Structural Basis of Anthracycline Selectivity for Unilamellar Phosphatidylcholine Vesicles: An Equilibrium Binding Study†

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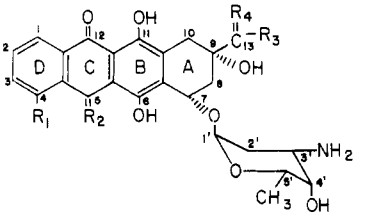
ABSTRACT: Fluorescence anisotropy titration was used to determine the equilibrium binding affinities of several anthracycline antitumor antibiotics for sonicated dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) vesicles at 27.5 °C. Eight daunomycin analogues, all differing from the parent by one structural change in the aglycon portion of the molecule, as well as four anthracycline congeners modified in the amino sugar were studied. Double-reciprocal plots were used to determine overall binding affinities (K). It was shown that structural changes in both the aglycon and amino sugar portions of the daunomycin molecule strongly modulated K values for DMPC and DPPC bilayers. For modifications in the aglycon portion of an anthracycline, a correlation between drug hydrophobicity and membrane affinity was observed. The number of binding sites per phospholipid molecule (n) and the apparent association constant (K_{app}), where $K = nK_{app}$, were determined at several temperatures for adriamycin, daunomycin, and carminomycin. The n values were found to be independent of temperature for fluid-phase DMPC or solid-phase DPPC bilayers. The K_{app} values (25 °C) ranged from (0.82–4.4) × 10^5 M^{-1} for DMPC vesicles to (4.4–7.3) × 10^5 M^{-1} for DPPC vesicles. Although the K_{app} values for the three drugs were similar for a particular bilayer, major differences were noted in the values of n and, therefore, in the overall vesicle affinities (nK_{app}). van't Hoff plots showed that anthracycline binding was exothermic; in all cases but one binding was accompanied by a decrease in entropy. Published data on cellular transport and in vitro cytotoxicities of the anthracyclines appear to correlate with the K values measured here. The dependence of K values as well as thermodynamic parameters on drug structure and bilayer type indicates that the cell surface membrane could serve as a prime target for improving the cytotoxic selectivity of the anthracyclines.

The anthracycline antitumor antibiotics are an important class of agents used in the treatment of human cancer. Numerous reviews concerning the chemistry and pharmacology of the anthracyclines have appeared (Arcamone, 1981; Young et al., 1981; Gianni et al., 1983). Adriamycin, the most widely used congener of this series, has been shown to influence many properties of cellular membranes and model membrane systems [see Tritton & Hickman (1985) for a review]. The importance of these membrane changes to the cytotoxic action of adriamycin compared to its ability to interfere with the DNA of the cell, considered the classical paradigm for the mechanism of action, has not been clearly established. The strongest evidence that the cell surface membrane is a primary target for adriamycin comes from experiments with polymer immobilized drug (Tritton & Yee, 1982; Wingard & Tritton, 1983; Wingard et al., 1983; Tokes et al., 1982; Rogers et al., 1983), where the nonpenetrating form of adriamycin was shown to be 2–3 orders of magnitude more potent than an equimolar concentration of free drug. Although it has been shown that the drug can exert its biological activity solely by a plasma membrane interaction, the more difficult problem of the molecular mechanism remains unsolved.

The anthracyclines are amphipathic molecules consisting of an aglycon (a red-pigmented, dihydroxyanthraquinone nucleus) linked through a glycosidic bond to an amino sugar (commonly daunosamine). Table I shows the molecular structures of several daunosamine-containing anthracyclines, all of which differ from daunomycin by one structural modification. The 6,11-dihydroxy-5,12-dioxoanthraquinone nucleus of daunomycin has been shown by X-ray analysis to be nearly planar with only the alicyclic ring A being out of plane (Neidle & Taylor, 1977). The anthracyclines can exist in either neutral or ionized forms. At physiological pH, the main ionization occurs at the amine group of the sugar and the daunosamine residue of adriamycin was shown to have a pK_a of 7.6–8.2, depending upon ionic strength (Dalmark & Storm, 1981). *N*,*N*-Dimethyladriamycin, 4′-deoxyadriamycin and 4′-epiadriamycin are analogues included in this study that differ from the parent molecule by the replacement of the daunosamine moiety with rhodosamine, 4-deoxydaunosamine,

Table I



compound	R_1	R_2	R_3	R_4
daunomycin	OCH_3	O	CH_3	O
4-demethoxydaunomycin	H	O	CH_3	O
carminomycin	OH	O	CH_3	O
5-iminodaunomycin	OCH_3	NH	CH_3	O
adriamycin	OCH_3	O	CH_2OH	O
5-iminoadriamycin	OCH_3	NH	CH_2OH	O
rubidazone	OCH_3	O	CH_3	$NNHCOC_6H_5$

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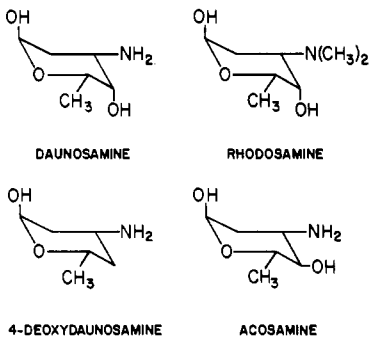

FIGURE 1: Structures of four amino sugars contained in anthracycline congeners.

and acosamine residues, respectively (Figure 1).

Because amphipathic molecules can be expected to interact with the phospholipid bilayer of biological membranes and because the surface membrane of the cell is the first barrier encountered by the anthracyclines, drug–membrane interactions should play an important role in determining their bioactivities, irrespective of whether the anthracyclines elicit their antitumor activity or cardiac toxicity at the DNA of the cell or the plasma membrane. The anthracyclines listed in Table I, in addition to adriamycinol and daunomycinol (which vary from their parents by the reduction of the C-13 carbonyl), all differ from daunomycin by one structural change in the aglycon portion of the molecule. These nine drugs provide a means of systematically studying how such structural changes affect membrane binding. Seven of the corresponding aglycons, where the daunosamine moiety has been replaced by a hydroxyl group, are also included in this study to assist in evaluating the effect of the amino sugar in binding. Four anthracyclines with amino sugar residues other than daunosamine were also studied. We report here a study characterizing the dependence of binding affinities and thermodynamic parameters on anthracycline structure for two types of phosphatidylcholine bilayers. This information in turn is used to search for relationships between membrane binding and the biological properties of cellular uptake and in vitro cytotoxicity.

Materials and Methods

Chemicals. Adriamycin, daunomycin, 4-demethoxydaunomycin, rubidazone, *N,N*-dimethyldaunomycin, *N,N*-dimethyladriamycin, 4′-deoxyadriamycin, and 4′-epiadriamycin were the gift of Dr. Leonard Kedda of the Division of Cancer Treatment, National Cancer Institute. Adriamycinol and daunomycinol were supplied by Dr. Mervyn Israel of the University of Tennessee Health Sciences Center, Memphis, TN. Carminomycin was provided by Bristol Laboratories, Syracuse, NY. 5-Iminoadriamycin and 5-iminodaunomycin were the gift of Dr. Edward M. Acton, SRI International, Menlo Park, CA. All of the above anthracyclines were in their hydrochloride form and were used without further purification since thin-layer chromatographic (TLC) analysis on silica gel, using a solvent system of chloroform–methanol–water (40:10:1), showed them to be >98% pure. L-α-Dimyristoylphosphatidylcholine and L-α-dipalmitoylphosphatidylcholine were obtained from Sigma Chemical Co. and were used without further purification.

Preparation of Aglycons. Adriamycinone, daunomycinone, carminomycinone, 4-demethoxydaunomycinone, adriamycinol aglycon, and daunomycinol aglycon were prepared by acid hydrolysis (0.2 N HCl, 60 °C, 48 h) of the respective parent anthracycline (Arcamone et al., 1964) to yield the 7-hydroxyl aglycon species. TLC analysis on silica gel, using a solvent system of chloroform–methanol–water (40:10:1), showed that the resultant aglycons were free of contamination by their unhydrolyzed parents. Confirmation that acid hydrolysis of a parent anthracycline yielded the aglycon (with a hydroxyl group at C-7) was accomplished by ^{1}H NMR at 500 MHz for adriamycinone, daunomycinone, and 4-demethoxydaunomycinone in $CDCl_3$ (data not shown). 5-Iminodaunomycinone was synthesized from daunomycinone by the same method used to convert daunomycin to 5-iminodaunomycin (Tong et al., 1979). Allowing 10 mg of daunomycinone to stand in 10 mL of saturated methanolic ammonia (5 °C, dark, 72 h) resulted in a 99% yield of 5-iminodaunomycinone. 7-Deoxydaunomycinone was synthesized from daunomycin by the reductive cleavage of the glycosidic bond by sodium dithionite (Smith et al., 1977). The structures of 5-iminodaunomycinone and 7-deoxydaunomycinone were confirmed by ^{1}H NMR at 500 MHz.

Preparation of Stock Solutions. Stock solutions of the anthracyclines and the aglycons were prepared in methanol and stored in the dark at −20 °C. The same extinction coefficient was used for an aglycon as for its corresponding anthracycline. The following absorption wavelengths and extinction coefficients were used: daunomycin and adriamycin, 480 nm, 12 200 M^{-1} cm^{-1} (Calendi et al., 1965); carminomycin, 490 nm, 8200 M^{-1} cm^{-1} (Brazhnikova et al., 1974); 5-iminodaunomycin, 592 nm, 19 800 M^{-1} cm^{-1} (Tong et al., 1979); 5-iminoadriamycin, 592 nm, 19 600 M^{-1} cm^{-1} (Acton & Tong, 1981); rubidazone, 480 nm, 12 960 M^{-1} cm^{-1} (Skovsgaard, 1977); 4-demethoxydaunomycin, 480 nm, 9900 M^{-1} cm^{-1}. The extinction coefficients of other daunomycin derivatives modified in ring A or the amino sugar were assumed to be the same as that of daunomycin.

Vesicle Preparation. Small unilamellar vesicles were prepared the day of an experiment by the method of Poste et al. (1976). Lipid was weighed and suspended in phosphate-buffered saline, pH 7.4 (PBS),[1] at a typical concentration of 10 mg/mL. Lipid dispersions were prepared by Vortex mixing for 5–10 min above the T_M of the lipid. The lipid dispersion was then subjected to ultrasound with a Laboratory Supplies Co. (Hicksville, NY) bath-type sonicator for 3–4 h until no further optical clearing of the solution was observed. TLC analysis on silica gel, using a solvent system of chloroform–methanol–acetic acid–water (25:15:4:2), revealed that no decomposition of either lipid had occurred during sonication. The sonicated lipid suspension was annealed above the T_M for 0.5–1 h and then cooled to ambient temperature prior to use. Differential scanning calorimetry studies of the sonicated DPPC vesicles showed a main phase transition centered at 36.9 °C, in agreement with results from Suurkuursk et al. (1976).

Fluorescence Instrumentation. All fluorescence measurements were obtained on a SLM 4800 subnanosecond spectrofluorometer with a thermostated cuvette compartment. This instrument was interfaced to a Hewlett-Packard 9825 data processor Excitation and emission spectra were recorded in the ratio mode with an excitation resolution of 4 nm and an emission resolution of 8 nm; spectra were corrected for background fluorescence and scatter from unlabeled lipids or

[1] Abbreviations: DMPC, L-α-dimyristoylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; K, overall association constant; n, maximum number of binding sites per phospholipid molecule; K_{app}, apparent association constant; r, number of bound drugs per lipid; TLC, thin-layer chromatography; ^{1}H NMR, proton nuclear magnetic resonance; PBS, phosphate-buffered saline containing 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, and 1 mM KH_2PO_4 (pH 7.4); T_M, gel to liquid-crystalline phase transition temperature; a, anisotropy; C_f, free drug concentration.

from solvents by subtraction of the spectrum of a blank. Steady-state fluorescence intensity measurements were made in the ratio mode without polarizers. Steady-state polarization spectra were recorded with the instrument operated in the "L format" (i.e., with single photomultiplier detection). Steady-state anisotropy (a) measurements were determined with the instrument in the "T format" for simultaneous measurement of two polarized intensities. The anisotropy is calculated from $a = (I_{vv} - GI_{vh})/(I_{vv} + GI_{vh})$, where $G = I_{hv}/I_{hh}$ and the subscripts refer to vertical and horizontal orientations of the excitation and emission polarizers, respectively. In each of the instrumental formats the fluorescence emission was observed through long pass filters (filters are specified below). The alignment of the polarizers was routinely checked by using a 2 mg/mL suspension of glycogen in water (anisotropy values of >0.99 were obtained). With the exception of the 5-iminoanthracycline derivatives, steady-state anisotropy measurements on anthracycline analogues were conducted with an excitation wavelength of 470 nm and band-pass of 4 nm, two 500-nm short pass filters (Melles Griot) in the excitation beam to prevent transmission of stray light from the excitation monochromator, and a 550-nm long pass filter (Schott) for each emission channel to isolate fluorescence from scattered light. Experiments on 5-iminoanthracycline derivatives involved an excitation wavelength of 564 nm and a band-pass of 4 nm, a 600-nm short pass filter (Melles Griot) in the excitation beam, and a 650 ± 20 nm interference filter (Melles Griot) for each emission channel. The background fluorescence from unlabeled lipids or from solvents was typically less than 2% of the total intensity. All experiments were conducted in 1-cm quartz cuvettes. The temperature in the cuvette compartment was monitored by a thermistor placed in a blank cuvette.

Equilibrium Binding Measurements. Samples were prepared by adding vesicle solution to tubes containing identical amounts of a stock solution of drug in PBS such that the final drug concentration was 1×10^{-6} M, unless noted otherwise. The samples were equilibrated for 30 min prior to measurement. Fluorescence anisotropy titration was used to determine the concentration of free and bound drug according to

$$a = f_F a_F + f_B a_B + f_S a_S \quad (1)$$

where a equals the measured anisotropy and a_F, a_B, and a_S are the anisotropies of free drug, bound drug, and scatterer, respectively. The terms f_F, f_B, and f_S refer to the fraction of the total detected signal that originates from free drug, bound drug, and background scatter, respectively, and $f_F + f_B + f_S = 1$. In most cases, f_S did not exceed 2% of the total signal. The value of a_S is dependent upon the turbidity of the sample as well as the instrumental conditions (i.e., excitation wavelength, emission filters) used for a particular measurement. With the same experimental conditions, the value of a_S was evaluated by direct measurement of the scatter from a buffer solution or liposome suspension without fluorophore. Alternatively, a_S was measured by increasing the amount of background scatter through dilution and monitoring the change in the measured anisotropy. Results from both approaches were in close agreement. The anisotropy value for free drug was evaluated from the simplified form of eq 1 by using a buffer blank to determine f_S. The bound anisotropy value was determined by extrapolation to infinite total lipid concentration from a $1/a$ vs. 1/total lipid concentration plot, using only the samples of highest lipid content. In determining the amount of bound drug during a titration, we assumed that the term $f_S a_S$ increased linearly with increasing lipid concentration and values for this term at the minimum and maximum lipid concentration were determined for each experiment. Equation 1 was solved for f_F in terms of f_B, and the absolute amount of each species was calculated on the basis of the total drug concentration and the factor by which the fluorescence intensity of the drug increased upon binding.

Analysis of Binding Data. Plots of C_f/r vs. C_f, where C_f represents the concentration of free drug and r represents the concentration of bound drug/total lipid concentration, were used to determine apparent association constants and site stoichiometries. For a single class of binding sites, or mulitple classes of independent, noninteracting sites with identical binding constants, this plot gives a straight line with a slope of $1/n$ and a y intercept of $1/(nK_{app})$, where n is the maximum number of binding sites per lipid molecule, K_{app} is the apparent binding constant, and the overall association constant (K) equals nK_{app} (Klotz & Hunston, 1971). The best linear least-squares fit of C_f/r vs. C_f was determined, from which the n and K_{app} values were calculated. K values obtained from C_f/r vs. C_f plots and double-reciprocal plots (see Results) were in agreement. All calculations were performed on a Northstar Horizon microcomputer equipped with a hardware floating point processor.

Octanol–Buffer Partition Coefficient Measurements. Partition coefficients of fluorophores between 1-octanol and PBS were determined at ambient temperature. Anthracycline (2×10^{-8} mol) was placed in silanated glass tubes and subsequently dissolved in 4.0 mL of 1-octanol. PBS buffer solution (4.0 mL) was added, and the samples were vigorously mixed for 5 min and equilibrated for 24 h with continuous shaking. Partition coefficients were determined by quantitating the amount of drug in each phase of triplicate samples by using fluorescence intensity measurements compared to a standard curve.

RESULTS

Fluorescence Properties of Free and Bound Anthracyclines. Figure 2 shows the fluorescence excitation and emission spectra of free daunomycin and 4-demethoxydaunomycin and the emission spectra of the drugs when bound to fluid-phase DMPC bilayers. Binding of the two drugs to the bilayer resulted in a slight shift of the emission spectra to shorter wavelengths, indicative of drug relocation to a less polar environment; a 30–40% increase in the fluorescence intensity also accompanied binding. The excitation and emission spectra of carminomycin or daunomycin analogues that differ from the parent molecule by structural changes in ring A of the amino sugar occurred in the same spectral regions as shown in Figure 2, and binding was accompanied by a 10–40% increase in intensity. 5-Iminoadriamycin and 5-iminodaunomycin exhibited quite different spectra from the other analogues studied, with an excitation and emission maximum for free drug around 564 and 630 nm, respectively. Excitation polarization spectra of 3×10^{-6} M solutions of daunomycin, carminomycin, 4-demethoxydaunomycin, and 5-iminodaunomycin immobilized in aqueous solutions containing 72% sucrose at 1.5 °C (data not shown) demonstrated that the anisotropy values for these fluorophores were invariant across the absorption ranges described above.

Overall Binding Affinities for Fluid-Phase and Solid-Phase Bilayers. Fluorescence anisotropy titration was used to determine the overall binding affinity of an anthracycline or aglycon for unilamellar DMPC and DPPC vesicles at 27.5 °C. At this temperature, DMPC vesicles exist as fluid-phase bilayers while the DPPC vesicles are below their T_m and exist as solid-phase bilayers. Titration of anisotropy proved to be a sensitive means of determining the extent of fluorophore

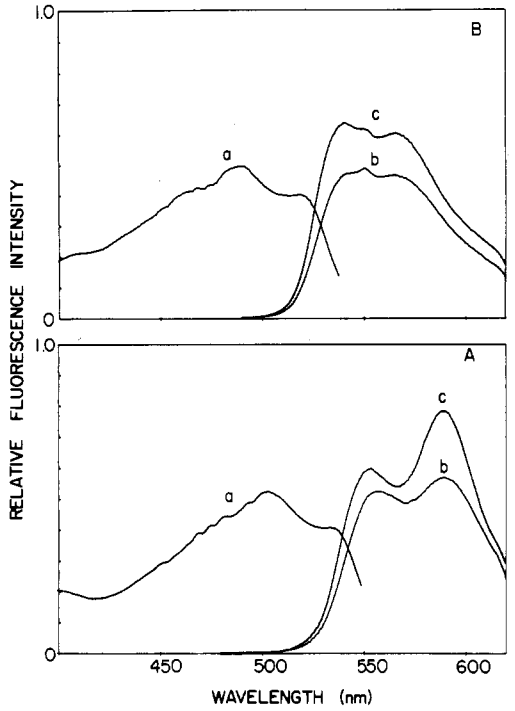

FIGURE 2: Fluorescence excitation and emission spectra of daunomycin (A) and 4-demethoxydaunomycin (B). The relative fluorescence intensity of 2×10^{-6} M drug in PBS buffer, 27.5 °C, is shown. The uncorrected excitation spectra (a) of free drug were recorded with λ_{em} = 592 nm for daunomycin and λ_{em} = 566 nm for 4-demethoxydaunomycin. The uncorrected emission spectra for free drug (b) and for bound drug in a solution containing unilamellar DMPC vesicles at a lipid concentration of 14.7 mM (c) were recorded with λ_{ex} = 470 nm for both daunomycin and 4-demethoxydaunomycin.

binding due to the 4–10-fold change in anisotropy that accompanied binding. In contrast, the fluorescence intensity of some of the fluorophores increased by only 10% upon binding. It has been previously shown that the half-life for the leakage of daunomycin out of vesicles is less than 1%/h (Juliano et al., 1978); we therefore assume that during the time course of these experiments the anthracyclines are essentially on the exterior of the vesicle.

The overall association constants are defined as

$$K = [A_B]/[A_F][L] \quad (2)$$

where $[A_B]$ represents the concentration of bound drug, $[A_F]$ represents the concentration of free drug, and [L] represents the total lipid concentration in the vesicle suspension. Equation 2 is valid when the concentration of free lipid is approximately equal to the concentration of total lipid (i.e., the concentration of free lipid is in great excess over the concentration of bound drug). Provided this condition is met, K may be determined from the inverse of the slope of a double-reciprocal plot (1/fraction of total drug bound vs. 1/[L], with y intercept = 1). Figure 3 is such a plot for the binding of four anthracyclines and their aglycons to fluid-phase DMPC bilayers. The linearity of these plots, as well as the corresponding plots for solid-phase DPPC bilayers (plots not shown), indicates that fluorophore binding at these lipid concentrations was adequately described by eq 2.

Table II summarizes the overall association constants for anthracyclines and aglycons interacting with unilamellar DMPC and DPPC vesicles at 27.5 °C. The fluorophores are divided into three categories: anthracyclines containing a daunosamine residue, anthracyclines containing an amino sugar residue other than daunosamine, and aglycons. The daunosamine-containing anthracyclines shown in Table I differ

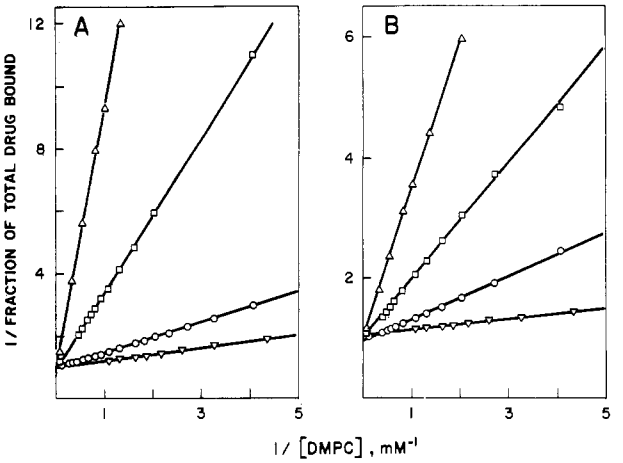

FIGURE 3: Double-reciprocal plots for fluorescence anisotropy titration of 1×10^{-6} M solutions of four anthracyclines (panel A) and their corresponding aglycons (panel B) by DMPC vesicles at 27.5 °C. Free and bound drug concentrations at a given lipid concentration were determined from the measured anisotropy value as described under Materials and Methods. The fluorophores are adriamycinol (△), adriamycin (□), daunomycin (○), and carminomycin (▽).

Table II: Overall Association Constants [K (M^{-1})] for Anthracyclines and Aglycons Interacting with Unilamellar DMPC and DPPC Vesicles at 27.5 °C[a]

compound	fluid-phase bilayer[b]	solid-phase bilayer[b]
Anthracyclines Containing a Daunosamine Residue		
carminomycin	4900	6000
5-iminodaunomycin	3000	3500
4-demethoxydaunomycin	3000	3200
daunomycin	2100	2800
rubidazone	1700	5400
5-iminoadriamycin	750	1600
adriamycin	410	970
daunomycinol	440	710
adriamycinol	120	340
Anthracyclines Containing Other Amino Sugar Residues		
4′-deoxyadriamycin (4-deoxydaunosamine)	880	1900
4′-epiadriamycin (acosamine)	850	2100
N,*N*-dimethyldaunomycin (rhodosamine)	680	2000
N,*N*-dimethyladriamycin (rhodosamine)	250	800
Aglycons (Replacement of Amino Sugar at C-7 with Hydroxyl Group)		
carminomycinone	11600	9700
4-demethoxydaunomycinone	5800	3600
5-iminodaunomycinone	3900	3000
daunomycinone	2800	3000
adriamycinone	1000	1200
daunomycinol aglycon	930	930
adriamycinol aglycon	400	590
7-deoxydaunomycinone	9900	9400

[a] The overall association constant, K, was determined in each case from the inverse of the slope of a double-reciprocal plot as described under Results. A fixed drug concentration of 1×10^{-6} M was used for each fluorophore with the following exceptions due to reduced aqueous solubility: rubidazone (5×10^{-7} M); carminomycinone (2×10^{-7} M). K values were independent of drug concentration over this range since they were dominated by the lipid concentration, which was in great excess throughout the experiments. [b] At 27.5 °C, DMPC vesicles represented fluid-phase bilayers and DPPC vesicles represented solid-phase bilayers. In each compound category, the fluorophores are ranked in order of decreasing affinity for fluid-phase bilayers.

from daunomycin by a single structural modification. Major differences in the values of K were found for these analogues for both types of bilayers. Because of the aromatic nature of the B and D rings of an anthracycline chromophore and because substituents at C-5 of ring C often interact through

hydrogen bonding with substituents at C-4 or C-6, it is difficult to predict how a structural modification in rings B–D will affect the polarity of the molecule. To address this issue, octanol–buffer partition coefficient measurements at ambient temperature were conducted on carminomycin, 5-iminodaunomycin, 4-demethoxydaunomycin, and daunomycin in order to evaluate how structural changes in the B–D rings alter drug polarity. These measurements demonstrated that the relative hydrophobicity of these drugs decreases in the order carminomycin (43) > 4-demethoxydaunomycin (32) > 5-iminodaunomycin (8.9) ~ daunomycin (9.0). The manner in which drug polarity varies with structural changes at alicyclic ring A is straightforward and predictable (i.e., in decreasing order of hydrophobicity: 5-iminodaunomycin > 5-iminoadriamycin, daunomycin > adriamycin ~ daunomycinol > adriamycinol). Rubidazone was found to have a higher octanol–buffer partition coefficient than daunomycin (Skovsgaard, 1978).

A general correlation between drug hydrophobicity and the overall binding affinities for both types of bilayers is apparent for the daunosamine-containing anthracyclines. Carminomycin, the most hydrophobic drug in this series, exhibited the highest affinity for both types of bilayers, followed by 4-demethoxydaunomycin and daunomycin. Insertion of a hydroxyl group at C-14 of daunomycin (adriamycin) or 5-iminodaunomycin (5-iminoadriamycin) results in a more polar drug. If drug hydrophobicity correlates with overall membrane affinity, then the more polar analogues should have lower K values; this prediction was in fact experimentally observed. Likewise, reduction of the C-13 carbonyl of daunomycin (daunomycinol) or adriamycin (adriamycinol) resulted in decreased membrane binding for both bilayers. A similar correlation between hydrophobicity and affinity for fluid-phase and solid-phase bilayers was observed for the anthracycline aglycons that were studied.

Anomalies in the drug hydrophobicity–membrane affinity correlation occur for two congeners—rubidazone and 5-iminodaunomycin. Rubidazone, unlike the other analogues that differ from one another by structural changes that introduce significant polarity changes with correspondingly small steric changes, differs from daunomycin by a bulky benzoylhydrazone substituent at C-13 that not only alters drug polarity (more hydrophobic as measured by octanol–buffer partition coefficient measurements) but also introduces a steric consideration. Rubidazone was found to bind fluid-phase and solid-phase bilayers to a lesser and much greater extent, respectively. It thus appears that the presence of the bulky hydrophobic substituent disfavors fluid-phase bilayer binding but results in an enhanced affinity for solid-phase bilayers. The other anomaly occurs for 5-iminodaunomycin, which exhibits greater bilayer affinities than daunomycin despite having a similar octanol–buffer partition coefficient. This finding suggests the possibility of a specific interaction between the C-5 imino group and the phospholipid, possibly a hydrogen bond, that is not present in the carbonyl-containing parent.

The data presented in Table II also show that the amino sugar structure of an anthracycline can modulate membrane binding affinities. Replacement of the daunosamine residue of adriamycin or daunomycin by rhodosamine resulted in decreased membrane affinities for both types of bilayers, with the decrease being more prominent for the fluid-phase bilayers. In contrast, substitution of acosamine or 4-deoxydaunosamine into the adriamycin molecule resulted in an approximately 2-fold enhancement in binding for both types of bilayers. The presence of an ionizable amine group whose pK_a is sensitive to structural changes precludes any type of simple polarity–membrane affinity correlation for structural changes in the amino sugar portion of the drug molecule.

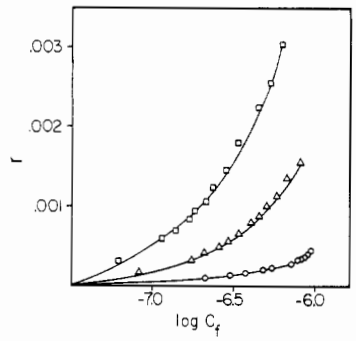

FIGURE 4: Plot of r vs. log C_f for the equilibrium binding of anthracyclines to DMPC vesicles at 27.5 °C. C_f is the molar concentration of free anthracycline and r is the molar ratio of bound anthracycline to total lipid. The anthracyclines are adriamycin (○), daunomycin (△), and carminomycin (□). Total drug concentrations of 1×10^{-6} M were used.

As previously stated, an aglycon differs from its parent anthracycline by the replacement of the amino sugar at C-7 with a hydroxyl group. Comparison of the binding data for 7-deoxydaunomycinone, daunomycinone, and daunomycin allows for the evaluation of how the C-7 substitution of a proton, hydroxyl, or amino sugar, respectively, alters membrane affinity. For both types of bilayer, 7-deoxydaunomycinone displayed more than a 3-fold higher K value than daunomycinone, whereas daunomycinone had a slightly higher affinity than daunomycin. These data indicate that the presence of a polar substituent at C-7 (be it a hydroxyl group or a sterically large amino sugar) significantly reduces the overall membrane affinity of an anthracycline chromophore.

For fluid-phase bilayers, each anthracycline aglycon that was studied showed a higher overall affinity than its corresponding parent, regardless of the amino sugar substituent. This trend was not always observed for solid-phase bilayers where 4′-deoxyadriamycin and 4′-epiadriamycin exhibited K values 1.5- and 1.7-fold greater than adriamycinone, respectively. Another trend apparent in the data indicates that the anthracyclines, regardless of the amino sugar substituent, exhibit greater affinities for solid-phase bilayers than for fluid-phase bilayers. This trend did not hold true for the aglycon series, especially those that exhibited the highest membrane affinities.

Binding Site Stoichiometries. In order to determine the stoichiometry of anthracycline binding to the membranes, C_f/r vs. C_f plots were constructed. By use of such a plot, the overall association constant (K) can be divided into two terms: n, the maximum number of hypothetical receptor sites per mole of receptor, and K_{app}, the apparent association constant, where $K = nK_{app}$. The range of the experimental binding data can be demonstrated by the construction of an r vs. log C_f plot, where r is the number of drug molecules bound per total lipid and C_f is the concentration of free fluorophore (Klotz, 1982). This type of plot is expected to yield an S-shaped curve, approaching n as log C_f approaches infinity with an inflection point at $n_{1/2}$. Figure 4 shows the range of experimental binding data used in the determination of n and K_{app} for the interaction of adriamycin, daunomycin, and carminomycin with fluid-phase bilayers. The data for each drug are representative of only the lower portion of the expected S curve. While attempting to gather binding data at high r values, we observed phenomena consistent with the self-association of anthracyclines at the vesicle surface (Burke & Tritton, 1984). Hence, the anthracycline–liposome system is not amenable to the

Table III: Thermodynamic Parameters and Binding Site Stoichiometries for Anthracyclines Interacting with Unilamellar DMPC and DPPC Vesicles[a]

	fluid-phase DMPC bilayer					solid-phase DPPC bilayer				
anthracycline	ΔH (kcal/mol)	ΔS (kcal mol^{-1} deg^{-1})	ΔG (kcal/mol)	K_{app}[b] (M^{-1})	n	ΔH (kcal/mol)	ΔS (kcal mol^{-1} deg^{-1})	ΔG (kcal/mol)	K_{app}[b] (M^{-1})	n
adriamycin	−3.8	13.0	−7.7	4.4×10^5	0.0013	−21.0	−44.5	−7.7	4.4×10^5	0.0029
carminomycin	−9.6	−8.7	−7.0	1.4×10^5	0.040	−10.5	−8.5	−8.0	7.3×10^{-5}	0.0111
daunomycin	−7.4	−2.3	−6.7	8.2×10^4	0.028	−15.8	−27.1	−7.7	4.4×10^5	0.0083

[a] Free energy (ΔG), enthalpy (ΔH), and entropy (ΔS) were obtained from the van't Hoff plots of anthracycline binding of fluid-phase DMPC bilayers (Figure 6A) and solid-phase DPPC bilayers (Figure 6B). The n values, or number of binding sites per phospholipid molecule, were obtained from the binding data shown in Figure 6; they are the average of measurements for four to six temperatures. The n, ΔG, ΔH, and ΔS values are all subject to 10% uncertainty. [b] The K_{app} (25 °C) values were calculated from the ΔG values.

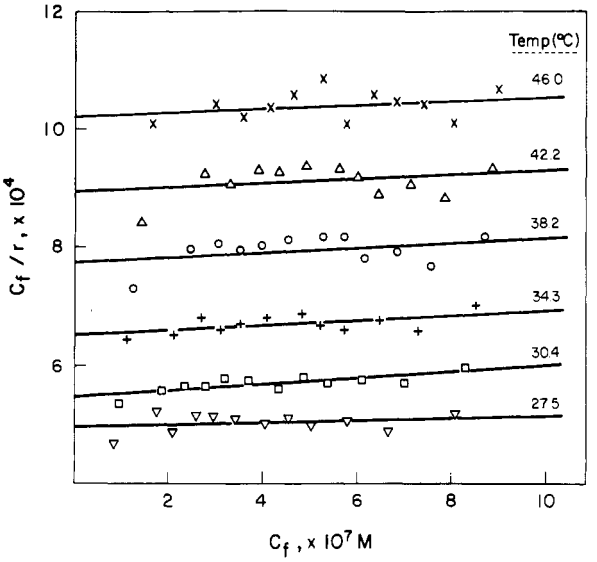

FIGURE 5: Plot of C_f/r vs. C_f for the binding of daunomycin to DMPC vesicles at various temperatures above the gel–liquid-crystalline phase transition temperature (T_m) of the lipid. A total drug concentration of 1×10^{-6} M was used.

collection of binding data at high r values (self-association occurs for daunomycin–DMPC binding, 27.5 °C, at r values in excess of 0.0023). The binding data shown in Figure 4 represent uncomplicated equilibria where both bound and free forms of the drug are in the monomeric form (Burke & Tritton, 1984; Chaires et al., 1982; Menozzi et al., 1984).

Figure 5 shows a C_f/r vs. C_f plot for the binding of daunomycin to DMPC bilayers at several temperatures above the T_m of the lipid. For such a plot, n equals 1/slope and K or nK_{app} equals $1/y$ intercept. These data demonstrate that the value of nK_{app} was dependent upon temperature whereas the value of n, since the lines are parallel, was not. For the binding of adriamycin, daunomycin, and carminomycin to fluid-phase DMPC bilayers or solid-phase DPPC bilayers, the K_{app} term was found to decrease with increasing temperature while n was found to be invariant with temperature within the limits of error. Table III summarizes the binding site stoichiometries for the interaction of adriamycin, daunomycin, and carminomycin with fluid-phase and solid-phase bilayers. A single class of binding sites was found in each case. Major differences in the values of n were noted for the anthracyclines studied, and the value of n was found to increase in the order adriamycin > daunomycin > carminomycin for both types of bilayers. At saturation, assuming 3000 phosphatidylcholine molecules per liposome (Huang & Mason, 1978), there are approximately 4, 84, and 120 molecules per fluid-phase DMPC vesicle for adriamycin, daunomycin, and carminomycin, respectively. For solid-phase DPPC vesicles at saturation, there are 9 adriamycin molecules per vesicle, 33 daunomycin molecules, and 25 carminomycin molecules.

Anthracycline Binding Is Exothermic. The temperature dependence of the K_{app} values of adriamycin, daunomycin, and carminomycin for fluid-phase DMPC and solid-phase DPPC bilayers is presented in Figure 6 in the form of a van't Hoff plot. The linearity of the plots for both types of bilayers indicates that ΔH and ΔS are independent of temperature over the ranges studied. Thermodynamic parameters (ΔH, ΔS, ΔG) were determined from the slope ($-\Delta H/R$) and y intercept ($\Delta S/R$) of the data fit by linear least-squares analysis; these values are presented in Table III. K_{app} (25 °C) values were calculated from the ΔG values. The binding of all three anthracyclines was found to be exothermic, irrespective of the type of bilayer. The binding of adriamycin, daunomycin, and carminomycin to solid-phase bilayers as well as the binding of daunomycin and carminomycin to fluid-phase bilayers was found to proceed with a decrease in entropy.

Discussion

Binding Thermodynamics and Site Stoichiometries. Interactions of adriamycin, daunomycin, and carminomycin with fluid-phase DMPC and solid-phase DPPC unilamellar vesicles have been shown here to exhibit either a single class of binding sites or multiple classes of independent, noninteracting sites with similar binding constants. As shown in Table III, the change in free energy that accompanied binding was similar for the three drugs and two bilayers studied (ΔG values ranged from −6.7 to −8.0 kcal/mol). The significant differences in the overall binding affinities (nK_{app}) of the anthracyclines for a particular bilayer were due to large variations in the value of n rather than K_{app}. Similarly, a recent study on the interactions of polyene antibiotics with sonicated phosphatidylcholine–sterol vesicles (Witzke & Bittman, 1984) showed little variation in K_{app} values with vesicle type or drug structure but major differences in the values of n.

An increase in the value of n for polyene antibiotics (Witzke & Bittman, 1984) or for the free acid form of A23187 (Kauffman et al., 1983) that accompanied changes in the phospholipid membrane structure (i.e., composition and phase transition, respectively) was taken to indicate increasing penetration of the drug molecule into the bilayer. Iodide quenching experiments with adriamycin, daunomycin, and carminomycin bound to excess fluid-phase DMPC and solid-phase DPPC liposomes have shown the order of accessibility to membrane-impermeable iodide to be adriamycin > daunomycin > carminomycin for both bilayers (Burke and Tritton, unpublished results). For each bilayer, the order of decreasing n values was found to be carminomycin > daunomycin > adriamycin. Therefore, it appears that increasing values of n correlate with increasing penetration of the anthracycline molecule into the bilayer. For daunomycin and carminomycin, the n value is greater for the fluid-phase bilayer than the solid-phase bilayer, suggesting deeper penetration of these

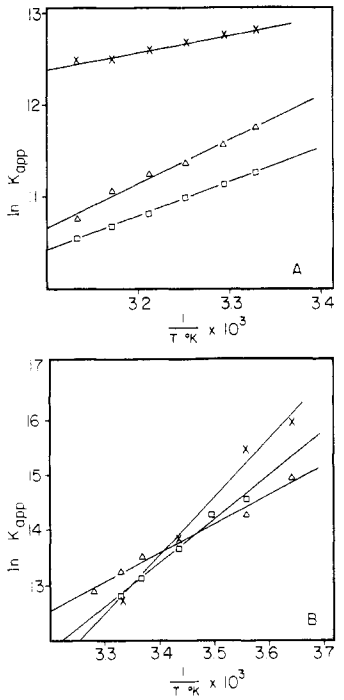

FIGURE 6: van't Hoff plots for the binding of anthracyclines to DMPC vesicles at temperatures above the T_m of the lipid (panel A) and to DPPC vesicles at temperatures below its T_m (panel B). The anthracyclines are adriamycin (×), daunomycin (□), and carminomycin (△). Total drug concentrations of 1×10^{-6} M were used. Plots of C_f/r vs. C_f were used to evaluate K_{app} values from the fluorescence anisotropy titration data. The thermodynamic parameters derived from these plots are summarized in Table III.

anthracyclines in the fluid-phase bilayer. Consistent with this trend, Woolley (1979) observed that *N*-phenylnaphthylamine was totally excluded from the interior of crystalline methylphosphatidic acid bilayers but, after the transition to the more fluid liquid-crystalline phase, the probe penetrated into the hydrocarbon chains.

The binding of adriamycin, daunomycin, and carminomycin to fluid-phase DMPC bilayers and solid-phase DPPC bilayers was found to be exothermic. In each case, with the exception of adriamycin–DMPC binding, membrane association of the anthracyclines was enthalpy driven and opposed by entropy. The observation that binding proceeds with a decrease in entropy suggests that anthracycline association results in an ordering of the bilayer. This prediction has been confirmed for adriamycin–DPPC binding by spin-labeling studies (Constantinides and Tritton, unpublished results). Such a finding is provocative because it raises the possibility that, in addition to local contacts with immediately adjacent lipid molecules, a bound drug can exert longer range membrane effects by organizing (i.e., ordering) neighborhoods of lipids.

Relationship between Drug Structure and Membrane Affinity. In general, it is shown here that structural changes in the aglycon portion of a daunosamine-containing anthracycline that reduce drug hydrophobicity result in a corresponding reduction in the overall affinity of the anthracycline for fluid-phase and solid-phase bilayers. An exception to the hydrophobicity–membrane affinity correlation was noted for rubidazone, a daunomycin analogue that differs from its parent by both polarity (more hydrophobic) and steric considerations. It appears that the sterically bulky benzoylhydrazone substituent resulted in a reduced affinity for fluid-phase bilayers compared to daunomycin. It was also shown here that the *K* values of the anthracyclines were sensitive to structural changes in the amino sugar portion of the molecule. Substitution of the daunosamine residue of adriamycin by acosamine or 4-deoxydaunosamine resulted in greater membrane binding whereas rhodosamine substitution into adriamycin or daunomycin resulted in reduced affinities.

Examination of the manner in which the overall affinities listed in Table II vary with fluorophore structure for a given type of bilayer renders insight into the physical forces that control binding for that particular bilayer. It was observed that placement of a polar substituent (i.e., a hydroxyl group or amino sugar) at C-7 of 7-deoxydaunomycinone significantly reduces the overall membrane affinity of the anthracycline chromophore for fluid-phase bilayers. Because of the hydrophobic nature of rings B–D of 7-deoxydaunomycinone (or any of the other anthracycline derivatives studied) relative to that of ring A that contains a polar hydroxyl moiety at C-9 and because of the planar nature of rings B–D relative to that of alicyclic ring A, it seems logical to expect the probe, if it intercalates into the bilayer, to do so in such a way that ring A is closest to the membrane–solvent interface. Ring A may thus be located at the lipid–solvent interface or in the polar head-group region of the bilayer. In this manner, hydrophobic interactions between rings B–D and the hydrocarbon chains are maximized. The reduction in the *K* value of 7-deoxydaunomycinone accompanied by the substitution of a polar group at C-7 can be explained by reduced hydrophobic interactions because of reduced bilayer penetration. Similarly, the substitution of a polar hydroxyl group at C-13 or C-14 or both for any anthracycline or aglycon resulted in reduced membrane affinities, presumably due to reduced penetration. Consistent with this notion was the observation that the order of iodide accessibility to DMPC-bound anthracycline decreased in the order adriamycin > daunomycin > carminomycin. It was also noted that each aglycon had a higher *K* value for fluid-phase bilayers than its corresponding parent anthracycline, regardless of the amino sugar residue. It appears then that the presence of a large polar substituent at C-7 (relative to the smaller OH functionality) reduces membrane affinities, most likely because it further hinders bilayer penetration of the chromophore. Hence, the data on the interactions of anthracyclines and aglycons with fluid-phase DMPC bilayers indicate that overall affinities are dominated by hydrophobic interactions between rings B–D and the hydrocarbon chains; reduction of the hydrophobicity of these rings as well as placement of polar functionalities on ring A reduces membrane binding.

For solid-phase DPPC bilayers, substitution of a polar group at C-7 of 7-deoxydaunomycinone also resulted in reduced membrane affinities. Similar to a trend observed for fluid-phase bilayers, substitution of a polar group into ring A at C-13 or C-14 of an anthracycline resulted in reduced *K* values for solid-phase bilayers. This trend was also observed for the binding of the aglycon series to solid-phase bilayers. The data therefore indicate that hydrophobic interactions between the chromophore and the hydrocarbon interior of solid-phase bilayers were an important determinant in binding. However, in contrast to what was observed for fluid-phase bilayers, two anthracyclines (4′-epiadriamycin and 4′-deoxyadriamycin) exhibited higher *K* values than their corresponding aglycon, adriamycinone. The observation that the substitution of an amino sugar into adriamycinone results in an enhanced overall membrane affinity indicates that forces other than hydrophobic interactions (i.e., electrostatic interactions) had favorable influences in anthracycline binding. Whereas favorable membrane–anthracycline interactions for *fluid-phase* DMPC bilayers appear to be dominated largely by hydrophobic forces,

both hydrophobic and electrostatic interactions appear to be involved in the interaction of anthracyclines with *solid-phase* DPPC bilayers.

The overall membrane affinities for solid-phase bilayers exceeded those for fluid-phase bilayers for all of the anthracyclines studied. The nature of anthracycline interactions with these membranes is such that the composite of n and K_{app} is always greater for solid-phase bilayers than for fluid-phase bilayers; this does not always hold for the individual n and K_{app} values. The degree of preference for solid-phase bilayers was greater for the anthracyclines that exhibited the poorest membrane affinities. Biological membranes are thought to be inhomogeneous with respect to phospholipid distribution, with domains of differing composition and phase properties coexisting in the bilayer (Jain, 1980). Consequently, the higher overall affinities of the anthracyclines for solid-phase bilayers suggest that these drugs may preferentially locate in the more ordered lipid domains of biomembranes.

Relationship between Membrane Affinity and Cellular Transport. The exact mechanism by which anthracyclines traverse the plasma membrane of cells is uncertain, although three models have been proposed: (1) a "leak and pump" mechanism involving diffusion of an anthracycline into the cell with selective removal through an "efflux pump" (Dano, 1973); (2) facilitated diffusion via a carrier molecule (Skovsgaard, 1978); (3) passive diffusion. The strongest evidence for the passive diffusion model has come from experiments that examined the pH dependency of adriamycin efflux from loaded human erythrocytes into drug-free media (Dalmark, 1981). It was concluded that adriamycin transport in erythrocytes takes place by simple Fickian diffusion of the electrically uncharged anthracycline molecule through the lipid domain of the cell membrane.

Assuming anthracyclines are transported across a cellular membrane by the passive diffusion of the electroneutral molecule, two properties of the drugs are important in determining their relative transport rates in a given cell line: (1) general membrane affinity and (2) pK_a of the amino sugar. Since a greater affinity will result in greater membrane concentrations of the unprotonated form of the drug, enhanced membrane binding of an anthracycline should result in greater cellular uptake. Unfortunately, the literature is lacking in data that lead to a complete understanding of how the pK_a of the amino sugar affects cellular uptake, and we will not pursue this aspect further. The daunosamine-containing anthracyclines have been shown here to exhibit a wide range of membrane affinities. Since the properties of the amino sugar residue are relatively constant for the daunosamine-containing anthracyclines, these molecules serve as a useful set to examine how general membrane binding correlates with cellular transport. Because fluid-phase bilayers are considered to be more relevant models of biomembranes than solid-phase bilayers (Jain, 1980), we will limit our discussion to fluid-phase bilayers when comparing anthracycline membrane affinities with their biological properties (i.e., transport and cytotoxicities). The order of decreasing membrane affinities for the daunosamine-containing anthracyclines was carminomycin > 5-iminodaunomycin ~ 4-demethoxydaunomycin > daunomycin > rubidazone > 5-iminoadriamycin > adriamycin ~ daunomycinol > adriamycinol.

In order to establish a general order of preference for the transport of the anthracyclines by tumor cells in vitro, it was necessary to gather published data from several different cell lines. Bachur et al. (1976) showed the order of uptake in L1210 cells to be daunomycin > rubidazone > adriamycin ~ daunomycinol > adriamycinol. The order of uptake in L1210 (Kessel, 1979) and Ehrlich ascites (Seeber et al., 1980) cells was found to be carminomycin > daunomycin > adriamycin. The uptake of 4-demethoxydaunomycin exceeded that of daunomycin in HeLa cells (Casazza et al., 1983). Hence, there appears to be a good correlation between the rates of cellular transport determined by various other workers and the membrane affinities presented in this paper for the daunosamine-containing anthracyclines. Since greater binding affinities predict greater membrane concentrations for the anthracyclines, the membrane affinity–uptake correlation is consistent with the involvement of some type of general membrane phenomenon in transport; this is to be expected for the passive diffusion model but not necessarily for a carrier-mediated process. The observation that rubidazone exhibited reduced influx relative to daunomycin in Ehrlich ascites cells, despite having a higher octanol–buffer partition coefficient than daunomycin, was used as evidence against the passive diffusion model and supportive of the structural specificity expected from a facilitated transport process (Skovsgaard, 1978). We have shown here that rubidazone has a lower membrane affinity than daunomycin, and therefore, the uptake data have been reconciled with the passive diffusion model.

Relationship between Membrane Affinity and Biological Activity. The anthracyclines have two major types of biological activities, namely, cardiac toxicity and inhibition of cell growth. We are interested in the possibility that these physiological effects may be related to membrane binding and thus have searched the literature for comparison of the existing biological data with the physical results reported here. No significant relationship has been discerned between membrane binding and cardiac toxicity, probably because this side effect is determined by a number of biological factors. Comparison of the overall membrane affinities of the daunosamine-containing anthracyclines with their in vitro cytotoxicities suggests that enhanced membrane binding may correlate with increased potencies. Such comparisons between anthracycline congeners are made difficult by the lack of comprehensive data and the use of several different cell lines, but the existing literature does provide a useful data base for preliminary analysis. 4-Demethoxydaunomycin was 27–100 times more effective than daunomycin at inhibiting the cloning efficiency of exponential phase HeLa cells (Supino et al., 1977). In a similar study, adriamycin and daunomycinol were found to be less potent than daunomycin (DiMarco et al., 1973). Studies measuring the ability of anthracyclines to inhibit the growth of human lymphoblastic leukemia cells (CCRF-CEM) showed the following order of potency: carminomycin > 4-demethoxydaunomycin > daunomycin > adriamycin (Schwarz & Kanter, 1979). Thus, for these various anthracyclines, a qualitative correlation is apparent between membrane affinity and cytotoxicity. An exception should be noted in the case of 5-iminodaunomycin, which was found to be 2–3-fold less potent than adriamycin, as assessed by inhibition of L1210 colony formation (Zwelling et al., 1982). We should also stress that since enhanced membrane affinity results in both greater membrane drug concentrations and superior transport properties, a correlation between membrane affinity and cytotoxicity is consistent with both the cell interior (presumably DNA) and the surface membrane as sites of cytotoxic action.

We have shown in this report that the overall membrane affinities of the anthracyclines as well as the thermodynamic nature of anthracycline–membrane interactions vary markedly with drug structure and bilayer type. Model membranes therefore have the inherent ability to discriminate between

anthracyclines; conversely, the anthracyclines exhibit selectivity between different bilayers. Table II shows that the anthracyclines that bind with lower affinities exhibit a 2–3-fold preference for solid-phase bilayers whereas the drugs with the higher affinities were significantly less selective. Since differences in structure and composition between normal and neoplastic cell membranes have been well documented (Wallach, 1975), it seems quite reasonable to expect that the cell surface membrane could serve as a prime target site for improving the selectivity of the anthracyclines regardless of the site of the primary toxic mechanism. In addition, changes in surface membrane composition and structure have also been proposed as an explanation of anthracycline resistance developed by tumor cells [see Siegfried et al. (1984) and references cited therein]. Thus, the ability of the drug to recognize and discriminate among cell types based on surface membrane structure may play a role in the expression of drug resistance. Experiments on model membranes such as those described here provide an essential foundation for the more demanding studies concerning the effects of drug structure and membrane composition in anthracycline selectivity for neoplastic cells. Such studies should also provide information concerning the molecular mechanism(s) of cytotoxic action.

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Registry No. DMPC, 18194-24-6; DPPC, 63-89-8; adriamycin, 23214-92-8; daunomycin, 20830-81-3; carminomycin, 50935-04-1; 5-iminodaunomycin, 72983-78-9; 4-demethoxydaunomycin, 58957-92-9; rubidazone, 54083-22-6; 5-iminoadriamycin, 84275-95-6; daunomycinol, 28008-55-1; adriamycinol, 54193-28-1; 4′-deoxyadriamycin, 63521-85-7; *N,N*-dimethyldaunomycin, 67508-87-6; *N,N*-dimethyladriamycin, 70222-95-6; carminomycinone, 52744-22-6; 4-demethoxydaunomycinone, 60660-75-5; 5-iminodaunomycinone, 94889-78-8; daunomycinone, 21794-55-8; adriamycinone, 24385-10-2; daunomycinol aglycon, 28008-51-7; adriamycinol aglycon, 56149-23-6; 7-deoxydaunomycinone, 32384-98-8; acosamine, 41094-24-0.

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