem of mediation of the drug action either through binding to the membrane proteins or modifications promoted in the lipid structure.

Dipyridamole, 2,6-bis(diethanolamino)-4,8-dipiperidino-pyrimido-[5,4-*d*] pyrimidine (DIP), has been used in medicine as a coronary vasodilator and as a coactivator of antitumor compounds [15,16]. The biological activity of DIP (and its derivatives) depends upon the nature of the substituents in different positions of the molecule [17–19]. Since DIP structure presents a pyrimido-pyrimidine nucleus it can be protonated and exist in solutions in neutral and/or charged forms. The pyrimido-pyrimidine nucleus is also responsible for the fluorescence properties of the drug. The fluorescence properties depend significantly on drug forms (neutral or protonated) and on the characteristics of the environment [20].

In previous work, we have studied the interaction of DIP with different micelles and lipid monolayers [20–24]. It was shown that the molecular mechanisms of DIP interaction imply both non-polar and polar interactions with these systems. It was concluded that the polarizable heteroaromatic cycle of DIP is located at the border of polar heads and hydrophobic tails, while its hydrophobic side groups are oriented towards the hydrophobic phase. The results obtained show that these interactions depend on the surfactant headgroups and the pH of the medium. In the case of micelles, the effect of the microheterogeneous environment upon the optical properties of the drug was assessed and binding constants and drug localization were obtained. On the other hand, the studies with monolayers allowed the effect of the drug upon the properties of the lipid, such as packing and electric surface potential, to be assessed. The presence of the drug in the monolayer induced a condensation as monitored by pressure-area isotherms.

DIP has also been reported to have quite significant antioxidant activity both in model and natural membrane systems [19,25]. This is a strong indication of the importance of DIP–membrane interaction in the mediation of its biological activity.

In the present work, the interaction of DIP with the zwitterionic lipids dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylcholine (DMPC) was investigated. The aim of this study is to use a more realistic membrane model system as compared to previously used micelles [20–23] and to further elucidate the effect of drug–membrane interaction in order to obtain a better understanding of the mechanisms of drug action in the organism. DIP–vesicle interaction is analyzed by fluorescence studies involving both association of the drug to vesicles and quenching of the fluorescence by quenchers at different depth of the bilayer, which allow the localization of the drug to be obtained.

2. Experimental section

Dipalmitoylphosphatidylcholine (DPPC), dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylglycerol (DPPG), lysophosphatidylcholine (LPC) and dipyridamole (DIP) were purchased from Sigma. RA25 was a kind gift from Dr. Karl Thomae

GmbH (Biberach am Riss, Germany). The fluorescence quenchers TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl radical) and spin labeled stearic acids with the nitroxide groups at different carbon atoms of the chain, 5-DSA, 12-DSA and 16-DSA, were purchased from Aldrich. These reagents were used without further purification. All the solutions were prepared in ultrapure water from a Millipore system and all the other solvents were analytical grade.

In fluorescence measurements, solutions of DIP were prepared in ultrapure water or in appropriate buffers (0.02 M). The concentrations in phosphate (pH 7 and 8) and acetate (pH 4) buffers were 1.1×10^{-5} M. The experiments were performed at temperatures below and above the phase transitions of the lipids (23°C for DMPC and 41°C for DPPC) [8].

The pH titrations were carried out in water using HCl or NaOH concentrated stock solutions. The suspension of vesicles containing the drug was added to the fluorescence cuvette and then, small aliquots of acid or base were added directly to the cuvette and the emission intensities were monitored as a function of the pH.

Binding constants were obtained by titrations of the drug solutions in appropriate buffers and pHs. These experiments were performed by additions of aliquots of vesicle (or micelle) concentrated stock solutions (5 mM) to the solution of the drug, directly to the fluorescence cuvette, and the emission intensities were registered after each addition. Data were analyzed as described previously [20,21,23].

In the determination of binding constants and pK_a value, DIP was excited at 415 nm and the emission was monitored at 460 nm, the wavelength where the maximal changes were observed in the presence of saturating lipid.

The fluorescence quenching experiments of the drug were performed in the presence of vesicles using as quenchers sodium iodide, the nitroxide radical TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl radical) or the spin labeled stearic acids with the nitroxide groups at different carbon atoms of the chain (5-DSA, 12-DSA and 16-DSA). Aliquots of concentrated solutions of the quenchers (10 mM) were added directly to the drug/vesicle solution (with saturating amount of lipid) contained in the fluorescence cuvette and the fluorescence intensities were monitored at maximum emission wavelength (485 nm); data were analyzed by Stern–Volmer plots [26].

Fluorescence measurements were performed on a Perkin–Elmer LS-5B spectrofluorometer equipped with a thermostated bath (Fischer 801), the absorption was monitored on a Hitachi U-3000 spectrophotometer. The pH values were measured with a Corning 130 pH meter equipped with a glass Ag/AgCl semimicro combination electrode.

2.1. Vesicle preparation

The lipids were dissolved in chloroform and the solvent was removed with a stream of nitrogen and then under vacuum at room temperature. The dispersion was prepared by the addition of an appropriate aqueous buffer solution, followed by shaking in a Vortex. The lipids were left to hydrate for 2 h at a temperature above the phase transition temperature. Then the suspension of unilamellar vesicles was prepared by the extrusion technique through polycarbonate filters (Nucleopore) with 0.1 μm pore size, using a Lipex Extruder. The vesicles were extruded through the two filters ten times at a temperature above the phase transition [27]. All fluorescence experiments were performed immediately after vesicle preparation to avoid sample deterioration. The size of the vesicles was monitored by light scattering and was shown to be uniformly distributed as a single peak around 0.11 μm, very close to the pore size of the filter [28,29].

3. Results and discussion

3.1. Spectral properties in the presence of phospholipids

Spectral properties of dipyridamole have been extensively studied [20–23], $\pi \rightarrow \pi^*$ and $\pi \leftarrow \pi^*$ transitions in the heteroaromatic cycle are responsible for the optical absorption (DIP shows absorption bands with maxima around 280 and 410 nm) and for the intense emission of fluorescence, centered around 500 nm [20–23]. The quantum yield of DIP fluorescence is high in neutral solution (pH 7.0), but the solubility is low at this pH; in acid solutions the solubility increases, but the quantum yield is reduced [20]. In Fig. 1, the fluorometric titration of a mixture

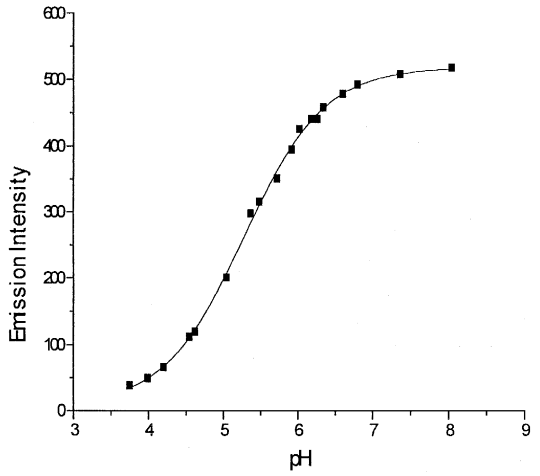

Fig. 1. Dependence of emission intensity of DIP in aqueous solutions in the presence of DMPC as a function of pH. Excitation wavelength 415 nm, emission wavelength 460 nm. Concentrations: DIP, 1×10^{-5} M; and DMPC, 2×10^{-3} M.

of DIP and DMPC vesicles at fixed concentrations is shown, in which the dependence of DIP emission intensity on the medium pH is observed. The emission intensities obtained as a function of pH were treated using the equation of Henderson–Hasselbach, described in a previous paper [30], and the titration curve was fitted using this equation in order to obtain the pK_a value.

In the presence of the vesicles the pK_a obtained for DIP was 5.30 ± 0.02. This value is lower than the value for DIP in water, which is 5.8 [20,23]. The change in pK_a gives evidence of the interaction of the drug with the phospholipid membrane. This value is higher than that obtained for zwitterionic micelles (4.3 for HPS) [20,23] suggesting a more superficial localization of the drug in the lipids.

The binding constants of DIP to vesicles were determined by the titration method in which the vesicle concentration is varied at constant drug concentration. In Fig. 2, the emission spectra of DIP are presented as a function of DMPC concentration. The maximum emission wavelength is shifted to the blue denoting the effective localization of the drug in an hydrophobic environment, with a gradual increase in the fluorescence intensity.

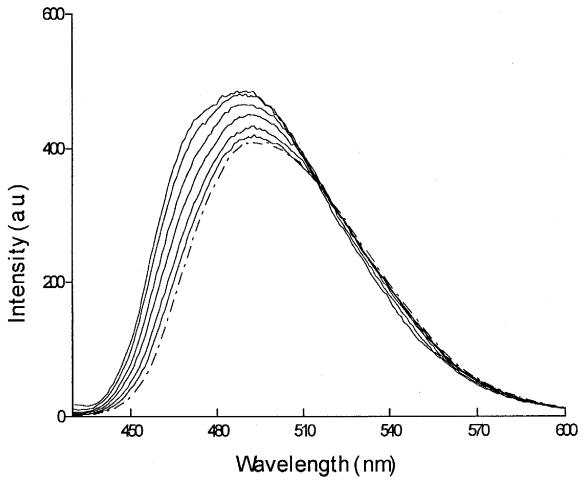

Fig. 2. Fluorescence emission spectra of DIP in aqueous solution pH 7 in the absence (– · – · –) and in the presence of DMPC vesicles (————). Concentrations of DMPC are (in order of increasing intensity, in mM): 0.146, 0.239, 0.414, 0.654, 1.16 and 1.96. Concentration of DIP, 1×10^{-5} M; excitation wavelength, 415 nm.

3.2. Binding constants to phospholipids

Considering the equilibrium:

$$D + L \rightleftarrows D - L \qquad (1)$$

where D represents the drug and L the studied lipid, the total fluorescence observed is due to the free and bound species of D. ΔF represents the difference between the fluorescence emission intensity of the drug at a certain concentration of the lipid (F) and the initial fluorescence intensity (F°), in the absence of the lipid. This difference (ΔF) is related to the quantity of the drug associated with the lipid.

In order to obtain quantitatively the association constants for binding of DIP to phospholipid vesicles, data for titrations of the drug with phospholipid were treated using the mass-action law [23] and by the method of the double reciprocal plot [21,23]. This latter treatment is based on the following equation:

$$\frac{1}{\Delta F} = \frac{1}{\Delta F_{\max}} + \frac{1}{\Delta F_{\max}} \frac{1}{K_b} \frac{1}{[L]} \qquad (2)$$

A plot of $1/\Delta F$ as a function of $1/[L]$ may be used to find K_b, the binding constant. The results obtained for both treatments are listed in Table 1. Some typical results of these titrations are presented in Figs. 3 and 4. Fig. 3 presents the binding of DIP to DPPC above the phase transition temperature (50°C) at several

Table 1
Binding constants of dipyridamole in phospholipid vesicles at different pH values and temperatures, calculated by the method of the double reciprocal plot (a) and by the mass-action law (b)

	pH	T (°C)	(a) K_b (M^{-1})	K_d (10^{-3} M)	(b) K_b (M^{-1})
DMPC	7	30	951 ± 22	1.06 ± 0.04	943
	8	30	1090 ± 60	0.94 ± 0.02	1064
DPPC	4	30	195 ± 60	–	–
		50	424 ± 77	–	–
	7	30	633 ± 22	1.52 ± 0.08	658
		50	1149 ± 16	0.85 ± 0.02	1176
	8	30	694 ± 19	1.44 ± 0.06	694
		50	1302 ± 16	0.76 ± 0.02	1316
DPPC + DPPG (11%)	7	30	460 ± 22	2.04 ± 0.09	490
		50	828 ± 19	1.15 ± 0.02	869
LPC	7	30	5380 ± 260	0.18 ± 0.01	5530
		50	4350 ± 130	0.23 ± 0.01	4340

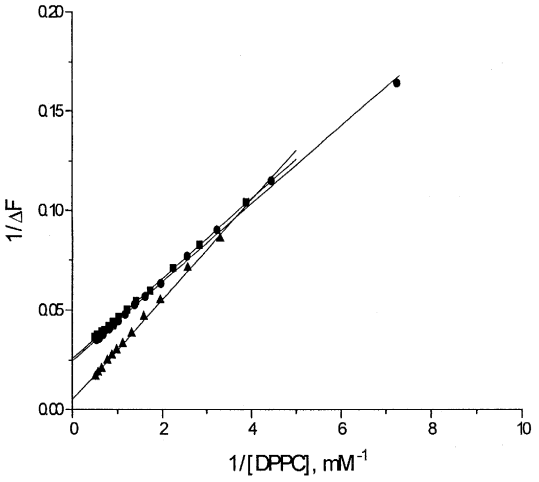

Fig. 4. Double reciprocal plot of the change in the fluorescence emission vs. concentration of DPPC for DIP at 50°C. ▲, pH 4; ●, pH 7.0; and ■, pH 8.0. The full lines are the fits to the experimental data. Concentration of DIP, excitation and emission wavelengths are the same as in Fig. 3.

pHs, while Fig. 4 corresponds to the same data presented as double reciprocal plots.

For DMPC and deprotonated DIP above the transition temperature, K_b is around 1×10^3 M^{-1} (Table 1), a value similar to those found for SDS and HPS micelles [20]. At pH 8.0 K_b increases around 15% as compared to pH 7.0. The values obtained from both treatments of data, direct fitting of binding isotherm or double reciprocal plot, are very similar. In the case of DPPC, measurements were performed both above and below the lipid phase transition and at different pH values. It is seen that at all studied pHs, the value of K_b is by a factor of 1.8–2.2 greater above the phase transition. Comparison of K_b for DIP in DPPC at pH 7.0 and 8.0 shows an increase around 14% at pH 8.0, similar to the result with DMPC. At pH 4.0, where the drug is protonated, the association constant to DPPC is reduced by a factor of 3 as compared to pH 8.0. So, the neutral drug has an association constant greater than the protonated one, this result being similar to that found previously for micellar systems [20], suggesting that an electrostatic interaction occurs between the phospholipid headgroup and the protonated form of DIP, making the association unfavorable [23]. The protonation also reduces the drug affinity to the hydrophobic portion of the membrane, leading to an increase in the drug solubility in the water phase. This fact was observed previously for zwitterionic HPS micelles [22,23]. In the micelle the reduction of K_b is considerably greater than in the vesicle consistent with a greater change in pK_a of the drug [23].

In the case of the neutral drug, the values of K_b

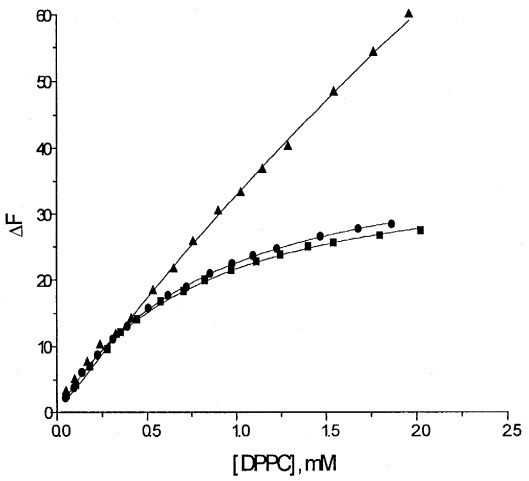

Fig. 3. Change in the fluorescence emission of DIP in the presence of DPPC vesicles at 50°C. ▲, pH 4.0; ●, pH 7.0; and ■, pH 8.0. The full lines are the fits to the experimental data. Concentration of DIP, 1×10^{-5} M; excitation at 415 nm and emission at 460 nm.

for DPPC are similar (20% higher) to those obtained for DMPC, but they are lower than those obtained for LPC (by a factor of 3.8), where the binding is more effective. Binding constants to phospholipid vesicles tend to increase as a function of temperature, this fact being due to the transition of an ordered (rigid) gel phase to a liquid crystalline phase, where the chains become more disordered and so the packing is not so dense [6,8,9]. Above the phase transition temperature, the bilayer is more fluid and this effect promotes the increase in partitioning of the drug in the bilayer. This fact is clearly observed for the DPPC system and it is not so obvious for DMPC, probably due to the relatively low transition temperature for the latter (around 23°C) which would require experiments at lower temperatures. This effect was not observed in LPC micelles where the K_b value only decreases with temperature increase (decrease of 19% at the higher temperature). The higher value of K_b and its weak temperature dependence for LPC as compared to DPPC is probably related to the more dense packing of the lipids in the bilayer which leads to a reduction in binding constant for the drug due to the restrictions imposed on its accommodation in the bilayer. In the more flexible micellar system, the association is facilitated and the binding constant is significantly higher. These results are also in agreement with the changes in pK_a for the drug, which are much smaller in the phospholipid system as compared to the micelles. This is also consistent with the fact that the polarity of both systems, LPC and DPPC, as monitored by ESR spectra of 5-DSA is quite similar (the isotropic hyperfine constants are the same).

A preliminary experiment was also performed to check if the surface charge in the membrane affects the binding of the drug. A mixture of DPPC and DPPG (11%) at pH 7.0 was used, and the results obtained suggest that the negative charge in the bilayer or the change in the lipid packing leads to a reduction of the association constant for neutral DIP of around 28%. Studies of interaction of DIP with phospholipid monolayers have shown that at low concentrations of neutral drug, a condensation of the monolayer is observed both for DPPC and DPPG. A higher surface pressure is required for the coexistence of liquid expanded and liquid condensed phases and this increase is greater in DPPC [31]. Despite the difficulties in directly comparing the data on monolayers with those in vesicles, we believe the reduction in K_b could be due to a more dense lipid packing in the mixture of DPPC/DPPG since the effect of the neutral drug is quite similar for both phospholipids. This increase in packing is also supported by the shift of the phase transition temperature to higher values (45°C) observed for DPPC and DPG (12%) mixtures [32].

3.3. Binding of RA25 derivative

In order to compare the values of association constants, a DIP derivative, RA25, was used. This compound is known to be considerably more soluble in water than DIP and to associate to a less extent to micelles [20]. Besides this, its biological activity is lower than that of DIP [17–19]. Data for binding constants of RA25 are presented in Table 2. It is clearly seen that the constant is reduced from around 1150 M^{-1} for DIP (Table 1) to around 295 M^{-1} for RA25 at pH 7.0, a trend similar to that observed for micelles [20]. In this case, no pH effect on K_b is observed, the values being the same within the experimental error for all pH values. The side substituents determine the hydrophobic properties of the DIP derivative molecules and, hence, their interaction with the lipid system are probably related to their biological activity [18,19]. For RA25, the solubility in the aqueous phase is quite significant in the explored pH range consistent with a lower solubility in the membrane and a lower activity [17–19]. So, our data are in agreement with the previous results of interaction of the drug with micelles and our proposal that the biological activity correlates with membrane solubility.

Table 2
Binding constants of RA25 in phospholipid vesicles at 50°C and different pH values, calculated by the method of the double reciprocal plot (a) and by the mass-action law (b)

	pH	(a) K_b (M^{-1})	K_d (10^{-3} M)	(b) K_b (M^{-1})
DPPC	4	306 ± 107	5.58 ± 2.00	179
	7	295 ± 70	4.32 ± 1.12	232
	8	267 ± 56	3.46 ± 0.36	289

3.4. Fluorescence quenching in phospholipids

Fluorescence quenching studies may be employed to investigate the position of the drugs in a bilayer. The fluorescence quenching of DIP was analyzed using different quenchers, like I^-, TEMPO and compounds with a nitroxide radical at a shallow, medium or deep location in the bilayer. Solutions of the drug saturated with lipids were monitored by their fluorescence emission as a function of quencher concentration. In Figs. 5 and 6 Stern–Volmer plots obtained in typical experiments are shown. The plots are linear in the concentration range of quencher used and the quenching constants are presented in Table 3.

Iodide is an anion and does not penetrate the hydrophobic region of the bilayer; TEMPO dissolves in water and in the bilayer interface and its partition in DPPC and DMPC is influenced by changes in temperature [33]; the stearic acids are almost insoluble in water; however, they can be solubilized in the hydrophobic region of the bilayer. Due to their differential localization and solubilization in the bilayer, these quenchers were used to characterize the position of DIP in the vesicles [34].

The Stern–Volmer constants for DIP in DMPC reported in Table 3 show that in the case of TEMPO, an increase of around 30% occurs on going from 19 to 30°C. This result is probably due to the fact that

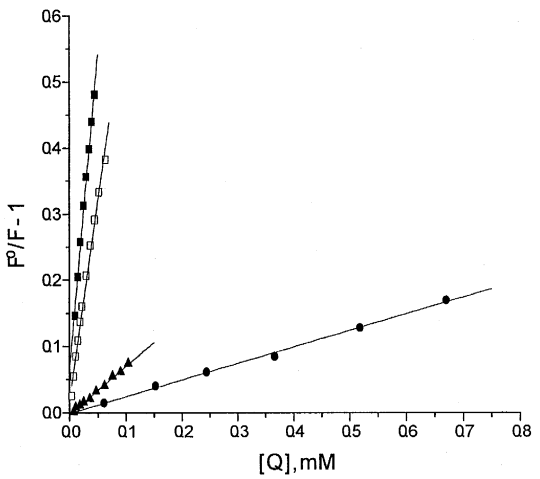

Fig. 5. Stern–Volmer plots for DIP fluorescence quenching in DPPC vesicles, pH 7.0 and at 30°C. ●, TEMPO; ■, 5-DSA; ▲, 12-DSA; and □, 16-DSA. Concentration of DIP, 0.6×10^{-5} M; excitation at 415 nm, emission at 485 nm; concentration of DPPC 2×10^{-3} M.

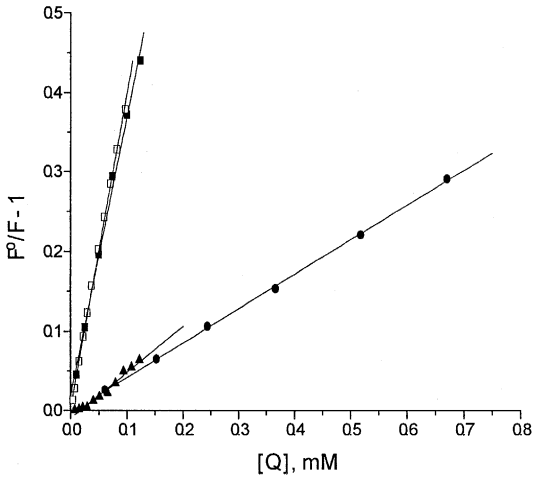

Fig. 6. Stern–Volmer plots for DIP fluorescence quenching in DPPC vesicles, pH 7.0 and at 50°C. ●, TEMPO; ■, 5-DSA; ▲, 12-DSA; and □, 16-DSA. Concentrations of DIP and DPPC, excitation and emission wavelengths are the same as in Fig. 5.

Table 3
Stern–Volmer constants obtained by quenching of dipyridamole fluorescence in phospholipid vesicles at several conditions of pH and temperature

	pH	T (°C)	Quencher	K_{sv} (M^{-1})
DMPC	7	19	TEMPO	254 ± 1
			5-DSA	7903 ± 183
		30	I^-	1.67 ± 0.07
			TEMPO	332 ± 3
			5-DSA	4290 ± 52
	8	30	TEMPO	386 ± 6
DPPC	4	50	TEMPO	317 ± 4
			5-DSA	3623 ± 100
			12-DSA	1690 ± 55
	7	30	TEMPO	254 ± 2
			5-DSA	9560 ± 280
			12-DSA	750 ± 20
			16-DSA	6480 ± 150
		50	TEMPO	411 ± 4
			5-DSA	3530 ± 100
			12-DSA	490 ± 40
			16-DSA	3960 ± 50
	8	30	5-DSA	9915 ± 388
		50	TEMPO	350 ± 3
			5-DSA	3884 ± 56
DPPC + DPPG (11%)	7	30	5-DSA	9890 ± 360
		50	TEMPO	370 ± 4
			5-DSA	3930 ± 40

the partitioning of TEMPO in the vesicle above the phase transition is enhanced in agreement with ESR data [33]. Increase of pH from 7.0 to 8.0 gives an additional 16% increase in K_{sv} for TEMPO. In the case of 5-DSA, it is observed that below the phase transition, K_{sv} is by a factor of 1.8 greater than above the phase transition. This could be related to a greater static quenching at lower temperatures, where the mobilities of both fluorophore and quencher are restricted at the bilayer interface maintaining both of them in close proximity and facilitating the quenching. This is supported by recent data on pressure effects on quenching reactions [35]. The authors found a considerable static quenching of diphenylhexatriene by a nitroxide radical in DMPC both in the fluid and gel states; in the gel state, the small contribution from dynamic quenching tends to become considerably smaller (around 1% of total quenching). Our data for quenching of DIP in DPPC are quite similar: the quenching by TEMPO is somewhat lower below the phase transition as compared to its quenching above the transition (60% lower at pH 7.0); the quenching by TEMPO above the phase transition varies with pH, being 30% greater at pH 7.0 as compared to pH 4.0, but decreasing 17% at pH 8.0 as compared to pH 7.0. For 5-DSA, which is located closer to the bilayer surface, the quenching is reduced by a factor of 2.5–2.7 on going from 30 to 50°C, showing, as for DMPC, a more effective quenching below the lipid transition temperature, this effect being practically independent of pH or presence of DPPG in the bilayer. This would imply a similar location of DIP in the bilayer independently of pH or presence of DPPG. In the case of 12-DSA the quenching is significantly less effective than for 5-DSA and the effect of the phase transition is not so strong (at pH 7.0 a 50% reduction in K_{sv} occurs on going from 30 to 50°C). The difference in K_{sv} for 5-DSA and 12-DSA which is a factor of 12.7 at 30°C and pH 7.0 reduces to a factor of 7.2 at 50°C. So, above the lipid phase transition, 5-DSA is still more efficient than 12-DSA, but their difference is reduced as compared to the gel phase. Finally, data for 16-DSA suggest that although this nitroxide has its paramagnetic fragment located deeper in the hydrophobic bilayer interior it is almost as efficient to quench the DIP fluorescence in the gel phase (its K_{sv} is 68% of that for 5-DSA), and even more efficient in the fluid phase (ratio of K_{sv} values is 1.12). Two possibilities arise to explain this result: one is that due to the high probability of *trans*-gauche isomerization in the membrane interior and especially above the phase transition the paramagnetic fragment of 16-DSA is able to approach to a close contact with DIP located close to the bilayer interface; the second possibility would imply that DIP resides part of the time in the membrane hydrophobic interior, which is not consistent with the relatively small spectral changes observed above for DIP in the presence of phospholipid vesicles, the small change in pK_a for the drug or the data for interaction with micelles which imply a localization in close proximity to the border of hydrophobic and polar interface [22]. The effect of accessibility of the paramagnetic fragment of 16-DSA to fluorophores located in close proximity to the membrane surface has been previously reported [36]. In this study, the authors observed a considerable dynamic quenching of 8-anilinonaphthalene-1-sulfonic acid fluorescence in DMPC vesicles above the lipid phase transition due to the presence of nitroxide radicals. This quenching was very efficient both for 5-DSA and 16-DSA.

Above the phase transition temperatures, the bilayer approaches the behavior of a non-restricted, non-viscous solution, thus the quencher has difficulty in reaching the fluorophore because it moves to a different region of the bilayer. In the gel phase, the fluorophore and the quencher find themselves in close proximity, so the suppression becomes more probable [35]. Another recent paper has also reported a considerable static quenching of transparinaric acid fluorescence by nitroxides (5- and 16-DSA) in DPPC bilayers, especially below the lipid phase transition [37]. In the bilayer, the quencher molecules are not distributed homogeneously relative to the position of the fluorophore. This behavior is observed in Table 3 for the stearic acid nitroxides, but for TEMPO, the effect is different: in this case there is an increase in quenching above the phase transition. This would be consistent with a higher solubilization of the nitroxide in the bilayer and more effective quenching by enhancing the accessibility of the drug to the quencher [33]. At the moment it is not possible to discriminate quantitatively the contributions of static and dynamic quenching in our results for DIP.

The effect of different positional isomers of stearic acid nitroxides is demonstrated in the above experi-

ments. The K_{sv} values decrease following the sequence 5-DSA > 16-DSA > 12-DSA > TEMPO below the phase transition temperature, but this sequence may be altered at a temperature above the phase transition and the new order may be described as follows: 5-DSA ~ 16-DSA > 12-DSA ~ TEMPO > I^-. This result suggests that DIP is localized closer to the paramagnetic fragment of 5-DSA. The increase in DIP solubility due to protonation is not sufficient to overcome the hydrophobicity of the molecule [22].

3.5. Fluorescence quenching of RA25 emission

In Table 4, data are presented on the quenching of RA25 fluorescence emission; they suggest a different localization of this derivative as compared to DIP. RA25 is more hydrophilic and accessible to the water solvent [20]. The results obtained for the quenching by 5-DSA are significantly reduced and suggest that RA25 molecules may be located more superficially closer to the polar heads in the region of the structured water. This behavior is similar to that observed for RA25 in micellar systems [20]. Quenching with TEMPO also suggests a more superficial external location for RA25 in the bilayer.

3.6. Biological activity

In the work of Von Gerlach et al. [17], over one hundred dipyridamole derivatives were studied with regard to their inhibitory activity upon adenosine and phosphate ions transport in red blood cells. It has been shown that DIP has a strong inhibitory effect on both processes, while RA25 has no effect at all. These results were only qualitative since no quantitative values of defined parameters were measured. Later, in the work by Ramu and Ramu [18], an attempt to correlate the chemical structure with the biological activity was performed by monitoring the circumvention of adriamycin resistance in cells by dipyridamole and a considerable number of DIP analogues. The result of this study was the assumption that the pyrimido-pyrimidine nucleus is not responsible for the biological effect by itself, but besides this structural element, the need for substituents was demonstrated. For instance, the pyridine substituents seemed to be relevant. Since the work was based on drug effects upon cells, precise quantitative data were also difficult to obtain. Recently, we have studied another important biological effect of DIP, namely its antioxidant effect regarding oxidation of mitochondria by iron ions [19]. In this work, we showed that the inhibitory effect of DIP was very significant with the concentration to obtain 50% inhibition of 1 μM. At the same time, this concentration for RA25 was by two orders of magnitude greater, 100 μM. This result is in agreement with data on inhibition of adenosine and phosphate transport through red blood cells where a strong effect was found for DIP and no effect for RA25. In our present work, the fact that the association constant for binding of RA25 to DPPC vesicles is only a factor of 4 lower than that for DIP suggests that the pyrimido-pyrimidine nucleus could be essential for the insertion of the drug in the membrane, but that the key element for biological activity is indeed associated to the substituents as suggested by Ramu and Ramu [18].

Table 4
Stern–Volmer constants obtained by quenching RA25 in phospholipid vesicles at several conditions of pH and at 50°C

	pH	Quencher	K_{sv} (M^{-1})
DPPC	4	TEMPO	152 ± 3
		5-DSA	434 ± 39
	7	TEMPO	180 ± 1
		5-DSA	343 ± 17
	8	5-DSA	269 ± 28

4. Conclusions

In the present work, the interaction of dipyridamole and RA25 with phospholipid vesicles was investigated through the use of fluorescence.

A value of association constants of 1150 M^{-1} was obtained for DPPC and 950 M^{-1} for DMPC above the lipid transition temperature at pH 7.0. In the case of DPPC, the association constant below the transition temperature decreases by a factor of around 2 (1.8–2.2). So, the binding is more effective in the liquid crystalline lipid phase. Also, the neutral drug has an association constant higher as compared to the protonated drug (a factor of 3).

Quenching experiments are suggestive of the localization of the drug in the hydrophobic medium close to the phospholipid polar headgroup. Interestingly, the behavior of TEMPO and 5-DSA is quite different, suggesting the possibility of very effective static quenching for 5-DSA below the transition temperature, while in the case of TEMPO, a significant contribution to quenching could be due to a dynamic mechanism.

Results with the DIP derivative RA25 are in close agreement with the observations in micelles: much lower association constant ($295\ M^{-1}$) independent of pH and more superficial localization with great accessibility to the aqueous phase. So, it can be assumed that the DIP interaction with phospholipid vesicles depends on the nature of the side substituents and the state of protonation of the tertiary nitrogens. The side substituents, which define the hydrophobicity of the molecule, are responsible for the drug location in these systems. Our data also indicate that the pyrimido-pyrimidine nucleus might be important for the insertion of DIP and derivatives in the membrane since despite the significant difference in biological activity of DIP and RA25 (more than two orders of magnitude [19]) only a 4-fold reduction in binding constant to DPPC is observed.

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