Anthracycline Binding to Synthetic and Natural Membranes. A Study Using Resonance Energy Transfer†

Elizabeth A. Griffin, Jane M. Vanderkooi,* Grzegorz Maniara, and Maria Erecińska
Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6059

Received June 25, 1986; Revised Manuscript Received August 22, 1986

ABSTRACT: The binding of adriamycin and its two analogues 4′-epidoxorubicin and 4′-deoxydoxorubicin to synthetic and mitochondrial membranes was investigated by using resonance energy transfer between these drugs and two fluorescent probes, diphenylhexatriene (DPH) and tryptophan. The fluorescence of the lipid probe DPH in both types of membranes and tryptophan in mitochondria was quenched by the anthracyclines in a dose-dependent manner. In sonicated, fluid-phase dimyristoyl-L-α-phosphatidylcholine (DMPC) vesicles, the half-quenching concentration (K_{50}) of adriamycin was 17 ± 1 μM, whereas in bilayers containing a 1:1 molar ratio of DMPC to cardiolipin (CL), the value was 8 ± 1 μM. In liver and heart mitochondria, the K_{50} values were 8 ± 2 and 11 ± 3 μM, respectively. Similar results were obtained for the other two drugs. Replacing a nonionic with an ionic medium or decreasing the pH from pH 7.7 to pH 6.9 increased the K_{50} value of adriamycin for DPH in DMPC/CL (1:1 molar) liposomes and in mitochondria. Higher concentrations of anthracycline were needed to quench the fluorescence of tryptophan. The results suggest that these drugs interact with both phospholipids and proteins and that the cardiotoxicity of adriamycin is unlikely to be related to the amount of drug bound to heart mitochondria.

Adriamycin, a cytotoxic antibiotic in the anthracycline family, is a widely employed and very effective antineoplastic agent. However, its use is limited due to a major side effect: a specific, dose-dependent toxicity to the heart. Recently, investigations into the cardiotoxicity of this drug have focused on the interactions of adriamycin and other anthracyclines with synthetic, plasma, and mitochondrial membranes. Several techniques have been employed for this purpose. With synthetic membranes, fluorescence anisotropy of the anthracyclines (Burke & Tritton, 1984, 1985a,b), fluorescence quenching of membrane-bound drug by membrane-impermeable iodide (Karczmar & Tritton, 1979; Burke & Tritton, 1985b; Samuni et al., 1986), and circular dichroism (Henry et al., 1985) have been used. However, the first two approaches provide information only on the relative location and dynamics of the drug within the membrane, whereas the third one examines changes in anthracycline conformation and self-association as a function of binding of the drug to the membrane. With both intact mitochondria and mitochondrial membranes, the cellular content of the drug has been examined (Nicolay et al., 1984). However, this approach is incapable of distinguishing between binding and transport. Still, many questions remain as to how anthracyclines bind to and are transported into cells (Skovsgaard & Nissen, 1982; Siegfried et al., 1985).

In the present study, the interaction of anthracyclines with synthetic and mitochondrial membranes has been investigated by observing the fluorescence quenching of two probes, diphenylhexatriene (DPH)[1] and/or tryptophan, as a function of anthracycline concentration. DPH, situated in the lipid bilayer, and tryptophan in membrane proteins will transfer its emitted energy to anthracycline provided that the drug and the probe are sufficiently close (<4–5 nm, Förster, 1948). The quenching of the probe's fluorescence will effectively occur only when the anthracycline is on or within the membrane and not free in solution. Thus, this technique provides direct information on the binding of the drug to the membrane, independent of transport.

Experimental Procedures

Materials. Dimyristoyl-L-α-phosphatidylcholine and cardiolipin from bovine heart were obtained from Sigma Chemical Co. (St. Louis, MO); diphenylhexatriene was from Aldrich (Milwaukee, WI); adriamycin, 4′-epidoxorubicin, and 4′-deoxydoxorubicin were a kind gift from Adria Laboratories (Columbus, OH).

Membrane Preparations. Vesicles consisting of DMPC, DMPC/CL (1:1 molar ratio), or DMPC/CL (1:0.5 molar ratio) were prepared by suspending the dried phospholipids in either buffer A (15 mM NaCl/10 mM phosphate at pH 6.2, 7.4, or 7.8), buffer B (200 mM NaCl/10 mM phosphate at pH 6.2 or 7.8), or buffer C (225 mM mannitol/75 mM sucrose/10 mM Tris-HCl at pH 6.9 or 7.7) to a final concentration of 20 mg/mL. The lipids were sonicated for 30 s in a Branson sonicator. The liposomes were incubated in DPH (1.5 μM) for at least 1 h to ensure complete penetration of the fluorescent probe into the lipid bilayer. Two other concentrations of DPH (4.5 and 0.5 μM) were also used.

Phospholipid vesicles, suitable in size for FRAP measurements, were prepared according to the procedure of Fragata et al. (1984). Essentially, the solvent of a 50-μL aliquot of DMPC (20 mg/mL) was evaporated under nitrogen to ensure dryness. The residue was placed in a desiccator for 2 h.

† This work was supported by U.S. Public Health Service Grant GM-21524 and National Institutes of Health Predoctoral Training Grant HL-67502 (to E.A.G.).

* Correspondence should be addressed to this author.

[1] Abbreviations: DPH, diphenylhexatriene; DMPC, dimyristoyl-L-α-phosphatidylcholine; CL, cardiolipin; FRAP, fluorescence recovery after photobleaching; MSE, 225 mM mannitol, 75 mM sucrose, and 1 mM EGTA/Tris; Mops, 3-(*N*-morpholino)propanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/86/0425-7875$01.50/0

Adriamycin (200 μL of a 2 mM saline solution) was added to the dried phospholipid, and the mixture was hydrated in the presence of 15 mM NaCl/10 mM phosphate, pH 7.1, for 45 min at 25–30 °C. After hydration, the sample was diluted to 1 mL with the same buffer.

Rat liver and heart mitochondria were prepared according to the procedures of Schneider (1948) and of Tyler and Gonze (1967), respectively. The pellets were suspended in MSE/10 mM Mops, pH 7.4, to a final concentration of approximately 30 mg/mL. Aliquots of mitochondria were diluted to a concentration of 0.25 mg/mL in either MSE/10 mM Mops (pH 6.4, 7.4, or 7.8) or 140 mM KCl/10 mM Hepes (pH 7.4). DPH (1.5 μM) was added to a fraction of the mitochondria with an incubation time of 1 h.

Protein was determined by the biuret method, using bovine serum albumin as the standard (Gornall et al., 1949).

Spectral Studies. The absorption spectrum of adriamycin (100 μM in buffer A, pH 7.4) was measured in a Perkin-Elmer/Hitachi 200 UV–vis spectrophotometer. The fluorescence emission spectra of DPH and tryptophan and of adriamycin (100 μM) were obtained in a Perkin-Elmer 650-10S fluorescence spectrophotometer. From the spectral overlap of adriamycin absorption with DPH and tryptophan fluorescence emission, the Förster distances (R_0), at which the transfer efficiency is 50%, were calculated by using the equation

$$R_0 = 9.79 \times 10^2 (k^2 n^{-4} \phi_d J)^{1/6} \text{ nm}$$

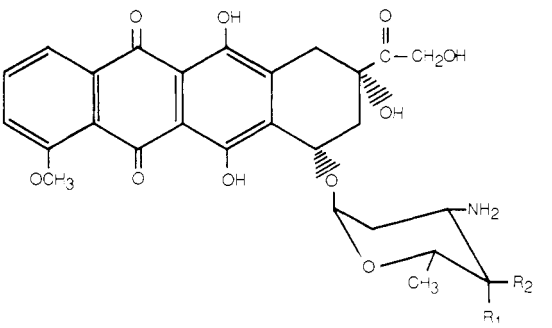
where $k^2 = {}^2/_3$ (random orientation factor, verified by a fluorescence polarization experiment), $n = 1.5$ (refractive index for lipids; Weast, 1977), and $\phi_d = 0.2$ (experimentally determined quantum yield of DPH, based on quinine bisulfate in 1 N H_2SO_4 as a standard with a quantum yield of 0.55; Melhuish, 1961) or $\phi_d = 0.1$ (quantum yield for tryptophan, which can range from 0.04 to 0.20 in proteins; Longworth, 1971). J is calculated from the equation

$$J = \int_0^\infty \epsilon_A(\lambda) f_D(\lambda) \lambda^4 \, d\lambda \text{ M}^{-1} \text{ cm}^3$$

where ϵ_A is the extinction coefficient of the acceptor and f_D is the normalized fluorescence of the donor at wavelength λ (Förster, 1948). For DPH–adriamycin (spectral overlap from 390 to 500 nm), $J = 2.68 \times 10^{-14}$ M^{-1} cm^3; for tryptophan–adriamycin (spectral overlap from 300 to 400 nm), $J = 3.98 \times 10^{-15}$ M^{-1} cm^3. The values of R_0 calculated in this manner are approximate due to the fact that the calculations assume that the donor and acceptor are point charges.

Resonance Energy Transfer Studies. A 1-mL sample of phospholipid vesicles (1 mg/mL phospholipid) at 27.5 ± 0.5 °C or mitochondria (0.25 mg/mL protein) at 24.0 ± 0.5 °C in the appropriate buffer was titrated with increasing concentrations of adriamycin, 4′-epidoxorubicin, or 4′-deoxydoxorubicin (0–180 μM) from a saline stock solution. A 0.3 cm by 1 cm cuvette was used to avoid absorption artifacts. The decrease in fluorescence of DPH (excitation, 380 nm; emission, 440 nm) and/or tryptophan (excitation, 280 nm; emission, 340 nm) was measured after each addition in a Perkin-Elmer LS-5 fluorescence spectrophotometer.

Fluorescence Recovery after Photobleaching. The fluorescence photobleaching apparatus and the method of data analysis have been previously described (Vanderkooi et al., 1985). FRAP measurements were performed on the prepared sample at 10, 15, 20, 24, 25, 30, and 37 °C. A 2-W argon ion laser from Coherent Inc. (Palo Alto, CA) served as a light source. The line at 514.5 nm, unattenuated at 30–40 mW for the bleached pulse, was employed. The bleaching time was



Compound	R_1	R_2
Adriamycin (doxorubicin)	OH	H
4′-epidoxorubicin	H	OH
4′-deoxydoxorubicin	H	H

FIGURE 1: Structure of adriamycin, 4′-epidoxorubicin, and 4′-deoxydoxorubicin.

0.001 s. The spot size was determined by using the convolution scan method on a film of DiI in collodion as described by Schneider and Webb (1981). The $1/e^2$ intensity radius was 3.2 μm.

Interactions of Adriamycin with Mitochondria. Solutions of varying concentrations of adriamycin, ranging from 0 to 400 μM in either MSE/10 mM Mops or 140 mM KCl/10 mM Hepes, pH 7.4, were prepared. Two milliliters of each solution was added to an aliquot of rat liver mitochondria (suspended in MSE/10 mM Mops, approximately 30 mg/mL) to a final concentration of 1 mg/mL. After a 2-min incubation, the suspension was centrifuged at 8000 rpm for 7 min. The absorbance of the supernatant and of the adriamycin solutions (each appropriately diluted) was measured at 500 nm in a Perkin-Elmer/Hitachi 200 UV–vis spectrophotometer.

RESULTS

Three drugs, adriamycin and its two analogues, 4′-epidoxorubicin and 4′-deoxydoxorubicin, were used in the present study. Figure 1 shows that the structural differences among these analogues are very small, and consequently, the absorption spectra for all three drugs, as well as the fluorescence emission spectra (excitation, 440 nm), were similar.

Properties of Adriamycin Fluorescence with and without Lipid. The fluorescence intensity of adriamycin was monitored as a function of its concentration in the presence and absence of membrane vesicles composed of fluid-phase DMPC/CL (1:0.5 molar ratio). The measurements were done at pH 6.2 and 7.8 in both low (15 mM NaCl) and high (200 mM NaCl) ionic strength media. Both with and without lipid, the fluorescence intensity of adriamycin initially increased linearly with increasing adriamycin concentration (Figure 2). At concentrations higher than 10 μM, the fluorescence intensity began to saturate. (The pH and the ionic strength of the medium had little effect on these fluorescence intensity profiles.) This saturation suggests that adriamycin self-associates both in solution and on the surface of membrane vesicles. Similar behavior has been observed with daunomycin (Barthelemy-Clavey et al., 1974; Chaires et al., 1982; Burke & Tritton, 1984) and was thought to result from the stacking of the anthracycline rings (Chaires et al., 1982).

Resonance Energy Transfer. To observe interactions of anthracyclines with membranes, two fluorescent probes, DPH and tryptophan, were employed. Since the fluorescence

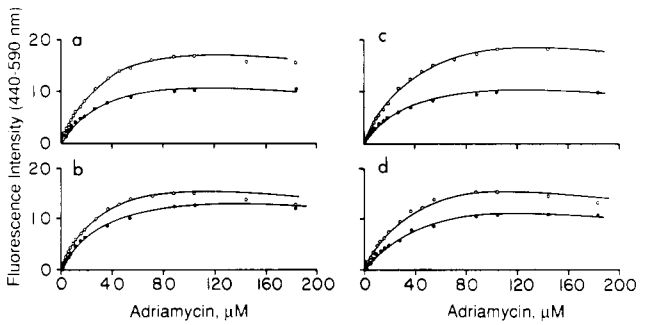


FIGURE 2: Fluorescence of adriamycin as a function of its concentration in the presence and absence of lipids. Increasing concentrations of adriamycin (0–200 μM) were added to buffer B at pH 6.2 (a) and 7.8 (c) and to buffer A at pH 6.2 (b) and 7.8 (d) in the presence (○) and absence (●) of DMPC/CL (1:0.5 molar ratio) vesicles (1 mg/mL phospholipid). The fluorescence intensity of the suspension was recorded upon each addition of adriamycin (excitation, 440 nm; emission, 590 nm; slits at 10 nm).

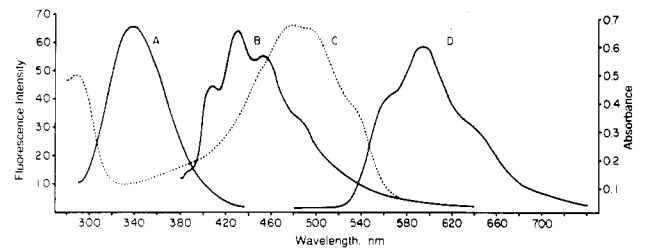


FIGURE 3: Fluorescence emission (—) spectra of tryptophan (A), DPH (B), and adriamycin (D) and the absorption (···) spectrum of adriamycin (C). In spectrum A, rat liver mitochondria at a protein concentration of approximately 1 mg/mL were suspended in MSE/10 mM Mops, pH 7.4; excitation was at 280 nm. In spectrum B, DMPC vesicles containing DPH were suspended in buffer A, pH 7.4; excitation was at 380 nm. In spectra C and D, the fluorescence emission (excitation, 440 nm) and absorption spectra of adriamycin were obtained at a concentration of 100 μM in buffer A, pH 7.4.

emission spectrum of the donor (DPH or tryptophan) overlaps with the absorption spectrum of the acceptor (an anthracycline) (Figure 3), a transfer of energy, which results in the quenching of the fluorescence of the donor, is possible if the donor and acceptor are within the Förster quenching distance, R_0. From the spectral overlap, approximate R_0 values for DPH–adriamycin and for tryptophan–adriamycin were calculated to be 2.9 and 1.9 nm, respectively.

To determine whether quenching of the fluorescence of the donor follows a Förster energy transfer mechanism, the excitation spectra of adriamycin (10 μM, emission at 590 nm) with DMPC liposomes in the presence and absence of DPH (1.5 μM) were recorded (data not shown). The two additional peaks between 330 and 400 nm in the excitation spectrum of adriamycin observed in the presence of DPH indicate that the excitation of the latter at 380 nm is responsible for the fluorescence of adriamycin at 590 nm.

Quenching of DPH Fluorescence in Synthetic Membranes. Interactions between anthracyclines and the lipid bilayer were first investigated in synthetic membranes composed of the neutral phospholipid DMPC or of DMPC in a 1:1 molar ratio with negatively charged cardiolipin. Figure 4 shows the percent fluorescence intensity of DPH as a function of adriamycin concentration. The concentration of adriamycin necessary to quench 50% of the DPH fluorescence (K_{50}) was 17 ± 1 μM in DMPC liposomes but only 8 ± 1 μM in DMPC/CL vesicles. Similar results were obtained for the other two drugs. At high concentrations, all three drugs completely quenched the fluorescence of DPH. These results were independent of the DPH concentration employed.

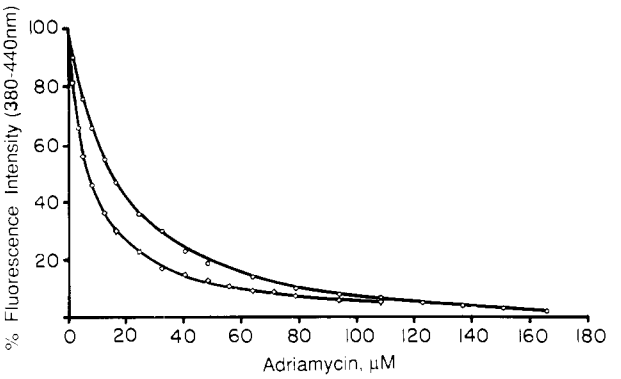


FIGURE 4: Quenching of DPH fluorescence by adriamycin in membrane vesicles composed of DMPC (○) and DMPC/CL (◇). A 1-mL suspension of DMPC or DMPC/CL (1:1 molar ratio) vesicles (1 mg/mL phospholipid) in buffer A, pH 7.4, containing 1.5 μM DPH, was titrated with increasing amounts of adriamycin (0–180 μM). Upon each addition, the fluorescence intensity of DPH was monitored (excitation, 380 nm; emission, 440 nm; slits at 5 nm). K_{50} values of 17 and 8 μM were obtained for adriamycin in DMPC and DMPC/CL liposomes, respectively.

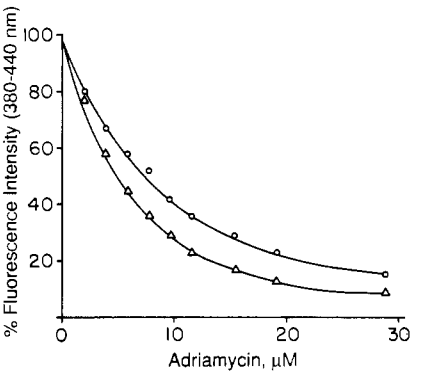


FIGURE 5: Effect of pH on the fluorescence quenching of DPH by adriamycin in DMPC/CL (1:1 molar ratio) liposomes. A 1-mL suspension of DMPC/CL vesicles (1 mg/mL phospholipid) in buffer C at pH 6.9 (○) and 7.7 (△), containing 1.5 μM DPH, was titrated with increasing concentrations of adriamycin (0–30 μM). The fluorescence intensity of DPH (excitation, 380 nm; emission, 440 nm; slits at 5 nm) was measured upon each addition of the drug. K_{50} values of 8 and 5 μM were obtained at pH 6.9 and 7.7, respectively.

The effect of ionic strength, pH, and Ca^{2+} on DPH quenching in DMPC and DMPC/CL (1:1 molar ratio) membrane vesicles by adriamycin was also investigated. In a high ionic strength medium (140 mM KCl/10 mM phosphate, pH 7.4), the K_{50} value for adriamycin binding to DMPC liposomes was unaltered, whereas K_{50} was decreased by approximately 20% in DMPC/CL vesicles. Increasing the pH from 6.9 to 7.7 (buffer C) had no effect on the fluorescence quenching of DPH in DMPC liposomes but did result in a slight increase in DMPC/CL vesicles (Figure 5). The addition of 10 mM Ca^{2+} to the same buffer decreased the fluorescence quenching of DPH at a given adriamycin concentration in both liposome preparations. However, the effect was much greater in DMPC/CL vesicles (data not shown).

Lateral Motion of Adriamycin in Lipid Membranes. FRAP measurements were carried out to characterize further adriamycin interaction in neutral phospholipid vesicles as a function of temperature. Figure 6 shows the recovery curves in DMPC vesicles at 10 and 37 °C. At 10 °C, the calculated diffusion coefficient was 8.9×10^{-10} cm^2/s (90% recovery), while at 37 °C, the value was 1.4×10^{-8} cm^2/s (94% recovery). A discontinuity in the temperature dependence was seen at approximately 24 °C, which is the temperature at which DMPC undergoes a phase transition.

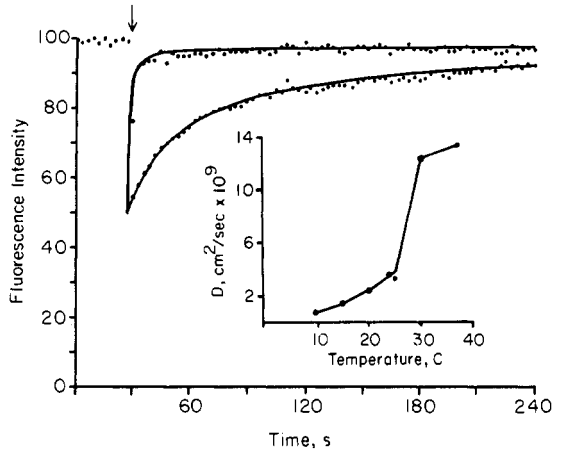


FIGURE 6: Effect of temperature on FRAP profiles of adriamycin bound to DMPC vesicles. The top curve shows the recovery profile at 37 °C; the bottom curve is the recovery profile at 10 °C. Solid lines are the computer fit; the arrow indicates the time of the bleach pulse. Inset: mean value of the diffusion coefficient for four separate FRAP measurements plotted vs. temperature. Experimental conditions are described under Experimental Procedures.

Interaction of Anthracyclines with Natural Membranes. Using resonance energy transfer, we investigated the interactions between drugs and lipid components of natural membranes by observing fluorescence quenching of DPH incorporated into mitochondria isolated from heart and liver. Figure 7A illustrates the DPH quenching profiles as a function of anthracycline concentration in liver mitochondria. A K_{50} value of 8 ± 2 μM was obtained for adriamycin, whereas those for the other two drugs were slightly smaller. The DPH fluorescence was completely quenched by all three drugs. Similar results were found for DPH quenching in heart mitochondria (K_{50} = 11 ± 3 μM). Interestingly, the K_{50} values for both types of mitochondria were the same as those obtained in membrane vesicles consisting of mixed phospholipids.

Interactions between anthracyclines and membrane proteins were followed by measuring the quenching of tryptophan fluorescence in mitochondria from heart and liver (Figure 7B). The fluorescence profiles were similar for mitochondria from both tissues. However, unlike DPH, tryptophan fluorescence was only partially quenched at the concentrations of anthracyclines employed.

The data for both DPH and tryptophan were also analyzed by plotting the ratio of the fluorescence intensity of the donor in the absence of anthracycline (F_0) to the fluorescence of the donor in the presence of the drug (F) vs. the concentration of anthracycline (Figure 7, inset). Straight lines were obtained for both the DPH and tryptophan quenching curves. The ratios of the slopes for DPH to those of tryptophan were 16, 20, and 21 for adriamycin, 4′-epidoxorubicin, and 4′-deoxydoxorubicin, respectively.

Quenching of the fluorescence of DPH and tryptophan was also used to determine the effect of high salt on interactions of drugs with mitochondrial membranes. For DPH quenching, Figure 8 shows that the K_{50} value obtained in MSE was 12 μM, but in KCl, it increased to 27 μM. For tryptophan quenching, the presence of KCl resulted in only a slight decrease in the percent fluorescence quenching.

In addition, interactions of adriamycin with mitochondria were investigated by utilizing the absorption properties of the drug. Figure 9 shows the amount of adriamycin bound at a given drug concentration in nonionic or ionic medium. Over the range of 0–400 μM, the binding of adriamycin was hyperbolic in both media, with saturation at high adriamycin

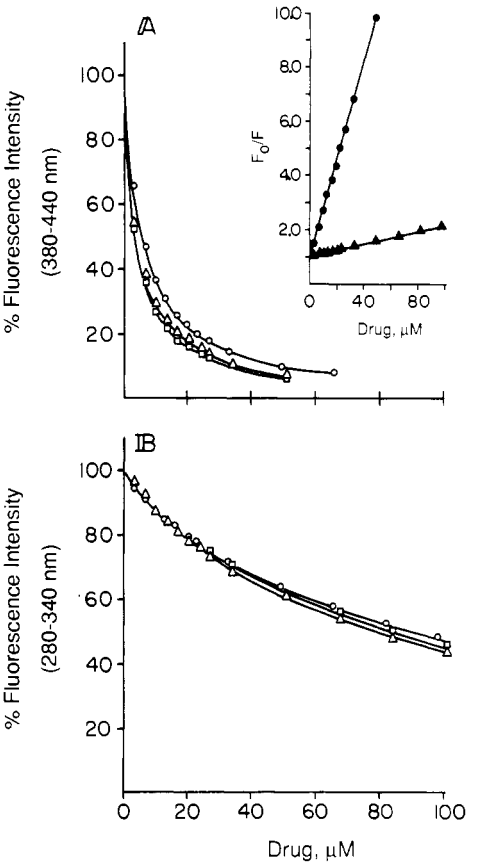


FIGURE 7: Quenching of DPH and tryptophan fluorescence by anthracyclines in rat liver mitochondria. Rat liver mitochondria suspended in MSE/10 mM Mops, pH 7.4 (0.25 mg/mL protein), containing 1.5 μM DPH, were titrated with increasing amounts of adriamycin (○), 4′-epidoxorubicin (△), and 4′-deoxydoxorubicin (□), and the fluorescence intensity of DPH (excitation, 380 nm; emission, 440 nm; slits at 10 nm) was recorded (A). The fluorescence intensity of tryptophan in rat liver mitochondria without DPH, as a function of increasing amounts of anthracycline, was also monitored (B). K_{50} values for quenching of DPH fluorescence were 6 μM for adriamycin and 4 μM for 4′-epidoxorubicin and 4′-deoxydoxorubicin. Inset: ratio of the fluorescence intensity in the absence of adriamycin (F_0) over the fluorescence intensity in the presence of adriamycin (F) as a function of adriamycin concentration. The ratios of the slope of the line generated from the data of DPH fluorescence quenching (●) over the slope of the line for tryptophan (▲) are 16, 20, and 21 for adriamycin, 4′-epidoxorubicin, and 4′-deoxydoxorubicin, respectively.

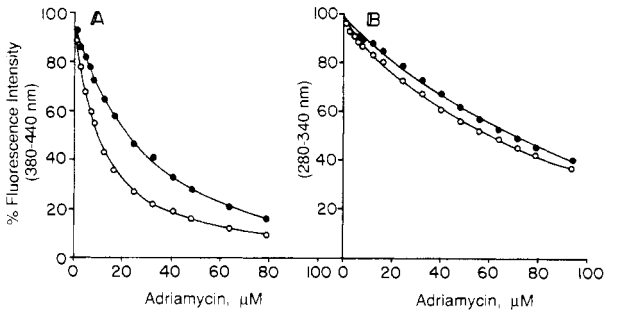


FIGURE 8: Effect of salt on quenching of DPH fluorescence by adriamycin in rat liver mitochondria. Rat liver mitochondria, suspended in either MSE/10 mM Mops, pH 7.4 (○), or 140 mM KCl/10 mM Hepes, pH 7.4 (●) (0.25 mg/mL protein), were titrated with increasing amounts of adriamycin (0–100 μM) in the presence (A) and absence (B) of DPH. The fluorescence intensity of tryptophan (excitation, 280 nm; emission, 340 nm; slits at 10 nm) and of DPH (excitation, 380 nm; emission, 440 nm; slits at 10 nm) was determined. For the quenching of DPH fluorescence, K_{50} values of 12 μM in MSE and 27 μM in KCl were obtained.

concentrations. However, there was less binding in KCl than in MSE. Addition of either rotenone plus FCCP (to collapse

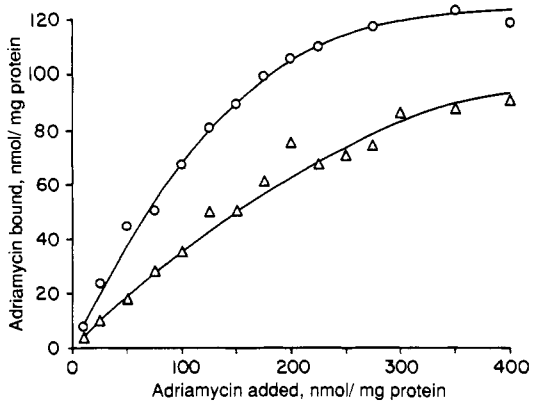


FIGURE 9: Adriamycin binding to rat liver mitochondria suspended in MSE (○) or KCl/Hepes (△) as a function of drug concentration. Experimental conditions are described under Experimental Procedures.

the membrane potential) or 10 mM acetate (to collapse the pH gradient) had no effect on adriamycin binding to mitochondria suspended in the nonionic medium.

Discussion

The goals of this study were to investigate the mechanism(s) of the interactions of anthracyclines with synthetic and natural membranes and to determine whether preferential binding of adriamycin to cardiac mitochondria may be responsible for the cardiotoxicity of this drug. To explore further the latter hypothesis, 4′-epidoxorubicin and 4′-deoxydoxorubicin, two analogues of adriamycin which are thought to be less cardiotoxic (Casazza et al., 1978, 1980; Bonfante, 1981), were used.

To examine the binding of these drugs to the membrane, without interference from transport, advantage was taken of the interactions between the fluorescence of anthracyclines and those of two probes: the extrinsic lipid probe DPH and the intrinsic protein probe tryptophan. The fluorescence of both probes was quenched by the anthracyclines in a dose-dependent manner via a Förster energy transfer mechanism. Since the quenching of fluorescence occurs when the drugs are within a distance of ≤ 3 nm from the probes, these results provide direct evidence that anthracyclines bind to both the lipid and protein components of membranes.

Detailed analysis of the results obtained with the lipid probe DPH suggests that the interactions between anthracyclines and phospholipids in membranes have both a hydrophobic and an electrostatic component. The former is supported by experiments which demonstrate that DPH fluorescence in uncharged DMPC liposomes is quenched by all three drugs. The calculations show that these drugs approach the probe, located throughout the hydrophobic domain of the lipid bilayer (Shinitzky & Inbar, 1974; Andrich & Vanderkooi, 1976), within the Förster quenching distance of 2.9 nm. Since the bilayer is about 6.0 nm wide, the observations that the quenching of DPH fluorescence is complete allow the conclusion that the drugs traverse the membrane to reach the inner leaflet of the bilayer. These findings confirm the earlier postulates of Tritton and co-workers (Karczmar & Tritton, 1979; Burke & Tritton, 1985a,b), Samuni et al. (1986), and Henry et al. (1985) that hydrophobic interactions between the anthracycline ring and the hydrocarbon interior of the membrane may be important in the association of the drug with phospholipid bilayers.

There are several lines of experimental evidence which suggest that electrostatic forces are involved in interactions between lipids and anthracyclines. First, the K_{50} value for a given drug in DMPC/CL liposomes is, by a factor of 2, smaller than that in DMPC vesicles. Second, the K_{50} values in cardiolipin-containing vesicles are larger in ionic KCl medium than in nonionic mannitol/sucrose medium. Third, the amount of adriamycin necessary to quench the fluorescence of DPH at pH 6.9 in DMPC/CL liposomes is less than that at pH 7.7. Fourth, the K_{50} values in liver and heart mitochondria, which are known to contain negatively charged lipids, are close to those for liposomes containing cardiolipin. Fifth, there is less binding of adriamycin to mitochondria suspended in a KCl medium than in the mannitol/sucrose buffer, as determined by both quenching of DPH fluorescence and direct assessment of the drug associated with the mitochondrial pellet (Figure 9). These results are consistent with earlier suggestions (Nicolay et al., 1984; Goormaghtigh et al., 1980; Henry et al., 1985) that the protonated amino group of adriamycin (pK of 7.6–8.2, depending on the ionic strength; Dalmark & Storm, 1981) interacts with the ionized phosphate residue of cardiolipin. Competition between the positively charged adriamycin and the H^+, K^+, or Ca^{2+} ions may account for the decreased binding observed at the higher pH value and in the high-salt medium.

It is interesting to note that the quenching profiles for DPH fluorescence in mitochondria look very similar to those for the DMPC/CL system. Of the total phospholipid content, the inner mitochondrial membrane has been reported to contain 24% and 20% cardiolipin for liver and heart, respectively (Cheneval et al., 1985). A 2:1 molar electrostatic complex of adriamycin and cardiolipin, which is stabilized by the electronic interactions between the anthracycline rings, has been proposed to exist in both synthetic and mitochondrial membranes (Nicolay et al., 1984; Goormaghtigh et al., 1980). In fact, Carafoli and co-workers have suggested the use of adriamycin as a specific probe for determining the transversal distribution of cardiolipin in the inner mitochondrial membrane of liver and heart (Cheneval et al., 1985). Our data are in agreement with these studies in that they indicate that cardiolipin provides an important site of binding for the anthracycline drugs.

In addition to lipids, we find that anthracyclines interact also with proteins. The fluorescence of tryptophan was quenched by all three drugs in liver and heart mitochondria. There was a small decrease in the quenching of tryptophan fluorescence in the high-salt medium, which suggests that binding of these drugs to proteins may also include an electrostatic component. This is not unreasonable, since the carboxylic group of amino acids and negatively charged sugar residues on membrane glycoproteins may be involved in such interactions.

Thus far in our discussion we have assumed that the quenching of DPH and tryptophan fluorescence occurs only by a static mechanism; i.e., the quenching results because the donor and acceptor are within the interaction radius at the time of excitation. We believe that a dynamic process, in which diffusion of the acceptor into the sphere of interaction of the donor occurs during the lifetime of the excited state, does not influence the quenching. The reasons for this are as follows. First, the fluorescence lifetimes of DPH and tryptophan in mitochondria, while not a single exponential, are less than 10 ns (our unpublished observations). Since the lateral diffusion coefficient of adriamycin in membranes above the phase transition is approximately 1.0×10^{-8} cm^2/s (Figure 6), one can calculate that within the lifetime of the excited state of the donor, the probability of drug molecules, which were initially outside the sphere of interest, diffusing into the quencher radius is very small (<1%). Second, plots of F_0/F

(fluorescence intensity in the absence of quencher/fluorescence intensity in the presence of quencher) vs. added anthracycline are linear (Figure 7). If *both* static quenching and dynamic quenching were occurring, an upward curvature would be expected (Lakowicz, 1983).

Accepting the fact that the mitochondrion is an extremely complex membrane system, we can, nevertheless, ask whether the results presented in Figure 7 indicate preferential binding to lipid or protein. Shaklai et al. (1977) have dealt with the general problem of energy transfer between a probe randomly adsorbed to the surface of a membrane and a buried chromophore. Under conditions of excitation with a steady beam of light, the quenching profile can be described by

$$F_0/F = 1 + K_qA \quad (1)$$

where A is the density of quencher molecules on the membrane. The value of K_q, the effective quencher constant, is derived by these authors to be

$$K_q = \pi R_0^6/2R_m^4 \quad (2)$$

where R_m is the effective minimal distance. It then follows that

$$\frac{K_q(\text{DPH})}{K_q(\text{Trp})} = \frac{R_0^6(\text{DPH})/R_m^4(\text{DPH})}{R_0^6(\text{Trp})/R_m^4(\text{Trp})} \quad (3)$$

If the effective minimal distances of adriamycin to DPH and tryptophan are the same, eq 3 predicts that the ratio of the slopes should be about 7. The ratio of the slopes for DPH to tryptophan is 16; thus, we conclude that tryptophan is less accessible to adriamycin than DPH. Until there is more detailed knowledge about the location of tryptophan in mitochondrial proteins, the most straightforward explanation is that adriamycin binds preferentially to lipids.

The considerations presented above may offer some insight into the possible mechanism(s) of transport of these drugs across plasma and mitochondrial membranes. Three alternatives have been entertained recently to explain the accumulation of anthracyclines by cells: passive diffusion through the lipid bilayer with or without the selective removal of the drugs via an efflux pump or facilitated diffusion involving a protein (Skovsgaard & Nissen, 1982; Siegfried et al., 1985). Although adriamycin and its analogues can reside in proximity of membrane proteins, the preferential binding of these drugs to lipids and their ability to interact with the hydrophobic domain of the bilayer lend support to the hypothesis that anthracyclines diffuse through the membrane passively.

It has been suggested that damage to heart mitochondria induced by adriamycin is responsible for the cardiotoxicity of the drug (Goormaghtigh & Ruysschaert, 1984). Although our results do not provide evidence for or against such a possibility, they do show clearly that adriamycin does not bind preferentially to heart as compared to liver mitochondria. Moreover, two adriamycin analogues, which are thought to be less cardiotoxic, exhibit the same binding properties. Hence, if heart mitochondria are indeed the preferred target for adriamycin, the amount of the drug that is bound does not seem to be related directly to the damage that it causes.

Registry No. Adriamycin, 23214-92-8; 4′-epidoxorubicin, 56420-45-2; 4′-deoxydoxorubicin, 63521-85-7.

REFERENCES

Andrich, M. P., & Vanderkooi, J. M. (1976) *Biochemistry 15*, 1257–1261.

Barthelemy-Clavey, V., Maurizot, J.-C., Dimicoli, J.-L., & Sicard, P. (1974) *FEBS Lett. 46*, 5–10.

Bonfante, V. (1981) in *Anthracyclines: Current Status and Future Developments* (Mathe, G., Maral, R., & De Jager, R., Eds.) pp 149–152, Masson, New York.

Burke, T. G., & Tritton, T. R. (1984) *Anal. Biochem. 143*, 135–140.

Burke, T. G., & Tritton, T. R. (1985a) *Biochemistry 24*, 1768–1776.

Burke, T. G., & Tritton, T. R. (1985b) *Biochemistry 24*, 5972–5980.

Casazza, A. M., Di Marco, A., Bertazzoli, C., Formelli, F., Giuliani, F., & Pratesi, G. (1978) *Curr. Chemother. 2*, 1257–1260.

Casazza, A. M., Di Marco, A., Bonadonna, G., Bonfante, V., Bertazzoli, C., Bellini, O., Pratesi, G., Sala, L., & Ballerini, L. (1980) in *Anthracyclines: Current Status and New Developments* (Crooke, S. T., & Reich, S. D., Eds.) pp 403–430, Academic Press, New York.

Chaires, J. B., Dattagupya, N., & Crothers, D. M. (1982) *Biochemistry 21*, 3927–3932.

Cheneval, D., Miller, M., Toni, R., Ruetz, S., & Carafoli, E. (1985) *J. Biol. Chem. 260*, 13003–13007.

Dalmark, M., & Storm, H. H. (1981) *J. Gen. Physiol. 78*, 349–364.

Förster, T. (1948) *Ann. Phys. (Leipzig) 2*, 55–75.

Fragata, M., Ohnishi, S., Asada, K., Ito, T., & Takahashi, M. (1984) *Biochemistry 23*, 4044–4051.

Goormaghtigh, E., & Ruysschaert, J. M. (1984) *Biochim. Biophys. Acta 779*, 271–288.

Goormaghtigh, E., Chatelin, P., Caspero, J., & Ruysschaert, J. M. (1980) *Biochim. Biophys. Acta 597*, 1–14.

Gornall, A. G., Bardawill, C. J., & David, A. M. (1949) *J. Biol. Chem. 177*, 751–766.

Henry, N., Fantine, E. O., Bolard, J., & Garnier-Suillerot, A. (1985) *Biochemistry 24*, 7085–7092.

Karczmar, G. S., & Tritton, T. R. (1979) *Biochim. Biophys. Acta 557*, 306–319.

Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, pp 257–301, Plenum Press, New York.

Longworth, J. W. (1971) in *Excited States of Proteins and Nucleic Acids* (Steiner, R. F., & Weinryb, I., Eds.) pp 319–484, Plenum Press, New York.

Melhuish, W. H. (1961) *J. Phys. Chem. 65*, 229–235.

Nicolay, K., Timmers, R., Spoelstra, E., Van der Neut, R., Fok, J. J., Huigen, Y. M., Verkleij, A. J., & De Kruijff, B. (1984) *Biochim. Biophys. Acta 778*, 359–371.

Samuni, A., Chong, P. L.-G., Barenholz, Y., & Thompson, T. E. (1986) *Cancer Res. 46*, 594–599.

Schneider, M. B., & Webb, W. W. (1981) *Appl. Opt. 20*, 1382–1388.

Schneider, W. C. J. (1948) *J. Biol. Chem. 176*, 259–266.

Shaklai, N., Yguerabide, J., & Ranney, H. M. (1977) *Biochemistry 16*, 5585–5592.

Shinitzky, M., & Inbar, M. (1974) *J. Mol. Biol. 85*, 603–615.

Siegfried, J. M., Burke, T. G., & Tritton, T. R. (1985) *Biochem. Pharmacol. 34*, 593–598.

Skovsgaard, T., & Nissen, N. I. (1982) *Pharmacol. Ther. 18*, 293–311.

Tyler, D. D., & Gonze, J. (1967) *Methods Enzymol. 10*, 75–77.

Vanderkooi, J. M., Maniara, G., & Erecińska, M. (1985) *J. Cell Biol. 100*, 435–441.

Weast, R. C., Ed. (1977) *CRC Handbook of Chemistry and Physics*, 58th ed., pp D216–D217, CRC Press, West Palm Beach, FL.