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# EVIDENCE OF A SPECIFIC COMPLEX BETWEEN ADRIAMYCIN AND NEGATIVELY-CHARGED PHOSPHOLIPIDS

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## Summary

Membrane-model systems (monolayers, small unilamellar vesicles) were used to study the interaction between adriamycin (ADM) and phospholipids.

Adsorption of $^3$H-labeled adriamycin on different phospholipid monolayers demonstrated the specificity of adriamycin for negatively-charged phospholipids (cardiolipin, phosphatidylserine, phosphatidic acid). The stoichiometry has been found to be approx. 2 mol (1.8) adriamycin per mol cardiolipin and approx. 1 mol (0.75) adriamycin per mol phosphatidylserine and phosphatidic acid. No adsorption was detected with neutral lipids.

Surface-potential measurements confirm the formation of a complex stabilized by electrostatic interactions without penetration of the drug into the lipid lipophilic phase.

Some adriamycin derivatives were used to discriminate between the ionized hydrophilic and hydrophobic contributions in the complex formation.

The absorption spectrum of adriamycin in the presence of cardiolipin resembles the behavior of the ADM-DNA complex. Moreover, the association constants of the two complexes are very similar (cardiolipin-ADM, $1.6 \cdot 10^6 \cdot M^{-1}$; ADM-DNA, $2.4 \cdot 10^6 \cdot M^{-1}$).

To explain the high affinity of cardiolipin for adriamycin, we proposed that two essential interactions are responsible for the complex stabilization: an electrostatic interaction between the protonated amino groups of the sugar residues and the ionized phosphate residues, and an interaction between adjacent anthraquinone chromophores. These data strongly suggest competitive behavior between a membrane site and the target. Consequently, it must be assumed that the lipidic components of the cell membrane structure may be an impor-

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tant determinant in the behavior of adriamycin. This observation should be kept in mind in the building of new derivatives.

## Introduction

The anthracycline aminoglycosides play a prominent role in the treatment of leukemias and solid tumors in man [1,2]. Among this class of compounds, adriamycin (Fig. 1) is one of the most promising. Its mechanism of action is considered to involve an intercalation in DNA [3] and subsequent inhibition of DNA and RNA synthesis [4,5]. However, adriamycin cardiotoxicity places a limit on the total dose of adriamycin that may be given since its toxicity is cumulative [6]. Histologically, cardiac changes due to adriamycin are mainly characterized by a disarray of myofilaments, vacuolization of sarcoplasmic reticulum and degeneration of mitochondria with increased electron density and myelin-like figures formation [7,8] generally related to the formation of a drug-lipid complex [9—11]. If the adriamycin mechanism of action is generally considered to involve intercalation into double-helical DNA, a number of observations have implicated a direct interaction of anthracycline drugs with cell membranes [12]. Moreover, studies on model membranes suggest that adriamycin exhibits a specific affinity to membrane lipid domains [13—15] and modifies the lipid thermotropic properties [16,17].

It is the purpose of the present paper to demonstrate in model membranes, the existence of a complex between adriamycin and acidic phospholipids. Lipids were spread at the air/water interface and adriamycin was injected into the aqueous subphase. Surface-potential and surface-radioactivity measurements allowed the definition of the specificity and stoichiometry of the complex formed. Adriamycin analogs were tested to discriminate between the hydrophobic contribution and the charge effects in the complex formation. Studies of the absorbance spectra of adriamycin incubated with small unilamellar vesicles allowed the definition of the interactions involved in the complex formation.

## Materials and Methods

DL-α-Dipalmitoyl phosphatidylcholine, phosphatidic acid and cardiolipin were purchased from Sigma Chemical Co. Phosphatidylserine was supplied by Koch Light Laboratories. Adriamycin, rubidazone and steffymycin (Fig. 1) were generously supplied by Dr. J. Hildebrand (Institut Jules Bordet) and by Dr. C. Deslover (Farmitalia). *N*-Acetyl-adriamycin was a gift of Drs. A. Trouet and R. Baurin (Laboratoire de Chimie Physiologique, Université de Louvain). Adriamycin was $^3H$-labeled in pure $^3H_2$ gas. To increase the rate of exchange between $^3H$ and $^1H$, the sample was subjected to a silent electric discharge. The $^3H$-labeled adriamycin was repurified by preparative thin-layer chromatography with a $CHCl_3/CH_3OH/H_2O$ mixture (65 : 25 : 4, v/v/v) as eluant. Specific activity of $^3H$-labeled adriamycin was 45.4 mCi/g.

Surface radioactivity was measured with a gas-flow counter [18]. The vibration-electrode technique was employed to measure the surface potential [19,

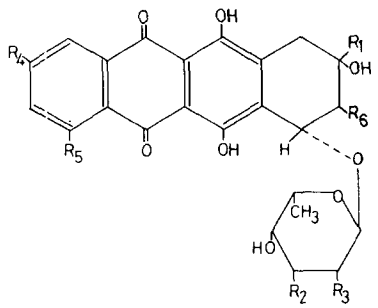

Fig. 1. Structure of adriamycin and its analogs.

| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | $R_6$ |
|---|---|---|---|---|---|---|
| (a) Adriamycin | $-CO-CH_2OH$ | $-NH_2$ | H | H | $-OCH_3$ | H |
| (b) *N*-Acetyl-adriamycin | $-CO-CH_2OH$ | $-NHCOCH_3$ | H | H | $-OCH_3$ | H |
| (c) Rubidazone | $-C(CH_3)=N-NHCO-C_6H_5$ | $-NH_2$ | H | H | $-OCH_3$ | H |
| (d) Steffimycin | $-CH_3$ | $-OH$ | $-OCH_3$ | $-OCH_3$ | H | $OCH_3$ |

20]. The accuracy is approx. ±5 mV. During the surface-potential measurements, the aqueous subphase was stirred but surface-radioactivity measurements were recorded, for technical reasons, without stirring.

Phospholipids were spread at the air-water interface from a $CHCl_3$ solution using an Agla Microlitre Syringe. Drugs were injected into the subphase at a final concentration of $1 \cdot 10^{-5}$ M. All experiments were carried out at 25°C.

All chemicals were of analytical grade and water was triple-distilled. Buffered solutions (Tris-HCl; $1 \cdot 10^{-2}$ M, pH 7.4) were used to prepare the subphase.

Absorbance spectra were recorded with a double-beam Unicam SP 1800 spectrophotometer using 1 cm and 1 mm path-length cells.

To prepare small unilamellar vesicles, lipids were dissolved in $CHCl_3$ in a spherical flask. The solution was evaporated to dryness and further dried under vacuum. Multilamellar vesicles were obtained by mechanical stirring (vortex mixer) of the lipid film in buffer at a temperature ($T_c$) above the corresponding lipid phase transition [21].

Small unilamellar vesicles were obtained by sonication of the multilamellar vesicle dispersion (Branson Sonifier B12). The temperature was kept above $T_c$ during sonication.

## Results

*Surface radioactivity*

Lipid monolayers (DL-α-dipalmitoyl phosphatidylcholine, phosphatidylserine, phosphatidic acid and cardiolipin) were spread at the air-water interface in the close-packed structure (60, 60, 60 and 120 Å$^2$/molecule, respectively). $^3$H-labeled adriamycin was injected into the aqueous subphase.

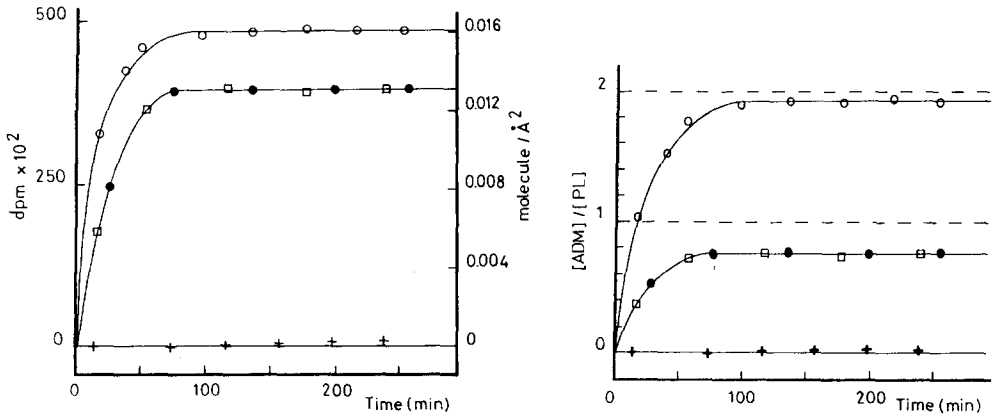


Fig. 2. Evolution as a function of time of the surface radioactivity (dpm/$10^2$) after injection of $^3$H-labeled adriamycin beneath lipid monolayers: cardiolipin, (○———○); phosphatidylserine, □———□; phosphatidic acid, (●———●); DL-α-dipalmitoyl phosphatidylcholine, (+———+). Subphase adriamycin concentration is $1 \cdot 10^{-5}$ M. Buffered solutions (Tris-HCl, pH = 7.4, $1 \cdot 10^{-2}$ M; KCl, $1 \cdot 10^{-1}$ M) were used as subphase. Temperature was kept constant at 25°C. The right-hand scale gives the corresponding surface concentration in molecules per $Å^2$.

Fig. 3. Evolution of the adriamycin : lipid ([ADM]/[PL]) ratio as a function of time. At equilibrium (100 min), the molar ratio of adriamycin to cardiolipin (○———○) is close to 2 : 1 (upper broken line) and the ratio of adriamycin to phosphatidylserine (□———□) and phosphatidic acid (●———●) is close to 1 : 1 (lower broken line). Experimental conditions are identical to those described in Fig. 2.

Typical plots of the increase of surface radioactivity as a function of time are given in Fig. 2. Adriamycin adsorption is related to the charge of the spread lipid. No adsorption was detected in the presence of the neutral DL-α-dipalmitoyl phosphatidylcholine monolayer, but the surface radioactivity increases with respect to time with negatively-charged lipids (cardiolipin, phosphatidylserine and phosphatidic acid).

From the surface radioactivity at equilibrium, the surface concentration of complexed adriamycin can be estimated. Indeed, the appendix demonstrates that the contribution of the uncomplexed adriamycin to the surface radioactivity is quite negligible compared to the contribution of the complexed adriamycin. The molar ratio of complexed adriamycin to the spread lipid has been found to be approx. 2 mol (1.8) adriamycin per mol cardiolipin and approx. 1 mol (0.75) adriamycin per mol phosphatidylserine and phosphatidic acid (Fig. 3). The fact that cardiolipin has two phosphate residues and phosphatidylserine and phosphatidic acid (PA) only one each suggests a charge-charge complex. It should be stressed at this point that the presence of $Ca^{2+}$ ($1 \cdot 10^{-3}$ M) in the buffer does not modify the adriamycin adsorption on a cardiolipin monolayer. This result suggests that the adriamycin-cardiolipin association constant must be higher than the $Ca^{2+}$-anionic lipid association constant ($1 \cdot 10^4$ $M^{-1}$) [22].

A complexation reaction between a lipid anionic site (P) and the ionized adriamycin (ADM) [23] can be written as:

ADM + P → complex

with an association constant:

$$K = \frac{[\text{complex}]}{[P]} \cdot \frac{1}{[ADM]_s} \quad (1)$$

The concentration of the surface complex is directly obtained from the radioactivity measurements. The surface charge-concentration of the monolayer (P) is the difference between the lipid surface charge-concentration before and after complexation. $[ADM]_s$ is the molar concentration of the uncomplexed adriamycin. Subscript, s, indicates that the concentration has to be considered at the level of the surface reaction site. $[ADM]_s$ is related to the bulk concentration ($[ADM]_\infty = 1 \cdot 10^{-5}$ M) through a Boltzmann distribution:

$$[ADM]_s = [ADM]_\infty \cdot \exp(-e\psi/kT) \quad (2)$$

where $e$ is the electronic charge, $k$ the Boltzmann constant, $T$ the absolute temperature and $\psi$ the remaining negative surface-potential after complexation. At 25°C, the electrostatic potential, $\psi$, can be determined by means of the Gouy-Chapman relation [20]:

$$\psi = 50.5 \cdot sh^{-1} \cdot \frac{134\sigma}{C^{1/2}} \quad (3)$$

where $C$ is the concentration of monovalent ions in the subphase (mol/l) and $\sigma$ is the surface charge-density (charge/Å$^2$). $K$ values are reported in Table I.

*Surface potential*

Since the stoichiometry established by surface radioactivity seems to point to a complexation between opposite charges, surface-potential measurements were made in order to confirm the electrostatic nature of the complex. Indeed,

TABLE I

ASSOCIATION CONSTANTS BETWEEN ADRIAMYCIN AND PHOSPHOLIPIDS: SURFACE-RADIOACTIVITY MEASUREMENTS

ADM, adriamycin; PS, phosphatidylserine; PA, phosphatidic acid; DPPC, DL-α-dipalmitoyl phosphatidylcholine. Adriamycin concentration, $1 \cdot 10^{-5}$ M. Composition of the subphase, buffered solution (Tris-HCl, pH 7.4; KCl, $1 \cdot 10^{-1}$ M). Lipids were spread in the close-packed state (CL, 120 Å$^2$/molecule; PS, PA and DPPC, 60 Å$^2$/molecule). $T$ = 25°C. (1), surface-radioactivity measurements; (2), Eqn. 3; (3), Eqn. 2; (4), Eqn. 1.

| | Cardiolipin | PS | PA | DPPC |
|---|---|---|---|---|
| Complex (molecules/Å$^2$)(1) | $1.6 \cdot 10^{-2}$ | $1.3 \cdot 10^{-2}$ | $1.3 \cdot 10^{-2}$ | 0 |
| Surface charge-density ($\sigma_0$) before injection of ADM (charge/Å$^2$) | $1.7 \cdot 10^{-2}$ | $1.7 \cdot 10^{-2}$ | $1.7 \cdot 10^{-2}$ | 0 |
| Surface charge-density ($\sigma$) after injection of ADM (charge/Å$^2$) | $9.6 \cdot 10^{-4}$ | $4.6 \cdot 10^{-3}$ | $4.6 \cdot 10^{-3}$ | 0 |
| $\psi$ (mV)(2) | 20 | 70 | 70 | 0 |
| $[ADM]_s$(M)(3) | $2.1 \cdot 10^{-5}$ | $14.6 \cdot 10^{-5}$ | $14.6 \cdot 10^{-5}$ | $1 \cdot 10^{-5}$ (bulk conc.) |
| Association constant $K$ ($M^{-1}$)(4) | $1.6 \cdot 10^6$ | $1.8 \cdot 10^4$ | $1.8 \cdot 10^4$ | — |

the surface potential ($\Delta V$) of a lipid monolayer can be described by:

$$\Delta V = \psi + \frac{12\pi\mu_z}{A} \tag{4}$$

where $\psi$ is the electrostatic potential expressed in mV, $\mu_z$ is the contribution due to the vertical component of the total dipole moment expressed in mdebye and $A$ is the area occupied per molecule, in Å$^2$/molecule. After adriamycin-injection into the subphase, the increase of the surface potential $\Delta(\Delta V)$ will describe quantitatively the cancelling of the negative potential generated by the acidic phospholipids (Fig. 4) if it is assumed that the dipole term of Eqn. 4 remains constant during complexation (in these conditions $\Delta(\Delta V)$ will be equal to $\Delta\psi$).

The difference between the theoretical increase of potential $\Delta\psi$ corresponding to a 100% electrostatic complex formation (Eqn. 3 with $\sigma = \sigma_0$) and the effective observed increase, $\Delta\psi_{exp}$, allows us to calculate the surface potential, $\psi$ (Eqn. 3), after complexation (Table II). Since the complexation is not complete, a surface potential remains in each case. It must be pointed out that the surface potential remaining after complexation as estimated by surface radioactivity (Table I) and the surface potential remaining as observed by surface-potential measurements (Table II) are in fairly good agreement. This strongly suggests that the dipolar term of Eqn. 4 is not modified by the complexation, and thus, that no penetration of the drug into the hydrophobic lipidic region occurs. Moreover, a complexation constant (Table II) can be calculated [24] from:

$$K = \frac{\sigma_0 - \sigma}{\sigma} \cdot \frac{1}{[\mathrm{ADM}]_s} \tag{5}$$

where $\sigma$ and $\sigma_0$ are, respectively, the surface charge-density after and before

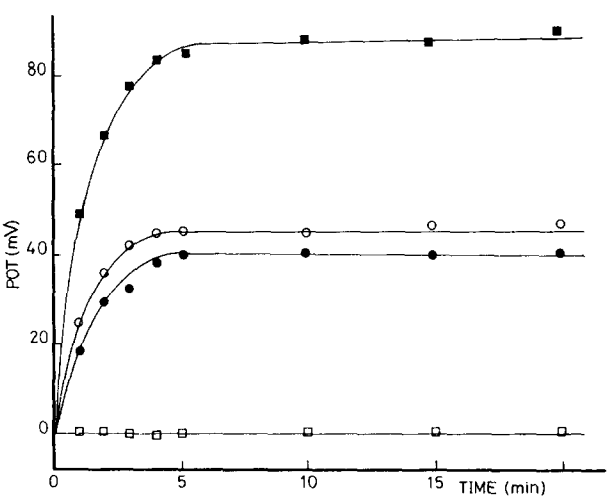


**Fig. 4. Evolution of the surface potential, in mV, as a function of time, in min. Zero-time corresponds to the injection of adriamycin in the subphase (final conc., $1 \cdot 10^{-5}$ M). Experimental conditions are identical to those described in Fig. 2. Cardiolipin, (■———■); phosphatidylserine, (○———○); phosphatidic acid, (●———●); DL-α-dipalmitoyl phosphatidylcholine, (□———□).**

TABLE II

ASSOCIATION CONSTANTS BETWEEN ADRIAMYCIN AND PHOSPHOLIPIDS: SURFACE POTENTIAL MEASUREMENTS

ADM, adriamycin; PS, phosphatidylserine; PA, phosphatidic acid; DPPC, DL-α-dipalmitoyl phosphatidylcholine. Adriamycin concentration, $1 \cdot 10^{-5}$ M. Composition of the subphase, buffered solution (Tris-HCl, pH 7.4; KCl, $1 \cdot 10^{-1}$ M). Lipids were spread in the close-packed state, except for cardiolipin (275 $Å^2$/molecule). (1), surface-potential measurements; (2), Eqn. 3; (3), Eqn. 3; (4), Eqn. 2; (5), Eqn. 5.

| | Cardiolipin | PS | PA | DPPC |
|---|---|---|---|---|
| $\Delta\psi$ exp. (mV)(1) | 85 | 45 | 40 | 0 |
| $\Delta\psi$ theor. (mV)(2) | 95 | 118 | 118 | — |
| $\Delta\psi$ theor. $-\Delta\psi$ exp. (mV) | 10 | 73 | 78 | — |
| $\sigma$ (charge/$Å^2$)(3) | $4.7 \cdot 10^{-4}$ | $4.9 \cdot 10^{-3}$ | $5.4 \cdot 10^{-3}$ | — |
| $[ADM^+]_s$(M)(4) | $1.5 \cdot 10^{-5}$ | $14.9 \cdot 10^{-5}$ | $15.5 \cdot 10^{-5}$ | $1 \cdot 10^{-5}$ (bulk conc.) |
| $K$ ($M^{-1}$)(5) | $1.8 \cdot 10^{6}$ | $1.2 \cdot 10^{4}$ | $1.4 \cdot 10^{4}$ | |

complexation, and $[ADM]_s$ has been previously described (Eqn. 2).

To check how a structural change in the adriamycin molecule can modify its complexation capacity, some analogs were studied using the surface-potential approach. The structures of these analogs are shown in Fig. 1. *N*-Acetyl-adriamycin which bears no charge, does not modify the $\Delta V$ value (Table III) and does not form any complex with cardiolipin or DL-α-dipalmitoyl phosphatidylcholine. Steffimycin, another uncharged adriamycin analog, reacts with the lipid layer but without specificity. Indeed, the surface-potential increase, $\Delta(\Delta V)$, was identical to that of a neutral lipid monolayer (DL-α-dipalmitoyl phosphatidylcholine) or a charged lipid monolayer (cardiolipin) (Table III). This nonspecific adsorption was already observed with drug-induced lipidosis [9—11] and corresponds to the nonspecific penetration of the drug into the lipid layer. No attempt was made, for these two compounds, to calculate an association constant. The situation is quite different with the positively-charged adriamycin analog, rubidazone ($K = 6 \cdot 10^4$ $M^{-1}$). Indeed, it shows a specific activity for anionic lipids ($\Delta V = 48$ mV) and no affinity for neutral lipids ($\Delta V = 0$ mV) (Table III). The $\Delta V$ modification corresponds, in this case, to a specific charge-to-charge interaction.

The results obtained with these three analogs suggest that the complexing capacity of adriamycin is strongly associated not only to the adriamycin ionizable amino group but also to its well-defined hydrophilic-hydrophobic balance.

TABLE III

SURFACE POTENTIAL CHANGE (mV) AFTER INJECTION OF ADRIAMYCIN DERIVATIVES BENEATH CARDIOLIPIN AND DIPALMITOYL PHOSPHATIDYLCHOLINE MONOLAYERS

Experimental conditions were identical to those described in Table II. All values are expressed in mV. DPPC, DL-α-dipalmitoyl phosphatidylcholine.

| | Adriamycin | *N*Ac-adriamycin | Rubidazone | Steffimycin |
|---|---|---|---|---|
| Cardiolipin | 85 | 0 | 48 | 82 |
| DPPC | 0 | 0 | 0 | 87 |

*Absorption spectra*

In Fig. 5 are reported the spectral changes of adriamycin in the presence of cardiolipin small unilamellar vesicles. The substantial reduction of the molar extinction coefficient resembles the behavior of the ADM-DNA complex [5] stabilized by two essential interactions: an electrostatic interaction between the protonated amino groups of the sugar residues and the ionized phosphate residues, and an interaction between the adriamycin molecule intercalated into the double strand and the adjacent base-pairs. The same kind of organization has been proposed to describe the ADM-mucopolysaccharides interaction [25]: a parallel orientation of the anthraquinone chromophores along the tertiary structure of the mucopolysaccharide would contribute to the stabilization of this complex in addition to the stabilization derived from the electrostatic binding between the basic group of the amino sugar moeity and the poly-anionic macromolecule. Moreover, the shoulder in the absorption spectrum of free adriamycin at about 550 nm disappears if adriamycin is mixed with cardiolipin vesicles (Fig. 5). Interestingly, the same behavior was observed with the ADM-DNA mixture [5]. This analogy suggests, again, the same kind of organization in the two complexes. Indeed, it is not unlikely that this kind of interaction occurs between neighboring adriamycin molecules in the complex phospholipid-ADM since all adriamycin molecules are excluded from the hydrophobic part of the membrane model as shown by comparison of the surface-potential and surface-radioactivity measurements. Moreover, fluorescence titration of adriamycin-binding to egg lecithin-dicetylphosphate (9 : 1, w/w) vesicles showed that the intercalation region of the anthracycline moeity is the hydrocarbon/water interface [16].

An electrostatic interaction between adriamycin and lipids is essential in order to observe a spectral change. Indeed, in the absence of negatively-charged

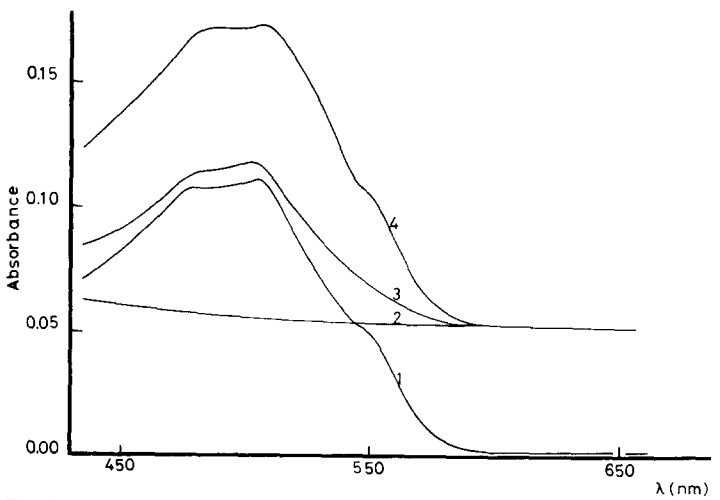


**Fig. 5. Absorption spectra of: (1) free adriamycin (conc., $1 \cdot 10^{-5}$ M, (2) small unilamellar cardiolipin vesicles (lipid conc., $1 \cdot 10^{-5}$ M), (3) a mixture of small unilamellar vesicles of cardiolipin and of adriamycin at the same final concentration and (4) theoretical values of the absorption of mixture 3 obtained by addition of curves 1 and 2. Experimental conditions were identical to those described in Fig. 2.**

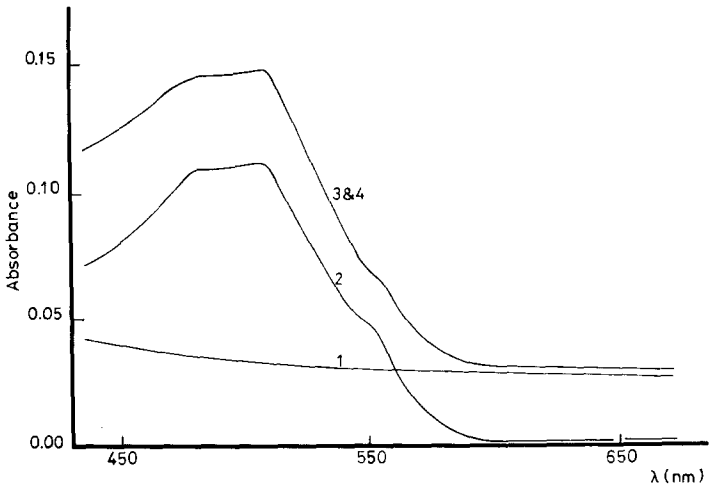


Fig. 6. Absorption spectra of: (1) small unilamellar DL-α-dipalmitoyl phosphatidylcholine vesicles (lipid conc., $1 \cdot 10^{-5}$ M), (2) free adriamycin (conc., $1 \cdot 10^{-5}$ M), (3) a mixture of small unilamellar vesicles of DL-α-dipalmitoyl phosphatidylcholine and of adriamycin at the same final concentration and (4) theoretical values of the absorption of mixture 3 obtained by addition of curves 1 and 2. Experimental conditions were identical to those described in Fig. 2.

lipids, i.e., with DL-α-dipalmitoyl phosphatidylcholine small unilamellar vesicles, no spectral change was observed (Fig. 6). In this case, the experimental spectra clearly correspond to the values expected (Fig. 6). The spectra (Figs. 5 and 6) were also run in a range of wavelengths where there is no absorbance due to adriamycin. The identical absorbance obtained in this range for the liposome alone and for the liposome-adriamycin mixture allowed us to eliminate the possibility of a modification in the baseline of the liposome spectrum due to a light-scattering change. The proximity of the adriamycin molecules at the surface of the vesicles will enhance a dimerization process as already demonstrated for other dyes bound to negatively-charged liposomes [26] and for free anthracycline derivatives in concentrated solutions [31]. Let us consider the dimerization reaction:

monomer + monomer → dimer

with an association constant $K_D$:

$$K_D = \frac{[\text{dimer}]}{[\text{monomer}]^2} \tag{6}$$

If $\epsilon_M$ is the molar extinction coefficient of the monomer and $\epsilon_D$ the molar extinction coefficient of the dimer, then for a 1 cm path-length cuvette containing adriamycin and small unilamellar vesicles, the absorbance of the adriamycin is given by:

$$A = \epsilon_M[\text{monomer}] + \epsilon_D[\text{dimer}]$$

If the analytical adriamycin concentration is

$$[\text{ADM}]_0 = [\text{monomer}] + 2[\text{dimer}] \tag{7}$$

it can be written as:

$$A = \epsilon_M([ADM]_0 - 2[dimer]) + \epsilon_D[dimer]$$

or with $A_0 = \epsilon_M [ADM]_0$

$$[dimer] = \frac{A - A_0}{\epsilon_D - 2\epsilon_M} \tag{8}$$

$\epsilon_M$ (= 13 200 $M^{-1} \cdot cm^{-1}$) was determined at a final concentration of $1 \cdot 10^{-6}$ M. The experimental evaluation of the molar extinction coefficient as a function of the concentration of adriamycin in the absence of lipid small unilamellar vesicles permits us to evaluate the dimerization constant, $K_D$, and the molar extinction coefficient, $\epsilon_D$, of the dimer [27].

Combining Eqns. 6 and 7, we obtain:

$$\frac{1}{K_D} = \frac{([ADM]_0 - 2[dimer])^2}{[dimer]} \tag{9}$$

Substituting Eqn. 8 into Eqn. 9:

$$\frac{1}{K_D} = [ADM]_0^2 \cdot \left(\frac{\epsilon_D - 2\epsilon_M}{A - A_0}\right) - 4[ADM]_0 + \frac{4(A - A_0)}{\epsilon_D - 2\epsilon_M} \tag{10}$$

This last equation was resolved for a great number of adriamycin concentrations and gave the same values of $\epsilon_D$ and $K_D$ in all the ranges of concentration between $1 \cdot 10^{-5}$ and $1 \cdot 10^{-3}$ M. This agrees well with a dimerization process (an *n*-merization process, is, however, possible if $\epsilon_D = \epsilon_{n\text{-mer}}$: in this case, *n*-mers will be seen, like dimers).

Numerical values obtained were; $K_D = 1.7 \cdot 10^3$ $M^{-1}$, and $\epsilon_D$ = 13 700 $M^{-1} \cdot cm^{-1}$.

From Eqn. 8, it was possible to evaluate the number of adriamycin molecules organized in dimers. Results are listed in Table IV as percentages of the total amount of adriamycin molecules.

Again, it can be seen that only negatively-charged lipids show an association with adriamycin subsequently followed by a dimer formation. However, although the surface charge-density was identical for each of the three phospholipids tested, the proportion of paired molecules is much higher in the case of cardiolipin (CL). Indeed, the cardpack-stacking formation of adriamycin molecules when bound to close enough anionic sites would involve not only a

TABLE IV

CALCULATION OF THE AMOUNT OF ADRIAMYCIN DIMERS IN THE PRESENCE OF SMALL UNILAMELLAR VESICLES

$\Delta A$ is the difference between the absorbance of free adriamycin ($1 \cdot 10^{-5}$ M/l) and adriamycin in the presence of small unilamellar vesicles. Buffer composition: Tris-HCl, $1 \cdot 10^{-3}$ M, pH 7.4; KCl, $1 \cdot 10^{-1}$ M. $T$ = 25° C. ADM, adriamycin; PS, phosphatidylserine; DPPC, DL-α-dipalmitoyl phosphatidylcholine.

| | Cardiolipin | PS | DPPC | No lipid |
|---|---|---|---|---|
| $\Delta A$ | 0.066 | 0.039 | 0.002 | 0 |
| Paired ADM (%) | 34 | 17 | 0 | 0 |

free-energy term for the electrostatic interaction, but also a free-energy term describing the interaction between neighboring dyes. We suggest that the interactions between paired adriamycin molecules stabilize the complex, CL-ADM. The free energy of association between one adriamycin molecule and one phosphatidylserine (PS) molecule is given by:

$$\Delta G_1^0 = -RT \cdot \ln K_{PS} \quad \text{(Tables I and II)} \tag{11}$$

$$= -5.5 \text{ kcal/mol}$$

and the free energy of association between one adriamycin molecule and one anionic site of the cardiolipin molecule is:

$$\Delta G_2^0 = -RT \cdot \ln K_{CL} \quad \text{(Tables I and II)} \tag{12}$$

$$= -8.4 \text{ kcal/mol.}$$

Moreover, the free energy of pair-formation between adriamycin molecules is:

$$\Delta G_3^0 = -\tfrac{1}{2} \cdot RT \cdot \ln K_D \tag{13}$$

$$= -2.8 \text{ kcal/mol.}$$

The increase in stability of the complex formed with cardiolipin as compared to the PS-ADM complex could, thus, be quantitatively explained by a preferential formation of cardpacked complexes when the phospholipid is cardiolipin.

## Discussion

Interaction of adriamycin with phospholipids has been examined with special reference to the ionic association between the polar heads of various phospholipids and the positively-charged antibiotic.

By means of surface-radioactivity measurements, we demonstrated the specificity of adriamycin for negatively-charged phospholipids and established the stoichiometry of the complex. The surface-potential approach has confirmed the electrostatic nature of the binding and the non-penetration of the adriamycin molecule into the lipidic phase. Both approaches allowed us to calculate an association constant which agrees with the following sequence:

$$K_{CL} >> K_{PS} \simeq K_{PA} >>> K_{DPPC}$$

Studies of adriamycin analogs have confirmed not only the essential role of the adriamycin amine residue in the complex formation, but also the importance of a well defined hydrophobic-hydrophilic balance.

To explain the particularly high affinity of adriamycin for cardiolipin, it has been proposed that two molecules of adriamycin in interaction with one molecule of cardiolipin are sufficiently close to form a cardpack dimer. This structure would stabilize the complex and thus explain the high $K$ value. Quantitative evaluation of this stabilization shows that this organization could explain the differences in the association constants.

Experimental results implicate cellular membrane alterations in the cytotoxic action of adriamycin. Even if the plasma membrane is not the primary target for the cytotoxic action of this agent, an interaction at this level is

absolutely required for entry into the cell. It is of interest to compare the association constant between adriamycin and cardiolipin ($1.6 \cdot 10^6$ $M^{-1}$) with that of adriamycin and DNA nucleotides ($2.4 \cdot 10^6$ $M^{-1}$) [3]. Clearly, cardiolipin could play the role of a competitive target for adriamycin. These data provide a rational hypothesis for the pathogenesis of the mitochondrial lesions, one of the major and most specific subcellular changes characterizing adriamycin cardiotoxicity. The rather selective toxicity of adriamycin for mitochondria may be due to the high concentration of cardiolipin in mitochondria membranes. This phospholipid is, indeed, an almost characteristic component of the inner membranes of mitochondria which are abundant in the cardiac muscles. The uptake of $^3$H-labeled adriamycin by mitochondria [28] is in favor of this hypothesis. Moreover, it has been suggested that cardiolipin may be found in cellular membranes upon malignant transformation [29]. This could explain the susceptibility of neoplastic cells to adriamycin.

Consequently, one must assume that the cell membrane structure may be an important determinant in the mechanism of action of adriamycin. This competition between membrane sites and target should be kept in mind in the building of new derivatives.

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## Appendix

The purpose of this appendix is to examine whether or not the contribution of the uncomplexed $^3$H-labeled adriamycin to the total surface radioactivity is negligible in comparison with the contribution of the complexed $^3$H-labeled adriamycin.

This contribution is really negligible if $^3$H-labeled adriamycin is distributed homogeneously in the aqueous phase. Indeed, in the presence of a DL-$\alpha$-dipalmitoyl phosphatidylcholine monolayer, no increase of the surface radioactivity was observed. If the monolayer spread is negatively-charged, the concentration of $^3$H-labeled adriamycin dramatically increases in the aqueous phase immediately adjacent to the monolayer. We will calculate the contribution to the total surface radioactivity of $^3$H-labeled adriamycin in the neighborhood of the monolayer when a surface electrostatic potential of 78 mV remains after complexation. This is the highest value found after complexation (Tables I and II).

If $n_e^+(x)$ is the number of positive ions in excess per Å depth per cm$^2$, then:

$$n_e^+(x) = n^+(x) - n_\infty^+ \tag{14}$$

where: $n_\infty^+$ is the number of positive ions per Å depth per cm$^2$ in the bulk and $n^+(x)$ is the number of positive ions per Å depth per cm$^2$ at a distance, $x$, from

the spread monolayer. If the complexation does not reach 100%, $n^+(x)$ will be different from $n^+_\infty$ since an electrostatic field modifies the repartition of positive ions. $n^+(x)$ can be described with a Boltzmann distribution:

$$n^+(x) = n^+_\infty \exp(-e\,\psi(x)/kT) \tag{15}$$

where $e$ is the electron charge, $k$ the Boltzmann constant and $T$ the absolute temperature. $\psi(x)$ is calculated from the Gouy-Chapman equation (Eqn. 3). To calculate $N$, the excess of positive ions/cm$^2$ which will be counted, we integrated between 0 and 100 Å. Indeed, at a distance of 100 Å from the surface, the distribution of ions does not depend on the surface potential, and:

$$N = \int_0^{100\text{Å}} n^+_e(x) \cdot cf(x) \cdot dx \tag{16}$$

where $cf(x)$ is a coefficient giving the fraction of $\beta$-rays crossing effectively the depth, $x$, of water. It is given by [30]:

$$cf(x) = \frac{A(x)}{A(0)} = exp(-\mu l(x)) \tag{17}$$

with $A(x)$ and $A(0)$, respectively, the intensities of rays at distances $x$ and 0 from the emission; $\mu$ = 23 000 cm$^2$/g for $^3$H in water at 20°C and $l(x)$ is the volumic mass of water per $x$ cm. Integration was obtained by approximating the function by third-order Spline functions. The total excess of effectively-counted adriamycin molecules has been found to be $3.9 \cdot 10^8$ molecules per cm$^2$ for a surface potential of 78 mV. In these conditions, $4.5 \cdot 10^{13}$ molecules of adriamycin per cm$^2$ are in the electrostatic complexed form. Comparison of these two values demonstrates clearly (factor $10^5$) that the contribution of the uncomplexed adriamycin is negligible.

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