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DNA-Containing Liposomes as a Model for the Study of Cell Membrane Permeation by Anthracycline Derivatives†

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ABSTRACT: The uptake of anthracycline derivatives into large unilamellar vesicles (LUV) in response to a driven force provided by DNA encapsulated inside the LUV has been investigated. Four anthracyclines have been used: adriamycin, 4′-*O*-tetrahydropyranyladriamycin (THP-ADR), daunorubicin (DNR), and carminomycin. No quenching of the drug fluorescence is observed through interaction of the drugs with the lipidic bilayer. Rapid quenching of drug fluorescence occurs when drugs intercalate between the base pairs of DNA. The kinetics of the decay of anthracycline fluorescence in the presence of DNA-containing liposomes can thus be used to follow the diffusion of the drug through the membrane. The initial rates of uptake, as a function of pH, and lipid bilayer permeability coefficients have been calculated for the neutral forms of THP-ADR and DNR. This system suggests that anthracycline may gain access to cells by passive diffusion of the neutral form of the drug under the action of a driven force provided by DNA in the nucleus.

The anthracycline antibiotic adriamycin (ADR)[1] is one of the most potent anticancer drugs in clinical use. It is active against a wide range of malignancies, including sarcomas, carcinomas, melanomas, leukemias, and lymphomas (Arcamone, 1981). Other anthracycline derivatives such as daunorubicin (DNR), THP-adriamycin (THP-ADR), and carminomycin (CAR) also have outstanding antitumor activity. One of the major obstacles of chemotherapy is that, after repeated treatments, cellular resistance to the drug appears. A particular phenotype of resistant cells, called multidrug resistance (MDR), has been recognized and encompasses a broad pattern of resistance to anticancer drugs derived from natural products (Bradley & al., 1988; Kessel, 1988). A common feature of MDR cells is a net decrease intracellular accumulation of drug that has been ascribed to an increased efflux pump mechanism (Dano, 1973; Inaba & Johnson, 1977; Skovsgaard, 1978; Riordan & Ling, 1985) and associated with the overexpression of an integral membrane glycoprotein, the P-glycoprotein (Juliano & Ling, 1976; Beck et al. 1979; Riordan & Ling, 1979). It has been proposed that P-glycoprotein may function as an energy-dependent drug efflux pump.

The determination of the precise role of P-glycoprotein involves the determination and the comparison of the mechanism of drug uptake and release by resistant and sensitive cells. We have recently developed a new spectrofluorometric method based on the observation that the fluorescence of an

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[1] ADR, adriamycin; THP-ADR, 4′-*O*-tetrahydropyranyladriamycin; CAR, carminomycin; DNR, daunorubicin; LUV, large unilamellar vesicles; PC, egg phosphatidylcholine; PA, egg phosphatidic acid; PS, phosphatidylserine; CHOL, cholesterol; FCCP, carbonyl cyanide [*p*-(trifluoromethoxy)phenyl]hydrazone.

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anthracycline molecule is quenched by intercalation between the base pairs of DNA but not by interaction with the membrane (Tarasiuk et al. 1989; Frezard & Garnier-Suillerot, 1991a,b). We have shown that the monitoring of the decay of fluorescence of anthracycline in the presence of cells is a means to measure the kinetics as well as gain insight into the mechanisms of drug transport through the plasma membrane. We have thus shown that the transport of anthracycline occurs by passive diffusion of the neutral form of the drug through the lipid bilayer.

Once inside the cell, the drugs intercalate between the base pairs of DNA in the nucleus, which are strong binding sites for these molecules. This suggests that anthracycline may gain access to cells by the action of a driven force provided by DNA. This gave us the idea that a very simple system composed of DNA encapsulated into large unilamellar vesicles could mimic cells and be used to study the mechanism of diffusion of anthracycline derivatives through a lipid bilayer under the effect of a driven force provided by DNA.

In this paper we present this model and use it to establish a lipid bilayer coefficient for the permeation of daunorubicin and THP-adriamycin.

Materials and Methods

Drugs and Chemicals. Purified adriamycin and 4′-*O*-tetrahydropyranyladriamycin were kindly provided by Laboratoire Roger Bellon (France); daunorubicin and carminomycin were from Laboratoire Rhône-Poulenc (France). Concentrations were determined by diluting stock solutions to approximately 10^{-5} M and using $\epsilon_{480} = 11\,500\ M^{-1}\ cm^{-1}$. As anthracyclines are sensitive to light and oxygen, stock solutions were prepared just before use. Egg phosphatidylcholine, egg phosphatidic acid, phosphatidylserine, cholesterol, calf thymus DNA, valinomycin, gramicidin, carbonyl cyanide [*p*-(trifluoromethoxy)phenyl]hydrazone (FCCP) and deoxyribonuclease (DNase I) were from Sigma. Carboxyfluorescein was from Eastman Kodak. Unless otherwise stated the experiments were performed in PBSK buffer: 2.25 mM NaH_2PO_4/7.7 mM Na_2HPO_4/0.15 M KCl at pH ranging from 6 to 8.5. All other reagents were of the highest quality available, and deionized double-distilled water was used throughout the experiments.

Absorption spectra were recorded on a Cary 219 spectrophotometer and fluorescence spectra on a JY 3CS spectrofluorometer. Experiments were conducted in a 1-cm quartz cuvette containing 2 mL of buffer. The temperature was controlled by use of a circulating thermostated water bath. Potentiometric measurements were performed with a Metrohm pH meter, Model E603, at 37 °C using a Metrohm EA 147 combined glass electrode.

Preparation of DNA-Containing Liposomes. DNA was dissolved in PBSK buffer at pH 7.2. The base pairs concentration of the solution was determined by measuring the optical density at 260 nm and using $\epsilon_{260} = 13\,200\ cm^{-1}\ M^{-1}$ (bp). In the following, a 2.5 mM DNA (bp) solution was used. In order to get a solution of homogeneous small fragments of DNA, the solution was sonicated for 10 min at 0 °C (power 2) and filtered with a 0.2-μm pore size filter.

Liposomes were prepared as described previously (Barchfeld & Deamer, 1988). Egg phosphatidylcholine, phosphatidic acid, and cholesterol were combined at a PC/PA/CHOL molar ratio equal to 78/2/20. The small amount of PA was added in order to avoid LUV aggregation. Organic solvents were removed by evaporation under nitrogen gas and vacuum. A 100 μmol phospholipid containing film was obtained. The lipids were redissolved with 6 mL of diethyl ether and 2.5 mL of DNA solution. The suspension was sonicated for 2 min at 0 °C to produce a homogeneous dispersion, and then the diethyl ether phase was removed by rotatory dispersion under reduced pressure for about 30 min according to the method described by Szoka et al. (1980). The suspension thus obtained was diluted by addition of buffer to obtain a final volume of 2.5 mL. To eliminate the nonencapsulated DNA, the liposome suspension was passed over Sephacryl S 1000 that was equilibrated with the buffer solution. The dimensions of the column were 70-cm height and 2-cm diameter. The rate of flow was 0.5 mL/min. The optical density at 260 nm was recorded for each eluted fraction (2 mL). The plot of the optical density at 260 nm as a function of the elution volume shows two peaks: peak I (elution volume equal to 55 mL), corresponding to DNA encapsulated into the LUV, and peak II (elution volume equal to 85 mL), corresponding to free DNA. This was confirmed by the following procedure: 2 mL of a 1 μM adriamycin solution was placed in the cuvette of the spectrofluorometer. The temperature was regulated at 4 °C. A 200-μL sample of each eluted fraction was added to the solution under continuous stirring. This resulted in quenching of adriamycin fluorescence, which was monitored as a function of time at $\lambda_{em} = 590$ nm through excitation at 480 nm. It is well documented that the intercalation of adriamycin between the base pairs of DNA occurs within less than 1s. The addition of fractions corresponding to peak II yielded an immediate quenching of adriamycin fluorescence and the subsequent addition of 0.05% Triton X-100 did not modify fluorescence intensity. We thus inferred that free DNA was present in fractions corresponding to peak II. However, when fractions corresponding to peak I were added to the adriamycin solution, the quenching of fluorescence occurred very slowly and the subsequent addition of 0.05% Triton X-100 yielded an immediate quenching of fluorescence. We thus inferred that these fractions corresponded to DNA-containing liposomes and that the phospholipid bilayer was permeabilized by the addition of Triton X-100.

Determination of the Amount of DNA Encapsulated inside the LUV. A total of 2 mL of 1 μM adriamycin was placed in the cuvette (t = 25 °C) in the spectrofluorometer. Small aliquots of the DNA solution, used to prepare DNA-containing liposomes, were added stepwise to the adriamycin solution resulting in quenching of the fluorescence intensity at 590 nm ($\lambda_{ex} = 480$ nm). A calibration curve was obtained by plotting the fluorescence intensity at 590 nm as a function of DNA concentration (bp). In exactly the same conditions, a small and known amount of the suspension of DNA-containing liposomes (peak I) was added to a 1 μM adriamycin solution in the presence of Triton X-100. With use of the calibration curve, the decrease of fluorescence intensity at 590 nm allowed the determination of the overall concentration of DNA (C_e) encapsulated into the LUV.

Determination of the Concentration of Phospholipid. The method of Marshall Stewart (1980) was used to determine the concentration of phospholipid (C_{PL}) in peak I. We have checked that the presence of DNA does not interfere with this measurement.

Determination of the Volume V_e of the DNA Solution Encapsulated inside the LUV. The DNA solution used to prepare DNA-containing liposomes was 2.5×10^{-3} M (bp). This means that the local concentration of DNA inside the LUV was $C_i = 2.5 \times 10^{-3}$ M. The overall DNA concentration C_e determined as described above was equal to C_iV_e. Knowing $V_e = C_e/C_i$ and C_{PL}, we calculated the mean volume encapsulated per phospholipid $\langle V_e \rangle = V_e/C_{PL}$. We obtained $\langle V_e \rangle$

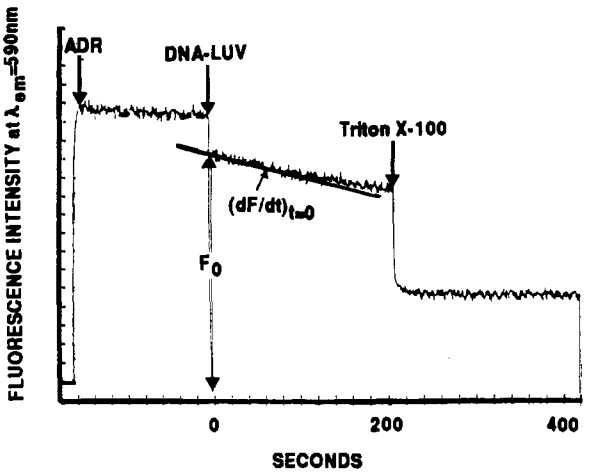


FIGURE 1: Fluorometric determination of the kinetics of uptake of anthracycline derivatives by DNA-containing liposome. The fluorescence intensity F at 590 nm (λ_{ex} = 480 nm), is recorded as a function of time. A total of 2 mL of a 1 μM anthracycline solution in PBSK buffer is placed in a cuvette under vigorous stirring. At t = 0, 400 μL of DNA containing LUV is added yielding a C_T = 0.83 μM anthracycline solution; the fluorescence is then F_0. The slope of the tangent to the curve $F = f(t)$ at t = 0 is $(dF/dt)_{t=0}$, and the initial rate of uptake $V_+ = (dF/dt)_{t=0}C_T/F_0$ (M s^{-1}). After about 5 or 10 min, in order to check the amount of DNA present, 0.05% (w/v) of Triton X-100 is added.

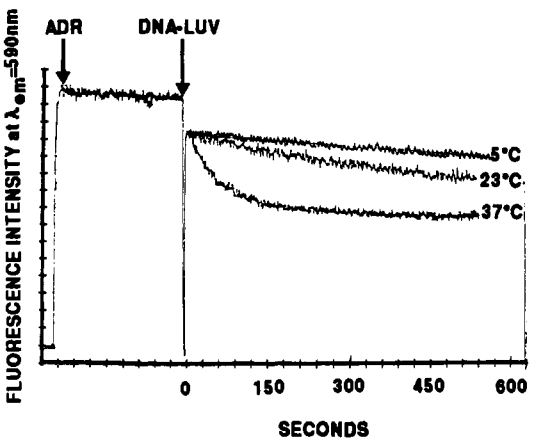


FIGURE 2: Kinetics of uptake of adriamycin by DNA-containing liposome as a function of temperature. A total of 2 mL of a 1 μM adriamycin solution in PBSK buffer at pH 7.2 was placed in the cuvette at the desired temperature. At t = 0, 400 μM DNA-containing LUV suspension was added. The final concentrations were 0.83 μM ADR and 0.2 mM PC.

$\approx$ 7 μL/mg of PC. This is in agreement with previous authors who reported values between 5 and 15 μL/mg of phospholipid for liposomes prepared using the reverse-phase method.

Determination of the Initial Rate of Uptake of the Drugs by DNA-Containing Liposomes. Anthracycline uptake by DNA-containing liposomes was followed by use of a new fluorometric method developed to follow the uptake of anthracycline by cells (Tarasiuk et al., 1989; Frezard & Garnier-Suillerot, 1991a,b). With use of this method, it is possible to follow the kinetics of drug uptake during the incubation with drugs. This method is based on the observations (i) that fluorescence of anthracycline is only quenched by intercalation of the drug between the base pairs of DNA and (ii) that transport across the lipid bilayers is the rate-limiting step, as fluorescence quenching occurs immediately after the bilayer is permeabilized by the addition of Triton X-100.

All experiments were conducted in 1-cm quartz cuvette. In a typical experiment, 2 mL of a 1 μM anthracycline solution PBSK buffer was placed in the cuvette. A total of 400 μL of the suspension of DNA-containing liposomes was quickly added. The concentrations of the various components in the solution were [PC] = 0.2 mM, [DNA] = 2.5 μM (bp), C_T = [anthracycline] = 0.83 μM. The decrease of fluorescence intensity F at 590 nm was followed as a function of time. The initial rate of uptake V_+ can be determined from the equation $V_+ = (dF/dt)_{t=0}C_T/F_0$ where $(dF/dt)_{t=0}$ is the slope of the tangent to the curve $F = f(t)$. As shown in Figure 1, F_0 is the fluorescence intensity at t = 0 of a C_T μM anthracycline solution. The step decrease of the fluorescence intensity obtained through addition of liposome was due to the dilution. In order to eliminate pH and potential gradients, experiments were performed in the presence of FCCP and valinomycin.

We have checked that DNA was well entrapped into liposomes by the following experiment. ADR (1 μM) was incubated with DNA-containing LUV at 37 °C. Once the steady state was reached (after about 300 s, as shown in Figure 2) DNase I (1 mg/mL) was added to the solution. This did not yield modification of the fluorescence signal. However, the subsequent addition of Triton X-100 gave rise to a recovering of the initial fluorescence signal. In that case permeable liposomes admitted DNase, which hydrolyzed the entrapped DNA.

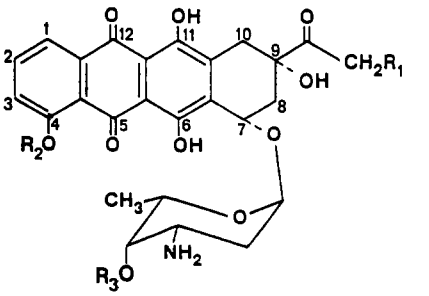
We have checked that entrapped DNA does not perturb the permeability properties of the bilayer by examination of the efflux of entrapped carboxyfluorescein. It is well known that the fluorescence of carboxyfluorescein is quenched at high dye concentrations inside the vesicles but appears when the dye is released and diluted into the external medium (Weinstein et al., 1981). Carboxyfluorescein (40 mM) was entrapped, in either the presence or absence of 2.5 mM DNA (bp), by use of the method described above. A total of 2 mL of PBSK buffer solution was placed in the cuvette of the spectrofluorometer. The temperature was controlled to 25 °C. A 50 μL sample of the eluted fraction was added to the solution under continuous stirring, and the fluorescence intensity at 520 nm (λ_{ex} = 490 nm) was followed as a function of time. Under these conditions, no modifications of the fluorescence intensity, ie., no release of carboxyfluorescein, were observed in both cases. In the presence of gramicidin, a slow release of carboxyfluorescein was observed. In both cases, within 10 min about 10% of the dye entrapped was released. No significant difference was observed in the presence or absence of entrapped DNA.

Results

The structures of the four anthracycline derivatives used in the present study are shown in Scheme I. At acidic pH, the four drugs are once positively charged at the level of the amino group on the sugar. We have previously determined the pK_a of deprotonation of these drugs (Frezard & Garnier-Suillerot, 1990). AT 37 °C the pK_a of deprotonation of the amino group is 8.6 for ADR, DNR, and CAR and 7.7 for THP-ADR. In addition, the carminomycin molecule bears a hydroxyl group on C_4 whose pK_a of deprotonation is 7.6 at 37 °C. The other hydroxyl groups present in positions C_6 and C_{11} undergo deprotonation at pH higher than 10, and therefore, they will not be deprotonated in the pH range 6–8 used in the following study. In the pH range 6–8, the anthracycline derivatives are either positively charged or neutral (zwitterionic in the case of CAR).

Most of the experiments were performed in order to measure the kinetics of uptake of the drugs as a function of the concentration of drug either in the neutral or in the positively charged form. However, the kinetics of uptake depends not

Scheme I



	R_1	R_2	R_3
Adriamycin	OH	CH_3	H
Daunorubicin	H	CH_3	H
Carminomycin	H	H	H
THP-Adriamycin	OH	CH_3	

Table I: Initial Rate V_+ of Uptake of Anthracycline Derivatives by DNA-Containing LUV[a]

drug	ADR	DNR	THP-ADR	CAR
V_+ (nM s^{-1}) at				
5 °C	0.06	1.7	22	25
23 °C	0.4	3.0		
37 °C	4.2			
P_+^0 (cm s^{-1})		2.6×10^{-4}	2.7×10^{-4}	

[a] Experimental conditions: PBSK buffer at pH 7.2; [DNA] 2.5 μM (bp) inside the LUV; [PC] = 0.22 mM; [Drug] = 1 μM, t = 5 or 23 or 37 °C. P_+^0 is the permeability constant for the neutral form of the drug.

only on the pH but also on the temperature and on the nature of the anthracycline derivatives. A first set of experiments was performed to determine the influence of the temperature on the kinetics of uptake of adriamycin and daunorubicin.

Kinetics of Uptake of ADR and DNR at pH 7.2 as a Function of Temperature. Figure 2 shows typical results of experiments performed with ADR at pH 7.2 at 37, 23, and 5 °C, respectively. At these temperatures, the PC bilayer is in the liquid crystal state, as the temperature of transition is lower than 0 °C. The kinetics of uptake greatly increased as the temperature increased. The values of the initial rates of uptake of ADR and DNR at these temperature are shown in Table I.

Kinetics of Uptake of THP-ADR and CAR by DNA-Containing LUV at pH 7.2 and at 5 °C. Strictly analogous experiments were performed with THP-ADR and CAR. The values of the initial rates of uptake determined at 5 °C are reported in Table I. As can be seen, the kinetics of uptake follows the order V_+(ADR) < V_+(DNR) < V_+(THP-ADR) < V_+(CAR).

At higher temperatures, the kinetics of uptake was so rapid, especially in the cases of THP-ADR and CAR, that it was impossible to record the kinetics. In order to get accurate measurements, most of the experiments were performed at 5 °C.

Kinetics of Uptake of THP-ADR and DNR by DNA-Containing LUV as a Function of pH. The pH of the buffer was varied from 6 to 8, and these experiments were performed at 5 °C. For each pH value, the initial rate of uptake V_+ of the drug was recorded as described in Figure 1. Figure 3 shows the plot of V_+ as a function of pH, for THP-ADR and DNR.

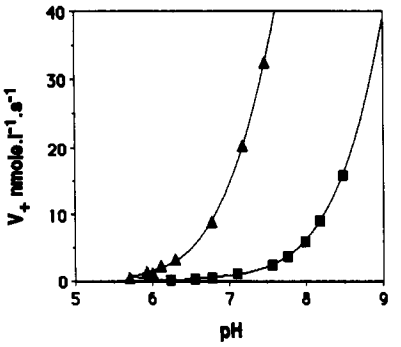


FIGURE 3: Kinetics of uptake of THP-ADR and DNR by DNA-containing LUV as a function of pH. The initial rate of uptake V_+ of THP-ADR (▲) and DNR (■) has been plotted as a function of pH. Experimental conditions: 2 mL of a 1 μM THP-adriamycin solution in PBSK buffer was placed in the cuvette thermostated at 5 °C. At t = 0, 400 μL of the DNA-containing LUV suspension was quickly added. The final concentrations were 0.83 μM THP-ADR and 0.2 mM PC, and the pH was varied from 6.5 to 8.

As can be seen in both cases V_+ increased as pH increased, suggesting that V_+ mainly depends on the concentration of drug in the neutral form.

Discussion

The problem of uptake and release of drugs, including anthracycline derivatives, by tumor cells is very important. This problem is particularly crucial in the phenomenon of resistance to antitumor compounds because a common feature of MDR cells is a net decrease in intracellular accumulation of drug. If one wants to gain insight into the mechanism of uptake and release of drugs by cells, it is necessary to accurately determine the physicochemical parameters of the drugs and of the membrane that may influence the transport of the drug across the membrane.

Anthracycline derivatives used in this study are lipophilic amine compounds whose pK_a in aqueous solution range between 7.7 and 8.6. Therefore, in the pH range 6.8–8 used in the present work, they existed in two forms in equilibrium: neutral and cationic (Frezard & Garnier-Suillerot, 1990). The lipophilic properties of anthracycline can allow relatively free permeation through lipid bilayers.

The uptake of adriamycin by LUV has been accurately studied by Mayer et al. (Bally et al., 1990; Mayer et al., 1985, 1986, 1989). These authors have shown that, in LUV systems exhibiting pH gradients, rapid uptake levels resulting in high interior concentrations can be achieved. This results from rapid permeation of the neutral form of the lipophilic amine and subsequent transmembrane redistribution dictated by the Henderson–Hasselbach relation. Alternatively, uptake in response to ΔΦ transmembrane potential proceeds more slowly and cannot be directly accounted for by ΔΦ-induced pH gradients. In conclusion, the rate of uptake in response to ΔpH is much faster than that observed for ΔΦ, which is consistent with the relatively high membrane permeability of the neutral species. Thus, uptake of the protonated form may be sufficiently slow to escape detection.

The intracellular transport of anthracyclines is a complex process. Numerous experiments suggest that the uptake of anthracycline derivatives by cells occurs by passive diffusion of the neutral form of the molecule through the lipid domains of the plasma membrane (Dano, 1973; Skovsgard, 1980; Dalmark, 1981; Skovsgard & Nissen, 1982; Frezard & Garnier-Suillerot, 1991a,b). However, the affinity of the positively charged form of the drug for negatively charged phospholipids such as cardiolipin and phosphatidylserine

(Henry et al., 1985; Goormaghtigh & Ruysschaert, 1984; Garnier-Suillerot & Gattegno, 1988) should probably play a role in the uptake. On the other hand, once inside the cell, the distribution of anthracycline to intracellular organelles such as lysosomes and compartments has not been defined.

We have previously measured the initial rate of uptake of various anthracyclines by different types of cells where lipid composition should be different: lymphocytes (Tarasiuk et al., 1989), U937 (unpublished data), and K562 cells (Frezard & Garnier-Suillerot, 1991a,b) were used. In all three cases, the uptake occurs through diffusion of the neutral form of the drug and to our knowledge, up to now, the presence of specific transporters for anthracyclines has not been reported.

We have recently brought new insight to that problem (i) by measuring the initial rate of uptake of the drug by the cells at different pH values and showing that the rate was proportional to the concentration of drug in the neutral form and (ii) by observing that, at the steady state, in drug-sensitive cells, the concentration of drug in the neutral form outside the cell is the same as in the cytoplasm following a Henderson–Hasselbach distribution. This second observation is not verified in drug-resistant cells that possess in their membranes the P-glycoprotein, which actively pumps the drug out of the cell. Once inside the cell, anthracycline derivatives accumulate in the nucleus, which exhibits strong binding sites for these compounds.

In most cells, the pH gradient between the exterior of the cells and the cytoplasm is rather low, the cytoplamic pH value being about 7.2. This is, for instance, the value that has been determined for K562 cells (Frezard & Garnier-Suillerot, 1991a). This strongly suggests that the accumulation of drug inside the cells occurs under the effect of a driven force provided by DNA in the nucleus. Therefore, our idea was that uptake of anthracycline by cells could be mimicked by DNA encapsulated inside LUV.

The fluorescence intensity of anthracycline is only slightly modified through interaction with the phospholipid bilayer; the strong decrease of fluorescence intensity observed through interaction with DNA-containing LUV can unambiguously be assigned to the intercalation of the drug between the base pairs of DNA. The fluorescence signal measured can thus be assigned to anthracycline molecules that are not intercalated between the base pairs of DNA, i.e., (i) extravesicular free drug, (ii) intravesicular free drug, (iii) drug molecules bound to the phospholipid bilayer, or (iv) drug molecules bound to the negatively charged phosphate of DNA but not intercalated. Taking into account the points (i) that the ratio of the total volume of the solution to the volume encapsulated into the LUV is high ($\sim$1000), (ii) that the affinity of anthracycline for "external" binding sites of DNA is low as compared to the affinity for intercalative binding sites, and (iii) that the affinity of anthracycline for the neutral phospholipids PC is low and in our assays the concentration of phospholipid is low, we can infer that in the initial conditions the fluorescence intensity measured is that of the free extravesicular anthracycline and that the decrease of fluorescence observed during the first minutes can be assigned to the decrease of the free extravesicular drug concentration.

In these conditions

$$V_+ = (dC_T/dt)_{t=0} = (dF/dt)_{t=0}C_T/F_0$$

Within our experimental conditions, the interaction of anthracycline with naked DNA is instantaneous ($\Delta t < 1$ s). When Triton X-100, which permeabilizes the lipid bilayer, is added to DNA-containing LUV, the quenching of fluorescence intensity of anthracycline occurs at once. From these observations, we inferred that the transport of anthracycline through the lipid bilayer is the limiting step of the measured kinetics.

At the begining of the interaction of DNA-containing LUV with drug ($t = 0$), the drug concentration in the compartment toward which net flow is proceeding is zero. In this situation, the flux J_+ in the case of simple diffusion is given by the equation (Fick's law)

$$J_P = P_+^0[D^0]$$

where P_+^0 is the permeability constant for the neutral form of the drug and $[D^0]$ the neutral drug concentration. The initial rate of uptake is then

$$(V_+)_{t=0} = J_+S_T$$

where S_T is the surface of bilayer exchange

$$(V_+)_{t=0} = (P^0{}_+S_T)[D^0]$$

S_T was calculated by use of data in the literature (Szoka et al., 1980); values of 55 Å^2 and 38 Å^2 per polar head of phosphatidylcholine (or phosphatidic acid) and cholesterol, respectively, have been used.

In the case of 0.2 mM PL and 0.02 mM CHOL concentrations, the total liposome surface area in a 1-cm^3 suspension is 730 cm^2. Taking into account that we are dealing with a bilayer, the area of lipids for the influx is $S_T = 365$ cm^2 in 1 cm^2 of suspension.

The concentration of drug in the initial form $[D^0]$ was calculated using the Hendersen–Hasselbach equation

$$pH = pK_a + \log [D^0]/[DH^+]$$

where DH^+ stands for the drug in the protonated form, pK_a is the pK_a of deprotonation of the drug at 5 °C. This yields

$$[D^0] = C_T/(1 + 10^{pK_a-pH})$$

with $C_T = [D^0] + [DH^+]$.

The expression of J_+ as a function of pH is given by the equation

$$J_+ = P_+^0C_T/(1 + 10^{pK_a-pH}) \quad (1)$$

where J_+ is expressed in mol s^{-1} cm^{-2}, C_T (the total concentration of drug) is in mol cm^{-3}, and P_+^0 is in cm s^{-1}.

The equation can be written as

$$J_+ = -10^{pK_a}(J_+ \times 10^{-pH}) + P_+^0C_T$$

and the plot of J_+ as a function of $J_+ \times 10^{-pH}$ yields P_+^0 and pK_a.

Figure 4 shows such as a plot in the case of THP-ADR and DNR. One obtains for THP-ADR $P_+^0 = 2.7 \times 10^{-4}$ cm s^{-1} and $pK_a = 7.7 \pm 0.1$ and for DNR $P_+^0 = 2.6\ 10^{-4}$ CM S^{-1} and $pK_a = 8.9 \pm 0.1$.

The pK_a of deprotonation of DNR has been measured by potentiometric titrations at 25 and 37 °C. Values equal to 8.6 (Kiraly & Martin, 1982) and 8.4 (Frezard & Garnier-Suillerot, 1990) have been obtained, respectively. Using van't Hoff's equation, we have calculated the pK_a value that can be expected at 5 °C: a value of 9.0 was found in agreement with the value that we have obtained. In the case of THP-ADR, we have previously determined the pK_a at 25 and 37 °C (Frezard & Garnier-Suillerot, 1990). The same value, 7.7, has been obtained experimentally at both temperatures. The value 7.7 ± 0.1 obtained at 5 °C is thus in agreement with these data.

In conclusion, in this work, we demonstrate that anthracycline derivatives can rapidly accumulate into DNA-containing LUV systems, exhibiting neither a membrane potential nor a pH gradient. This leads to the suggestion that anthracycline may gain access to cells by passive diffusion of the

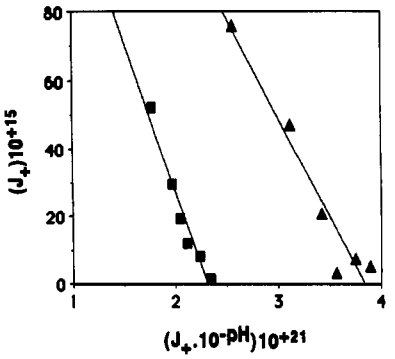


FIGURE 4: Determination of the permeability coefficient P_+^0 for the neutral form of DNR (▲) and THP-ADR (■). The flux J_+ determined at various pH values has been plotted as a function of $J_+ \times 10^{-pH}$. The experimental conditions are the same as those described in Figure 3. J_+ is expressed in mol cm^{-2} s^{-1}. The lines drawn have been least-squares fitted to the data. The correlation coefficients are higher than 0.93.

neutral form of the drug under the action of a driven force provided by DNA in the nucleus: a mechanism that does not depend on the presence of specific carrier proteins. In addition, such a simple system allows the determination of physicochemical parameters such as permeability coefficients.

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