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ADRIAMYCIN INHIBITS THE FORMATION OF NON-BILAYER LIPID STRUCTURES IN CARDIOLIPIN-CONTAINING MODEL MEMBRANES

E. GOORMAGHTIGH [a], M. VANDENBRANDEN [a], J.M. RUYSSCHAERT [a] and B. DE KRUIJFF [b]

[a] *Laboratoire de Chimie Physique des Macromolécules aux Interfaces, Université Libre de Bruxelles, Campus Plaine CP. 206/2, 1050 Bruxelles (Belgium) and* [b] *Institute of Molecular Biology, State University of Utrecht, Padualaan 8, 3508 TB Utrecht (The Netherlands)*

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The effect of adriamycin on cardiolipin-containing model membrane systems have been studied by ^{31}P-NMR, freeze-fracture electron microscopy and binding experiments. Adriamycin effectively inhibits the formation of non-bilayer lipid structures induced by Ca^{2+} and cytochrome *c* in cardiolipin-containing liposomes. This drug also strongly inhibits the uptake of Ca^{2+} by cardiolipin into an organic phase. These results are discussed in relation to the cardiotoxic effect of adriamycin and the possible importance of non-bilayer lipid structures for the functioning of the mitochondrion.

Introduction

Adriamycin is a now widely used as antineoplastic drug [1,2]. Its mechanism of action is generally admitted to involve intercalation into the double helical DNA and subsequent inhibition of DNA and/or RNA synthesis [3]. Cardiotoxicity, however, is very specific and places a limit on the total dose of adriamycin that may be given, since the effect is cumulative [4]. The development of the cardiac failure is characterized by a good correlation with the impairment of mitochondria functions (ATP synthesis and proportional O_2 consumption [5]). Cardiolipin, a phospholipid specific of the inner mitochondrial membrane has been proposed as an additional target for adriamycin [6,7], which could be responsible for the toxicity at the mitochondria level. In fact, a good correlation between the cardiotoxicity of a series of seven adriamycin derivatives and their affinity for cardiolipin was pointed out elsewhere [8]. However, the molecular mechanism by which cardiolipin-adriamycin complexation can inhibit the mitochondria functions is still unknown.

An interesting feature of cardiolipin is that aqueous dispersions of this lipid show polymorphism. In the absence of divalent cations the lipid organizes itself in bilayers whereas in the presence of divalent cations the hexagonal H_{II} phase is preferred [9]. In mixtures with bilayer-forming lipids such as phosphatidylcholine [10,11], the Ca^{2+} induced non-bilayer preference of cardiolipin can also be expressed in an alternative structure: 'the lipidic particle' (intrabilayer inverted micelle). In addition, cytochrome *c* addition to cardiolipin containing model membrane systems specifically leads to the formation of non-bilayer structures for part of the lipids [12].

The ability of cardiolipin to adopt these non-bilayer lipid structures under a variety of conditions have led to suggestions that these structures might play important functional roles in the inner mitochondrial membrane of which Ca^{2+} transport [13] and functioning of the terminal part of the respiratory chain [14] are the most prominent ones. A possible explanation of the impairment of mitochondrial functioning by adriamycin might be that this drug by interacting with cardiolipin would change its phase properties.

In this paper therefore we report on the effect

of adriamycin on the phase behaviour and ionophoric properties of cardiolipin in model membrane systems. It will be shown that adriamycin effectively inhibits the formation of non-bilayer structures by Ca^{2+} and cytochrome *c* leading to the suggestion that the cardiotoxicity of adriamycin might be related to its ability to interfere with the formation of functionally important non-bilayer structures in the inner mitochondrial membrane.

Materials and Methods

Materials

DL-α-Dipalmitoylphosphatidylcholine (DPPC), DL-α-dipalmitoylphosphatidic acid salt (DPPA), L-α-phosphatidylethanolamine (PE) from bovine brain, DL-α-dipalmitoylphosphatidylethanolamine (DPPE), Na salt of cardiolipin from bovine heart (CL), L-α-phosphatidylglycerol (PG) salt from egg yolk, and L-α-phosphatidylinositol (PI) salt from soybean were purchased from Sigma Chemical Co. L-α-Phosphatidylserine (PS) salt from bovine brain was supplied by Koch Light Laboratories. Egg yolk phosphatidylcholine (egg PC) was isolated from hen eggs. Adriamycin was generously supplied by Dr. J. Hildebrand (Institut J. Bordet) and Dr. C. Deslover (Farmitalia).

All chemicals were of analytical grade and water was triple distilled. Buffered solutions (10 mM Tris-HCl, pH 7.4) were used.

Methods

Phospholipid-dependent Ca^{2+} uptake into chloroform. The procedure was described elsewhere [13]. Briefly, phospholipid corresponding to 1 μmol of phosphorus as estimated by the method of Bartlett [15] was dissolved in 1 ml of chloroform. Subsequently, 1 ml of aqueous buffer (100 mM NaCl, 10 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.4) was added to the chloroform phase, followed by the addition of 1 μCi $^{45}CaCl_2$ (NEN 9 Ci/g of Ca) in 10 μl. Increasing amount of adriamycin were added (from 0 to 1 μmol). The resulting two phase system was gently shaken for 3 h at 20°C. Subsequently, the $CHCl_3$ and aqueous phases were separated by brief centrifugation (15 min, 800 × *g*) and 100 μl of the chloroform phase was transferred to a scintillation vial for counting. Phosphorus content was determined as described [15] for the $CHCl_3$ phase.

The dissociation of the adriamycin-cardiolipin complex by different cations was studied on the following system: cardiolipin liposomes (0.05 μmol of phosphorus) were incubated with adriamycin (0.1 μmol) in 1 ml buffer. In order to eliminate excess of adriamycin, liposomes were centrifuged (15 min, 800 × *g*). The pellet was washed once and resuspended in 1 ml buffer. The appropriate salt was added and the volume adjusted to 2 ml. No change in pH could be detected. Next 1 ml of $CHCl_3$ was added. After shaking and phase separation, the phosphorus and adriamycin content of the $CHCl_3$ phase was measured. The adriamycin concentration was determined by measuring its absorbance at 500 nm.

Cytochrome c-adriamycin competition experiments. Multilamellar vesicles were obtained as described elsewhere [9]. Cardiolipin (0.15 μmol of phosphorus) and cytochrome *c* (0.05 μmol) were first incubated for 10 min. When mentioned, cytochrome *c* was reduced with dithionite. Excess of dithionite was eliminated by gel filtration (Sephadex G-25). Adriamycin was added (0 to 1 μmol; final volume 2 ml) and liposomes were centrifuged (800 × *g*, 15 min). The washed pellet was resuspended in 1 ml buffer. The amount of phosphorus was determined as described [15] and the respective amounts of adriamycin and cytochrome *c* were optically determined after physical separation. To determine the amounts of adriamycin and cytochrome *c*, 100 μl of HCl 34% (v/v) was added to the incubation mixture. After heating for 15 min at 90°C in a water-bath, the glycosidic linkage of adriamycin was specifically hydrolysed and the anthracycline chromophore was quantitatively extracted from the aqueous phase by 1 ml of chloroform. Adriamycin was then monitored at 500 nm in the $CHCl_3$ phase and cytochrome *c* was monitored at 405 nm in the aqueous phase.

NMR (nuclear magnetic resonance). The effect of adriamycin, Ca^{2+} and cytochrome *c* on the structure of cardiolipin containing multilamellar vesicles was investigated by high power proton decoupled ^{31}P-NMR at 36.4 or 81.0 MHz as described before [9]. All free induction decays were exponentially filtered, resulting in a 50 Hz line broadening. For other technical details see the figure legends. The 0 ppm position in the ^{31}P-NMR

spectra indicates the chemical shift of phospholipids undergoing isotropic motion, e.g. the chemical shift of the ^{31}P-NMR resonance of sonicated egg phosphatidylcholine vesicles in buffer.

Freeze-fracturing. Freeze-fracture studies were carried out as described elsewhere [16] on samples to which 25% (by volume) glycerol was added as a cryo-protectant. The samples were quenched from 20°C.

Results

Effect of adriamycin and Ca^{2+} on the structure of cardiolipin containing model membranes.

^{31}P-NMR is a convenient method for the determination of the structure of aqueous phospholipid dispersions [17]. Phospholipids organized in extended bilayers give rise to characteristic asymmetrical ^{31}P-NMR spectra with a low-field shoulder and a high-field peak. Phospholipids organized in hexagonal phases such as the H_{II} phase give rise to spectra with a reversed asymmetry and a reduced width. Finally, for phospholipids organized in structures in which the molecules can undergo rapid isotropic motion, such as (inverted) micelles or small vesicles, a narrow symmetrical resonance is observed.

In agreement with previous data [9] cardiolipin when dispersed in a 100 mM NaCl buffer at pH 7.0 is mainly organized in extended bilayers (Fig. 1). Approximately 10% of the signal intensity is present in an isotropic signal most likely originating from some small vesicles, which can be detected by freeze-fracture electron microscopy in the preparation. The effective chemical shift anisotropy ($\Delta\sigma$), which is a measure of the local order of the phosphate region [18], as measured from the 'bilayer' component of the spectrum was 24.7 ppm. Addition of increasing amounts of Ca^{2+} to the cardiolipin dispersion leads to a gradual conversion from a bilayer to a hexagonal type of ^{31}P-NMR spectrum such that in the presence of stoichiometric amounts of Ca^{2+} all the cardiolipin is organized in the H_{II} phase, as was shown before [9]. Addition of increasing amounts of adriamycin

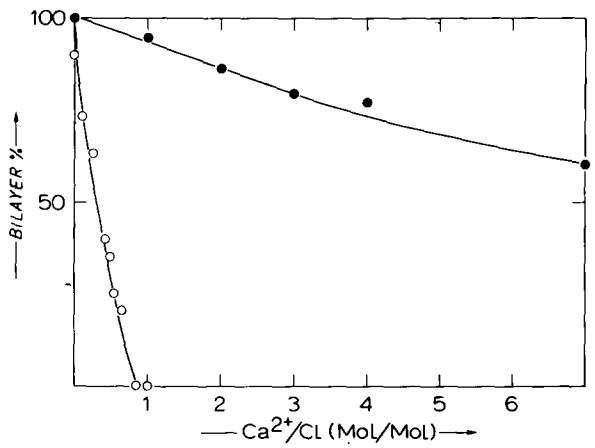

Fig. 1. Effect of Ca^{2+} and adriamycin on the structure of cardiolipin liposomes at 30°C. 30 μmol cardiolipin (CL) were dispersed in 0.8 ml 100 mM NaCl, 10 mM Tris-HCl, 0.2 mM EDTA, pH 7.0 buffer. Ca^{2+} was added as aliquots of a 1 M $CaCl_2$ solution whereafter the phospholipid structure was determined by ^{31}P-NMR at 36.4 MHz. O——O, in the absence of adriamycin; ●——●, after the addition of 0.4 ml buffer containing 40 μmol of adriamycin. Only in the 0–0.2 Ca^{2+}/cardiolipin range, in the absence of adriamycin, isotropic peaks were observed. In the other spectra only 'bilayer' or 'hexagonal' peaks were observed. ^{31}P-NMR spectra were obtained from 10000 transients using 5 μs 45° radiofrequency pulses and a 0.17 s inter-pulse time. NMR spectra were identical when obtained immediately after the addition of Ca^{2+} or adriamycin or after 3 h of incubation with these reagents.

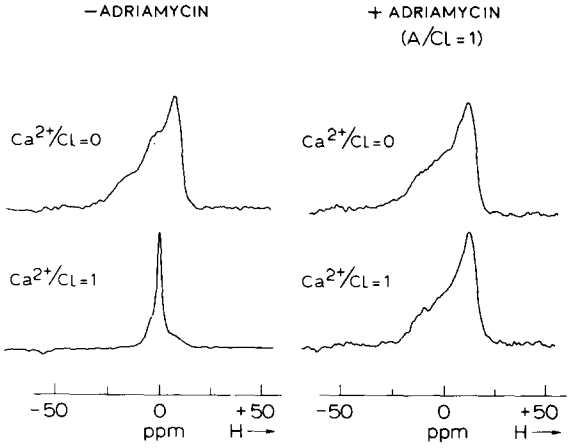

Fig. 2. Effect of Ca^{2+} and adriamycin on the 36.4 MHz ^{31}P-NMR spectra of phosphatidylcholine/cardiolipin (1 : 1) liposomes at 30°C. 40 μmol of phospholipid were dispersed in 1.2 ml 100 mM NaCl, 10 mM Tris-HCl, 0.2 mM EDTA, pH 7.0 buffer. Either 20 μl of 1 M $CaCl_2$ or 400 μl buffer containing 20 μmol adriamycin were added to the liposomes whereafter the NMR spectrum was recorded as described in the legend of Fig. 1. Ca^{2+} was always added after the adriamycin. A line broadening of 50 Hz was applied to the free induction decay.

to the cardiolipin liposomes in the absence of Ca^{2+} results in visual precipitation of the lipids. The only change observed in the ^{31}P-NMR spectrum was a decrease in intensity of the small isotropic signal which was totally absent for an adriamycin/cardiolipin molar ratio of 1.3 (Fig. 1). The $\Delta\sigma$ in this case was 24.6 ppm. This shows that the adriamycin/cardiolipin complex has a lamellar organization in which the local order of the phosphate region of the cardiolipin is not affected by the presence of the drug. However, the presence of adriamycin causes a strong inhibition of the Ca^{2+} induced hexagonal H_{II} phase formation of cardiolipin (Fig. 1). Even in presence of a large excess of Ca^{2+}, still bilayer structure is observed.

In mixed egg phosphatidylcholine/cardiolipin bilayers Ca^{2+} addition leads to the formation of both the H_{II} phase and of a structure which is characterized by isotropic motion of the phospholipid molecules and is associated with the occurrence of lipidic particles [10,11]. This is shown for mixed phosphatidyl/cardiolipin (1 : 1) bilayers in Figs. 2 and 3. Addition of adriamycin (equimolar to cardiolipin) to the mixture does not greatly affect the ^{31}P-NMR spectrum (Fig. 2) or the freeze-fracture morphology (data not shown). However, as in the pure cardiolipin system, the presence of adriamycin strongly inhibits the formation of non-bilayer lipid structures in this system as can be detected both by ^{31}P-NMR (Fig. 2) and by the smooth freeze-fracture faces (Fig. 3).

Effect of adriamycin on the ionophoric properties of cardiolipin

Non-bilayer structures, in particular inverted micellar ones, formed by the Ca^{2+}-cardiolipin

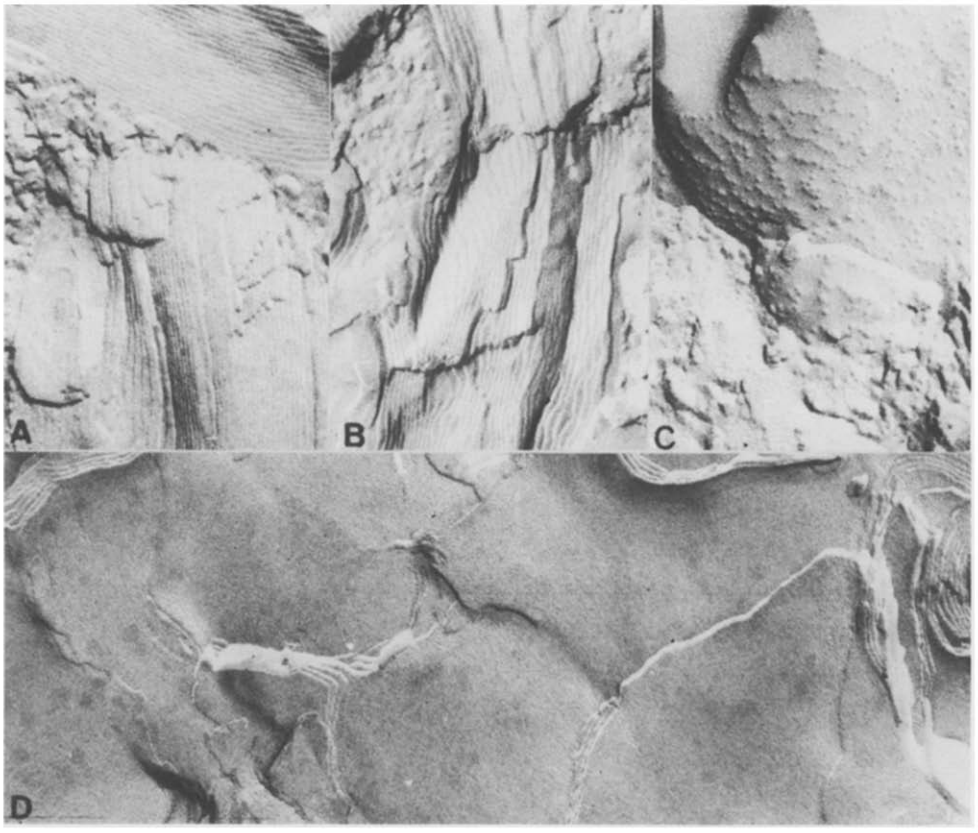

Fig. 3. Freeze-fracture morphology of phosphatidylcholine/cardiolipin (1 : 1) liposomes (A), (B) and (C) in the presence of equimolar Ca^{2+} (with respect to cardiolipin) and (D) in the presence of both adriamycin and Ca^{2+}. Experimental details see legend of Fig. 2. Final magnification 100000×.

complex might play a role in the Ca^{2+} transport across the inner mitochondrial membrane [13]. Cardiolipin would thus act as a Ca^{2+} ionophore. Any compounds exhibiting ionophoric properties must have the capability to form a lipid soluble complex with the agent to be transported. A first approximation to this biological situation is to measure the lipid mediated Ca^{2+} uptake into an organic phase. The ionophoric capabilities of cardiolipin have already been successfully investigated on such a system [13]. We therefore assayed the phospholipid facilitated uptake of Ca^{2+} from an aqueous phase into chloroform following this procedure (described in Materials and Methods). Results are reported in Fig. 4. These results agree with those of Tyson et al. [19] and show clearly that cardiolipin and PA sequester most effectively Ca^{2+}. This uptake is inhibited by adriamycin and this inhibition is almost complete as soon as the adriamycin concentration allows to complex all the negatively charged phospholipids. In order to determine more precisely the amount of Ca^{2+} needed to dissociate the adriamycin/cardiolipin complex, the adriamycin/cardiolipin complex was incubated with increasing amounts of Ca^{2+} and the amount of bound adriamycin was monitored as described in Materials and Methods. It is clear that monovalent cations cannot dissociate the adriamycin/cardiolipin complex and that a large excess of divalent cations is needed to displace the adriamycin from the cardiolipin molecule (Fig. 5).

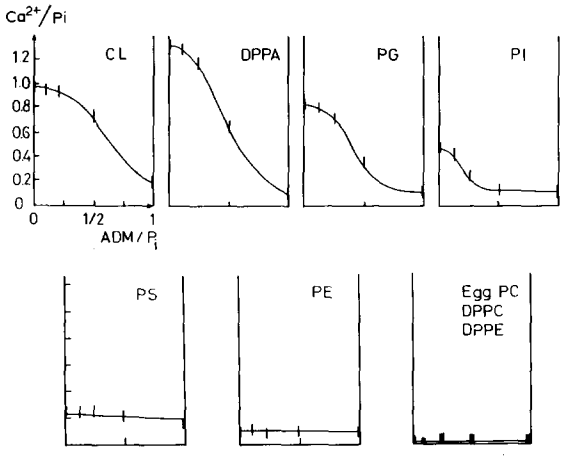

Fig. 4. Effect of adriamycin on the amount of Ca^{2+} taken up into an organic phase (chloroform) in presence of various phospholipids species. Phospholipids (1 μmol) were dissolved in 1 ml chloroform and Ca^{2+} (10 μmol) was added with adriamycin (from 0 up to 1 μmol) in 1 ml buffer. The molar ratio adriamycin/phospholipid phosphorus is reported in abscissa and the number of calcium ions per phospholipid phosphorus taken up into the chloroform after shaking of the two phase system is reported in ordinate.

Effect of adriamycin on the cytochrome c/cardiolipin interaction

Cytochrome *c* which in the inner mitochondrial membrane is the substrate of the cytochrome *c* oxidase has a strong interaction with negatively charged phospholipids. However, only in the case of cardiolipin, which is the only major negatively charged phospholipid in this membrane, this interaction leads to the formation of non-bilayer lipid structures [9]. As cardiolipin is strongly bound to the oxidase and appears to be required for its activity we suggested [14] that non-bilayer lipid structures might possibly play a role in the cytochrome *c*/cytochrome *c* oxidase reaction. As in a recent work we were able to demonstrate that both in mitochondria and in reconstituted systems adriamycin inhibits cytochrome *c* oxidase activity (Goormaghtigh, E., Brasseur, R. and Ruysschaert, J.M., unpublished data) by complexation of the essential cardiolipin [20] we thought it of interest to study the effect of adriamycin on the structure of cytochrome *c*/cardiolipin systems. In Fig. 6 it is

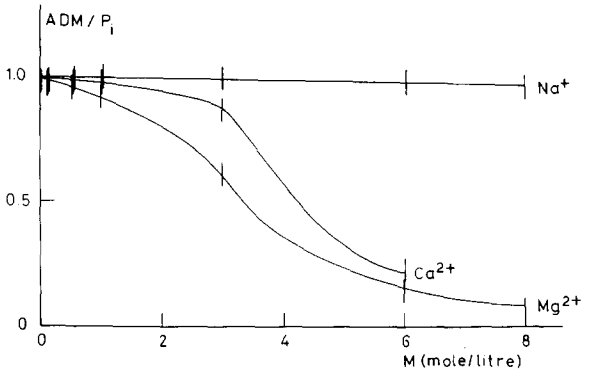

Fig. 5. Dissociation of the cardiolipin/adriamycin complex by monovalent and bivalent cations. Cardiolipin liposomes were incubated with adriamycin in 1 ml buffer in presence of mono- and bivalent cations. The amount of adriamycin molecules bound per cardiolipin phosphorus is indicated in ordinate and the salt concentration is given in abscissa. For Na^{+}, chloride and nitrate forms were tested, for Ca^{2+}, chloride was used and for Mg^{2+}, chloride and sulfate were employed.

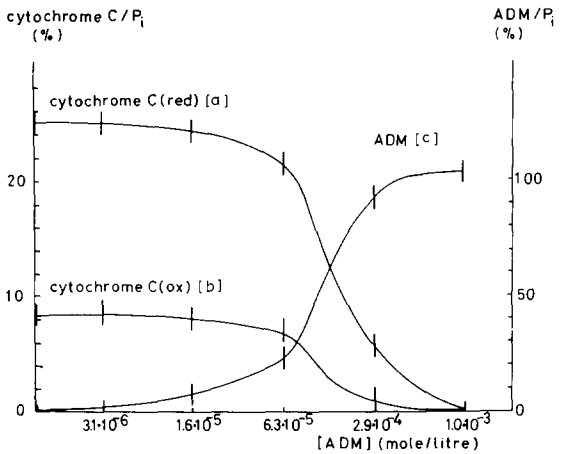

Fig. 6. Dissociation of the cytochrome *c*-cardiolipin complex by adriamycin. Adriamycin (ADM), at a final concentration indicated in abscissa, was added to cardiolipin liposomes ($1.5 \cdot 10^{-4}$ M) previously incubated in presence of cytochrome *c* (oxidized and reduced). The left ordinate gives the number of cytochrome *c* molecules bound per 100 cardiolipin phosphorus for cytochrome *c* reduced (a) and cytochrome *c* oxidized (b). The right ordinate gives the number of adriamycin molecules (c) bound per 100 cardiolipin phosphorus. The curves obtained for adriamycin binding on cardiolipin liposomes pre-incubated with reduced and oxidized cytochrome *c* are superposed.

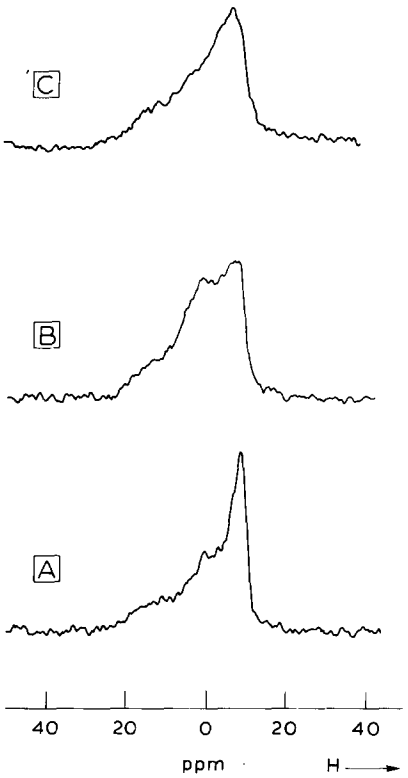

Fig. 7. Effect of cytochrome *c* and adriamycin on the 81 MHz ^{31}P-NMR spectra of cardiolipin at 30°C. (A) ^{31}P-NMR spectrum of 30 μmol cardiolipin dispersed in 100 mM NaCl, 10 mM Tris-HCl, 0.2 mM EDTA, pH 7.0 buffer. (B) After addition of 0.2 ml buffer containing 36 mg oxidized cytochrome *c*. (C) After subsequent addition of 20 μmol adriamycin in 0.2 ml buffer. NMR spectra were recorded immediately after the appropriate addition. Spectra recorded 2 h later were identical. A line broadening of 50 Hz was applied to the free induction decay. ^{31}P-NMR spectra were obtained for 1000 transients using 16 μs 90° radiofrequency pulses and a 1 s inter-pulse time.

shown that adriamycin can effectively displace both reduced and oxidized cytochrome *c* from cardiolipin containing liposomes as soon as the adriamycin concentration is sufficient to complex all the cardiolipin molecules. The effect of oxidized cytochrome *c* and adriamycin on the ^{31}P-NMR spectra of cardiolipin liposomes is shown in Fig. 7. In agreement with previous data [12] addition of cytochrome *c* to the liposomes results in the formation of an isotropic ^{31}p-NMR signal on the low-field side of which a shoulder is present indicating the presence of some H_{II} phase. Subsequent addition of adriamycin causes a strong reduction of these signals demonstrating that adriamycin eliminates the H_{II} phase and the isotropic structure induced by the cytochrome *c*. The reason for the differences in line width between the spectra shown in Fig. 7A and C is not understood.

Discussion

The main result of the present study is the finding that the adriamycin/cardiolipin interaction is very effective in blocking the formation of non-bilayer lipid structures in cardiolipin containing model membrane systems. This most likely is the result of the very strong and specific [6,8] electrostatic interaction between the monovalent positively charged drug and the negatively charged phosphates in the cardiolipin molecule. Ring stacking in the aqueous phase [7] might provide some additional stabilization of the complex.

These findings have interesting implications for two areas of mitochondrial functioning, e.g. Ca^{2+} transport and cytochrome *c* oxidase activity, which we shall discuss in turn.

It has been suggested that the mitochondrion might be a regulator of the cytosolic Ca^{2+} concentration because it is capable of a rapid, energy dependent uptake and release of Ca^{2+} [21,22]. The inner mitochondrial membrane is the barrier for this transport process which appears to be mediated by ionophoric molecules. Recently a protein-like ionophore has been isolated from this membrane [23]. Furthermore, there is now considerable evidence that cardiolipin by its ability to adopt non-bilayer lipid structures might act as a Ca^{2+} ionophore. Evidence for this suggestion includes the observation that Ca^{2+} induces the formation of lipidic particles in cardiolipin containing bilayers [10,11], that these particles facilitate lipid flip-flop [24] and transport Mn^{2+} [11,24], that cardiolipin can facilitate uptake of $^{45}Ca^{2+}$ into an organic phase [13,19] and that both this uptake [13,19] and the formation of non-bilayer lipid structures [13] is blocked by ruthenium red which is the classical inhibitor of the mitochondrial Ca^{2+} transport system. These data support the hypothesis [14,16] that cardiolipin is part of the Ca^{2+} transport system. The cardiotoxic effect of adriamycin might be related to an inhibition of this transport process. This suggestion which is supported by the finding that adriamycin blocks the uptake of Ca^{2+} into an organic phase by cardiolipin is presently under active investigation.

The role of cardiolipin in the activity of the cytochrome *c* oxidase has been the subject of considerable controversy. At present, most experimental data point to a catalytic role of some strongly bound cardiolipin molecules [20,25]. In line with these findings is the observation that adriamycin inhibits cytochrome *c* oxidase activity (unpublished). The specific induction of non-bilayer lipid structures by cytochrome *c* in cardiolipin containing model membranes [13] suggests that these structures (of the type shown in Fig. 13 of Ref. 14) might be essential for the activity of the oxidase. The inhibition of mitochondrial oxidative phosphorylation by adriamycin could thus possibly be understood by the inhibition of formation of essential non-bilayer lipid structures next to a displacement of the cytochrome *c* from the cardiolipin oxidase complex.

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