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Effects of adriamycin on lipid polymorphism in cardiolipin-containing model and mitochondrial membranes

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(1) The effects of the anti-tumor drug adriamycin on lipid polymorphism in cardiolipin-containing model membranes and in isolated inner mitochondrial membranes has been examined by ^{31}P-NMR. (2) Adriamycin binding does not affect the macroscopic structure or local order in the phosphate region of cardiolipin liposomes. (3) In cardiolipin liposomes and in cardiolipin-phosphatidylcholine (1:1) liposomes, the drug inhibits the ability of Ca^{2+} to induce the hexagonal H_{II} phase. (4) Adriamycin interaction with both dioleoylphosphatidylethanolamine-cardiolipin (2:1) and dioleoylphosphatidylethanolamine-phosphatidylserine (1:1) liposomes results in structural phase separation into a liquid-crystalline hexagonal H_{II} phase for the phosphatidylethanolamine and a liquid-crystalline lamellar phase for the negatively charged phospholipid. (5) Combined high-resolution ^{31}P-NMR, electron microscopy and light scattering studies reveal the prominent fusion capacity of adriamycin towards cardiolipin-phosphatidylcholine small unilamellar vesicles. (6) Addition of Ca^{2+} to total rat liver inner mitochondrial membrane lipids, dispersed in excess buffer, results in hexagonal H_{II} formation for part of the phospholipids. By contrast, the original bilayer structure is completely conserved when the above experiment is performed in the presence of adriamycin. (7) ^{31}P-NMR spectra of isolated inner mitochondrial membranes are indicative of a bilayer organization for the majority of the phospholipids. Approximately 15% of the signal intensity originates from phospholipids which experience isotropic motion. Adriamycin addition almost completely eliminates the latter spectral component. In the absence of adriamycin, Ca^{2+} addition greatly increases the percentage of the phospholipids giving rise to an isotropic signal possibly indicating the formation of non-lamellar lipid structures. Adriamycin which specifically binds to cardiolipin (K. Nicolay et al. (1984) Biochim. Biophys. Acta 778, 359–371) completely blocks the Ca^{2+}-induced structural reorganization of the lipids in this membrane.

Introduction

The anthracycline antibiotic adriamycin is one of the most important anti-cancer drugs used in the treatment of a broad range of malignancies [1]. The major side-effect of adriamycin treatment is its specific, dose-dependent cardiotoxicity [2]. There is increasing evidence suggesting that the mitochondrial membrane could be the target responsible for this cardiotoxicity (for a review, see Ref. 3).

In model systems, adriamycin displays a high affinity towards the negatively charged phospholipid cardiolipin [4], which, in normal tissue, is

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exclusively localized in the inner membrane of mitochondria. Recently, we demonstrated that the formation of a 2 : 1 complex between adriamycin and cardiolipin accounts for the extent of drug binding to mitochondrial membranes [5]. This led us to conclude that cardiolipin represents the major target for adriamycin in mitochondria.

It was demonstrated previously that adriamycin inhibits the formation of non-bilayer lipid structures in cardiolipin-containing model membranes [6]. Here, these studies are extended to cover a broader range of model membranes while also a quantitative description of the effects of adriamycin-cardiolipin complex formation on lipid polymorphism is presented. Furthermore, the present paper reports on the structural influence of the drug on inner mitochondrial membranes as studied with ^{31}P-NMR. The results will be discussed in terms of possible mechanisms for the adriamycin-induced inhibition of mitochondrial functions, such as Ca^{2+} transport and oxidative phosphorylation.

Materials and Methods

Materials

Adriamycin (NSC 123127) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bovine heart cardiolipin (disodium salt) was purchased from Avanti-Polar Lipids (Birmingham, MA, U.S.A.). Phosphatidylcholine was isolated from egg by standard procedures. 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine ($18:1_c/18:1_c$-phosphatidylethanolamine) was synthesized as described before [7]. Phosphatidylserine (sodium salt) was isolated from bovine brain using general procedures [8] with some modifications. The major change consisted of replacing the elution of the lipid from a DEAE-cellulose column with glacial acetic acid by column chromatography and high performance liquid chromatography under milder chemical conditions. Total mitochondrial inner membrane lipids were extracted from rat liver mitochondria as detailed before [5]. Inner membrane ghosts were prepared from rat liver mitochondria according to published procedures [9].

Methods

Membrane preparations. Aqueous lipid dispersions were prepared for ^{31}P-NMR by dispersing (by vortex mixing at room temperature) a dry film of the relevant phospholipid mixture in 0.8 ml 20% ^2H_2O-containing 100 mM NaCl, 10 mM Tris-HCl, 0.5 mM EDTA (pH 7.4).

Small unilamellar vesicles were obtained by sonicating liposome suspensions under nitrogen and on ice for 5 min using a Branson B-12 tip sonicator (power setting 50 W), followed by 15 min centrifugation at $25\,000 \times g$ to sediment large structures and metal particles originating from the tip.

The fatty acid composition of cardiolipin was determined by gas-liquid chromatography of the methyl esters according to established procedures.

The final pellet of inner membrane ghosts was resuspended in 20% ^2H_2O-containing 10 mM NaCl, 10 mM Tris-HCl, 0.5 mM EDTA (pH 7.4) and subsequently transferred to a 10 mm tube for ^{31}P-NMR studies.

Drug solutions. Adriamycin was added to the lipid dispersions and ghost preparations in 0.5 to 1.0 ml of the relevant buffers.

Nuclear magnetic resonance (NMR). ^{31}P-NMR studies were performed at 36.4 and 81.0 MHz on a Bruker WH90 and WP200 spectrometer, respectively. High-power proton decoupling was employed in all experiments. Unless otherwise indicated, the NMR studies were carried out at 30°C. Chemical shift scales are drawn with the ^{31}P-NMR peak of sonicated egg-phosphatidylcholine vesicles at 0 ppm.

The experimental conditions employed for assessing phospholipid structure by ^{31}P-NMR at 81.0 MHz were as follows. Typically, 3000 scans were recorded using 90° radio frequency pulses, a sweep width of 25 kHz and a 1.0 s repetition time. At 36.4 MHz, spectra (20 000 scans each) were obtained employing 9 μs 45° radio frequency pulses and a 0.17 s repetition time. The spectral width was 12 000 Hz. At both frequencies, free induction decays were exponentially filtered resulting in a 50 Hz line broadening.

The Ca^{2+}-induced conversion of phosphatidylserine in an originally liquid-crystalline state displaying a bilayer lineshape into crystalline, cochleated structures was monitored by ^{31}P-NMR at 36.4 MHz. By the choice of the spectral width (12 kHz) and a 90 μs delay between pulse and

data acquisition, phospholipids in a crystalline, immobilized organization, such as the Ca^{2+}-phosphatidylserine salt [10], do not significantly contribute to the NMR signal. The intensity of the remaining bilayer component at each Ca^{2+} concentration was determined by computer integration. To assure that intensities can be compared directly, all spectra were collected under exactly identical conditions.

Small unilamellar cardiolipin-phosphatidylcholine vesicles give rise to ^{31}P-NMR spectra with separate peaks for cardiolipin and phosphatidylcholine. The former phospholipid resonates at 1 ppm down field from the latter which is at 0 ppm. 36.4 MHz ^{31}P-NMR spectra of these small unilamellar vesicles were recorded at 30°C by collecting 500 scans with 16 μs 90° pulses, a repetition time of 1.7 s and a spectral width of 1200 Hz. Due to this choice of the spectral width only small structures significantly contribute to the NMR signal. Macroscopically large systems originating from aggregation or fusion of small vesicles are essentially NMR 'silent' in that their linewidth exceeds the spectral width, resulting in a (partial) elimination of their signal due to the use of cut-off filters. Free induction decays were exponentially filtered corresponding to a 2 Hz line broadening. Signal intensities were determined separately for the phosphatidylcholine and cardiolipin resonances by means of computer integration. The intensity of the peak from a triphenylphosphine solution in $CHCl_3$ in a capillary located in a central insert in the NMR tube served as an external standard.

Electron microscopy. Freeze-fracture electron microscopy studies were performed as described before [11].

Fusion of small unilamellar vesicles. Adriamycin-induced fusion of small unilamellar vesicles prepared from phosphatidylcholine-cardiolipin liposomes was studied by the following techniques: (i) The first method employed to monitor vesicle aggregation and/or fusion consisted of measuring drug-induced absorbance increases at 650 nm on a Perkin-Elmer type 356 double-beam spectrophotometer. At this wavelength light scattering could be measured without interference of adriamycin absorbance. Samples of small unilamellar vesicles are essentially translucent whereas aggregation and/or fusion to larger structures results in increased turbidity. 50 μl of a suspension of small unilamellar vesicles (3 μmol phospholipid) were diluted with the 100 mM NaCl buffer to a final volume of 1 ml. Adriamycin was added from a 5 mM stock solution. (ii) High-resolution ^{31}P-NMR, which yields separate peaks for cardiolipin and phosphatidylcholine (see above). Under the experimental conditions employed only small structures significantly contribute to the NMR signal. Therefore, this method provides a means to monitor adriamycin-induced conversion of small vesicles to large assemblies. (iii) Since the above two methods are indirect and cannot discriminate between drug-induced aggregation and fusion of vesicles, freeze-fracture electron microscopy was used to assess the exact nature of the resulting structures.

Results

The present paper deals with the consequences of adriamycin-cardiolipin interaction for the structural organization of cardiolipin-containing membranes, as studied by ^{31}P-NMR. The disodium salt of bovine heart cardiolipin, dispersed in excess buffer, is organized in extended bilayers [9], giving rise to characteristic asymmetrical ^{31}P-NMR spectra with a low-field shoulder and a high-field peak [12]. Addition of increasing amounts of adriamycin to cardiolipin liposomes leads to visual precipitation of the lipid. The chemical shift anisotropy, which is related to the local order of the phosphate region, was not significantly affected by adriamycin. It amounted to 25.5 ppm in the absence of the drug and to 26.2 ppm at an adriamycin/cardiolipin molar ratio of 2 : 1, as estimated from the distance of the low-field shoulder and high-field peak in the corresponding ^{31}P-NMR spectra (not shown). This implies that upon complexation with adriamycin cardiolipin retains its liquid-crystalline lamellar organization. However, the presence of adriamycin causes a strong inhibition of the Ca^{2+}-induced hexagonal H_{II} formation, as will be discussed next.

In agreement with previous data a small isotropic component is present in the ^{31}P-NMR spectrum of cardiolipin liposomes. This most likely is due to some smaller vesicles present in the pre-

paration. Addition of Ca^{2+} in aliquots form a concentrated solution greatly increases this isotropic component, in parallel with the formation of so-called lipidic particles in freeze-fracture replica's of the suspension. Above Ca^{2+}/cardiolipin = 0.15 an increasingly larger fraction of the lipid adopts the hexagonal H_{II} phase such that at a Ca^{2+}/cardiolipin ratio of 1.0 all cardiolipin molecules are organized in this phase. Since these data have already been reported in detail before [13], no further information on this aspect is presented here. In contrast to the situation described above, no isotropic spectral component is observed during similar Ca^{2+} titrations performed in the presence of adriamycin, i.e. only bilayer and/or hexagonal H_{II} type peaks contribute to the ^{31}P-NMR spectra. The reason for the complete absence of isotropic peaks when the drug is present, most probably is related to the capacity of adriamycin to increase the size of the lipid aggregates (see below) and to a reduction in the rate of lateral diffusion [14]. The results of Ca^{2+} titrations carried out in the presence of increasing amounts of drug are depicted in Fig. 1, where the percentage bilayer component is plotted against the molar ratio of Ca^{2+}/cardiolipin at different adriamycin/cardiolipin ratios. It is evident that adriamycin causes a progressive conservation of bilayer structure for cardiolipin, such that at a drug/lipid molar ratio of 2 : 1 an exclusive bilayer organization is retained at all Ca^{2+} concentrations indicated. At the latter drug/lipid ratio, a molar excess of Ca^{2+} as high as 3 is required to create a small hexagonal H_{II} component in the ^{31}P-NMR spectra (not shown), in agreement with published data [6]. It is importance to stress that adriamycin was added before Ca^{2+} in the above experiments. Recently, we have reported that when Ca^{2+} is added to cardiolipin liposomes leading to the formation of the hexagonal H_{II} phase subsequent addition of adriamycin is without any effect [5]. Apparently, cardiolipin organized in this non-bilayer structure is not accessible for the drug.

In view of the fact that adriamycin can support peroxidation of poly-unsaturated fatty acids in phospholipids [15], which in itself is known to drastically affect lipid polymorphism [16], we have determined whether the drug causes peroxidation of cardiolipin liposomes under our experimental conditions. It was found that even after incubation of 24 h in the presence of an excess of adriamycin the fatty acid composition of the treated cardiolipin was the same as the composition of the starting material. This excludes a possible contribution of lipid peroxidation to the effects described here.

Apart from cardiolipin, adriamycin also binds to phosphatidylserine be it with a much lower affinity [17]. In order to see whether this difference in affinity of the drug for the above lipids is expressed as a difference in the inhibition of Ca^{2+}-induced phase transitions, bovine brain phosphatidylserine liposomes were titrated with Ca^{2+} in the absence and in the presence of adriamycin (Fig. 2). The addition of Ca^{2+} to phosphatidylserine leads to precipitation of the lipid accompanied by the formation of crystalline cochleate structures [18]. These structures display very broad, powder-like ^{31}P-NMR spectra which, under our conditions, result in the disappearance of the bilayer signal. Fig. 2 demonstrates that in the control and also when adriamycin is present, the ^{31}P-NMR signal is reduced to approx. 20% of its original intensity at a Ca^{2+}/phosphatidylserine ratio of 0.5. This indicates that, in contrast to cardiolipin liposomes (Fig. 1), the drug does not block the Ca^{2+}-induced phase transition in phosphatidylserine liposomes. It should be realized, however, that the structures resulting from Ca^{2+} addition are largely different for the two lipid species: hexagonal H_{II} for cardiolipin and lamellar, crystalline for phosphatidylserine.

Cardiolipin constitutes approx. 20% of the phospholipids in the inner mitochondrial membrane, while phosphatidylcholine and phosphatidylethanolamine amount to approx. 40% and 35%, respectively [5,19]. Therefore, it is of interest to study the effects of adriamycin on the structure of cardiolipin/phosphatidylcholine and of cardiolipin/phosphatidylethanolamine mixtures. Fig. 3 shows the bilayer stabilizing effect of the drug towards Ca^{2+} in liposomes prepared from equimolar amounts of cardiolipin and egg phosphatidylcholine. Although, under physiological conditions, the latter lipid in isolated form always adopts the bilayer phase, it is known to be largely incorporated into the hexagonal H_{II} phase upon Ca^{2+} addition to equimolar cardiolipin/phosphatidylcholine mixtures [13]. As for pure

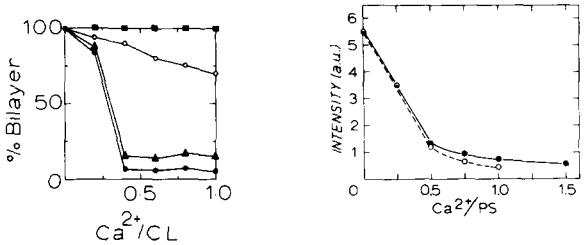

Fig. 1. Bilayer stabilizing effect of adriamycin towards Ca^{2+} in cardiolipin liposomes at 30°C. 30 μmol cardiolipin (CL) were dispersed as described in Materials and Methods. Thereafter, adriamycin was added from a concentrated stock solution to yield the following ratios of drug to cardiolipin phosphorus: 0.50 (●), 0.66 (▲), 0.80 (○), and 1.00 (■). Ca^{2+} was introduced in aliquots from a 1 M $CaCl_2$ solution. The ratio Ca^{2+}/CL refers to the molar ratio of Ca^{2+} added to cardiolipin present. The phospholipid structure was determined by ^{31}P-NMR at 36.4 MHz. The percentage bilayer intensity in the spectra was obtained by computer subtraction methods using reference spectra consisting of only one spectral component. The error in the percentages is estimated to be approx. 10%.

Fig. 2. ^{31}P-NMR signal intensities for phosphatidylserine as a function of added Ca^{2+} concentration, in the absence (○) and in the presence of adriamycin (●). In both experiments, 75 μmol bovine brain phosphatidylserine (PS) was dispersed as described in Materials and Methods. Adriamycin (75 μmol) in buffer was added afterwards. The ratio Ca^{2+}/PS refers to the molar ratio of Ca^{2+} added to phosphatidylserine present. For further details see also the legend to Fig. 1.

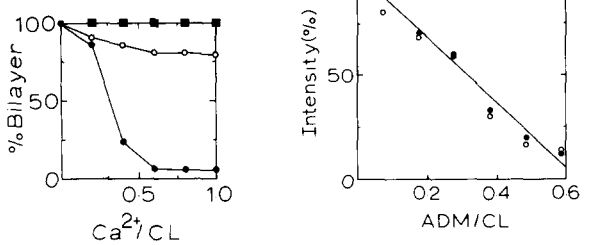

Fig. 3. Bilayer stabilizing effect of adriamycin towards Ca^{2+} in cardiolipin-phosphatidylcholine (1:1) liposomes at 30°C. 45 μmol phospholipid were dispersed as described in Materials and Methods. Thereafter, adriamycin was introduced to yield the following ratios of drug to cardiolipin phosphorus: 0.50 (●), 0.90 (○), and 1.00 (■). For further details see also the legend to Fig. 1.

Fig. 4. High resolution ^{31}P-NMR signal intensities for phosphatidylcholine (○) and cardiolipin (●) in phosphatidylcholine-cardiolipin (2:1) small unilamellar vesicles as a function of the added adriamycin (ADM) concentration. Small unilamellar vesicles were prepared from a dispersion of 40 μmol phospholipid as described in Materials and Methods. Adriamycin was added in aliquots from a 25 mM stock solution. The ratio ADM/CL refers to the molar ratio of adriamycin added to cardiolipin present.

cardiolipin liposomes, Ca^{2+} introduction in the absence of adriamycin gives rise to an isotropic component in the ^{31}P-NMR spectra with associated lipidic particles (see also Ref. 13). However, in the presence of the drug, only bilayer and/or hexagonal lineshapes are observed. Fig. 3 demonstrates that a pure bilayer spectrum is maintained in the range of Ca^{2+}/cardiolipin 0–1 when adriamycin is present in equimolar amounts relative to cardiolipin phosphorus. In summary, adriamycin effectively inhibits the formation of Ca^{2+}-induced non-lamellar lipid structures in cardiolipin-phosphatidylcholine (1:1) liposomes.

In the course of this study a number of observations were made indicative of prominent fusogenic properties of adriamycin towards cardiolipin-containing membranes. To study this phenomenon in more detail, we have performed light-scattering, ^{31}P-NMR and freeze-fracture electron microscopy experiments of the effects of adriamycin on small unilamellar vesicles prepared from different ratios of cardiolipin and phosphatidylcholine. As an example, Fig. 4 illustrates the decrease in intensity of the high-resolution ^{31}P-NMR resonances of phosphatidylcholine and cardiolipin when increasing amounts of drug were added to small unilamellar phosphatidylcholine-cardiolipin (2:1) vesicles. The decrease in signal intensity is the consequence of the formation of larger structures in which the lipid phosphates no longer experience isotropic motion and, therefore, are not detected within the small spectral width employed. It is evident that both resonances are equally affected by the drug. Freeze-fracture electron microscopy demonstrated that, indeed, adriamycin action leads to fusion of cardiolipin-phosphatidylcholine vesicles to multilamellar liposomes (not shown). It can be inferred from the data in Fig. 4 that essentially all unilamellar vesicles have undergone aggregation and/or fusion at a drug to cardiolipin phosphorus ratio of 0.3.

Light-scattering experiments revealed that adriamycin did not cause aggregation and/or fu-

sion below approx. 5% cardiolipin in the phosphatidylcholine vesicles and below 0.3 mM adriamycin, respectively (data not shown). It is obvious from the above data that adriamycin is a potent fusogen and that the drug-cardiolipin interaction is responsible for the fusion process.

Next, we have investigated the effects of adriamycin on the macroscopic organization of cardiolipin-phosphatidylethanolamine mixtures. The endogenous mitochondrial phosphatidylethanolamine adopts the hexagonal H_{II} phase upon dispersion under physiological conditions [9]. The same holds for soybean phosphatidylethanolamine [20]. However, when the latter unsaturated lipid species is mixed with cardiolipin in a 2 : 1 ratio in 100 mM NaCl buffer, a lamellar structure results [20] as a consequence of the bilayer stabilizing effect of the cardiolipin. A similar situation was created here by mixing cardiolipin with the synthetic $18:1_c/18:1_c$-(dioleoyl)phosphatidylethanolamine which has a bilayer-hexagonal H_{II} transition temperature of 10°C [21]. Fig. 5 shows a series of ^{31}P-NMR spectra of dioleoylphosphatidylethanolamine-cardiolipin (2 : 1) liposomes in the absence (Fig. 5A) and in the presence of adriamycin (Figs. 5B and C). The isotropic ^{31}P-NMR signal in Fig. 5A is caused by the fact that spontaneous small (lamellar) vesicles are formed in this system as evidenced by freeze-fracture electron microscopy (not shown). These structures allow significant motional averaging of the chemical shift anisotropy of the lipid phosphates. A completely different situation is encountered upon adriamycin introduction (Fig. 5B), which leads to visual precipitation of the lipid. The resulting NMR spectrum, recorded at 30°C, consists of a hexagonal H_{II} and a bilayer component amounting to 21% and 79% of the total intensity, respectively. The hexagonal H_{II} peak primarily originates from the phosphatidylethanolamine since at 5°C the H_{II} component in the spectrum is greatly reduced (Fig. 5C). Of the two lipids present only dioleoylphosphatidylethanolamine shows a bilayer to hexagonal phase transition around 10°C [21]. Control experiments demonstrated that the induction of the H_{II} phase does not arise from the increased ionic strength [22] due to drug addition since equivalent amounts of NaCl did not affect the ^{31}P-NMR spectrum (not shown).

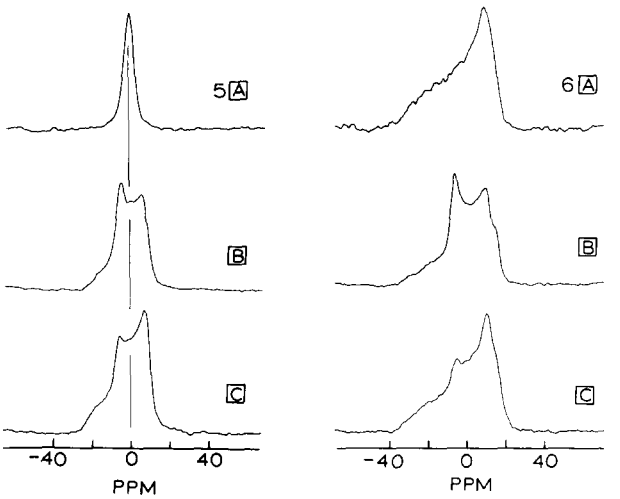

Fig. 5. 36.4 MHz ^{31}P-NMR spectra of dioleoylphosphatidylethanolamine-cardiolipin (2 : 1) liposomes. 60 μmol lipid were dispersed as described in Materials and Methods. (A) 30°C; (B) at 30°C, 5 min after the addition of 40 μmol adriamycin; (C) and (B) except that it was recorded at 5°C. Incubation of the adriamycin-containing sample did not further affect the ^{31}P-NMR spectrum. The vertical line indicates the chemical shift position of the isotropic peak at 0 ppm. Note that the hexagonal H_{II} component (Figs. 5B and C) resonates at approx. −7 ppm.

Fig. 6. 36.4 MHz ^{31}P-NMR spectra of dioleoylphosphatidylethanolamine-phosphatidylserine (1 : 1) liposomes. 80 μmol lipid were dispersed as described in Materials and Methods. (A) 30°C; (B) at 30°C, 5 min after the addition of 40 μmol adriamycin; (C) as (B) except that it was recorded at 5°C.

In order to see whether the hexagonal H_{II} phase induction by adriamycin described above for the dioleoylphosphatidylethanolamine-cardiolipin system might be a more general phenomenon in mixtures of the phosphatidylethanolamine and negatively charged phospholipids, we have performed similar experiments on dioleoylphosphatidylethanolamine-phosphatidylserine (1 : 1) liposomes (Fig. 6). The control suspension has a pure bilayer ^{31}P-NMR spectrum (Fig. 6A). Addition of the drug leads to precipitation of the lipid and, at 30°C, the NMR spectrum (Fig. 6B) consists of a hexagonal H_{II} (15%) and a bilayer component (85%). As for the cardiolipin system, a lowering in temperature to 5°C almost completely removes the H_{II} component from the spectrum (Fig. 6C) indicating that the hexagonal peak arises from the phosphatidylethanolamine species. This is sup-

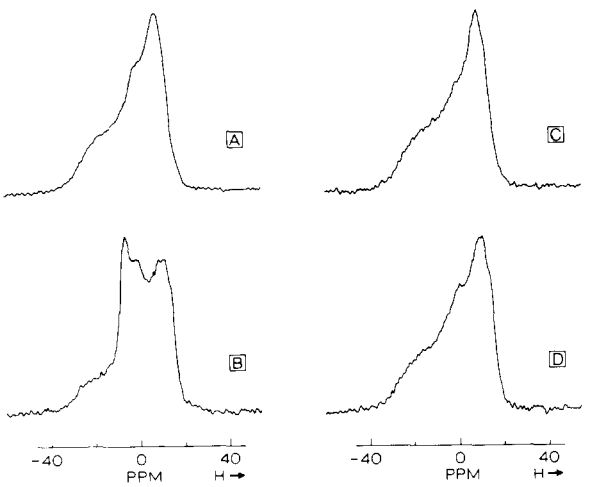

Fig. 7. 81.0 MHz ^{31}P-NMR spectra of dispersions of the total lipids extracted from rat liver inner mitochondrial membranes. 75 μmol lipid was dispersed as described in Materials and Methods. (A) Control; (B) in the presence of Ca^{2+} at a molar ratio of 2.0 Ca^{2+} per phospholipid; (C) as (A) in the presence of 15 μmol adriamycin; (D) as (C) after the addition of 2.0 Ca^{2+} per phospholipid present. All spectra (3000 scans each) were recorded at 30°C.

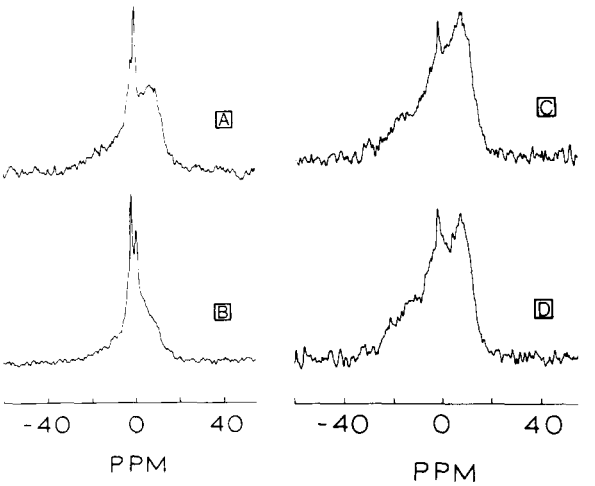

Fig. 8. 81.0 MHz ^{31}P-NMR spectra of isolated rat liver inner mitochondrial membranes. The protein content of the samples was 190 mg in 1.5 ml. (A) Control; (B) in the presence of 1.0 Ca^{2+} per phospholipid present; (C) as (A) after the addition of 12 μmol adriamycin; (D) as (C) after the addition of 1.0 Ca^{2+} per phospholipid present. All spectra (2000 scans each) were recorded at 37°C.

ported by close inspection of Fig. 6B, which reveals a shoulder at the high-field side, indicative of the larger chemical shift anisotropy of the phosphatidylserine component in the lamellar phase [23]. Since this shoulder was less pronounced in the starting condition, it suggests that the lamellar phase is enriched in phosphatidylserine. It is interesting to note that the doubling of the high-field peak in Fig. 6B is complemented by a doubling of the low-field shoulder.

The effects of adriamycin on Ca^{2+}-induced lipid polymorphism in dispersions of the endogenous inner mitochondrial membrane lipids are presented in Fig. 7. An essentially pure bilayer spectrum is observed in the 100 mM NaCl buffer (Fig. 7A). Ca^{2+} induces a hexagonal H_{II} phase corresponding to approx. 20% of the phospholipids (Fig. 7B), as published previously [9]. By contrast, adriamycin completely inhibits this Ca^{2+}-induced H_{II} phase formation (Figs. 7C and D) when present in equimolar amounts relative to cardiolipin phosphate.

Adriamycin also has intriguing effects on the membrane structure of isolated inner mitochondrial membranes, as studied by ^{31}P-NMR at 37°C (Fig. 8). Control spectra (Fig. 8A) of these so-called inner membrane ghosts demonstrate that the majority of the phospholipids is organized in extended bilayers giving rise to the characteristic bilayer lineshape. The significant isotropic component at 0 ppm indicates that part of the phospholipids undergo isotropic motion at 37°C, in agreement with previous data [9]. Adriamycin addition to a level corresponding to the cardiolipin phosphorus content of the preparation largely removes the isotropic component and, in addition, leads to a better definition of the low-field shoulder of the bilayer component (Fig. 8C). The sharp peak superimposed upon the bilayer-type peak in Fig. 8C, most probably originates from inorganic phosphate which has remained in the ghost preparation. When Ca^{2+} is introduced into the ghost preparation, a marked increase in the amount of isotropic signal is observed, as reported previously [9]. Fig. 8B shows the resulting ^{31}P-NMR spectrum when equimolar Ca^{2+} relative to phospholipid has been added. A completely different situation is encountered upon Ca^{2+} addition in the presence of adriamycin (Fig. 8D), where an essentially pure bilayer-type spectrum is retained. Adriamycin ad-

dition after Ca^{2+} has no further effect on the structural organization of the phospholipids in the ghost membranes, i.e. the resulting ^{31}P-NMR spectrum is identical to the spectrum in Fig. 8B (data not shown).

It is important to consider the possibility that isotropic ^{31}P-NMR peaks may either arise from bilayer regions with tight radius of curvature (e.g. small bilayer vesicles) or from non-bilayer lipid structures. We favour the latter possibility on the basis of the following considerations: (i) the isolation procedure employed includes extensive washing by centrifugation at relatively low centrifugal forces thereby eliminating the possibility of a significant contamination of the control ghost suspension with small membrane fragments (Fig. 8A). This was independently confirmed by freeze-fracture electron microscopy, which demonstrated that apart from mitochondrial ghosts with their well-known morphology (i.e. 0.5–0.8 μm diameter fracture faces, randomly covered with protein particles) insignificant amounts of small membrane fragments were present; (ii) ghost preparations sedimented by low-speed centrifugation in the presence of Ca^{2+} form a more densely packed pellet than control preparations and, in addition, show only a minor increase in the content of small membrane fragments upon examination by electron microscopy. This eliminates a significant contribution of small bilayer structures to the prominent isotropic peak in Fig. 8C.

Discussion

Adriamycin is known to form a complex with the negatively charged phospholipid cardiolipin. The complex has a stoichiometry of two drug molecules per cardiolipin [5,17], which is not only observed for dispersions of pure cardiolipin but also for more complex systems like dispersions of the endogenous inner mitochondrial membrane lipids, and intact mitochondria [5]. The above knowledge justifies the interpretation of the present data on the effects of adriamycin on membrane structure in terms of a specific adriamycin-cardiolipin interaction.

The ^{31}P-NMR studies reported here demonstrate that the complex between adriamycin and cardiolipin has a lamellar organization, in agreement with conclusions drawn previously from small-angle X-ray scattering data [5]. In addition, it could be concluded that the lamellar structure of the complex is of a liquid-crystalline nature. This could be inferred from the fact that the residual chemical shift anisotropy, which is sensitive to the occurrence of gel to liquid-crystalline phase transitions [37], of the cardiolipin phosphate (as measured from the ^{31}P-NMR spectra) is not affected by complex formation.

Ca^{2+}-induced phase transitions in all cardiolipin-containing membranes studied here are inhibited by adriamycin (Figs. 1, 3, 7 and 8). At a Ca^{2+}/cardiolipin ratio of 1.0, complete inhibition is achieved, i.e. bilayer stabilization by adriamycin towards Ca^{2+} is complete only when the the drug is added in equimolar amounts relative to cardiolipin phosphorus (e.g. Figs. 1 and 3). This again is indicative of the formation of a complex between drug and cardiolipin having a stoichiometry of 2 : 1. Interestingly, the presence of equimolar adriamycin has no effect on the Ca^{2+}-induced phase transitions in phosphatidylserine-containing membranes (e.g. Fig. 2). These results are fully consistent with the reported difference in affinities of adriamycin for phosphatidylserine and cardiolipin being two orders of magnitude higher for the latter phospholipid [17].

The bilayer stabilizing effect of adriamycin on cardiolipin-containing membranes is not confined to Ca^{2+}-induced phase changes. Also, the cytochrome *c*-induced formation of hexagonal H_{II} and isotropic phases in cardiolipin liposomes [20] is effectively blocked by the drug [6].

Before coming to an integration of the present data allowing the evaluation of the possible functional implications, the drug-induced fusion of and structural phase segregation in cardiolipin-containing model membranes will be discussed. The combination of experimental methods used here has yielded results in favour of strong fusogenic properties of adriamycin towards small unilamellar vesicles of cardiolipin and phosphatidylcholine (e.g. Fig. 4). Unfortunately, alternative spectrophotometric procedures for measuring fusion of vesicles, including fluorescence techniques for measuring mixing of aqueous contents [24] and resonance energy transfer between photo-labeled lipids [25], cannot be used to study adriamycin-in-

duced fusion due to the prominent spectroscopic properties of the drug itself [4]. By electron microscopy, we could demonstrate that non-saturating levels of adriamycin relative to cardiolipin are sufficient to initiate fusion of small unilamellar phosphatidylcholine-cardiolipin vesicles to multilamellar vesicles. In this context it is important to stress that in these vesicles not only approx. 70% of the total phospholipids but also 70% of the individual cardiolipin and phosphatidylcholine species are localized in the outer monolayer, as demonstrated by the use of shift reagents in ^{31}P-NMR (Ref. 26; Nicolay, K. and Van der Neut, R., unpublished data). This means that the observed dependence of fusion on the adriamycin-cardiolipin ratio (Fig. 4) cannot be explained on the basis of a preferential localization of the cardiolipin in the inner monolayer. A fusion mechanism based exclusively on surface charge neutralization due to drug-cardiolipin complex formation seems to be highly unlikely, since in that case Ca^{2+} would be expected to be a more potent fusogen than the monovalent cation adriamycin. On the contrary, the minimal Ca^{2+} concentration required for fusion in 1 : 1 cardiolipin/phosphatidylcholine vesicles is approx. 9 mM [24], whereas the threshold of adriamycin-induced fusion lies around 0.6 mM as monitored by electron microscopy under comparable conditions. This leads us to suggest that in addition to charge neutralization, also phase segregation of the adriamycin-cardiolipin complex in the plane of the bilayer might well play a role in the drug-induced fusion process. This phase segregation might result in bilayer instabilities which are responsible for the fusion process.

Structural phase segregation due to adriamycin action was demonstrated in dioleoylphosphatidylethanolamine-cardiolipin (Fig. 5) and -phosphatidylserine mixtures (Fig. 6). Binding of the drug led to the formation of specific domains, enriched either in phosphatidylethanolamine or in the complex between drug and negatively charged phospholipid. It is important to note that this phase segregated state is reached under fully liquid-crystalline conditions for all the lipids. Poly(L-lysine) is also capable of inducing a similar phase separation in soybean phosphatidylethanolamine-cardiolipin (2 : 1) liposomes [23]. However, this random coil peptide is a multivalent cation, whereas adriamycin carries only one positive charge at neutral pH. Therefore, apart from the electrostatic interaction between the lipid phosphates and the drug amino groups, another property of adriamycin is required to explain the segregation phenomenon. The driving force for the latter process most probably lies in the ability of neighbouring anthraquinone planes to undergo stacking interaction which has been reported to bring about an additional free energy of drug-phospholipid complex formation of -2.8 kcal/mol [17]. Recently, Huart et al. [27] also reported on adriamycin-cardiolipin complex segregation in the lipid matrix, as was concluded from differential scanning calorimetry experiments on liposomes made from cardiolipin/dimyristoylphosphatidylcholine mixtures.

Two aspects concerning the molecular details of the adriamycin-cardiolipin interaction are particularly evident from the model membrane studies presented here: (i) adriamycin-cardiolipin interaction blocks the formation of non-lamellar lipid structures by cardiolipin, (ii) adriamycin-cardiolipin complex formation causes lateral phase separation of a lamellar adriamycin-cardiolipin complex.

The ^{31}P-NMR experiments on isolated inner mitochondrial membranes deserve special attention. From the high concentration of non-bilayer lipids in this membrane and several functional properties of the system, it was suggested that non-bilayer lipid structures could occur in this membrane and would play an essential role in membrane function [28]. One of the arguments in favour of this suggestion is the observation that in isolated ghosts at 37°C an isotropic component occurred in the ^{31}P-NMR spectrum which intensity markedly increased upon Ca^{2+} addition [9]. It is important to realize that these isotropic peaks cannot originate from small membrane fragments, as indicated in Results. However, it cannot be excluded that the isotropic spectral component arises from localized regions of tight radius of curvature present in large vesicles. Here we have reproduced the NMR characteristics of the ghost preparation, but in addition have shown that due to the adriamycin-membrane interaction, which most likely involves the cardiolipin component of the membrane, the isotropic ^{31}P-NMR component

is greatly reduced in intensity. In view of the model membrane experiments these data suggest a specific interaction between cardiolipin and adriamycin resulting in a profound protection against the formation of non-bilayer lipid structures in the inner membrane. In intact, functionally active mitochondria Ca^{2+} addition did not result in the immediate appearance of an isotropic ^{31}P-NMR signal from the phospholipids suggesting that additional factors are involved in determining the Ca^{2+}-cardiolipin interaction in this system [38].

It is tempting to speculate on the possible functional implications of the adriamycin-induced effects on membrane structure presented here. Non-bilayer lipid structures formed as a result of Ca^{2+}-cardiolipin and cytochrome *c*-cardiolipin interaction have been proposed to be involved in Ca^{2+} transport, cytochrome *c* oxidase activity, and the formation of contact sites (for a review of the available evidence, see Refs. 6, 28, 39). Adriamycin is known to be an efficient inhibitor of these important mitochondrial functions [5,27,29]. It thus appears that the inhibition is due to the blocking of the formation of essential non-bilayer lipid structures by adriamycin. Also, the adriamycin-induced phase segregation observed in cardiolipin-containing model membranes, most probably, has important functional implications. A number of enzymes in the inner mitochondrial membrane have an absolute requirement for cardiolipin. These include cytochrome *c* oxidase [30] and the inorganic phosphate carrier [31]. Both enzymes are efficiently inhibited by adriamycin [15,32–34]. Moreover, it was shown that the drug-related inhibition is the consequence of adriamycin-cardiolipin complex formation rather than of a direct drug-enzyme interaction [15,32–34]. Lateral phase segregation of the drug-cardiolipin complex would lead to the removal of the phospholipid from the immediate environment of the enzymes, which might explain their inactivation. This mechanism would apply to both the phosphate carrier and cytochrome *c* oxidase. Another contribution to the severe inhibition of the latter enzyme might result from competition of the adriamycin with the substrate cytochrome *c*. There is ample evidence that cytochrome *c* acts as electron carrier between its reductase and oxidase by lateral diffusion while bound to cardiolipin [35,36]. Moreover, Goormaghtigh et al. [6] have shown that adriamycin displaces both the reduced and oxidized forms of cytochrome *c* from cardiolipin multilamellar liposomes.

At present we are investigating the effects of adriamycin on membrane structure in intact, functionally active rat liver mitochondria.

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