Effect of Doxorubicin on the Order of the Acyl Chains of Anionic and Zwitterionic Phospholipids in Liquid-Crystalline Mixed Model Membranes: Absence of Drug-Induced Segregation of Lipids into Extended Domains†

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ABSTRACT: We investigated the effect of the antineoplastic drug doxorubicin on the order of the acyl chains in liquid-crystalline mixed bilayers consisting of dioleoylphosphatidylserine (DOPS) or -phosphatidic acid (DOPA), and dioleoylphosphatidylcholine (DOPC) or -phosphatidylethanolamine (DOPE). Previous ^{2}H-NMR studies on bilayers consisting of a single species of di[11,11-$^2H_2$]oleoyl-labeled phospholipid showed that doxorubicin does not affect the acyl chain order of pure zwitterionic phospholipid but dramatically decreases the order of anionic phospholipid [de Wolf, F. A., et al. (1991) *Biochim. Biophys. Acta 1096*, 67–80]. In the present work, we studied mixed bilayers in which alternatively the anionic or the zwitterionic phospholipid component was ^{2}H-labeled so as to monitor its individual acyl chain order. Doxorubicin decreased the order parameter of the mixed anionic and zwitterionic lipids by approximately the same amount and did not induce a clear segregation of the lipid components into extended, separate domains. The drug had a comparable disordering effect on mixed bilayers of unlabeled cardiolipin and ^{2}H-labeled zwitterionic phospholipid, indicating the absence of extensive segregation also in that case. Upon addition of doxorubicin to bilayers consisting of 67 mol % DOPE and 33 mol % anionic phospholipid, a significant part of the lipid adopted the inverted hexagonal (H_{II}) phase at 25 °C. This bilayer destabilization, which occurred only in mixtures of anionic phospholipid and sufficient amounts of DOPE, might be of physiological importance. Even upon formation of extended H_{II}-phase domains, lipid segregation was not clearly detectable, since the relative distribution of ^{2}H-labeled anionic phospholipid and [^{2}H]DOPE between the bilayer phase and H_{II} phase was very similar. Our findings argue against a role of extensive anionic/zwitterionic lipid segregation in the mechanism of action and toxicity of doxorubicin.

Doxorubicin and related anthracyclines are important anticancer agents that interact with DNA (Gigli et al., 1989; Frederick et al., 1990; Frezard & Garnier-Suillerot, 1990), topoisomerase II (Capranico et al., 1989, 1990), and membranes (Nicolay et al., 1984; Griffin et al., 1986; Arancia et al., 1988; Escriba et al., 1990). Possibly in combination with drug-mediated radical generation (Sinha & Mimnaugh, 1990), these interactions can result in DNA damage (Capranico et al., 1989; Vichi et al., 1989) and inhibition of DNA synthesis (Crooke et al., 1978). On the other hand, anthracyclines can interfere with membrane-associated processes (Santone et al., 1986; Nicolay & De Kruijff, 1987; Eilers et al., 1989; Voelker, 1991), including those involved in signal transduction (Thompson et al., 1987; Posada et al., 1989; Oakes et al., 1990; Lanzi et al., 1991; Mustonen & Kinnunen, 1991). These effects can occur already at nanomolar to micromolar drug levels (Santone et al., 1986; Oakes et al., 1987, 1990; Posada et al., 1989). We have recently shown that in mixed membranes containing mainly zwitterionic phospholipid, doxorubicin binds specifically to polyphosphoinositides (De Wolf et al., 1991b). Neither the interplay between the various drug-induced changes in the cell (Vichi et al., 1989; Kharbanda et al., 1991) nor the relative importance of these changes for anthracycline action and toxicity is fully understood as yet [see, for example, Siegfried et al. (1983), Capranico et al. (1989), Lanks and Lehman (1990), and Papoian and Lewis (1991)]. However, the interaction of the anthracyclines with the plasma membrane is likely to be of major importance, not only in terms of drug transport (Burke et al., 1987; Frezard & Garnier-Suillerot, 1991) but probably also in terms of drug action and toxicity (Tritton & Hickman, 1990; Tritton, 1991).

Doxorubicin, which is positively-charged at physiological pH (Figure 1A), binds highly specifically to membranes containing anionic phospholipids (Goormaghtigh et al., 1980a; Burke et al., 1988; De Wolf et al., 1991a,b). This specificity cannot be explained merely by the electrostatic redistribution of the free drug close to the membrane surface (De Wolf, 1991; De Wolf et al., 1991a). The binding probably involves the formation of specific complexes with the anionic phospholipids (Goormaghtigh et al., 1980a,b; Henri et al., 1985). Hydrophobic interactions also play a role, and at least part of the bound drug molecules penetrates into the membrane (Henri et al., 1985; Constantinides et al., 1990; Dupou-Cézanne et al., 1989). The drug induces a decrease of acyl chain mobility as well as a dramatic decrease of acyl chain order in membranes consisting of anionic phospholipids (De Wolf et al., 1991a). In contrast, it has no effect on the order and mobility in membranes consisting of zwitterionic phospholipids, even at drug levels exceeding 10 mM, in accordance with the relatively low binding capacity of these membranes observed at lower overall drug levels (Burke & Tritton, 1985; De Wolf et al., 1991a,b).

There are some indications that these drug–lipid and mutual drug–drug interactions can result in segregation of the anionic and zwitterionic phospholipid components of mixed membranes into extended domains, each significantly enriched in

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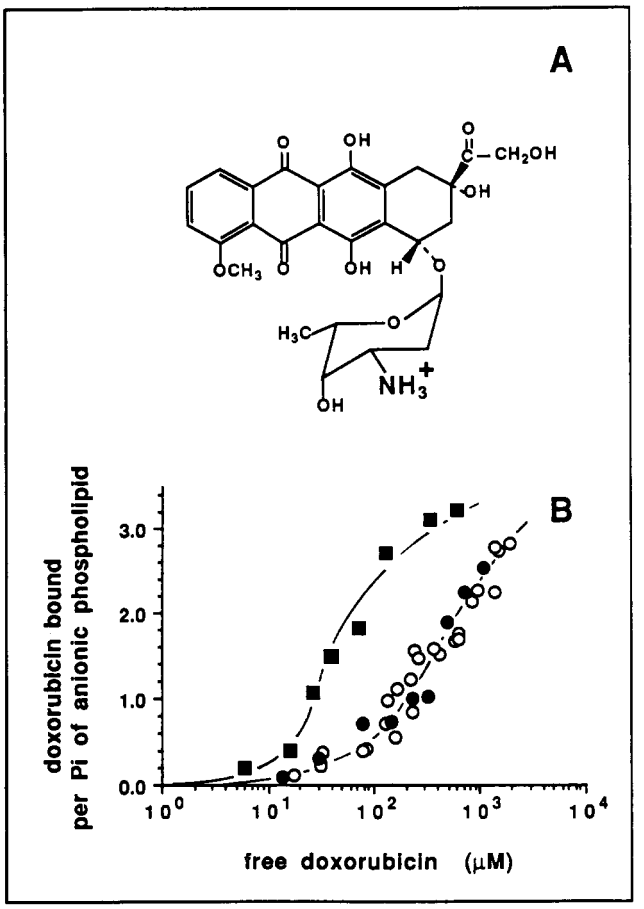

FIGURE 1: (A) Structure of doxorubicin in the single-protonated form. (B) Binding of doxorubicin to large unilamellar vesicles consisting of DOPA/DOPC mixtures at a molar lipid ratio of 1/1 (■) or 1/9 (●) or consisting of a mixture of phospholipids from *Escherichia coli* containing 73% PE, 15% cardiolipin–P_i, and 12% phosphatidylglycerol (○). Vesicles were prepared by extrusion of the hydrated lipid mixtures through polycarbonate filters (from Nuclepore, 400-nm pores) according to Hope et al. (1985). After a 1–3-h incubation with doxorubicin, binding was analyzed according to De Wolf (1991) by pelleting the vesicles (45 min at 436*g* in a Beckman TLA 100.2 rotor) and determining the free drug concentration in the supernatant.

one of the components. This is analogous to the effect of Ca^{2+} on mixed bilayers containing phosphatidylserine (PS)[1] or phosphatidic acid (PA) (Ohnishi & Ito, 1974; Jacobson & Papahadjopoulos, 1975; Haverstick & Glaser, 1987). Differential scanning calorimetry shows that in model membranes consisting of dimyristoyl- or dipalmitoylphosphatidylcholine (DPPC) and 6–15 mol % cardiolipin, and in the absence of drug, the transition of the PC from the gel to the liquid-crystalline state is largely obscured. The transition is partially reestablished upon addition of positively-charged anthracyclines, suggesting the formation of membrane domains that are significantly cardiolipin-depleted (Goormaghtigh et al., 1982, 1986, 1990). Another indication for a drug-induced segregation of lipids into extended separate domains is provided by studies on mixed bilayers containing phosphatidylethanolamine (PE). Although pure dioleoyl-PE (DOPE) is in the inverted hexagonal (H_{II}) phase at 20–25 °C (Cullis & de Kruijff, 1976; Tilcock et al., 1982; Farren et al., 1984), it can be incorporated into mixed bilayers containing anionic phospholipids (PS, cardiolipin). Addition of doxorubicin to such mixed bilayers induces a PE-dependent formation of the H_{II} phase (Nicolay et al., 1985, 1988). These observations are potentially very important, since lipid segregation provides a possible explanation for the antracycline-induced inhibition of cardiolipin-dependent mitochondrial energy transduction (Goormaghtigh et al., 1982, 1986; Nicolay & De Kruijff, 1987; Nicolay et al., 1985, 1988) and other vital anionic phospholipid-dependent processes.

However, an anthracycline-induced segregation of anionic and zwitterionic phospholipids has never been directly demonstrated in biological membranes. In fact, the evidence for drug-induced lipid segregation is entirely based on the occurrence of lipid-phase transitions in specially designed model membranes. The segregation may have occurred merely as a result of these phase transitions. It is not known whether anthracyclines can induce extensive lipid segregation in physiologically more relevant model systems that remain in the liquid-crystalline bilayer organization after the addition of the drugs.

We addressed this question by making use of the above-described acyl chain disordering effect of doxorubicin and its specificity for anionic phospholipids. We studied mixed membranes consisting of anionic and zwitterionic dioleoylphosphatidylcholine, and monitored the acyl chain order by ^2H-NMR. Each mixture was characterized in parallel experiments in which alternatively the anionic or the zwitterionic lipid was carrying a ^2H-label at the 11th carbon atom of its acyl chains. Doxorubicin does not appear to have a clear differential effect on the anionic and zwitterionic lipid components and does not induce a significant segregation of these components into extended, separate domains. The drug destabilizes mixed bilayers containing anionic phospholipid and sufficient amounts of PE, resulting in a partial transition to the H_{II} phase. Also in this case, we did not detect an extensive segregation of the lipids.

MATERIALS AND METHODS

Materials. Synthesis and purification of unlabeled and specifically di[11,11-$^2H_2$]oleoyl-labeled 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), -phosphoethanolamine (DOPE), -phosphoserine (DOPS), and -phosphate (DOPA) and isolation and purification of cardiolipin from bovine heart were according to published procedures (Van Deenen & de Haas, 1964; Comfurius & Zwaal, 1977; Farren et al., 1984; Chupin et al., 1987; Smaal et al., 1985). Unless stated otherwise, the following buffer was used: 100 mM NaCl, 25 mM Pipes, and 0.5 mM EGTA, pH 7.4, in ^2H-depleted water. Doxorubicin was purchased from Aldrich (Belgium) and shown to be pure by HPTLC according to Nicolay et al. (1984). All other chemicals were of analytical grade. Doxorubicin concentrations were determined by photometry at 480 nm, after dilution of samples to 5–10 μM drug in buffer (extinction coefficient = 10 600 M^{-1} cm^{-1}). Stock solutions in buffer were adjusted to the desired pH (usually 7.4) and to a final doxorubicin concentration of 20 mM and kept for maximally 1 day at 4 °C in the dark under N_2.

Model Membrane Preparation. Stock solutions of lipid were made in chloroform (cardiolipin in benzene), and the lipid concentration was determined on a phosphorus basis according to Böttcher et al. (1961). Amounts of stock solution containing 15, 20, or 30 μmol of lipid–phosphorus were rigorously mixed in a 10-mm NMR tube (total amount of lipid–phosphorus, 40–45 μmol). The solvent was evaporated

[1] Abbreviations: DOPA, DOPC, DOPE, and DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphate, -phosphocholine, -phosphoethanolamine, and -phospho-L-serine, respectively; $\Delta\nu_q$, residual ^2H quadrupolar splitting; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; NMR, nuclear magnetic resonance; Pipes, 1,4-piperazinediethanesulfonic acid; SAXS, small-angle X-ray scattering.

in vacuum, at 45 °C, while rotating the tube, so as to yield thin and homogeneously-mixed lipid films. After subsequent addition of fresh chloroform and rigorous mixing, the evaporation procedure was repeated, and solvent traces were removed in a desiccator under vacuum for at least 15 h, while keeping the sample dry over fresh P_2O_5.

The DOPC-containing mixed films were then hydrated by adding 0.5 mL of buffer and applying 10 cycles of slow freezing (−70 °C) and thawing (25 °C). Hydration of DOPE-containing mixed films (also with 0.5 mL of buffer) was carried out at 0 °C, normally without freeze-thawing. Hydration at 0 °C was necessary in order to prevent the occurrence of isotropic signals in the ^{31}P- and ^{2}H-NMR spectra of some of the mixtures. After approximately 1 h at 0 °C, the DOPE-containing samples were brought to 25 °C.

^{31}P- and ^{2}H-NMR showed that in the absence of doxorubicin and at 25 °C, all the phospholipid mixtures with molar anionic/zwitterionic phospholipid ratios of 1/2, 1/1, and 2/1 were organized in extended liquid-crystalline bilayers after dispersion and that the lipid molecules were preferentially oriented with their long axes perpendicular to the magnetic field. Small-angle X-ray scattering (SAXS) profiles of the dispersions revealed no sharp maxima, consistent with the well-established large swelling and the predominantly unilamellar characteristics of phospholipid dispersions containing anionic phospholipids (Hauser, 1984; Hauser et al., 1986).

Doxorubicin Titrations. The drug was slowly added to the lipid suspension at the bottom of the NMR tube from a 20 mM drug solution in buffer and mixed with the lipid using a vortex mixer until the suspension was homogeneously red-colored. After each addition of drug, a 1-h incubation was applied before spectra were recorded. Doubling this incubation time did not change the results. At the end of the titration, we observed that the residual quadrupolar splittings ($\Delta\nu_q$) did not change significantly after repeated freezing (−70 °C) and thawing (0.1 °C). On the basis of binding experiments carried out at 25 °C with membranes consisting of 50–90 mol % zwitterionic and 10–50 mol % anionic phospholipid (see examples in Figure 1B), we estimate that more than 90% of the doxorubicin added to the anionic phospholipid-containing membranes was bound, under the conditions used for NMR (≤2.5 mol of doxorubicin/mol of DOPA and ≤1.5 mol of doxorubicin/mol of DOPS or cardiolipin, the overall drug level always exceeding 2 mM).

The effect of the drug on DOPC-containing dispersions was studied at 25 °C. In DOPE-containing mixtures at 25 °C, doxorubicin can induce an H_{II} phase, which is largely abolished below 5 °C (Nicolay et al., 1985, 1988). Pure DOPE is subject to a bilayer-to-H_{II}-phase transition between 8 and 10 °C (Cullis & de Kruijff, 1976; Tilcock et al., 1982; Farren et al., 1984). In order to minimize the induction of H_{II} phase, the effect of the drug on DOPE-containing bilayers was studied at 0.1 °C. However, in mixtures of 33 mol % anionic phospholipid and 67 mol % DOPE, doxorubicin induced some H_{II} phase, even at this temperature. Care was taken that the drug/lipid mixtures remained *always* below 1 °C until the end of the experiment: mixing of the drug and lipid in the NMR tube and transfer of the tube to the NMR probe were carried out while keeping the tube continuously in ice; the temperature in the NMR probe was equilibrated to 0.1 °C before the tube was entered. The transition of the lipids to the gel state occurs well below 0.1 °C (Chupin et al., 1987; Smaal et al., 1987).

Nuclear Magnetic Resonance (NMR). ^{31}P-NMR spectra were recorded with ^{1}H decoupling on a Bruker MSL 300 spectrometer, basically according to Chupin et al. (1987). A total of 1000–3000 free induction decays (spectral width 200 ppm) were accumulated at 1-s intervals and exponentially filtered, resulting in a 50–100-Hz line broadening. ^{2}H-NMR spectra were recorded using the quadrupolar echo sequence (Davis et al., 1976); 10 000–20 000 free induction decays (spectral width 71.4 kHz) were accumulated at 0.1–0.5-s intervals and exponentially filtered, resulting in a 50–200-Hz line broadening. Only where indicated were the ^{2}H-NMR spectra recorded with ^{1}H decoupling, the decoupler power being gated to 0.3 W during 0.5 s and to 3.2 W during 14 ms including the acquisition time. Spin–lattice relaxation times (T_1) were determined at four to five different temperatures between 10 and 40 °C, using the inversion-recovery method and fitting the data according to a least-squares two-parameter procedure. The integrated intensity of the ^{31}P- and ^{2}H-NMR spectra remained approximately constant throughout the doxorubicin titrations, indicating that no component was escaping detection.

At all drug levels and after equilibration, the liquid-crystalline bilayer and H_{II} phase each apparently gave rise to a single component in the ^{2}H-NMR spectra. This suggests that within each phase, the ^{2}H-labeled lipid molecules were all equally affected. Thus, *within* each phase, the distribution of drug was apparently homogeneous and/or the exchange of the drug between the lipid molecules was rapid on the NMR time scale [not between the inverted hexagonal (H_{II}) and the bilayer phases]. We did not attempt to simulate the observed spectra in view of the many uncertainties in the assumptions that have to be made in such simulations (variable magnetic orientation of the lipids, unknown and drug-dependent distribution of vesicle sizes, possible drug-dependent vesicle aggregation, etc.). After addition of the doxorubicin, the SAXS profiles of the anionic phospholipid-containing mixtures, initially displaying a broad diffuse pattern, changed so as to display sharp maxima characteristic of closely-packed multilamellar and/or hexagonal structures.

Small-Angle X-ray Scattering (SAXS). SAXS was carried out as described previously (De Wolf et al., 1991a), using a temperature-controlled sample holder.

Differential Scanning Calorimetry. Lipid dispersions (0.3–1.7 mM anionic phospholipid) were prepared as described above, incubated with 0.5–2.0 mM doxorubicin during 1 h at room temperature, and pelleted (10 min at 37000*g*). About 2 μmol of lipid–phosphorus was transferred to an aluminum sample pan (Perkin-Elmer); thermograms were recorded on a Perkin-Elmer DSC-4 calorimeter (2 °C min^{-1}). For lipid quantification, the pan was reopened, and lipid was extracted in chloroform and determined according to Böttcher et al. (1961).

RESULTS

Effect on Mixed Bilayers Containing DOPC and Anionic Phospholipid. Examples of ^{2}H-NMR spectra, recorded from bilayers consisting of an equimolar mixture of DOPA and DOPC, are shown in Figure 2. The spectra on the left side are from a mixture of ^{2}H-labeled DOPA and unlabeled DOPC, those on the right side are from a mixture of unlabeled DOPA and ^{2}H-labeled DOPC. The splitting of the two peaks in each of the ^{2}H-NMR spectra (residual quadrupolar splitting, $\Delta\nu_q$) is directly proportional to the order parameters of the carbon–^{2}H bond and the ^{2}H-labeled acyl chain segment (Seelig & Niederberger, 1974; Seelig, 1977; Davis, 1983). In the absence of doxorubicin, the $\Delta\nu_q$ of the DOPA component was 7.7 kHz (Figure 2, upper left), and that of the DOPC

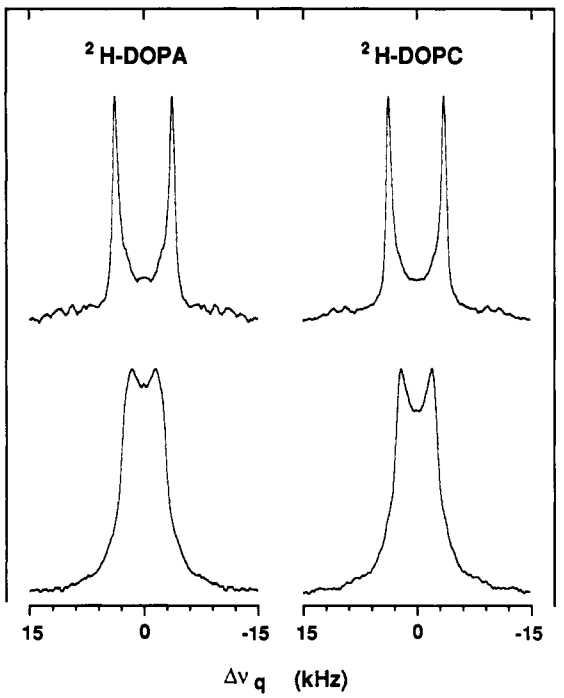

FIGURE 2: ^{2}H-NMR spectra recorded at 25 °C from equimolar mixtures of DOPA and DOPC dispersed in buffer. Either DOPA (left spectra) or DOPC (right spectra) was di[11,11-^{2}H]oleoyl-labeled. Top spectra: in the absence of drug. Lower spectra: after subsequent addition of 2.5 mol of doxorubicin/mol of DOPA (total doxorubicin concentration 16.7 mM).

component was 7.6 kHz (Figure 2, upper right). Doxorubicin decreased the $\Delta\nu_q$ of [^{2}H]DOPA and [^{2}H]DOPC to approximately the same extent. For example, at 2.5 mol of drug/DOPA, the $\Delta\nu_q$ was 3.6 and 4.1 kHz, respectively (Figure 2, lower spectra).

Figure 3 shows the doxorubicin-induced decrease of $\Delta\nu_q$ as a function of the overall drug level, in mixtures of anionic phospholipid and DOPC. In the DOPA- and DOPS-containing mixtures, doxorubicin changed the $\Delta\nu_q$ of the anionic lipid and the DOPC always precisely in parallel (Figure 3A–D). The net effect was much smaller than in pure anionic phospholipid and much larger than in pure DOPC. ^{2}H-Labeled cardiolipin was not available, but the effect of doxorubicin on mixtures of unlabeled cardiolipin and [^{2}H]-DOPC was similar to that on mixtures of unlabeled DOPS and [^{2}H]DOPC (Figure 3C–F). The presence of the cardiolipin was responsible for the drug-induced decrease of $\Delta\nu_q$.

In order to get some insight into the drug-induced changes of acyl chain mobility, ^{2}H spin–lattice relaxation times (T_1) were determined at several temperatures (Seelig, 1977; Davis, 1983). Upon addition of doxorubicin to an equimolar mixture of DOPA and DOPC (2.5 mol of drug/mol of PA), the T_1 of the [^{2}H]DOPA decreased from 21 to 17 ms at 25 °C. The corresponding activation energy decreased from 19 to 16 kJ mol^{-1}. The T_1 of [^{2}H]DOPC decreased from 20 to 17 ms, and the activation energy from 20 to 15 kJ mol^{-1}. Since under our conditions the T_1 decreased at decreasing temperatures, these data show (Seelig, 1977; Davis, 1983) that the T_1-determining motions of both the PA and PC molecules were slowed down. The effect on [^{2}H]DOPA and [^{2}H]DOPC in the mixture was approximately the same. It was much stronger in bilayers of pure DOPA, whereas it was not observed in pure DOPC (De Wolf et al., 1991a). Similar results were obtained with equimolar mixtures of DOPS and DOPC.

The above-described data indicate that in liquid-crystalline bilayers, doxorubicin does not induce a segregation of anionic phospholipid and DOPC into separate, extended domains with significantly different lipid composition and acyl chain order. We will discuss this in more detail at a later stage (see Discussion).

The absolute $\Delta\nu_q$ values obtained with [^{2}H]DOPS/DOPC mixtures were systematically higher than those obtained with DOPS/[^{2}H]DOPC mixtures, also in the absence of doxorubicin (Figure 3C). This was not due to differences in the composition of the corresponding samples, because in double-labeled [^{2}H]DOPS/[^{2}H]DOPC mixtures two spectral components were observed (Figure 4, top spectrum), which corresponded to the components of the single-labeled mixtures (Figure 4, lower spectra). Not only in DOPS/DOPC mixtures but also in the other phospholipid mixtures studied, the initial (control) $\Delta\nu_q$ of the anionic component was larger than the $\Delta\nu_q$ of the zwitterionic component (see Table I). (We verified that double spectral components were observed in equimolar double-labeled [^{2}H]DOPA/[^{2}H]DOPE and [^{2}H]DOPS/[^{2}H]-DOPE mixtures.)

The origin of the systematic difference between the anionic and zwitterionic components is not known, but will be discussed below (see Discussion). Note that in most DOPA/DOPE and DOPS/DOPE mixtures, the initial $\Delta\nu_q$ of at least one of the two mixed lipids is *not* intermediate between the $\Delta\nu_q$ values of the same lipids in pure form (Table I). Furthermore, the initial $\Delta\nu_q$ value of pure [^{2}H]DOPA or [^{2}H]DOPS is not higher, but *lower* than that of pure [^{2}H]DOPE (Table I). For example, the $\Delta\nu_q$ of equimolar [^{2}H]DOPA/DOPE and DOPA/[^{2}H]DOPE mixtures is 12.4 and 10.4, respectively, whereas the $\Delta\nu_q$ of pure [^{2}H]DOPA and pure [^{2}H]DOPE bilayers is 11.2 and 11.7, respectively, at 0.1 °C. Consequently, the initial difference between the $\Delta\nu_q$ of the anionic lipid and [^{2}H]DOPE in these mixtures cannot be simply explained by partial segregation of these lipids into separate domains. Also in equimolar DOPA/DOPC mixtures, the initial $\Delta\nu_q$ values of the two mixed lipids (7.7 and 7.6 kHz) are not intermediate between the values of the two lipids in pure form (7.3 and 6.0 kHz, Table I). Probably, the systematic difference between the initial $\Delta\nu_q$ of the anionic and zwitterionic lipids is not due to initial lipid segregation, in any of the lipid mixtures studied. This is consistent with the observation that, upon varying the relative composition of the lipid mixtures (Table I), or upon adding doxorubicin (Figures 3 and 5), the $\Delta\nu_q$ values of the anionic and zwitterionic lipid components always change in parallel.

Effect on Mixed Bilayers Containing DOPE and Anionic Phospholipid. The doxorubicin-induced decrease of $\Delta\nu_q$ in bilayers of anionic phospholipid and DOPE is shown in Figure 5. The data were obtained at 0.1 °C, in order to minimize the induction of H_{II} phase. The anionic phospholipid and the DOPE were subject to an approximately equal drug-induced change of $\Delta\nu_q$.

In mixtures of 33 mol % DOPS and 67 mol % DOPE, the doxorubicin-induced decrease of of $\Delta\nu_q$ was reversed at higher drug levels (Figure 5D; the maximal decrease was 1.2 and 0.8 kHz for [^{2}H]DOPS and [^{2}H]DOPE, respectively, at 1 mol of doxorubicin/mol of PS). The reason for this effect is not known. It is noteworthy that even in this case, doxorubicin changed the $\Delta\nu_q$ of the DOPS and DOPE precisely in parallel.

In agreement with the results obtained with DOPC-containing mixtures, these data indicate that in liquid-crystalline bilayers consisting of DOPE and either DOPA or DOPS, doxorubicin does not induce segregation of the lipid components into separate, extended domains with significantly differnt lipid composition and acyl chain order (we will discuss

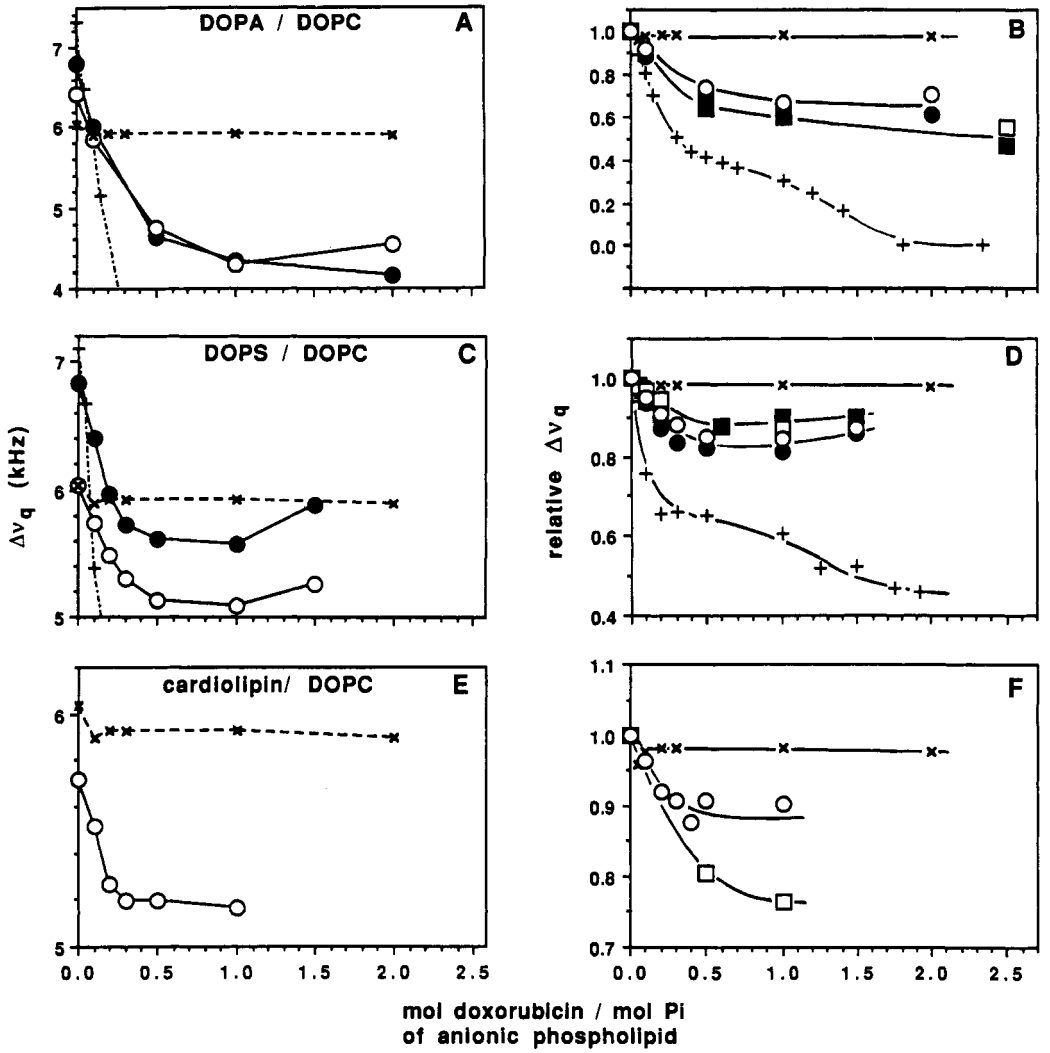

FIGURE 3: Effect of doxorubicin on $\Delta\nu_q$ in anionic phospholipid/DOPC mixtures at 25 °C. Either the anionic phospholipid (closed symbols) or the DOPC (open symbols) was di[11,11-$^2H_2$]oleoyl-labeled. The ratio of the anionic phospholipid–P_i/DOPC was 1/2 (●, ○) or 1/1 (■, □). For comparison: effect on pure ^{2}H-labeled anionic phospholipid (+) or DOPC (×) [data from De Wolf et al. (1991a)]; the abscissa of (×) indicates doxorubicin per PC. Left panels, absolute $\Delta\nu_q$ values; right panels, $\Delta\nu_q$ relative to the control value in the absence of doxorubicin. (The control values of all mixtures are shown in Table I.) Panels A and B both refer to the anionic phospholipid DOPA, panels C and D to DOPS, and panels E and F to cardiolipin. Total doxorubicin concentration: 2.6–15.0 mM.

this in more detail under Discussion). The results of Figure 5E,F suggest that this is also the case in mixtures of cardiolipin and DOPE.

Even at 0.1 °C, some H_{II} phase appeared to occur in the mixtures (not shown). The origin of this H_{II} phase is not clear, since at this temperature pure DOPE is organized completely in extended bilayers (see Discussion).

Mixtures Containing DOPE and Anionic Phospholipid under Conditions of H_{II}-Phase Formation. ^{31}P-NMR reveals that at 25 °C, doxorubicin induces a large H_{II} phase in mixtures of 67 mol % DOPE and 33 mol % DOPS, cardiolipin (Nicolay et al., 1985, 1988), or DOPA (present work). We have investigated the possibility that lipid segregation may occur under those conditions. The relative distribution of the individual lipid species between the bilayer phase and the H_{II} phase is not immediately clear from the ^{31}P-NMR spectra, but can be investigated using ^{2}H-NMR and anionic phospholipid/DOPE mixtures in which alternatively the anionic phospholipid or the DOPE is ^{2}H-labeled (Tilcock et al., 1982, 1984).

Examples of ^{31}P- and ^{2}H-NMR spectra of DOPS/DOPE mixtures are shown in Figure 6. The ^{31}P-NMR spectrum recorded in the absence of drug (top left) is characteristic of liquid-crystalline bilayers (Seelig, 1978). The presence of two high-field peaks arises from the difference between the residual chemical shift anisotropies of the DOPS (−54 ppm) and DOPE (−36 ppm) in the liquid-crystalline mixed bilayer. The corresponding ^{2}H-NMR spectra are shown on the right. After addition of doxorubicin, part of the lipid molecules adopted the H_{II} phase. From SAXS data, it appeared that the distance between the centers of neighboring H_{II} tubes was approximately 7.6 nm, at 25 °C. The occurrence of the H_{II} phase resulted in a second component in the NMR spectra (Figure 6, lower part). In the ^{31}P-NMR spectra, this component is characterized by a smaller residual chemical shift anisotropy, of reversed sign (+19 ppm). In the ^{2}H-NMR spectra, the temperature-dependent occurrence of the spectral component with the smallest $\Delta\nu_q$ (Figure 6, lower spectra) precisely coincided with the temperature-dependent occurrence of the H_{II} phase detected in the ^{31}P-NMR spectra (not shown). This ^{2}H-NMR component can thus be attributed to the H_{II} phase, rather than to bilayer domains with low acyl chain order. The ratio of the $\Delta\nu_q$ values originating from the bilayer and the H_{II} phase [$\Delta\nu_q$(bilayer)/$\Delta\nu_q$(H_{II})] was approximately 3.7 at all temperatures at which both phases were simultaneously detectable (the temperature dependence was studied by recording ^{2}H-NMR and ^{31}P-NMR spectra at 10 different temperatures between 0 and 25 °C; see Table II

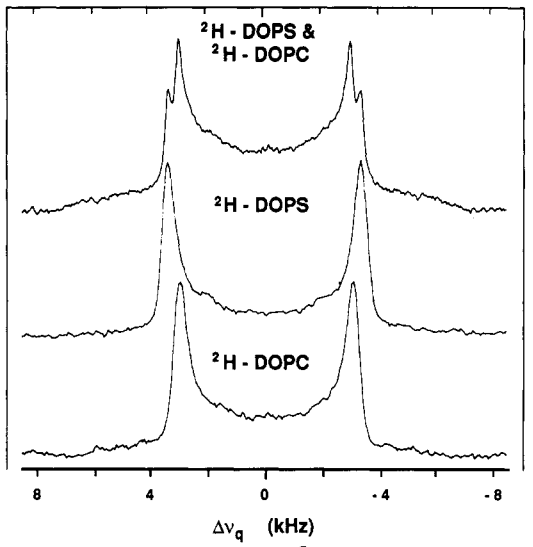

FIGURE 4: ^{2}H-NMR spectra recorded at 25 °C from equimolar mixtures of DOPS and DOPC dispersed in buffer. The following components of the mixtures were di[11,11-$^2H_2$]oleoyl-labeled: (top) both PS and PC; (middle) PS only; (bottom) PC only. Doxorubicin was absent. The top spectrum was recorded with proton decoupling; without decoupling, the outer peaks were detected as shoulders (not shown).

Table I: Control Values of $\Delta\nu_q$ in Mixed Bilayers Containing Di[11,11-$^2H_2$]oleoyl-Labeled Lipid, in the Absence of Doxorubicin

lipid mixture	lipid ratio (P_i/P_i)	temperature (°C)	$\Delta\nu_q$ (kHz) when the ^{2}H label is in: anionic phospholipid	$\Delta\nu_q$ (kHz) when the ^{2}H label is in: zwitterionic phospholipid
pure DOPC		25		6.0
DOPA/DOPC	1/2	25	6.8	6.4
	1/1	25	7.7	7.6
pure DOPA		25	7.3	
DOPS/DOPC	1/2	25	6.8	6.0
	1/1	25	6.8	6.1
pure DOPS		25	7.1	
cardiolipin/DOPC	1/2	25		5.7
	1/1	25		6.8
pure DOPE		0.1		11.7
DOPA/DOPE	1/2	0.1	12.8	11.0
	1/1	0.1	12.3	10.4
	2/1	0.1	12.4	10.3
pure DOPA		0.1	11.2	
DOPS/DOPE	1/2	0.1	12.0	10.3
	1/1	0.1	11.4	9.5
	2/1	0.1	10.8	8.8
pure DOPS		0.1	9.2	
cardiolipin/DOPE	1/1	0.1		9.0
	2/1	0.1		8.0

for data at 25 °C). Also in dispersions of pure DOPE, the $\Delta\nu_q$ originating from bilayers is approximately 3.7-fold larger than the $\Delta\nu_q$ originating from the H_{II} phase, both in the absence of doxorubicin (Tilcock et al., 1982; Farren et al., 1984; Chupin et al., 1987) and in the presence of the drug, at various temperatures. For example, in pure DOPE at 17 °C, where the bilayer and the H_{II} phase were simultaneously detectable in the presence of the drug, the corresponding $\Delta\nu_q$ values were 8.9 and 2.4 kHz, respectively.

It appeared from the ^{2}H-NMR spectra that in the mixtures the relative distribution of [^{2}H]DOPS between the bilayer phase and the H_{II} phase was very similar to that of [^{2}H]-DOPE (Figure 6, lower part). Comparable results were obtained with a mixture of 33 mol % DOPA and 67 mol % DOPE. Also at lower temperatures, when the contribution of the H_{II} component was gradually decreased, the relative distribution of the DOPA or DOPS among the bilayer phase and the H_{II} phase was always very similar to that of the DOPE (not shown).

Thus, extensive segregation of DOPE and DOPA or DOPS did not occur, even under conditions of H_{II}-phase formation. In this respect, the effect of doxorubicin on anionic phospholipid/DOPE mixtures is analogous to the effect of cholesterol on DOPC/DOPE mixtures, or the effect of Ca^{2+} on DOPS/DOPE/cholesterol mixtures (Tilcock et al., 1982, 1984). Consistent with the idea that neither doxorubicin nor anionic phospholipid is specifically excluded from the H_{II} phase, the $\Delta\nu_q$ of [^{2}H]DOPE in the H_{II} phase of the mixture (Table II) is 0.6–0.8 kHz smaller than the $\Delta\nu_q$ of the H_{II} phase of pure [^{2}H]DOPE (2.2–2.3 kHz in the presence or absence of doxorubicin, at 25 °C).

DISCUSSION

Absence of Extensive Doxorubicin-Induced Segregation of Lipids. Our aim was to test if doxorubicin is able to segregate the anionic and zwitterionic phospholipids in liquid-crystalline mixed bilayers into separate domains that (1) have a significantly different anionic phospholipid content and (2) are large enough to have a distinct acyl chain order and to prevent fast exchange of lipid molecules between the complementary domains (relative to the microsecond to millisecond NMR time scale). The occurrence of such an extensive doxorubicin-induced lipid segregation would imply the preferential partitioning of the anionic and zwitterionic phospholipid (either ^{2}H-labeled or unlabeled) into complementary domains with very different anionic phospholipid content (and acyl chain order). Doxorubicin has a relatively stronger disordering effect on lipid domains that are largely enriched in anionic phospholipid. In cardiolipin/DOPC, DOPA/DOPE, and DOPS/DOPE mixtures, the doxorubicin-induced effect is already significantly larger when the anionic phospholipid content is changed by only 17%, e.g., from 33 to 50% or from 50 to 67% (see Figures 3F, 5B,D, right panels). The preferential partitioning of the ^{2}H-labeled anionic and zwitterionic phospholipid into complementary domains would thus result in a differential effect of the drug on the complementary ^{2}H-labeled lipids.

Provided the complementary domains are of comparable size and the lipid composition is sufficiently different, the *average* effect on the two lipids can be expected to be different, even if the lipid exchange between the extended domains (each with a different physical composition and acyl chain order) would be fast on the NMR time scale (which is in the microsecond to millisecond range). This average effect would then be weighted so as to reflect the preference of the lipids for the different domains. Consider, for example, the following hypothetical situation, in which the following are assumed: (1) the effect of doxorubicin on ideal and homogeneous PA/PC mixtures is intermediate between the effects on pure PA and pure PC and linearly proportional to the relative amount of PA present; (2) in equimolar DOPA/DOPC mixtures, doxorubicin induces lipid segregation, resulting in two equally large domains, one containing 20% and the other 80% anionic phospholipid, each domain having a distinct acyl chain order; (3) within each domain, the two lipids are ideally and homogeneously mixed; (4) the exchange of lipid molecules between the domains is infinitely fast with respect to the NMR time scale. Since in pure DOPA and DOPC doxorubicin induces a 100% and 0% reduction of $\Delta\nu_q$, respectively, the drug should decrease the $\Delta\nu_q$ of [^{2}H]DOPA and [^{2}H]DOPC

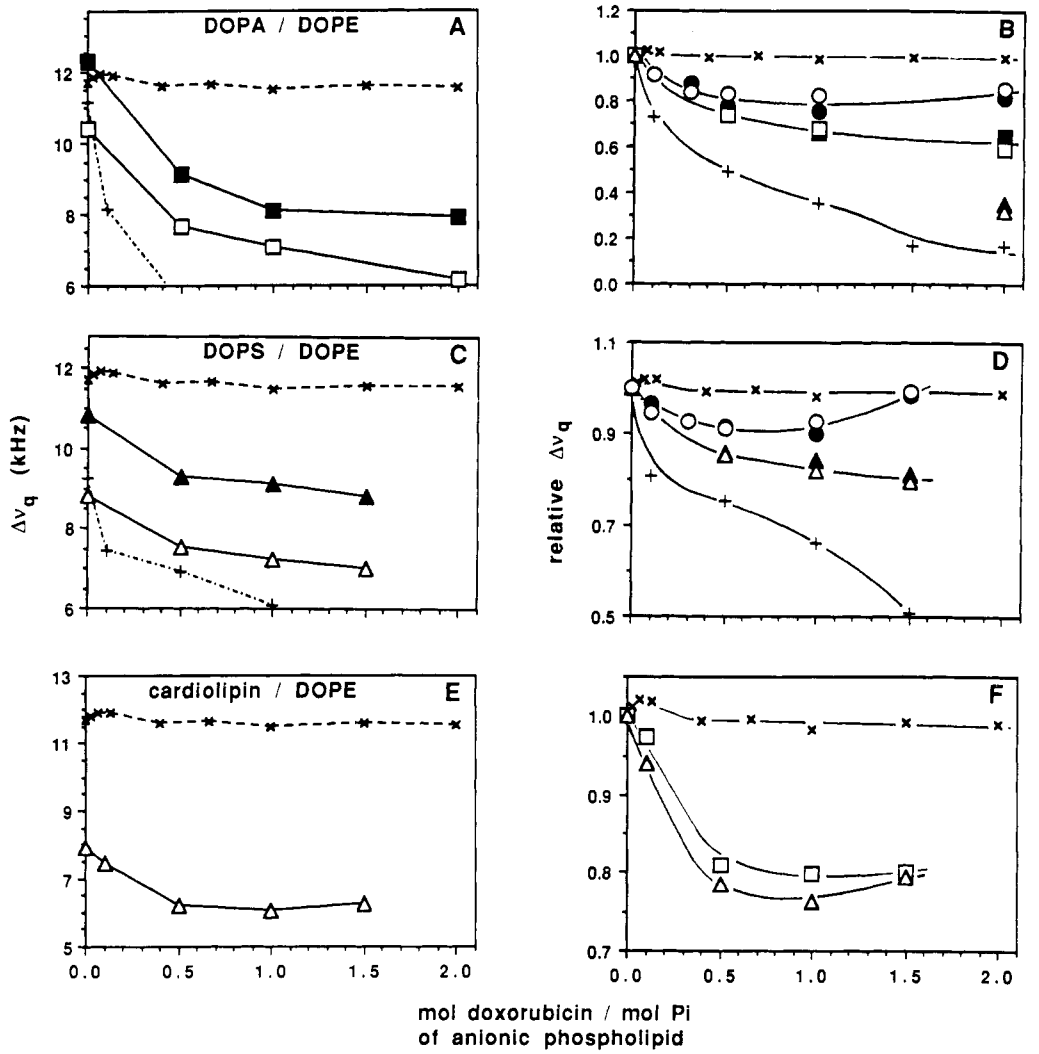

FIGURE 5: Effect of doxorubicin on $\Delta\nu_q$ in anionic phospholipid/DOPE mixtures at 0.1 °C. Either the anionic phospholipid (closed symbols) or the DOPE (open symbols) was di[11,11-$^2H_2$]oleoyl-labeled. The ratio of the anionic phospholipid–P_i/DOPC was 1/2 (●, ○), 1/1 (■, □), or 2/1 (▲, △). For comparison: effect on pure ^2H-labeled anionic phospholipid (+) or DOPE (×); the abscissa of (×) indicates doxorubicin per PE. Left panels, absolute $\Delta\nu_q$ values; right panels, $\Delta\nu_q$ relative to the control value in the absence of doxorubicin. (The control values of all mixtures are shown in Table I.) Panels A and B both refer to the anionic phospholipid DOPA, panels C and D to DOPS, and panels E and F to cardiolipin. All $\Delta\nu_q$ values refer to the spectral component originating from extended lipid bilayers. Total doxorubicin concentration: 4.6–17.1 mM.

by 72% and 36%, respectively, in the hypothetical DOPA/DOPC mixture (the effect on [^2H]DOPA would then be about 2.6 kHz larger than the effect on [^2H]DOPC). In an analogous DOPS/DOPC mixture, the $\Delta\nu_q$ of PS and PC should be decreased by 44% and 22%, respectively (the effect on [^2H]-DOPS would then be about 1.6 kHz larger than on [^2H]-DOPC).

Upon addition of doxorubicin, the $\Delta\nu_q$ of the anionic and zwitterionic lipid components always changed in parallel and to an approximately equal extent. Thus, our data show that in liquid-crystalline mixed bilayers, doxorubicin does not induce segregation of the anionic and zwitterionic phospholipid components into separate, large domains with significantly different lipid composition and acyl chain order.

We also observed that the anionic phospholipids DOPA and DOPS were not largely excluded from the doxorubicin-induced H_{II} phase in DOPE-containing mixtures. In previous studies on DOPS/DOPE and cardiolipin/DOPE mixtures, the extended doxorubicin-induced H_{II} phase was, on the contrary, considered to be significantly depleted of anionic phospholipid (Nicolay et al., 1985, 1988).

The absence of large-scale drug-induced segregation of anionic and zwitterionic phospholipid is remarkable in view of the fact that the drug elicited a gross rearrangement of the mixed bilayers, resulting in the formation of closely-packed multilamellar structures [bilayer repeat distance 7.1–7.6 nm, as monitored by SAXS; cf. Nicolay et al. (1984) and De Wolf et al. (1991a)] or in the formation of the H_{II} phase (Figure 6, Table II). In the absence of drug, aqueous dispersions of phospholipid mixtures containing significant amounts of anionic phospholipid are characterized by large swelling and a predominance of unilamellar structures (Hauser, 1984; Hauser et al., 1986); they display broad diffuse SAXS profiles.

Of course, the occurrence of microclusters consisting of only a few anionic phospholipid molecules cannot be excluded. For example, previous studies have shown that in mixed membranes to which the mitochondrial precursor protein apocytochrome *c* is bound, a small lipid microdomain in direct interaction with the protein is enriched in anionic phospholipid (Berkhout et al., 1987; Rietveld et al., 1986). This did not give rise to large differences between the $\Delta\nu_q$ of di[11,11-$^2H_2$]oleoyl-labeled anionic and zwitterionic phospholipids in those membranes (Jordi et al., 1990), possibly because the amount of lipid in direct interaction with the protein was only a relatively small fraction of the total amount of lipid present in those systems. In mixed bilayers such as we used at present,

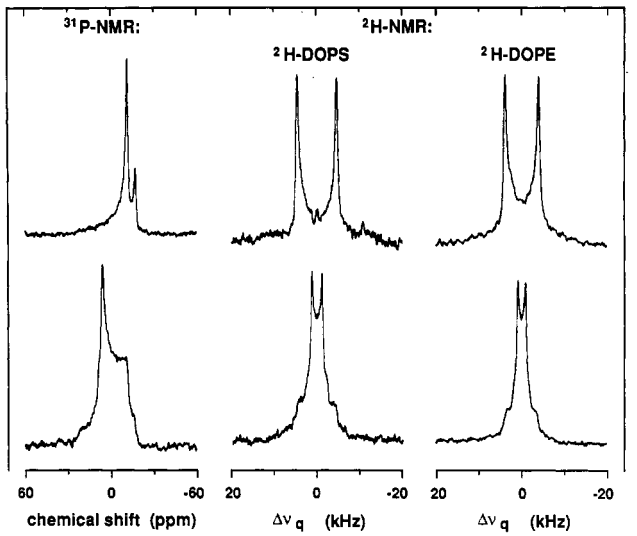

FIGURE 6: ^{31}P-NMR spectra (left) and ^{2}H-NMR spectra (middle and right) recorded at 25 °C from DOPS/DOPE mixtures (molar ratio 1/2) dispersed in buffer. Either DOPS (left and middle) or DOPE (right) was di[11,11-^{2}H]oleoyl-labeled. Top spectra: in the absence of drug. Lower spectra: after subsequent addition of 1.5 mol of doxorubicin/mol of DOPS; total doxorubicin concentration, 13.8 mM. Identical ^{31}P-NMR spectra were recorded from mixtures of unlabeled DOPS and [^{2}H]DOPE.

Table II: $\Delta\nu_q$ in Anionic Phospholipid/DOPE Mixtures (Molar Lipid Ratio 1/2) in the Bilayer Organization and in the Doxorubicin-Induced H_{II} Phase, at 25 °C

	$\Delta\nu_q$ (kHz)		
		+doxorubicin[a]	
lipid or lipid mixture	control without doxorubicin bilayer	bilayer	H_{II} phase
[^{2}H]DOPA/DOPE	9.5	7.0	2.1
DOPA/[^{2}H]DOPE	8.3	5.5	1.5
[^{2}H]DOPS/DOPE	9.3	8.5	2.3
DOPS/[^{2}H]DOPE	7.9	6.6	1.8

[a] 2 and 1.5 mol of doxorubicin was present per mole of DOPA and DOPS, respectively. The total drug concentration was 13.8–16.4 mM.

extremely small lipid microclusters (for instance, oligomeric complexes of anionic phospholipid and doxorubicin) may not even have a physically distinct acyl chain order: the orientations and motions of their acyl chains could be largely determined by the surrounding lipid molecules. At least our results show that doxorubicin does not induce *extensive* segregation of anionic and zwitterionic phospholipids.

This contrasts with previous data obtained with membranes that are subject to gel-to-liquid-crystalline-phase transitions. Addition of doxorubicin to mixed model membranes consisting of dimyristoyl- or dipalmitoylphosphatidylcholine (PC) and 6–15 mol % cardiolipin resulted in a sharpened transition of the PC from the gel to the liquid-crystalline state and a shift of this transition to slightly higher temperatures. This is most readily explained by asssuming that the drug induces extended membrane domains in which the cardiolipin concentration is largely reduced (Goormaghtigh et al., 1982, 1986, 1990; Nicolay et al., 1988). We have recently found that very similar results are obtained by calorimetry, when cardiolipin is replaced by DOPA, DOPS, or dioleoylphosphatidylglycerol (M. L. Verdonk, F. A. de Wolf, and B. de Kruijff, unpublished experiments). It is possible that the anionic lipid and the unsaturated PC become segregated only at temperatures at which the pure PC would be in the gel state (below 41 °C). Under those conditions, the specific binding of the drug to the anionic phospholipid and its penetration into the membrane might make lipid mixing energetically unfavorable. A drug-induced separation of the lipids is, however, not the *only* possible explanation for the results obtained with DSC. For example, the effect of the drug on the gel-to-liquid-crystalline transition could be due to a tighter packing of the anionic phospholipid/PC mixture in the gel state, resulting from a drug-induced neutralization of the negative surface charges [cf. Silvius (1991)]. The membrane-penetrating capacity of the drug is much lower in gel-state bilayers than in liquid-crystalline bilayers (Karczmar & Tritton, 1979; Constantinides et al., 1990).

Organization of Doxorubicin in the Membrane: Relation to Present Observations. Binding of doxorubicin to anionic phospholipid-containing membranes usually results in relatively high drug densities in the membrane and, consequently, in high self-association of the majority of the bound drug molecules (Goormaghtigh et al., 1980b; Henri et al., 1985; De Wolf et al., 1991a,b). It was originally thought that this self-association of doxorubicin, bound to anionic phospholipid, would induce extensive clustering of anionic phospholipid molecules in mixed membranes (Goormaghtigh et al., 1982, 1986; Nicolay et al., 1988). However, our data are not consistent with such a massive clustering of DOPS or DOPA in mixed bilayers, even though the conditions favored doxorubicin self-association. More than 1 mol of doxorubicin was bound per mole of anionic phospholipid at the end of the titrations, and the overall doxorubicin levels (bound + free) were always higher than 2–4 mM. Free doxorubicin, at 2–17 mM, forms large aggregates in buffer (Chaires et al., 1982; Confalonieri et al., 1991; Giomini et al., 1991; Hayakawa et al., 1991).

At comparable local drug density, i.e., at comparable distance between the drug molecules, membrane-bound doxorubicin has a relatively lower degree of self-association than free doxorubicin, as a result of the hydrophobic interactions of the drug with the membrane (Henri et al., 1985; De Wolf et al., 1991a,b). Possibly, it is due to these hydrophobic interactions that the self-association of bound doxorubicin is too weak to induce a detectable clustering of anionic phospholipid molecules in mixed membranes. The zwitterionic phospholipid in mixed membranes apparently provides more room for penetration of the electrostatically-bound drug (De Wolf et al., 1991b), as compared to membranes consisting of pure anionic phospholipid.

Systematic Difference between Anionic and Zwitterionic Phospholipids. Interestingly, the $\Delta\nu_q$ of the anionic phospholipid component was systematically higher than the $\Delta\nu_q$ of the zwitterionic component of the bilayer, in the absence as well as in the presence of the drug. Only in DOPA/DOPC mixtures, the difference was small and probably insignificant. The difference in the other mixtures was probably not due to an initial segregation of the lipid components, as can be inferred from the following observations.

In the first place, the $\Delta\nu_q$ of the anionic phospholipids in the mixture was *always* higher than that of the zwitterionic phospholipids, even in DOPE-containing bilayers, despite the fact that bilayers of *pure* DOPA or DOPS have a *lower* $\Delta\nu_q$ than bilayers of pure DOPE (Table I, Figures 3C and 5A,C). In the absence of drug, the $\Delta\nu_q$ of [^{2}H]DOPA in all DOPA/DOPE mixtures and the $\Delta\nu_q$ of [^{2}H]DOPS in a mixture of 33 mol % DOPS and 67 mol % DOPE (Table I, Figure 5A) were even slightly *higher* than the $\Delta\nu_q$ of pure DOPE. In the second place, the anionic and zwitterionic phospholipid components of the various bilayers were subject to an equally strong acyl chain disordering effect of doxorubicin. In the third place, the effect of the drug on the spin–lattice relaxation

times (T_1) of the PS and PC components of DOPS/DOPC mixtures was approximately equal: the T_1 (initially 22.5 ms) and activation energy (initially 20 kJ mol^{-1}) were decreased by approximately 10%. In the fourth place, the induction of an H_{II}-phase component by doxorubicin resulted in a very similar temperature-dependent distribution of the anionic phospholipid (DOPA, DOPS) and the DOPE among the bilayer phase and the H_{II} phase. Finally, in the H_{II} phase, the $\Delta\nu_q$ of [^{2}H]DOPE in the mixtures was systematically 0.6–0.8 kHz lower than the $\Delta\nu_q$ of [^{2}H]DOPA and [^{2}H]DOPS in the mixtures (see Table II), but it was also approximately 0.6–0.8 kHz lower than the $\Delta\nu_q$ of pure [^{2}H]DOPE (2.2–2.3 kHz in the presence or absence of doxorubicin, at 25 °C).

A possible explanation for the different order of the anionic and zwitterionic lipid components is that these components take slightly different positions along the normal to the lipid–buffer interface, the anionic headgroups being closer to the aqueous phase. It is well established that in a variety of natural and model membranes, the local order of the acyl chain segments gradually increases from the center to the surface of the bilayer (Seelig, 1977; Davis, 1983; Moser et al., 1989).

Effect of Doxorubicin on Cardiolipin. It has been previously shown that the interaction of doxorubicin with cardiolipin is very similar to the interaction with DOPA or DOPS, both in terms of binding affinity (De Wolf, 1991, De Wolf et al., 1991a,b) and in terms of the organization of the drug in the membrane (Henri et al., 1985; De Wolf et al., 1991a,b). Due to a lack of ^{2}H-labeled cardiolipin, we could not directly monitor th acyl chain order of cardiolipin in mixed membranes. The disordering effect of doxorubicin on membranes consisting of unlabeled cardiolipin and ^{2}H-labeled DOPC or DOPE indicates that doxorubicin has an acyl chain disordering effect on cardiolipin and that even at high drug levels significant amounts of cardiolipin remained mixed with the zwitterionic phospholipid. The disordering effect was approximately equal to that on DOPS-containing bilayers (Figures 3 and 5). Thus, the interaction of doxorubicin with cardiolipin appears to be comparable to the interaction with other anionic phospholipids, not only in terms of the binding affinity and in terms of the organization of the drug in the membrane but also in terms of the structural effects on the membrane.

Mechanism of Induction of the H_{II} Phase. The mechanism by which doxorubicin elicits the H_{II} phase in anionic phospholipid/DOPE mixtures is not clear. In the first place, a small drug-induced H_{II} component was already detectable at a temperature (0.1 °C) at which pure DOPE would still be fully organized in bilayers. Also at this temperature, both the anionic phospholipid and the DOPE were incorporated into the H_{II} phase. In the second place, we found that doxorubicin has a weak bilayer-*stabilizing* effect on membranes consisting of pure DOPE: it raises the temperature at which the bilayer/H_{II}-phase transition occurs by about 6 °C (not shown). Finally, the drug did not destabilize bilayers of pure DOPA, DOPS, and cardiolipin at low pH (6.0, 4.0, and 3.6, respectively; not shown). Below this pH, the lipids go over to the H_{II} phase in the absence of drug, at 25 °C [see De Kroon et al. (1990) and De Wolf et al. (1991a)]. Drug-free DOPA, DOPS, or cardiolipin dispersions are fully in the H_{II} phase at pH 4.4, 3.1, and 2.2, respectively, but addition of 1 mol of doxorubicin/mol of lipid–P_i (final doxorubicin concentration 13.3 mM) completely reorganized the lipids into bilayers (the drug was adjusted to pH prior to addition).

The induction of the H_{II} phase could involve several mechanisms: (1) neutralization of the negative surface charges by the positively-charged drug (Goormaghtigh et al., 1980a,b; Nicolay et al., 1988; Dupou-Cézanne et al., 1989); (2) deep penetration of the hydrophobic part of the drug between the acyl chains (Constantinides et al., 1990; De Wolf et al., 1991a), resulting in an increase of the hydrophobic volume of the membrane; (3) drug-induced changes in the orientation and/or hydration of the phospholipid headgroups [cf. Epand and Bryszewska (1988)].

A (pH-induced) neutralization of negative surface charges is known to induce an H_{II} phase in DOPS and DOPA dispersions (Hope & Cullis, 1980; Farren et al., 1983; De Kroon et al., 1990; De Wolf et al., 1991a). At low surface charge density, certain anionic phospholipids can induce the formation of H_{II} phase in DOPE-containing mixed membranes below the bilayer-to-H_{II} transition of pure DOPE, even in the absence of drug [see also Tari and Huang (1989)]. Thus, we observed spontaneous induction of the H_{II} phase at 0.1 °C in mixtures containing less than 30 mol % cardiolipin, 5 mol % DOPA, or 1 mol % DOPS, respectively, in the absence of drug (not shown).

Doxorubicin did not induce an H_{II} phase in mixtures containing maximally 50 mol % DOPE and at least 50 mol % DOPA, DOPS, or cardiolipin.[2] The inability of doxorubicin to induce an H_{II} phase in mixtures containing too much anionic phospholipid (50 mol % or more) is probably related to (1) the absence of sufficient amounts of DOPE as promotor of H_{II}-phase formation and (2) the high surface charge density of those bilayers, which implies that charge neutralization can occur only at high doxorubicin densities. As mentioned above, large amounts of drug molecules are apparently stacked at the membrane surface under those conditions. This may hinder the formation of type II structures. Moreover, the bulky drug stacks may not be easily incorporated into the lumen of the H_{II} tubes.

Concluding Remarks. An extensive doxorubicin-induced clustering of cardiolipin has been previously proposed to be the common mechanism responsible for the inhibition of various cardiolipin-dependent enzymes in the mitochondrial inner membrane (Goormaghtigh et al., 1982, 1986; Nicolay & de Kruijff, 1987; Nicolay et al., 1985, 1988). In view of the similarity of the interaction of doxorubicin with other anionic phospholipids, we originally expected that a comparable clustering would occur in the case of PA, PS, and other anionic phospholipids. This could then be expected to interfere with the role of these lipids in a variety of membrane-associated processes.

However, the present results argue against such a mechanism of action of the drug. It is more likely that the binding of the drug to the lipids, the local changes of acyl chain order and membrane structure, and possibly also the changes of surface charge density directly interfere with the binding of the lipids to membrane proteins and with the physiological role of the lipids in membrane-associated processes. It is noteworthy that a significant disordering of acyl chains can already occur at low binding levels. The relatively lower degree of self-association and the relatively increased penetration of doxorubicin in mixed membranes are likely to promote, in addition, long-range structural effects of the drug on the membrane and to allow a relatively easy permeation of the drug.

Although doxorubicin does not seem to induce anionic phospholipid clustering, it is obvious that preexisting clusters of anionic phospholipid can occur in the membrane, for

[2] A small H_{II}-phase component occurred sometimes at 25 °C in equimolar mixtures of cardiolipin and DOPE (67 mol % cardiolipin–P_i).

example, around membrane proteins such as protein kinase C (Bazzi & Nelsestuen, 1991). The local distribution of lipids and surface charges in/on the membrane (also the charges on proteins) will modulate the apparent binding affinity of the drug, the local drug density in the membrane, and the local disordering of acyl chains. Insight into these effects is still lacking and requires further investigations.

The doxorubicin-induced formation of type II structures in membranes containing relatively low amounts of anionic phospholipid and relatively high amounts of PE is interesting, since the cytoplasmic leaflet of the plasma membrane also has a low anionic phospholipid and high PE content (White, 1973; Op den Kamp, 1979; Devaux, 1991), and since the activity of protein kinase C is stimulated by agents that promote the formation of type II structures (Epand et al., 1988, 1991).

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