Location and Dynamics of Anthracyclines Bound to Unilamellar Phosphatidylcholine Vesicles†

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ABSTRACT: We have exploited the intrinsic fluorescence properties of the anthracycline antitumor antibiotics to study the dependence on drug structure of relative drug location and dynamics when the anthracyclines were bound to sonicated dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) vesicles at 27.5 °C. Iodide quenching experiments at constant ionic strength were used to evaluate the relative accessibilities of the bound fluorophores to membrane-impermeable iodide. Iodide was found to quench the fluorescence of anthracyclines in free solution by both static and dynamic mechanisms, whereas quenching of membrane-bound fluorophores was predominantly due to the dynamic mechanism. Modified Stern–Volmer plots of anthracyclines bound to fluid-phase DMPC bilayers were linear, and the bimolecular rate constant (k_q) values ranged from 0.6×10^9 to 1.3×10^9 M^{-1} s^{-1}. Modified Stern–Volmer plots of anthracyclines bound to solid-phase DPPC bilayers were curved, indicative of a heterogeneous-bound drug population. A strong correlation between drug hydrophobicity and penetration of the fluorophore into the bilayer was observed for the daunosamine-containing anthracyclines. Steady-state fluorescence anisotropy measurements under iodide quenching conditions were used to investigate the diffusive motions of anthracyclines in isotropic solvent and in fluid-phase DMPC bilayers. Anthracycline derivatives free in solution exhibited limiting anisotropy (α_∞) values which decayed to zero at times long compared to the excited-state lifetime, in contrast to anthracyclines bound to fluid-phase DMPC bilayers, which showed nonzero a_∞ values. Steady-state anisotropies of membrane-bound anthracyclines were found to be governed principally by a_∞ and not by the mean rotational rate (R). Spectral titration studies also showed that the membrane affinities of daunomycin increased with increasing ionic strength. The data presented in this paper are consistent with the hypothesis that anthracycline associations with neutral phospholipid bilayers (both fluid phase and solid phase) were driven by hydrophobic interactions between the aglycon portion of the anthracycline molecule and the hydrocarbon interior of the membrane. Whereas the ionic and electrostatic nature of the anthracycline amino sugar was previously found to be favorable for drug–DNA binding, anthracycline affinities for neutral membranes were apparently reduced by the ionization of the amino group.

The mechanism by which the anthracycline antitumor antibiotics exert their cytotoxic action has not been clearly established. Although the classical paradigm for their mechanism of action has been interference with DNA function, certain evidence indicates that the anthracyclines may exert their cytotoxic activity at the surface membrane of the cell [see Tritton & Hickman (1985) for a review]. The anthracyclines are amphipathic molecules consisting of a aglycon (a red-pigmented, dihydroxyanthraquinone nucleus) linked by a glycosidic bond to an amino sugar (commonly daunosamine). Structural changes in both the aglycon and amino sugar portion of an anthracycline molecule have been shown to strongly modulate equilibrium binding affinities and thermodynamic binding parameters for small unilamellar phosphatidylcholine vesicles (Burke & Tritton, 1985). Anthracycline binding to the lipid bilayers appeared to be dominated by hydrophobic interactions between the aglycon portion of the drug molecule and the hydrocarbon interior of the membrane.

In the present study, we have exploited the intrinsic fluorescence properties of the anthracyclines to reveal information concerning the relative drug location and dynamics when bound to fluid-phase L-α-dimyristoylphosphatidylcholine (DMPC)[1] and solid-phase dipalmitoylphosphatidylcholine (DPPC) small unilamellar vesicles. This allowed us to investigate the dependence on drug structure of anthracycline location and dynamics in lipid bilayers as well as the dependence of daunomycin binding on ionic strength. The interaction specificities of anthracyclines for neutral phospholipid membranes and DNA were compared.

Materials and Methods

Chemicals. The sources and drug stock preparation of the anthracycline derivatives have been previously described (Burke & Tritton, 1985). 1,4-Dihydroxyanthraquinone (Aldrich) was further purified by recrystallization in acetone. L-α-Dimyristoylphosphatidylcholine, L-α-dipalmitoylphosphatidylcholine, and glycogen were obtained from Sigma

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[1] Abbreviations: DMPC, L-α-dimyristoylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; TLC, thin-layer chromatography; PBS, phosphate-buffered saline containing 8 mM Na_2HPO_4, 1 mM KH_2PO_4, and the specified amount of KCl or KI (pH 7.4); T_M, gel- to liquid-crystalline-phase transition temperature; DPH, 1,6-diphenyl-1,3,5-hexatriene.

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Chemical Co. and were used without further purification. All chemicals were reagent grade and were used without further purification.

Vesicle Preparation. Small unilamellar vesicles were prepared the day of an experiment as reported previously (Burke & Tritton, 1984, 1985). Briefly, lipid was weighed and suspended at a typical concentration of 34 mg/mL in PBS buffer at a specified ionic strength. Lipid dispersions were prepared by vortex mixing, the lipid dispersion subsequently being subject to ultrasound with a bath-type sonicator. Vesicle preparations were annealed for 0.5–1.0 h above the T_M of the lipid prior to use.

Fluorescence Instrumentation. Fluorescence measurements were obtained by using an SLM Model 4800 subnanosecond spectrofluorometer equipped with EMI Industrial Electronics Ltd. (Ruislip, England) 9816A phototubes. Steady-state fluorescence intensity measurements, polarization spectra, and anisotropy measurements were obtained as previously described (Burke & Tritton, 1985). Lifetimes were measured by phase shift using exciting light modulated at 30 MHz (Spencer & Weber, 1969). A glycogen scattering solution (25 mg/mL) was used as reference, and the amount of scattered light was adjusted with neutral density filters (Schott) in order that signal levels from both the scatterer and the sample were approximately equal. The EMI 9816A phototubes were chosen in order to minimize color artifacts in the region of the spectrum where the anthracyclines emit. Since the emission for each anthracycline derivative was from the same region, this method allowed for a valid comparison of their relative bimolecular quenching constants and rotational rates. Lifetime determinations on anthracycline samples were conducted by using an excitation wavelength of 470 nm and a band-pass of 0.5 nm, two 500-nm short-pass filters (Melles Griot) in the excitation beam to reduce the transmission of stray light from the monochromator, and a 550-nm long-pass filter (Schott) for each emission channel to isolate fluorescence from scattered light. All experiments were conducted in 1-cm quartz cuvettes.

Equilibrium Binding Measurements. Fluorescence anisotropy titration was used to determine the concentrations of free and bound drug in liposome samples containing a total drug concentration of 1×10^{-6} M and varying lipid concentrations by a method previously described. The overall association constant (K) was determined from the slope of a double-reciprocal plot (Burke & Tritton, 1984, 1985).

Iodide Quenching Studies. All quenching studies were conducted in PBS buffer at a constant halide concentration of 1.0 M unless noted otherwise. These solutions contained 2×10^{-3} M sodium thiosulfate to prevent the oxidation of iodide. Samples of anthracycline derivatives free in solution at a concentration of 5.0×10^{-7} M were quenched over iodide concentrations ranging from 0 to 0.5 M. The shape and position of the emission spectrum of daunomycin were found to be essentially unaltered by the addition of iodide.

For the quenching studies of membrane-bound fluorophores, small unilamellar vesicle suspensions at a lipid concentration of 34 mg/mL were prepared in two PBS buffers containing either 1.1 M chloride or 1.1 M iodide. Identical amounts of aqueous drug stock solutions were added to aliquots of both of these liposome preparations such that the drug, lipid, and salt concentrations were 5.0×10^{-6} M, 30 mg/mL, and 1.0 M, respectively. A lifetime measurement was initially made on the bound fluorophore sample containing 1.0 M chloride. The excitation and emission polarizers were then installed, and steady-state anisotropy measurements were obtained on the sample. With the excitation polarizer horizontal, the individual horizontal (I_{HH}) and vertical (I_{HV}) polarized emission intensities were measured for one channel, the total fluorescence intensity being equal to $I_{HH} + 2I_{HV}$. Anisotropy and intensity measurements were then conducted in a similar manner for the 1.0 M iodide sample. A given amount of the chloride solution was then removed and replaced with an identical amount of the iodide stock solution. After each addition of iodide, the solution was gently agitated by inversion of the sealed cuvette, and the fluorescence anisotropy and intensity were measured after a 15-min equilibration.

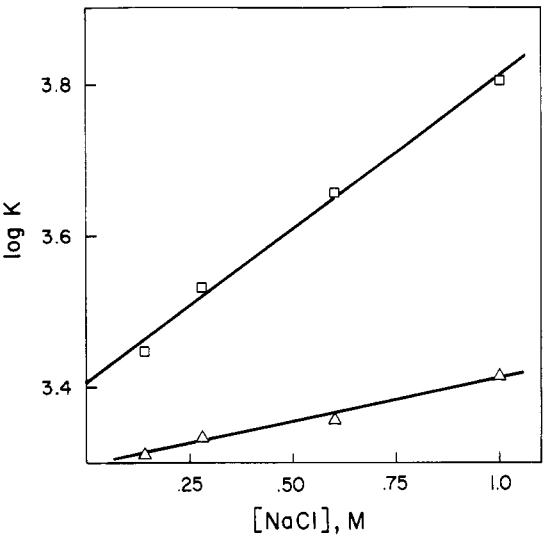

FIGURE 1: Ionic strength dependence of the overall binding affinity of daunomycin for fluid-phase DMPC (△) and solid-phase DPPC (□) bilayers. Liposomes were prepared in PBS buffer, pH 7.4, containing the specified concentration of NaCl. K values were determined from fluorescence anisotropy titration of a fixed drug concentration (1×10^{-6} M) with solutions of increasing lipid concentration at 27.5 °C as described under Materials and Methods.

Results

Dependence of Daunomycin Binding on Ionic Strength. Previously, we used fluorescence anisotropy titration to examine the effect of drug structure on the equilibrium binding affinities of anthracyclines for fluid-phase DMPC and solid-phase DPPC unilamellar phosphatidylcholine vesicles (Burke & Tritton, 1985). This technique was employed here to study the effect of ionic strength on daunomycin binding to fluid-phase and solid-phase bilayers, with the results shown in Figure 1. Daunomycin binding increased with increasing ionic strength for both types of bilayers, with the increase in binding being more pronounced for the solid-phase bilayers.

Static and Dynamic Iodide Quenching of Anthracyclines in Solution and Bound to Membranes. Iodide quenching studies were conducted here for a series of anthracycline derivatives bound to fluid-phase and solid-phase bilayers in order to determine how the relative membrane location varied with drug structure. Iodide, which has an immeasurably small permeation into the bilayer (Cranney et al., 1983; Jendrasiak, 1972), is thus able to discriminate between molecules bound to the hydrophilic surface from those in the hydrophobic interior of a membrane. The classical relationship used to describe the collisional quenching process is the Stern–Volmer equation

$$F_0/F = \tau_0/\tau = 1 + K_{SV}[Q] \quad (1)$$

where F_0, F and τ_0, τ are the fluorescence intensities and lifetimes in the absence and presence of Q, respectively. [Q] represents the quencher concentration, and K_{SV} is the collisional or dynamic quenching constant which equals $k_q\tau_0$ where

Table I: Summary of Fluorescence Lifetimes, Iodide Quenching Rate Constants, Steady-State Anisotropies, Limiting Anisotropies, and Rotational Rates for Free and Membrane-Bound Fluorophores[a]

	free in PBS solution					bound to fluid-phase DMPC bilayers				
compound	τ (ns)	V (M^{-1})	k_q ($\times 10^{-9}$ M^{-1} s^{-1})	a	R ($\times 10^{-8}$ s^{-1})	τ (ns)	k_q ($\times 10^{-9}$ M^{-1} s^{-1})	a	R ($\times 10^{-8}$ s^{-1})	a_∞
anthracyclines										
carminomycin	1.6	1.9	8.0	0.039	9.2	2.7	0.6	0.262	0.8	0.25
4-demethoxydaunomycin	1.7	1.9	8.1	0.039	8.8	2.5	0.7	0.268	0.7	0.25
daunomycin	1.1	1.9	9.4	0.060	8.3	1.8	1.0	0.301	0.5	0.28
rubidazone	1.2	1.3	8.8	0.079	5.5	1.7	1.0	0.318	2.7	0.31
adriamycin	1.1	1.9	9.5	0.065	7.6	1.6	1.2	0.295	1.3	0.29
daunomycinol	1.2	1.7	9.2	0.061	7.5	1.7	1.1	0.299	1.7	0.29
N,N-dimethyldaunomycin	1.2	1.7	9.1	0.061	7.5	1.6	1.3	0.296	0.8	0.28
aglycons										
carminomycinone	1.7	1.2	8.2	0.025	14.3	2.8	0.6	0.217	0.8	0.19
4-demethoxydaunomycinone[b]	1.8	1.2	8.0	0.025	13.7					
daunomycinone	1.2	1.8	8.4	0.042	11.7	1.9	0.7	0.270	0.6	0.25
adriamycinone	1.2	1.4	9.6	0.045	10.6	1.8	0.8	0.267	1.3	0.26
daunomycinol aglycon	1.2	1.5	8.8	0.043	11.2	1.8	0.8	0.268	1.4	0.26
7-deoxydaunomycinone	1.5	2.3	8.2	0.033	12.0	2.3	0.5	0.256	0.6	0.24
1,4-dihydroxyanthraquinone	1.5	1.2	9.2	0.008	49.0	2.8	0.6	0.123	0.9	0.10

[a] All experiments were conducted at 27.5 °C in PBS buffer at constant ionic strength (1.0 M halide), and small unilamellar liposomes were used. Lifetimes τ, in nanoseconds were determined at a chloride concentration of 1.0 M. Lifetime measurements have an uncertainty of ±0.1 ns. Experiments on fluorophores free in solution were conducted at concentrations of 1×10^{-6} M while studies of membrane-bound fluorophores were conducted at fluorophore concentrations of 5.0×10^{-6} M and lipid concentrations of 30 mg/mL, concentrations which assured essentially complete binding of all fluorophores. Quenching constants for compounds free in solution were obtained from eq 2 by using intensity data, where V is the rate constant for the static quenching process and k_q is the bimolecular rate constant for the collisional process. Eftink Ghiron (1976) estimated that this method of analysis results in limits of error for the assigned value of V of ±25% which leads to a variability in the resulting K_{SV} of ±10%. The k_q values for membrane-bound fluorophores were obtained from the slopes of modified Stern–Volmer plots with V assumed to be negligible. Steady-state anisotropy (a) values for fluorophores in 1.0 M chloride buffer have been corrected for scatter (<2% scatter in all cases). Rotational rates (R) for fluorophores free in solution were obtained from the Perrin equation by using an a_0 value of 0.39 for all fluorophores except 1,4-dihydroxyanthraquinone (r = 0.36). Rotational rates (R) and limiting anisotropies (a_∞) for membrane-bound compounds were obtained from lifetime-resolved anisotropy plots. The a values are subject to 3% uncertainty. [b] Membrane-bound 4-demethoxydaunomycinone was not studied due to problems with fluorophore aggregation during the preparation of 4.3×10^{-5} M aqueous stock solutions used in sample preparation.

k_q represents the experimentally observed rate constant for the collisional quenching process.

A more rigorous treatment of the kinetics of the quenching process includes consideration of a process referred to as "static" quenching. Static quenching results when a fluorophore is immediately adjacent to the quenching molecule at the instant of excitation. The following modified form of the Stern–Volmer relationship has been found to adequately account for the combination of static and dynamic quenching processes (Eftink & Ghiron, 1976):

$$F_0/Fe^{V[Q]} = 1 + K_{SV}[Q] \qquad (2)$$

where V is the static quenching constant. Quenching data were fit to eq 2 by determining the value of V which yielded the line with the best correlation coefficient for the plot of $F_0/Fe^{V[Q]}$ vs. [Q].

The Stern–Volmer plot for the iodide quenching of 4-dimethoxydaunomycin in PBS (Figure 2) shows a clear upward curvature for the F_0/F data whereas the τ_0/τ data are linear. Since the τ_0/τ plot reflects only the collisional quenching, we attribute the nonlinearity of the F_0/F plot to a static quenching component. By treatment of the data according to eq 2, the collisional and static quenching constants were dissected. The analysis yielded $V = 3$ M^{-1} and $K_{SV} = 14$ M^{-1}. The τ_0/τ data for 4-demethoxydaunomycin yielded $K_{SV} = 15$ M^{-1}. The agreement between the two types of plots demonstrated that K_{SV} and V were adequately determined by using eq 2. Similar static and dynamic quenching components were observed for the other anthracycline derivatives free in PBS solution, and the V and k_q values are summarized in Table I. We conclude here that no significant difference in the ability of iodide to quench fluorescence existed at a halide concentration of 1.0 M for the various anthracycline derivatives studied.

Figure 3 shows modified Stern–Volmer plots for several fluid liposome-bound anthracycline congeners which vary by alterations in the aglycon portion of the drug molecule (panel A) as well as daunomycin derivatives which differ by substitution changes at the amino sugar (panel B). The experimental conditions assured essentially complete binding for each of the anthracycline derivatives studied. The linearity of the plots is consistent with essentially one class of bound fluorophore whose predominant mode of quenching by iodide, since no upward curvature of the plots is apparent, was collisional quenching. Panel A demonstrtes how a structural change in the aglycon portion of daunomycin altered drug

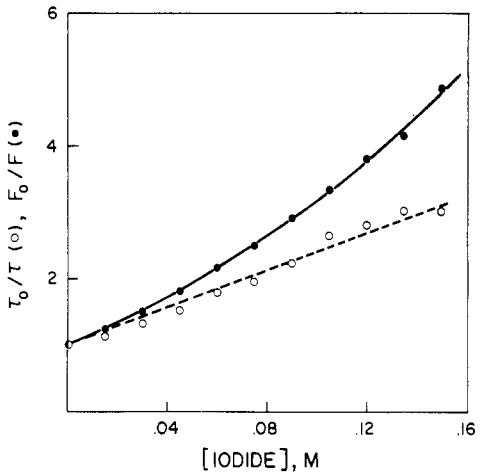

FIGURE 2: Iodide quenching of 2×10^{-6} M 4-demethoxydaunomycin in PBS at a constant halide concentration of 0.3 M (27.5 °C). The solid line reflects the static and dynamic quenching components as determined by the F_0/F values (●). The dashed line represents only the dynamic component as determined by using eq 2 with $V = 3.0$ M^{-1} and $K_{SV} = 14$ M^{-1}. (○) τ_0/τ values which also represent only dynamic quenching. The best linear least-squares fit of the τ_0/τ values yields $K_{SV} = 15$ M^{-1}. These results therefore demonstrate that the static and dynamic quenching components were accurately separated by using eq 2.

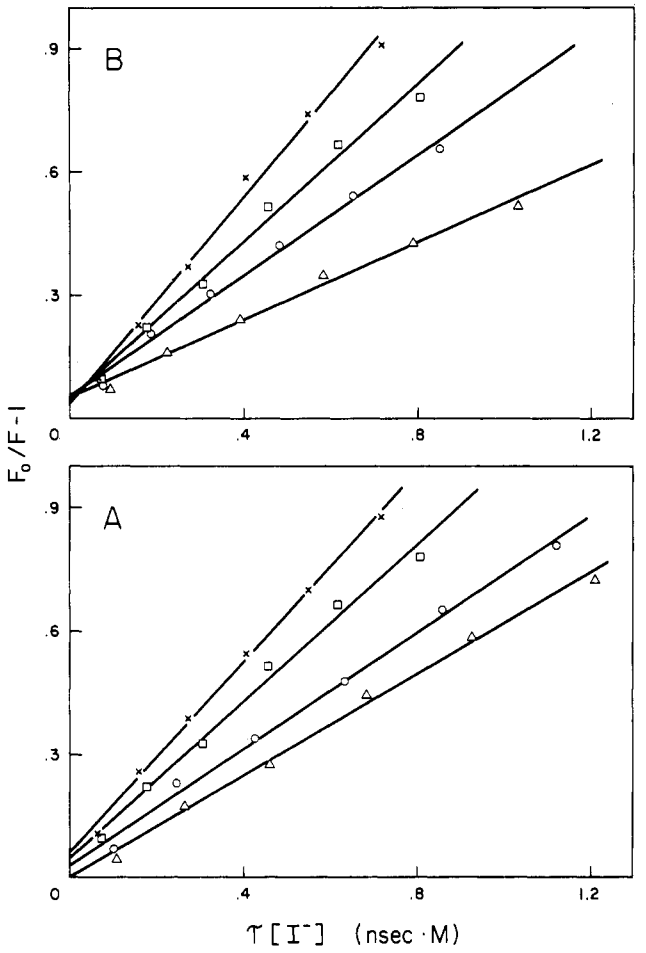

FIGURE 3: Modified Stern–Volmer plots for the iodide quenching, at constant ionic strength, of anthracyclines and aglycons bound to fluid-phase DMPC vesicles at 27.5 °C. Panel A shows how iodide accessibility varies with structural changes in the aglycon portion of an anthracycline molecule, with data shown for adriamycin (×), daunomycin (□), 4-demethoxydaunomycin (○), and carminomycin (△). Panel B shows how iodide accessibility varies with substitution changes for the amino sugar, with data shown for *N,N*-dimethyldaunomycin (×), daunomycin (□), daunomycinone (○), and 7-deoxydaunomycinone (△). The suspensions contained 4.42×10^{-2} M DMPC and 5.0×10^{-6} M drug at a halide concentration of 1.0 M which assured essentially complete binding for each derivative (see Results). F_0 equals the initial fluorescence intensity, F equals the fluorescence intensity in the presence of a given iodide concentration ($[I^-]$), and τ equals the initial excited-state lifetime. The slope of such a plot is equal to k_q, the collisional rate constant, and these values are summarized in Table I.

location in the bilayer, with the more hydrophobic derivatives being the least accessible. Panel B shows that penetration of the daunomycin chromophore decreased with substitution changes at C-7 in the following order: proton > hydroxyl > daunosamine > rhodosamine. Lifetime measurements on the membrane-bound carminomycin samples at the minimum and maximum iodide concentrations reflected a similar change as the intensity measurements (30% decrease in τ vs. 40% decrease in F), confirming the predominance of a dynamic mechanism for bound drug. The slopes of the modified Stern–Volmer plots are therefore treated as a measure of k_q, the bimolecular rate constant, and these values are summarized in Table I.

Table I demonstrates that all of the anthracycline derivatives studied showed an increase in excited-state lifetime and a decrease in their extent of collisional quenching by iodide upon binding to fluid-phase bilayers. A general correlation was observed between the previously measured binding affinities

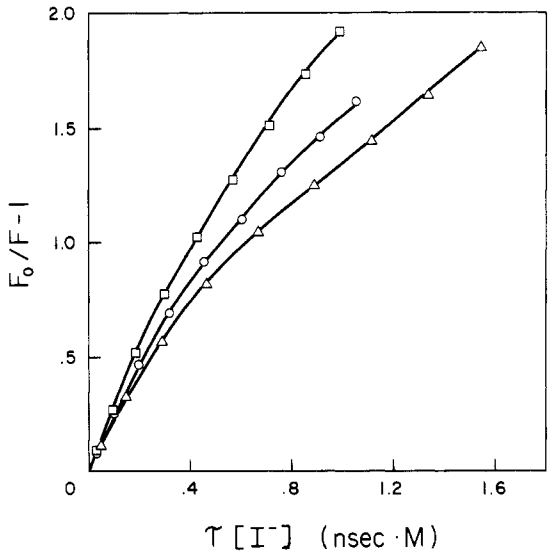

FIGURE 4: Modified Stern–Volmer plot for the iodide quenching, at constant ionic strength, of adriamycin (□), daunomycin (○), and carminomycin (△) bound to solid-phase DPPC vesicles at 27.5 °C. The F_0, F, τ, and $[I^-]$ terms and the experimental conditions are described in Figure 3. The nonlinearity of the plots for the anthracyclines is evidence that, in each case, more than one class of bound fluorophore was present.

of the daunosamine-containing anthracyclines and their penetration into the bilayer. The most hydrophobic drugs were apparently buried deeper in the membrane. It also appeared that aglycons, which have been previously shown to exhibit higher bilayer affinities than their corresponding parent, generally penetrated the membrane to a greater extent relative to the parent molecule.

Modified Stern–Volmer plots of adriamycin, daunomycin, and carminomycin bound to solid-phase DPPC bilayers are shown in Figure 4. Unlike the linear modified Stern–Volmer plots for drugs bound to fluid-phase bilayers, the plots in Figure 4 are curved for each of the drugs studied, indicative of a heterogeneous population of bound drug. The initial slopes of these plots are steeper than the corresponding plots for fluid-phase bilayers, indicating that at least a fraction of drug bound to solid-phase bilayers was more accessible than when bound to fluid-phase bilayers. Similar to findings for fluid-phase bilayers, a correlation was observed berween drug hydrophobicity and membrane penetration for the three anthracyclines bound to solid-phase bilayers. Because fluid-phase bilayers are considered to be more relevent models of biomembranes than solid-phase bilayers (Jain, 1980), and because the bound drug populations of fluid-phase bilayers were apparently less complicated as demonstrated by comparison of Figures 3 and 4, we decided to study anthracycline associations with the fluid-phase bilayers in greater detail.

Depolarizing Rotations of Anthracyclines in an Isotropic Solvent and in Fluid-Phase Bilayers. A steady-state fluoresence anisotropy measurement (a) is related to the rotational rate of the molecule by the Perrin equation:

$$a_0/a = 1 + 6R\tau \qquad (3)$$

where a_0 is the limiting fluorescence anisotropy observed in the absence of depolarizing rotations, τ is the excited-state lifetime, and R is the rotational diffusion rate of the fluorophore in radians per second. This equation is applicable in dilute nonviscous solution where the anisotropy is primarily determined by the rotational motions of the fluorophore and where the depolaizing rotational motions are unhindered and isotropic in nature (Lakowicz, 1983).

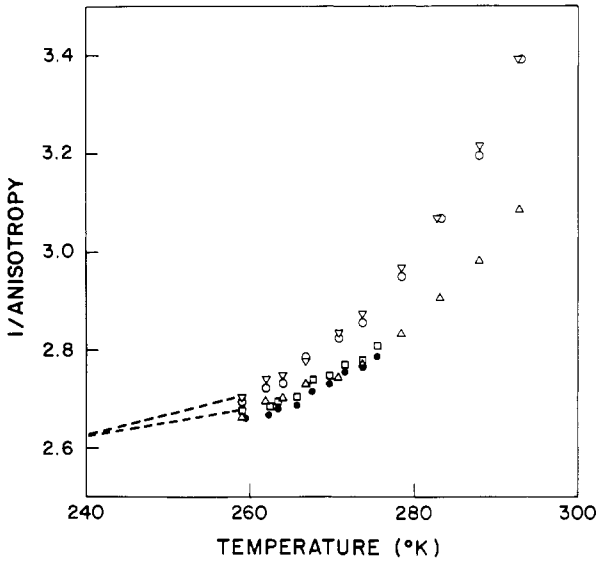

FIGURE 5: Plot of 1/anisotropy vs. temperature for 4-demethoxydaunomycin (▽), carminomycin (○), daunomycin (□), daunomycinol (△), and adriamycin (●) in a viscous aqueous solution containing 72.4% sucrose. Note that the 1/anisotropy values for these anthracyclines converge at low temperature and extrapolation to 240 K yields a limiting anisotropy (a_0) value of 0.39.

The Perrin equation can be modified to allow for the possibility that the depolarizing rotations are isotropic, but hindered, such that at times which are long compared with the fluorescence lifetime a nonzero limiting anisotropy (a_∞) is possible (Lakowicz et al., 1979a):

$$a = a_\infty + (a_0 - a)/6R\tau \quad (4)$$

For an isotropic but hindered rotator, a plot of a vs. $(a_0 - a)/\tau$ permits $1/6R$ and a_∞ to be obtained from the slope and a-axis intercept, respectively.

Lifetime-resolved anisotropy measurements using oxygen quenching have been used in the past to study the hindered depolarizing rotations of DPH (Lakowicz et al., 1976b) and perylene (Lakowicz & Knutson, 1980) in lipid bilayers. In a similar manner, we employed iodide quenching of anthracycline fluorescence here to provide a means of varying the excited-state lifetime (without perturbation of the process of interest) in order to study the anthracycline diffusive processes. Our interest was to further understand the dependence of anthracycline membrane dynamics on drug structure.

We measured a_0 values for the anthracycline fluorophores by two different approaches. Figure 5 shows the temperature dependence of the steady-state fluorescence anisotropies of several anthracyclines when in a viscous solution containing 72.4% sucrose. The isotherms for the various anthracyclines converge at low temperature or increasing solution viscosity. Capps & Vala (1981) have shown that the polarization excitation spectrum of daunomycin was constant across its absorption band (from 430 to 530 nm). We have observed for the other anthracyclines (in 72.4% sucrose solution at 1 °C) that the steady-state anisotropies were constant across their respective absorption bands; thus, the anisotropy measurements were not sensitive to the chosen excitation wavelength. Due to technical constraints, we were unable to achieve temperatures lower than −14 °C. Extrapolation of the data in Figure 5 to −40 °C yielded a_0 values of approximately 0.39 for each of the anthracyclines. As an alternative method for the a_0 determination, 3×10^{-6} M solutions of several anthracyclines and their aglycons were prepared in glycerol, and their steady-state anisotropies were measured at 1 °C; values of approximately 0.39 were obtained for each of the fluorophores with the exception of 1,4-dihydroxyanthraquinone, for which a value of 0.36 was obtained. We therefore chose to use an a_0 value of 0.39 for all of the anthracycline derivatives studied, while an a_0 value of 0.36 was used for 1,4-dihydroxyanthraquinone. Since these values closely approach the theoretical maximum of 0.4, the emission and excitation transition moments of the anthracyclines must be closely aligned.

We used the collisional quenching by iodide at constant ionic strength to lifetime-resolve the steady-state anisotropies of the anthracyclines in aqueous solution and in fluid-phase DMPC bilayers. For these measurements, the lifetimes of the samples at each iodide concentration were required. The lifetimes were calculated for a given iodide concentration from the Stern–Volmer equation by using the collisional quench constants listed in Table I. The lifetime-resolved anisotropies of 4-demethoxydaunomycin and its aglycon in PBS buffer are shown in Figure 6. A linear least-squares fit of the data yielded an a_∞ value of 0 for both fluorophores, indicating that the parent anthracycline and its aglycon were free rotators in aqueous solution. Table I summarizes the Perrin rotational rates for the anthracycline fluorophores in PBS buffer. It should be noted that each aglycon had a faster rotational rate than its corresponding parent, whose rotations in solution were apparently slowed by the presence of the amino sugar substituent.

Lifetime-resolved anisotropy measurements demonstrated significantly different rotational behavior for the anthracycline congeners when bound to fluid-phase DMPC bilayers than when free in solution (Figure 6). Unlike the results obtained for drug free in solution, the depolarizing rotations of membrane-bound anthracyclines and their aglycons appeared to be hindered (i.e., the anisotropy values did not decay to zero at infinitely long lifetimes). The limiting anisotropy (a_∞) values were determined by extrapolation to the a axis, and these values are summarized in Table I, along with the rotational rates obtained from the slopes of these plots.

The membrane-bound a_∞ values ranged from 0.25 to 0.31 for the anthracyclines and from 0.19 to 0.26 for the aglycons. 1,4-Dihydroxyanthraquinone, a relatively small, symmetrical molecule, was shown to have the lowest membrane-bound a_∞ value of the fluorophores studied, with a_∞ equal to 0.10. Since each anthracycline had a higher limiting anisotropy value than its corresponding aglycon, it appears that the presence of the amino sugar substituent resulted in more hindered rotations of the fluorophore in fluid-phase bilayers. Comparison of the a_∞ and a values for membrane-bound fluorophores listed in Table I clearly indicates that steady-state anisotropies were primarily governed by a_∞ and not the mean rotational rate. The rotational rates of the anthracyclines were apparently reduced on the average by a factor of 10 upon binding to fluid-phase bilayers. R values for membrane-bound anthracyclines ranged from 0.5×10^8 to 2.7×10^8 s^{-1}.

Thermal Dependence of the Steady-State Anisotropies of DPPC-Bound Anthracyclines. According to the hindered rotator model (eq 4), changes in the steady-state fluorescence anistropy of a membrane-bound fluorophore with temperature may be due to changes in the a_∞, τ, or R values. The anistropy values of the DPPC-bound fluorophores shown in Figure 7, where lipid concentrations were carefully chosen to assure essentially complete binding, all reported on the gel- to liquid-crystalline-phase transition which these small unilamellar vesicles are known to undergo around 37 °C (Suurkuusk et al., 1976). Of the fluorophores studied, DPH demonstrated the greatest relative change in steady-state anisotropy during the phase transition. Differential polarized phase measure-

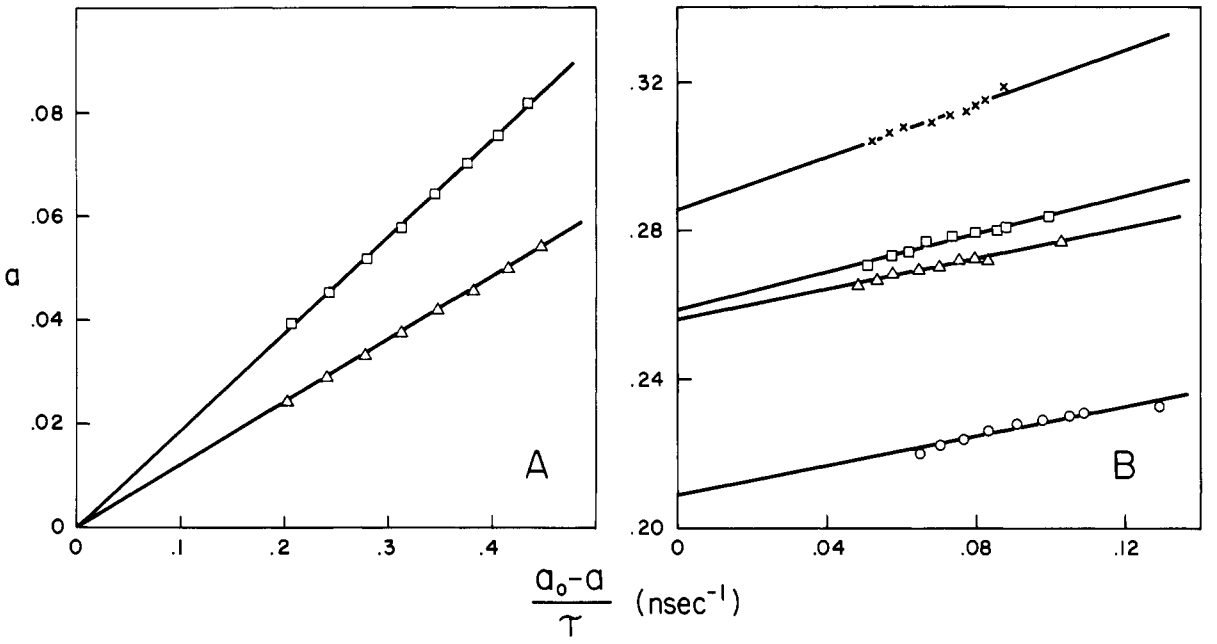

FIGURE 6: Lifetime-resolved anisotropy plots obtained by iodide quenching at constant ionic strength. The terms a and a_0 refer to the measured and limiting fluorescence anisotropies, respectively, and τ represents the excited-state lifetime at a given iodide concentration. Panel A shows the data for free 4-demethoxydaunomycin (□) and 4-demethoxydaunomycinone (△) at an ionic strength of 0.3 M. A fluorophore concentration of 2.0×10^{-6} M was used, and background scatter due to solvent did not exceed 1% of the total signal. A K_{SV} value of 15 M^{-1} was used to predict τ values from the iodide concentration according to the Stern–Volmer equation. The linearlity of these plots in combination with y intercepts of zero indicates that both fluorophores had essentially unhindered and isotropic rotations when free in solution. Panel B shows data for daunomycin (×), 4-demethoxydaunomycin (□), carminomycin (△), and carminomycinone (○) bound to fluid-phase DMPC bilayers under conditions described in Figure 3. Data for iodide concentrations ranging from 0.04 to 0.45 M are shown. The nonzero y intercepts indicate that the diffusive motions of these membrane-bound fluorophores were hindered.

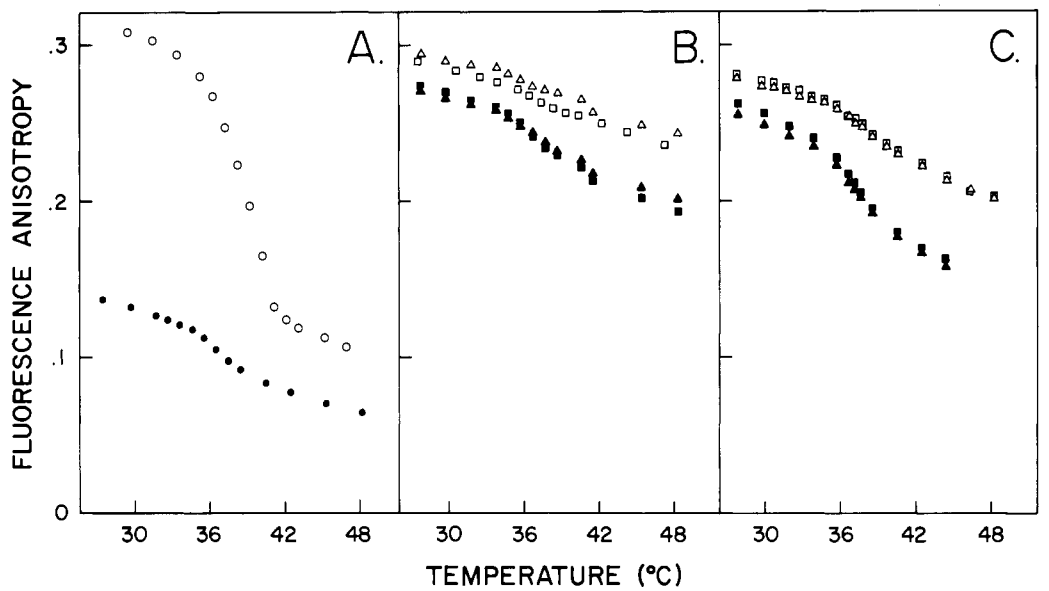

FIGURE 7: Steady-state anisotropy values of fluorophores bound to unilamellar DPPC vesicles in PBS buffer (0.14 M chloride) as a function of temperature. Panel A shows data for DPH (○) and 1,4-dihydroxyanthraquinone (●) (6.8×10^{-3} M DPPC and 1.0×10^{-6} M fluorophore); panel B shows data for adriamycin (□), daunomycin (△), adriamycinone (■), and daunomycinone (▲) (4.1×10^{-2} M DPPC and 5.0×10^{-6} M fluorophore); panel C shows data for carminomycin (□), 4-demethoxydaunomycin (△), carminomycinone (■), and 4-demethoxydaunomycinone (▲) (6.8×10^{-3} M DPPC and 1.0×10^{-6} M fluorophore). Vesicle and drug concentrations were chosen to assure >95% bound species for adriamycin, the congener with the lowest affinity, and a maximum background light scattering from unlabeled lipid and solvents of <2%. The anisotropy values are uncorrected for light scatter.

ments on DPPC-bound DPH (Lakowicz et al., 1979a) showed that the a_∞ values at 30 and 50 °C were approximately 0.32 and 0.04, respectively. It was concluded that changes observed in the steady-state anisotropy near the T_M derived principally from changes in the degree to which the probe's rotations were hindered and only to a small extent from changes in the rotational rate. 1,4-Dihydroxyanthraquinone, a small molecule and relatively symmetric in comparison to the anthracycline derivatives, also showed a relatively large change in steady-state anisotropy across the T_M, and its a_∞ value in fluid-phase bilayers was found here to be significantly less than those of the anthracycline fluorophores studied (Table I). Although the a vs. temperature plots of the anthracyclines and their aglycons are also sigmoid, their a values above the phase transition temperature are still high relative to DPH and 1,4-dihydroxyanthraquinone. The a_∞ values of the anthra-

cycline dervatives bound to fluid-phase DMPC bilayers (27.5 °C) were relatively high, in contrast to the low a_∞ value of approximately 0.06 for DPH bound under similar conditions as determined by differential polarized phase (Lakowicz et al., 1979a) and lifetime-resolved anisotropy (Lakowicz et al., 1979b) techniques. The τ values of several DPPC-bound anthracycline derivatives were found to be relatively invariant across the T_M of the vesicle (data not shown). Thus, it appears that the DPPC-bound anthracyclines did not show relatively large changes in their steady-state anisotropy values through the T_M presumably because the depolarizing rotations of these unsymmetrical molecules were still very hindered in the fluid-phase bilayers. The steady-state anisotropy value of a parent anthracycline was found to be higher than that of its corresponding aglycon across the entire temperature range of Figure 7.

Discussion

We have recently reported a study concerning the structural dependence of anthracycline binding to fluid-phase DMPC and solid-phase DPPC vesicles which indicated that hydrophobic interactions between the aglycon portion of an anthracycline molecule and the hydrocarbon interior of the membrane dominated drug binding to both types of bilayers (Burke & Tritton, 1985). Thermodynamic studies by Record et al. (1978) demonstrated that association reactions which are driven by the release of structured water (hydrophobic associations) are expected to show an increase in the observed association constant with increasing electrolyte activity. Accordingly, we have shown here that the overall binding affinity of daunomycin for both fluid-phase and solid-phase bilayers increased with increasing ionic strength. In a similar manner, the self-association of daunomycin in aqueous solution was enhanced with increasing ionic strength (Chaires et al., 1982a; Menozzi et al., 1984), and stacking of the aglycon portion of the molecule has been shown to occur in these aggregates (Chaires et al., 1982a). It thus appears that increased ionic strength of the solvent drove monomeric daunomycin to more hydrophobic environments, whether in lipid bilayers or molecular aggregates.

Our interest in using iodide as a quencher of anthracycline fluorescence was to establish how relative membrane location varied with drug structure. Because such structural changes could affect the intrinsic ability of iodide to quench the fluorescence of these drugs, it was necessary to characterize the iodide quenching of all the fluorophores in aqueous solution before studying their quenching in membranes. Iodide is an efficient quencher of anthracycline fluorescence, and by comparison of changes in fluorescence intensity with changes in fluorescence lifetime as a function of iodide concentration, it has been shown here to operate by both a collisional and a static mechanism. The static and dynamic quench constants of the anthracycline derivatives free in aqueous solution (Table I) indicated that, within the limits of error, a parent anthracycline and its corresponding aglycon were quenched to approximately the same degree. This result indicates that steric interactions due to the presence of the amino sugar did not significantly alter the ability of iodide to quench the fluorophore when free in solution of high ionic strength. Since the aglycons are electroneutral, it appears that the interaction between a quencher ion and an anthracycline moleucle was not due to simple electrostatic attraction. Moreover, the results show that the different anthracyclines exhibited similar degrees of quenching when free in solution. This last point is of significance because differences in the extent of quenching which exist between congeners when bound to membranes are therefore assumed to be due to differences in accessibility to the quencher ion located at the lipid–solvent interface.

The linearity of both the modified Stern–Volmer plots (Figure 3) and the lifetime-resolved anisotropy plots (Figure 6) for anthracycline derivatives bound to fluid-phase DMPC bilayers is consistent with the presence of one class of bound fluorophore. In contrast, the modified Stern–Volmer plots obtained for adriamycin, daunomycin, and carminomycin bound to solid-phase bilayers are curvilinear, indicative of a heterogeneous population of bound drug. Karczmar & Tritton (1979) have previously observed linear and curvilinear Stern–Volmer plots for adriamycin bound to fluid-phase and solid-phase DMPC bilayers, respectively. For each anthracycline studied, when bound to solid-phase bilayers, the initial slope of the modified Stern–Volmer plot is steeper than that observed when the drug was bound to fluid-phase bilayers, suggesting that at least a fraction of the bound drug population was more accessible in solid-phase than fluid-phase bilayers. A more interfacial membrane location of daunomycin bound to solid-phase bilayers relative to fluid-phase bilayers may, in part, explain the observation here that the increase in daunomycin binding with increasing ionic strength was more dramatic for the solid-phase bilayers. Since increasing ionic strength drove the free drug $\rightleftharpoons$ bound drug equilibrium to the right (apparently because hydrophobic associations were dominant), it seems logical to expect the binding of a more interfacial bound drug population to be more sensitive to electrolyte activity.

The V and k_q values for the iodide quenching of an anthracycline derivative free in PBS solution were approximately 1.6 M^{-1} and 9×10^9 M^{-1} s^{-1} respectively. Whereas the iodide quenching reaction of anthracycline derivatives free in solution occurred by both a collisional and a static mechanism, it appears that the quenching of these fluorophores bound to fluid-phase bilayers occurred predominantly by the collisional mechanism. Bound, daunosamine-containing anthracyclines showed k_q values ranging from 0.6×10^9 to 1.2×10^9 M^{-1} s^{-1} while their aglycons exhibited values of 0.5×10^9 to 0.8×10^9 M^{-1} s^{-1}. Since these k_q values are much higher than the negligible values observed for DPH and anthracene embedded in lipid bilayers (Cranney et al., 1983; Karczmar & Tritton, 1979), a more interfacial location is indicated relative to that of the hydrocarbon probes. In general, the aglycons were less accessible than their parent drug molecule, indicating that the presence of the amino sugar hindered penetration of the chromophore. The observation that *N*,*N*-dimethyldaunomycin was more accessible than daunomycin indicates that replacement of daunosamine with rhodosamine, considered to be a more ionic amino sugar (Siegfried et al., 1985), reduced membrane penetration of the chromophore.

For the binding of a series of daunosamine-containing anthracycline congeners to fluid-phase and solid-phase bilayers, a general correlation between drug hydrophobicity and binding affinity was previously observed (Burke& Tritton, 1985). These two properties have been shown in this report to further correlate with the penetration of the drug molecule into the bilayer. Structural changes in the aglycon portion of an anthracycline (in the aromatic chromophore or acyclic ring A) which increased drug polarity resulted in reduced membrane penetration and binding. The correlation of membrane affinity and membrane penetration with drug hydrophobicity, as well as the observation of the enhancement of membrane binding with increasing ionic strength, strongly indicates that anthracycline associations with neutral phospholipid bilayers (both fluid phase and solid phase) were driven by (1) favorable

hydrophobic interactions between the aglycon portion of the anthracycline molecule and the hydrocarbon interior of the membrane and (2) unfavorable interactions between the hydrophobic aglycon and the polar solvent molecules. The understanding of the physicochemical forces which control anthracycline interactions with neutral membranes which we have attained provides a basis for a systematic investigation of anthracycline interactions with negatively charged membranes which have been implicated in the unfavorable toxic properties of these drugs on cardiac tissues (Tritton et al., 1978; Karczmar & Tritton, 1979; Goormaghtigh et al., 1980).

We have used the collisional quenching of iodide at constant ionic strength to lifetime-resolve the steady-state anisotropies of the anthracyclines and their aglycons when free in solution and bound to fluid-phase DMPC bilayers. Unlike the essentially unhindered rotations of an anthracycline free in solution, a membrane-bound drug exhibited rotations that were hindered which led to nonzero limiting anisotropies (a_∞). Moreover, it appears that the steady-state anisotropies of membrane-bound anthracyclines were governed primarily by a_∞ and not by the mean rotational rate (R). Hydrocarbon probes such as perylene and DPH bound to fluid-phase DMPC bilayers have also been shown by other workers to exhibit steady-state anisotropies which were governed principally by a_∞ and not by R (Lakowicz et al., 1979a,b; Lakowicz & Knutson, 1980). However, the a_∞ values of DPH and perylene (approximately 0.08 and 0.03, respectively, at 28 °C) are significantly less than the a_∞ values observed here for the anthracycline derivatives. These differences in a_∞ between the hydrocarbon probes and the anthracycline derivatives must result from fundamental differences in probe shape and membrane location. As previously discussed, it appears that the anthracycline derivatives had a more interfacial membrane location than the hydrocarbon probes. The lack of correlation of R and a_∞ values with relative drug location (as measured by iodide quenching) for the membrane-bound anthracyclines can be taken as evidence that subtle changes in probe shape affect the determination of a_∞ and R values whereas collisional quenching is dominated by accessibility in the bilayer. A less subtle change in probe shape, the removal of the amino sugar of an anthracycline, was shown here to result in the reduction of the membrane-bound a_∞ value. This is expected both because the aglycon is more hydrocarbon-like in character and because of the deeper membrane penetration of the aglycon compared to the parent molecule.

Comparison of the interaction specificities of anthracyclines with membranes and DNA provides a potential basis for improving the site selectivity of these antitumor agents. Studies on the interaction specificities of DNA with various anthracyclines have provided evidence for an intercalation mode of binding of the anthracycline ring (Calendi et al., 1965; Kersten et al., 1966; Berg & Eckhart, 1970; Chaires et al., 1982b). Equilibrium binding studies have shown that ionic and electrostatic interactions between DNA and an anthracycline were strongly dependent upon the presence of the amino sugar moiety (Gabbay et al., 1976), the results being consistent with specific hydrogen bonding and electrostatic interaction of the primary amino group of the sugar moiety with the phosphate group of DNA as was proposed earlier by Pigram et al. (1972). Recent kinetic studies by Chaires et al. (1985) have led to a proposed three-step mechanism for daunomycin–DNA binding. The first and dominant form of binding was postulated as the rapid "outside" binding of daunomycin to DNA, thought to be an ionic, nonintercalated complex. The second and third steps of the mechanism were proposed to be intercalation of the aglycon portion of the molecule followed by conformational rearrangement of either the drug or the DNA binding site, or redistribution of bound daunomycin to preferred sites. Thus, the equilibrium and kinetic aspects of daunomycin–DNA interactions indicate that the ionizable amino group of the sugar moiety plays a crucial role in daunomycin binding to DNA.

Anthracycline affinities for neutral phospholipid membranes appeared to be dominated by hydrophobic interactions between the aglycon portion of the drug molecule and the acyl chains of the membrane. The function of the anthracycline amine in membrane binding, cellular transport, and cytotoxicity has recently been studied by using substitution at the amino group to vary the drug pK_a (Burke et al., 1985a,b). It was shown that reducing the drug pK_a by substitution at the amino group increased affinities for lipid bilayers. Whereas the ionic and electrostatic nature of the anthracycline amino sugar was shown to be favorable for drug–DNA binding, anthracycline affinities for neutral membranes were apparently reduced by the ionization of the amino group. Furthermore, the cellular accumulation of the anthracyclines was found to be very dependent on the drug pK_a, with the anthracyclines with nonbasic amino groups demonstrating reduced cellular accumulation. The basic differences in the physicochemical forces which influence anthracycline binding to membrane and DNA, as well as the dependence of cellular transport on the anthracycline pK_a, suggest a basis by which the anthracyclines may be made more site specific through rational drug synthesis.

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Registry No. Carminomycin, 50935-04-1; 4-demethoxydaunomycin, 58957-92-9; daunomycin, 20830-81-3; rubidazone, 54083-22-6; adriamycin, 23214-92-8; daunomycinol, 28008-55-1; *N,N*-dimethyldaunomycin, 67508-87-6; carminomycinone, 52744-22-6; 4-demethoxydaunomycinone, 60660-75-5; daunomycinone, 21794-55-8; adriamycinone, 24385-10-2; daunomycinol aglycon, 28008-51-7; 7-deoxydaunomycinone, 32384-98-8; 1,4-dihydroanthraquinone, 81-64-1.

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Long-Range Lipid–Protein Interactions. Evidence from Time-Resolved Fluorescence Depolarization and Energy-Transfer Experiments with Bacteriorhodopsin–Dimyristoylphosphatidylcholine Vesicles†

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ABSTRACT: The effect of monomeric bacteriorhodopsin on the lipid order and dynamics in dimyristoylphosphatidylcholine (DMPC) vesicles was monitored as a function of the protein to lipid ratio by time-dependent fluorescence anisotropy measurements with diphenylhexatriene (DPH). Energy transfer from the donor DPH to the acceptor retinal of bacteriorhodopsin was used as a spectroscopic ruler to estimate the range of the protein-induced perturbation of the lipid phase. The Förster distance for this donor–acceptor pair is approximately 45 Å. Since the effective radius of bacteriorhodopsin is about 17 Å, the labels within a neighborhood of radius R_0 around bacteriorhodopsin are strongly quenched and make a negligible contribution to the end value of the fluorescence anisotropy, from which the order parameter is calculated. Instead, the order parameter is mainly determined by the labels which are more than the Förster distance away from the retinal and which are consequently in the bulk lipid phase. The observed linear increase in order parameter from 0.29 for pure DMPC to 0.62 for a molar bacteriorhodopsin to DMPC ratio of 1/52 thus indicates that the order of the bulk lipids is increased by the interaction with bacteriorhodopsin and that the range of this perturbation is larger than 45 Å. In the absence of the acceptor retinal, no energy transfer occurs, and both bulk and boundary lipids are weighted equally in the determination of the order parameter. Only a very small change in the order parameter is observed upon removal of the acceptor, suggesting that bacteriorhodopsin affects the order of all the lipids in roughly the same way. The rotational diffusion constant of DPH determined from the initial slope of the anisotropy decay is independent of the surface concentration of bacteriorhodopsin and of the presence of the acceptor retinal. The viscosity calculated from the rotational diffusion constant is approximately 0.1 P at 35 °C and is an order of magnitude smaller than that determined previously from the rotational diffusion of bacteriorhodopsin. A comparison of the viscosities determined from the steady-state and time-resolved fluorescence anisotropy of DPH shows that the first method overestimates the viscosity by as much as a factor of 10.

When proteins are incorporated in lipid bilayers, the physical properties of the lipids are usually changed. The order and dynamics of the hydrocarbon chains will be affected, and the phase transition will be broadened and occasionally shifted. The considerable amount of work which has been done to date to characterize these aspects of lipid–protein interactions was recently reviewed (Jost & Griffith, 1983). One of the central questions remains unanswered, however. Is the perturbation

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