Role of Anionic Phospholipids in the Interaction of Doxorubicin and Plasma Membrane Vesicles: Drug Binding and Structural Consequences in Bacterial Systems†

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ABSTRACT: Anthracycline–membrane interactions play a role in the transport, the cytoplasmic distribution, and possibly also the activity of anthracyclines. Previous work on model membranes has shown that the widely-applied anticancer drug doxorubicin interacts specifically with anionic phospholipids [de Wolf, F. A., et al. (1991) *Biochim. Biophys. Acta 106*, 67–80]. We have now been able to investigate these interactions, and their selectivity for anionic phospholipids, directly in plasma membranes. Because of the recent availability of *Escherichia coli* mutants in which the anionic phospholipid content ranges from only 10% to as much as 100% of the total phospholipid content, we used this bacterium as a source of plasma membranes. We compared the interactions of the cationic anthracycline doxorubicin with (1) plasma membranes of different mutant strains, (2) total lipid extracts of these membranes, and (3) synthetic phospholipid mixtures in which a comparable fraction of the phospholipids was negatively charged. The results show that anionic phospholipids are important determinants of doxorubicin binding, not only in model membranes but also in plasma membrane systems. Only in plasma membranes with a very low anionic lipid content was the binding to the anionic phospholipid masked by other factors. Using an unsaturated fatty acid auxotroph grown on [11,11-$^2H_2$]oleic acid, it appeared from ^{2}H-NMR data that doxorubicin induces a disordering of acyl chains in bacterial plasma membranes and their total lipid extracts. This indicates that the binding is not purely electrostatic but involves the insertion of drug molecules into the lipid matrix, probably due to hydrophobic interactions.

The anthracycline anticancer drugs bind to several targets in the cell, DNA and DNA-associated enzymes being major targets in terms of cytostatic activity (Vichi et al., 1989; Capranico et al., 1990; Foglesong et al., 1992; Bachur et al., 1992). In addition, these drugs bind to various cellular membranes (Nicolay et al., 1984, 1986; Griffin et al., 1986; Arancia et al., 1988; Escriba et al., 1990). Incorporation into the lipid matrix of the membrane seems to govern passive drug transport (Dalmark & Storm, 1981; Burke et al., 1987; Frézard & Garnier-Suillerot, 1991; Tarasiuk & Garnier-Suillerot, 1992; De Gier, 1992), and it it may also be essential for active drug efflux (Raviv et al., 1990; Safa, 1992; Higgins & Gottesman, 1992). In view of the large amount of intracellular membranes, membrane binding will strongly influence the availability of free drug in the cytosol, especially when cells are only transiently exposed to the drugs and the drug distribution will not reach a steady-state [cf. Ramu et al. (1989)]. Drug–membrane interactions appear to disturb vital cellular functions, such as signal transduction (Thompson et al., 1987; Posada et al., 1989; Oakes et al., 1990), electron transfer (Nicolay & de Kruijff, 1987; Sun et al., 1992), or translocation of proteins and lipids (Eilers et al., 1989; Voelker, 1991). Accordingly, anthracyclines were found to be cytotoxic under conditions where they apparently do not enter the cell (Tritton & Yee, 1982; Tritton, 1991) or interact poorly with DNA (Israel et al., 1987).

Much insight into the interaction of anthracyclines and lipids has been obtained using model membrane systems. Moderately lipophilic and positively charged anthracyclines such as doxorubicin bind strongly to anionic phospholipids (Goormaghtigh et al., 1980; Henry et al., 1985; Burke et al., 1988; De Wolf et al., 1991a), resulting in partial penetration between the lipid molecules (Henry et al., 1985; Dupou-Cézanne et al., 1989; De Wolf et al, 1991a,b) and in disordering of the acyl chains (De Wolf et al., 1991a, 1992). As compared to pure anionic phospholipids, binding of doxorubicin to pure zwitterionic phospholipids is almost negligible (De Wolf et al., 1991a; Burke & Tritton, 1985). The apparent affinity of doxorubicin for the various anionic phospholipids is nearly identical (De Wolf et al., 1991a; De Wolf, 1991), but the amount of bound drug varies with the type of lipid and is specifically largest in polyphosphoinositide-containing model membranes (De Wolf et al., 1991b). As expected, the anionic lipid specificity is lost in anthracycline derivatives that are much more hydrophobic (Burke et al., 1990).

Little is known about the interactions of anthracyclines with lipids in true biological membranes such as the plasma membrane. For example, the role of anionic phospholipids as determinants for the binding of the widely applied drug doxorubicin has never been directly tested in a plasma membrane system. Such a test requires a membrane system that allows sufficient manipulation of the anionic lipid content. Although such a system should ideally be derived from mammalian (cancer) cells, appropriate mammalian systems do not yet exist. However, due to recent genetic developments, an excellent model plasma membrane system derived from *Escherichia coli* has become available (Heacock &

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Dowhan, 1989; Raetz & Dowhan, 1990; De Chavigny et al., 1991; Kusters et al., 1991).

In *E. coli* plasma membranes, cardiolipin and phosphatidylglycerol (PG)[1] are the main anionic phospholipids, and phosphatidylethanolamine (PE) is the main zwitterionic phospholipid (Raetz, 1978). The anionic phospholipid and PE contents of wild-type *E. coli* plasma membranes are similar to those in the cytoplasmic leaflet of mammalian plasma membranes (Op den Kamp, 1979; Devaux, 1991). Viable *E. coli* mutants do now exist in which PE is virtually absent (De Chavigny et al., 1991) or in which anionic phospholipid synthesis is under the control of the *lac* operon and can be varied at will from very low to normal levels (Heacock & Dowhan, 1989; Kusters et al., 1991; Asai et al., 1989).

By comparing membrane systems derived from these and other *E. coli* strains, we have been able to test the importance of anionic lipids as determinants of doxorubicin–membrane interactions directly in biological plasma membranes. The results show that such membranes bind significant amounts of doxorubicin even at low drug concentrations and that anionic lipids are indeed important for this binding. ^{2}H-NMR data obtained with a fatty acid auxotroph grown on [11,11-$^2H_2$]oleic acid showed that doxorubicin decreases the order of the acyl chains, both in plasma membranes and in total lipid extracts, indicating insertion of the drug into the lipid matrix.

MATERIALS AND METHODS

Chemicals. Doxorubicin was purchased from Pharmachemie (Haarlem, The Netherlands). The drug was shown to be pure by high-performance thin-layer liquid chromatography (HPTLC) according to Nicolay et al. (1984). Stock solutions were prepared shortly before use. Concentrations were routinely determined in Pipes buffer (see below), after dilution to 5–10 μM drug, by light adsorption at 480 nm using an extinction coefficient of 1.06×10^4 M^{-1} cm^{-1}.

Synthesis, isolation, and purification of 1,2-dioleoyl-*sn*-glycero-3-phosphate (phosphatidic acid, DOPA), -phosphocholine (DOPC), and -phosphoethanolamine (DOPE), and -phosphoglycerol (DOPG), bovine heart cardiolipin, and deuterated [11,11-$^2H_2$]oleic acid 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); 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and -phosphoglycerol (POPG) were purchased from Avanti Polar Lipids (Birmingham, AL). All lipids were shown to be pure by HPTLC.

The binding and NMR experiments were always carried out in the same buffer, containing 10 mM Pipes, 100 mM NaCl, and 0.5 mM EGTA, pH 7.4 (adjusted with NaOH).

Bacterial Strains and Growth Conditions. Escherichia coli strain MRE600 (Cammack & Wade, 1965) was grown in Luria broth [10 g L^{-1} bacto-tryptone (Sigma), 5 g L^{-1} yeast extract (Sigma), and 10 g L^{-1} NaCl]. Strain HDL11, in which anionic phospholipid synthesis is dependent on the sustained induction of the *lac* operon with isopropyl β-D-thiogalactoside (IPTG) (Kusters et al., 1991), was grown in L-broth supplemented with chloramphenicol (20 μg/mL), kanamycin (50 μg/mL), tetracycline (10 μg/mL), and, where indicated, 60 μM IPTG. Strain K1059, which is unable to synthesize or degrade unsaturated fatty acids (Overath et al., 1970), was grown in Cohen–Rickenberg (CR) mineral salts medium (Anraku, 1967) supplemented with 0.5% (v/v) glycerol, 0.3% (w/v) casamino acids (Difco, MI), 0.2% (w/v) Brij-35 (Janssen Chimica, The Netherlands), and 0.01% (v/v) [11,11-$^2H_2$] oleic acid (Killian et al., 1992). Finally, strain AH930, which is unable to synthesize PE (De Chavigny et al., 1991), was grown in L-broth supplemented with 20 mM $MgCl_2$. Cells were grown at 37 °C until late logarithmic phase (absorbance 0.6–0.8 at 660 nm in a Milton-Roy Spectronic 301 spectrometer), chilled on ice, and harvested by low-speed centrifugation at 4 °C.

Membrane Preparation. Spheroplasts and right-side-out inner membrane vesicles were prepared and isolated according to Kaback (1971). The final concentration of lysozyme (type 5281 from Merck, Darmstadt, Germany) during spheroplast formation was about 80 μg/mL. Inside-out inner membrane vesicles were prepared according to Müller and Blobel (1984) with modifications according to De Vrije et al. (1987). The total phosphorus (P_i) content of the isolated membranes was determined according to Rouser et al. (1970) and the protein content according to Lowry et al. (1951), Bradford (1976), or Smith et al. (1985), using bovine serum albumin as standard. In inner membrane vesicles, the protein/P_i ratio was determined to be 0.8 ± 0.2 (SD, 12 vesicle batches) g/mmol, without systematic differences between the various strains, vesicle types, and assay methods. In some cases, vesicles were treated with proteinase K (200 μg/mL) during 30 min at 37 °C, after which the reaction was stopped with 2 mM phenylmethanesulfonyl fluoride. This resulted in a 1.6-fold decrease of the protein/P_i ratio of the vesicles after washing. The DNA content of the isolated vesicles was determined after extraction of the samples with phenol and chloroform and precipitation of the DNA with ethanol according to Sambrook et al. (1989). The DNA was dried, dissolved in 10 mM Tris-HCl with 1 mM EDTA (pH 8.1), and quantified according to Rouser et al. (1970) or to its absorbance at 260 nm (extinction coefficient 1.33×10^4 cm^{-1} M^{-1} base pairs). The DNA–P_i was 2–6% of the total P_i in inside-out vesicles and about 7–9% in right-side-out vesicles. Free DNA binds maximally about 0.25 mol of doxorubicin/mol of P_i (0.5 mol/mol base pairs) (Chaires et al., 1982; Graves & Krug, 1983; F. A. de Wolf, unpublished results). This would correspond to about 0.005–0.023 mol of doxorubicin/mol of total P_i in inner membrane vesicles. 2-Keto-3-deoxyoctonate (KDO) was determined in vesicles according to Karkhanis et al. (1978) and amounted to 0.01–0.03 mol of KDO/mol of total P_i. Total lipid extracts were prepared from isolated inner membrane vesicles according to Killian et al. (1992) by extracting the lipids according to either Bligh and Dyer (1959) or Folch et al. (1957) and removing proteins and neutral lipids on a silica column. Both procedures resulted in the same overall P_i recovery in the final lipid extract (50–70% with respect to total P_i in the vesicles). Large unilamellar vesicles (LUVET) were prepared in Pipes buffer by extrusion (Hope et al., 1985) through polycarbonate filters with 400-nm pores (Costar-Nuclepore Europe, Badhoevedorp, The Netherlands) as previously described (De Wolf et al., 1991a). After extrusion, lipid concentration was determined on a P_i basis (Rouser et al., 1970). ^{2}H-depleted Pipes buffer was used to wash the spheroplasts and inner membrane vesicles isolated

[1] Abbreviations: DOPA, DOPC, DOPE, and DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphate, -phosphocholine, -phosphoethanolamine, and -phosphoglycerol, respectively; POPC and POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and -phosphoglycerol, respectively; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HPTLC, high-performance thin-layer liquid chromatography; IPTG, isopropyl β-D-thiogalactoside; LUVET, large unilamellar vesicles prepared by extrusion; NMR, nuclear magnetic resonance; Pipes, 1,4-piperazinediethanesulfonic acid; SDS, sodium dodecyl sulfate.

from the K1059 strain and to disperse the lipids from this strain.

Binding Assays. Doxorubicin binding was routinely assayed according to De Wolf et al. (1991a) by mixing 100 μL of membrane suspension with 900 μL of doxorubicin solution (both in Pipes buffer) to a known final P_i concentration of 0.3–1.0 mM, incubating duplicate samples for 1 and 4 h, respectively (in the dark and at room temperature), and pelleting the vesicles and bound drug (45 min at 388000*g* and 20 °C, Beckman TLA 100.2 rotor). The top 800 μL was collected for determination of P_i (not detectable, i.e., less than 0.5% of total P_i) and free drug; 90–100% of P_i was recovered in the pellets. Data were corrected for possible drug binding to the tubes (less than 5% of the overall binding levels) using blanks without lipid. Binding equilibrium was reached, since the two different incubation times normally yielded identical results, and application of freeze (–70 °C)–thaw (+40 °C) cycles did not change the results (checked for nearly all membrane preparations). Equilibrium dialysis was used at overall drug levels (free + bound) below 20–30 μM. The dialysis tube (8 cm Visking size 2, 18/32 in., from Medicell, U.K., boiled for 30 min in 0.1 M EDTA and thoroughly rinsed with water and buffer) was filled with 2 mL of membrane suspension in Pipes buffer (0.3–1.0 mM P_i), brought into a stirred 200-mL bath of the same buffer containing doxorubicin, and incubated for about 48 h in the dark under pure N_2 gas and at room temperature. We found that equilibrium was reached in less than 40 h. The bath and tube were sampled for doxorubicin and P_i determination. No P_i was found outside the tube. The total drug concentrations were determined by diluting the samples to an overall drug concentration of about 5–10 μM, mixing 1 mL of diluted sample with 4 mL of an SDS solution (7–10 mM) in buffer, and comparing the resulting doxorubicin fluorescence with a series of standard drug solutions in SDS (excitation at 490 nm, emission at 594 nm). The fluorescence depended linearly on the drug concentration up to at least 15 μM. Corrections for light scattering and inner filtering were negligible. In protein-free systems (LUVET), the total doxorubicin concentration could also be determined by mixing 1 mL of sample with 4 mL of a 1/3 chloroform/methanol mixture, yielding identical results as with SDS.

NMR. Preparation of lipid dispersions and NMR conditions were as described in De Wolf et al. (1991a). The P_i concentration in the NMR tube (before addition of doxorubicin) was about 20 mM, and doxorubicin was added from a 20–30 mM stock solution. The temperature was 30 °C.

RESULTS

Well-Defined Synthetic Phospholipid Mixtures as a Starting Point for Studies on Natural Membranes. In previous binding studies (De Wolf et al., 1991a,b), we concentrated on synthetic model membranes consisting of 100% anionic phospholipid. As an intermediate system, and for comparison with both natural plasma membrane-derived systems and synthetic single-component membranes, we first studied mixed membranes that consisted of well-defined mixtures of synthetic phospholipids with varying anionic lipid content. Figure 1 shows the structure of doxorubicin and the binding of this drug to a series of mixed membranes consisting of anionic DOPG and zwitterionic DOPC. Superimposable data were obtained with bovine heart cardiolipin/DOPC mixtures, in terms of cardiolipin–P_i or total P_i (data not shown).

It is clear from Figure 1 that the overall binding level is determined by the anionic lipid content. By relating the amount of bound drug to the amount of anionic lipid rather than to the total amount of lipid, we obtained the same binding level of 1.2–1.4 mol of doxorubicin/mol of anionic phospholipid–P_i at 2–3 mM free drug.

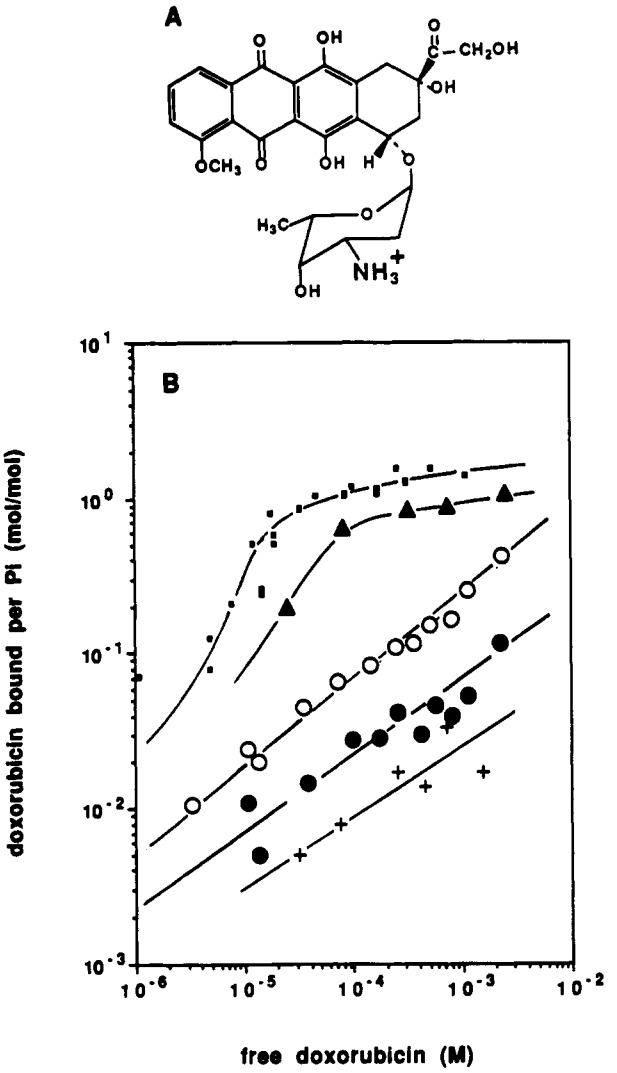

FIGURE 1: (A) Structure of doxorubicin. (B) Binding of doxorubicin to LUVET consisting of various DOPG/DOPC mixtures: 1/0 (■); 3/1 (▲); 1/3 (○); 1/9 (●); 0/1 (+). The (■) and (+) data are shown merely for comparison and are taken from De Wolf et al. (1991a). Binding is related to total P_i.

In addition, the binding affinity decreases at low anionic lipid content. For example, a 20-fold and a 70-fold higher free drug concentration is needed to reach a binding level of 0.7 doxorubicin/PG in membranes with 25% and 10% PG, respectively, as compared to 100% PG.

Two other features of Figure 1 are (1) a decrease of the anionic lipid content results in a decrease of the apparent degree of binding cooperativity, taken as the slope in the double-log plot at low binding levels, and (2) apparent binding saturation occurs at high binding levels (above 0.9 mol of doxorubicin/mol of lipid–P_i), observed in membranes with a high anionic lipid content.

Total Lipid Extracts of Bacterial Plasma Membranes. Essentially all the above-mentioned features emerge also from the data obtained with total lipid extracts of *E. coli* plasma membranes (Figure 2). The phospholipid compositions of the extracts used in Figure 2 are shown in Table I. The binding was again dependent on the anionic lipid content. The highest binding levels were obtained with extracts of strain AH930,

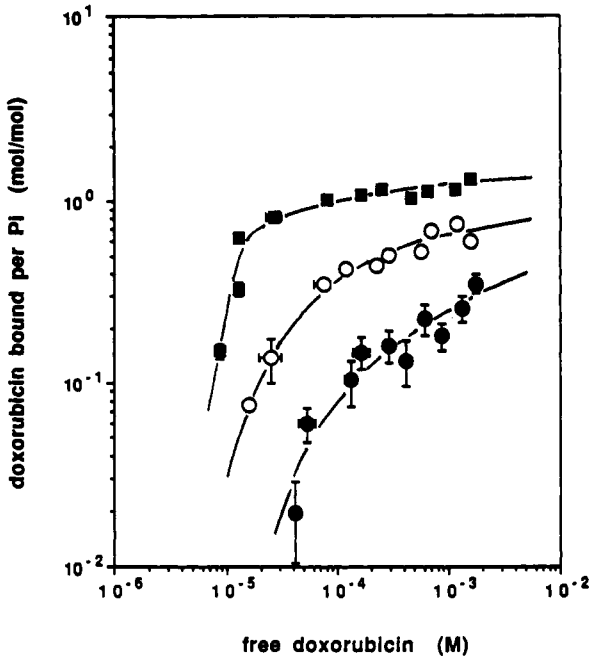

FIGURE 2: Binding of doxorubicin to LUVET consisting of total lipid extracts prepared from inner membrane vesicles of the *E. coli* strains AH930 (■) and HDL11 grown in the presence of 60 μM IPTG (○) or without IPTG (●). The error bars indicate the standard error of the mean (SEM). Average values from data of two (■, ○) or four (●) different experiments, each involving duplicate incubations at the various overall drug concentrations. At least two separately isolated lipid extracts were used. Binding is related to total P_i.

Table I: Phospholipid Composition of Total Lipid Extracts from Inner Membrane Vesicles[a]

	% P_i[b] in					
bacterial strain	PE	PG	cardiolipin	PA	rest[c]	anionic lipid[d]
AH930	0	35	47	13	5	95–100
MRE600	72	8	18	0	2	26–28
K1059	73	9	18	0	1	26–27
HDL11 (+IPTG[e])	66	8	20	1	5	29–34
HDL11 (–IPTG[e])	89	2	3	3	3	8–11

[a] Lipids were separated by two-dimensional high-performance thin-layer liquid chromatography (Kieselgel 60, Merck, Germany; 10 × 10 cm) with chloroform/methanol/ammonia/water (34/14/1/1, v/v) as the first eluent and chloroform/methanol/acetic acid (13/5/2, v/v) as the second eluent. Spots were visualized with I_2 and excised for P_i quantification according to Rouser (1970). [b] Standard deviation was in the range of 1–4 mol % (three to eight separate extracts from each strain). [c] P_i in one or more nonidentified minor spots in thin-layer chromatography. [d] Indicated is the range of uncertainty due to some P_i in nonidentified spots in thin-layer chromatography (see footnote *c*). [e] +IPTG or –IPTG: grown in the presence (60 μM) or absence of IPTG.

containing 95–100% anionic phospholipid–P_i, the lowest levels with extracts of strain HDL11 grown in the absence of IPTG (8–11% anionic lipid–P_i), and intermediate levels with extracts of strain HDL11 grown with 60 μM IPTG (29–34% anionic lipid–P_i). Total lipid extracts of the wild-type strain MRE600 (26–28% anionic lipid–P_i) and of strain K1059 (27% anionic lipid–P_i) were also investigated. Consistent with the results in Figure 2, these extracts bound 1.5–2-fold more doxorubicin than extracts of strain HDL11 with only 8–11% anionic lipid–P_i (the results are not shown in Figure 2, for the sake of clarity).

Thus, anionic phospholipids appear to be major determinants of doxorubicin binding also in total lipid extracts of bacterial plasma membranes. Nevertheless, the absolute binding levels in the total lipid extracts were often higher than in synthetic phospholipid mixtures with similar anionic lipid content (compare Figures 1 and 2). Accordingly, the binding

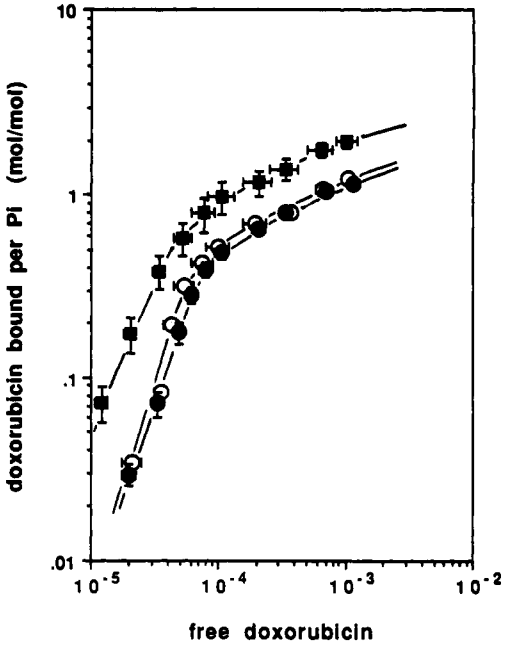

FIGURE 3: Binding of doxorubicin to inner membrane vesicles of the *E. coli* strains AH930 (■) and HDL11 grown with 60 μM IPTG (○) or without IPTG (●). Error bars indicate the SEM. The number of experiments was three (■, ○) or six (●), each with a separately isolated vesicle batch and involving duplicate incubations at each overall drug concentration. Binding is related to total P_i.

characteristics of the total lipid extracts were biphasic, first displaying positive cooperativity at moderate drug levels (slope higher than 1 in the double-log plot) and then apparent saturation (a decrease of the slope) in the range of 0.1–0.8 mol of doxorubicin bound/mol of lipid–P_i (Figure 2). Note that apparent saturation occurred at roughly the same binding levels in the synthetic membranes (Figure 1), but these were reached at higher free drug levels than in membranes consisiting of total lipid extract. Natural mixtures of more than three to four different lipid components with a variety of acyl chains are highly complicated systems in which unknown structural or cooperative effects can play a role. We cannot exclude that the acyl chain composition of the total lipid extracts influences the binding, although we found that DOPG/DOPC mixed membranes have the same binding characteristics as mixed membranes consisting of 1-palmitoyl-2-oleoyl-PG and -PC (POPG and POPC, not shown). As compared to POPG/POPC, DOPG/DOPC mixed membranes have a relatively lower acyl chain order (Seelig & Seelig, 1980; Gasset et al., 1988). The higher binding levels in total lipid extracts are not readily ascribable to the presence of PE, versus PC in the model membranes, because the binding levels in DOPG/DOPE mixed model membranes appeared to be the same as those in DOPG/DOPC mixtures [not shown, 33% PE or PC was used so as to prevent drug-induced and DOPE-dependent formation of type II structures; cf. De Wolf et al. (1992)].

Bacterial Plasma Membrane Vesicles. Figure 3 shows the binding levels in isolated plasma membrane vesicles, in terms of the total vesicle–P_i. For all strains and growth conditions, inside-out and right-side-out vesicles had the same binding characteristics, excluding the influence of membrane potential or ion gradients in these experiments (data are not shown separately). The similar binding characteristics of both types of vesicles are in agreement with the lack of effect of membrane-disrupting freeze–thaw cycles (see Materials and Methods) and with the notion that the drug is permeant and has access to both internal and external binding sites (Raviv

et al., 1990; Frézard & Garnier-Suillerot, 1991; Spoelstra et al., 1992; F. A. de Wolf et al., unpublished data).

The binding was again dependent on the anionic lipid content of the membranes, since vesicles of strain AH930 (95–100% anionic lipid–P_i) bound as much doxorubicin as LUVET of pure DOPG (Figures 1 and 3), and clearly more doxorubicin than vesicles of the other strains, which have a lower anionic lipid content (Figure 3). The binding levels obtained with strain HDL11 (Figure 3) were 15–30% higher than those obtained with the wild-type strain MRE600 (not shown). Although in Figure 3 a small difference can be discerned between vesicles of strain HDL11 with normal (29–34%) and low (8–11%) anionic lipid–P_i, the difference is not significant. The absence of a significantly decreased binding in the HDL11 vesicles with low anionic phospholipid content can be due to the presence of other binding sites as well as to unknown membrane structural effects. For example, a clustering of binding sites existing before addition of the drug can increase the binding affinity and the degree of binding cooperativity (De Wolf, 1991; De Wolf et al., 1991a). These vesicles bind 5-fold more drug than the corresponding lipid extracts, in terms of total P_i, and even 20-fold more than a synthetic binary mixture containing 10% anionic phospholipid (compare Figures 1, 2, and 3). Degradation of the extravesicular parts of the membrane proteins with proteinase K (see Materials and Methods) did not result in a decreased binding capacity of the HDL11 plasma membrane vesicles, or in a difference between vesicles with low and with normal anionic lipid–P_i (data not shown). Also, repeated treatment with ribonucleases and deoxyribonucleases did not have such an effect. Even before nuclease treatment, the DNA level in HDL11 vesicles grown in the absence of IPTG was low (3–6% of P_i).

We checked the possibility that binding to anionic phospholipids in HDL11 vesicles is masked only at high drug concentrations, as a result of low-affinity binding to other membrane components. The centrifuge assay used in Figures 1–3 is not reliable at low doxorubicin levels (below 5–10 μM free drug), because small amounts of drug bind to the centrifuge tubes. Therefore, we used equilibrium dialysis, which allows a more accurate determination of free and bound drug. A drawback of this method is the long time required to equilibrate the drug, and the resulting risk of chemical decomposition. However, under our conditions (drug equilibration in the dark, under N_2), and in the range of 10–20 μM free doxorubicin, both the dialysis and centrifuge assays yielded the same results, with pure DOPA LUVET (not shown), with DOPG/DOPC mixed LUVET containing 10–25% PG, and with plasma membrane vesicles. The results obtained at about 0.2–12 μM free doxorubicin with LUVET consisting of DOPG/DOPC mixtures are shown in Figure 4A. The binding level in LUVET with 25% PG was clearly higher than in LUVET with 10% PG, demonstrating the sensitivity of the assay. Panel B shows that AH930 vesicles bind clearly more drug than HDL11 vesicles, also at low doxorubicin concentrations. However, the difference between HDL11 vesicles with normal and low anionic lipid levels (approximately 30 and 10%, respectively) is very small and probably insignificant, at low drug levels just as at higher drug levels (Figures 3 and 4B).

Thus, anionic phospholipids appear to be important determinants for doxorubicin binding in bacterial plasma membrane vesicles, but the effect of anionic phospholipids is masked in plasma membranes of strain HDL11 with low anionic lipid content, at all drug levels.

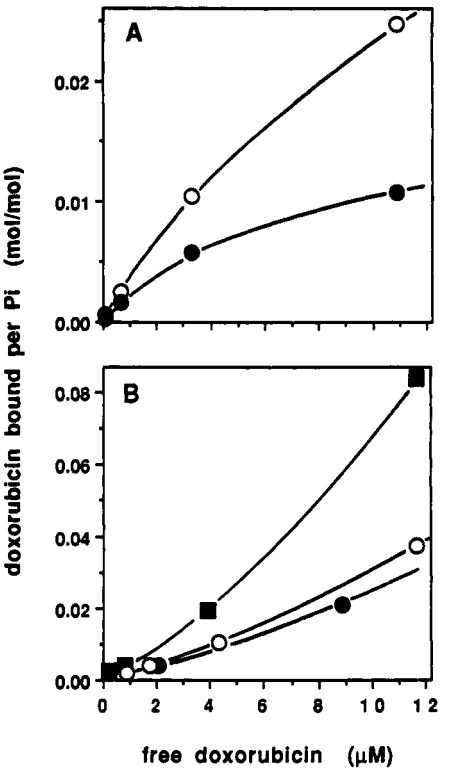

FIGURE 4: Binding of doxorubicin at low drug concentration. (A) Binding to LUVET consisting of DOPG/DOPC mixtures: 1/3 (○); 1/9 (●). (B) Binding to inside-out inner membrane vesicles of the *E. coli* strains AH930 (■) and HDL11 grown with 60 μM IPTG (○) or without IPTG (●). Binding was assayed by equilibrium dialysis.

Consequences of Doxorubicin Binding for Membrane Structure. In previous ^{2}H-NMR studies on model membranes of synthetic lipids with deuterated acyl chains, we observed that doxorubicin induces a dramatical disordering of the acyl chains and that this is absolutely dependent on the presence of anionic phospholipids (De Wolf et al., 1991a, 1992). The ^{2}H quadrupolar splitting determined from the ^{2}H-NMR spectra is a sensitive measure of the average acyl chain order at the position of the deuteron (Seelig, 1977). In order to investigate whether the drug is able to induce acyl chain disordering also in *E. coli* plasma membrane systems, we used strain K1059, which is unable to synthesize or degrade unsaturated fatty acids (Overath et al., 1970). It incorporates [11,11-$^2H_2$]oleic acid into its membrane lipids when it is grown on this fatty acid. Intact cells, plasma membrane vesicles, and other membrane systems derived from this strain have recently been characterized in detail in our laboratory with ^{2}H- and ^{31}P-NMR techniques (Killian et al., 1992). Figure 5 shows that doxorubicin induces a large decrease of the ^{2}H quadrupolar splitting in dispersions of total lipid extracts (27% anionic phospholipid–P_i), indicating penetration of the drug between the lipid molecules. The ^{2}H-NMR spectra (not shown) did not display multiple spectral components at any drug level, indicating homogeneous mixing of drug and lipids [cf. De Wolf et al. (1992)]. Consistent with the higher binding levels in total lipid extracts, as compared to synthetic lipid mixtures, the decrease shown in Figure 5 (approximately 23%) is relatively larger than the 10% decrease previously obtained with mixtures of synthetic lipids containing 33% anionic lipid–P_i (De Wolf et al., 1992).

Due to the small size of inside-out plasma membrane vesicles and the resulting increase of motional averaging of the ^{2}H-NMR signals (Killian et al., 1992), they could not be used to investigate the effect of doxorubicin on acyl chain order.

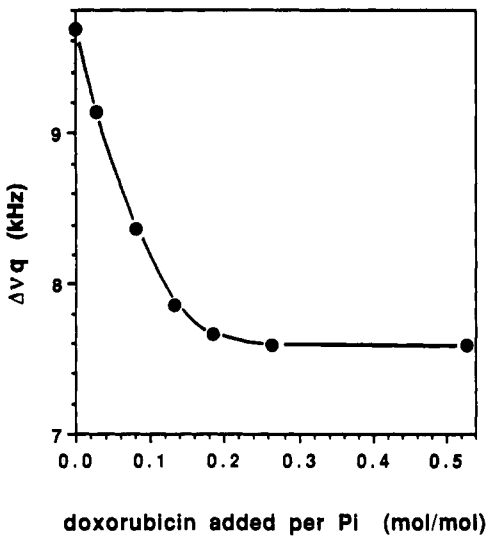

FIGURE 5: Effect of doxorubicin on the ^2H quadrupolar splitting ($\Delta\nu_q$) in dispersions of total lipid extract of strain K1059, grown on [11,11-$^2H_2$]oleic acid. Overall doxorubicin and lipid concentrations, 7–18 and 9–100 mM, respectively; temperature, 30 °C; 2×10^4 spectra were averaged.

Therefore, we used spheroplasts, which are larger and can be prepared relatively quickly in sufficient quantities. A 29% decrease of the ^2H quadrupolar splitting (from 6.5 to 4.6 kHz) was obtained at 1 mol of doxorubicin/total P_i in spheroplasts. This effect is comparable to that in total lipid extracts and indicates again that doxorubicin molecules become inserted in the lipid matrix of the plasma membrane.

^{31}P-NMR spectra of the total lipid extracts and spheroplasts were also recorded [not shown; cf. Killian et al. (1992)]. These spectra were characteristic of liquid-crystalline bilayers, displaying axial symmetry, with a high-field peak and low-field shoulder, due to fast rotation of the lipid molecules around their long axis. Doxorubicin did not change the ^{31}P-NMR spectra, and the lipids remained organized in bilayers after addition of the drug (not shown).

DISCUSSION

Importance of Anionic Phospholipids. The use of *E. coli* phospholipid synthesis mutants allowed us to directly test the role of anionic phospholipids in the binding of doxorubicin to plasma membrane systems. Our present data clearly show that anionic phospholipids are important determinants of doxorubicin binding in bacterial plasma membrane systems. The drug elicits a disordering of the acyl chains in plasma membrane systems, strongly indicating that it inserts between the lipid molecules. Drug penetration is also seen in phospholipid monolayers spread at an air–water interface (Goormaghtigh et al., 1980; Nicolay et al., 1988; Dupou-Cézanne et al., 1989; De Wolf et al., 1991b). Thus, the binding is probably not purely electrostatic, but involves hydrophobic interactions. The relevance of anionic phospholipids for doxorubicin–plasma membrane interactions is underlined by our recent observation that doxorubicin specifically inhibits anionic phospholipid-dependent protein translocation across plasma membranes of *E. coli* HDL11 whereas it has no effect on the anionic phospholipid-*in*dependent translocation of closely-related proteins (D. A. Phoenix, F. A. de Wolf, C. Hikita, S. Mizushima, and B. de Kruijff, unpublished results). This agrees with the previously observed inhibition of anionic phospholipid-dependent protein import in mitochondria (Eilers et al., 1989).

The *E. coli* system was used mainly because of the availability of phospholipid synthesis mutants. Although living *E. coli* cells are relatively insensitive to doxorubicin (personal communication, Prof. C. F. Higgins and Dr. D. R. Gill, Imperial Cancer Research Fund, University of Oxford, Oxford, U.K.), this is probably not due to the structure of their plasma membrane but due to their inaccessibility, resulting from the barrier formed by the outer membrane. In a *tolC* mutant in which the outer membrane is permeable (strain CS1562; C. J. Dorman, A. S. Linch, N. Ni Bhriain, and C. F. Higgins, Oxford, U.K.), we observed efficient and reversible growth inhibition at 1–5 μM doxorubicin (F. A. de Wolf et al., unpublished results). In agreement, we observed that doxorubicin does not elicit a disordering of acyl chains in intact cells, but only in spheroplasts of the fatty acid auxotroph K1059 (data not shown).

In view of the relevance of anthracycline–membrane interactions for drug transport (Tarasiuk & Garnier-Suillerot, 1992; Dordal et al., 1992) as well as drug action (Thompson et al., 1987; Oakes et al., 1990; Tritton, 1991; Vichi & Tritton, 1992), it is surprising that thus far relatively few studies are available that provide quantitative information about the binding of anthracyclines to mammalian plasma membranes (Mikkelsen et al., 1977; Goldman et al., 1978; Garnier-Suillerot & Gattegno, 1988; Escriba et al., 1990) or other membranes from mammalian cells (Nicolay et al., 1984; Cheneval et al., 1985; Griffin et al., 1986). Although in most of these papers the binding was studied in relation to the anionic lipid content, it has not been possible to vary the anionic lipid content of the membranes studied. Manipulation of the lipid acyl chain composition in cancer cells has been reported to affect drug uptake: an increased level of (poly)unsaturated acyl chains stimulated drug uptake (Burns & North, 1986; Burns et al., 1988), but this was probably the result of an increased passive drug transport rate [cf. Raviv et al. (1990)]. The membrane binding equilibria were not fully analyzed in the studies of Burns. As far as can be estimated from previously published results, there is no striking difference between the binding characteristics presently observed in *E. coli* plasma membranes with 10–30% anionic lipid–P_i and those previously seen in human erythrocyte plasma membranes (Mikkelsen et al., 1977; Goldman et al., 1978; Garnier-Suillerot & Gattegno, 1988) or mitochondrial inner membranes (Nicolay et al., 1984; Cheneval et al., 1985; Griffin et al., 1986). The binding levels of doxorubicin in *E. coli* membranes are close to those of the slightly more hydrophobic drug daunomycin in plasma membranes of drug-resistant P388 murine leukemia cells, and only a factor of 2–3 lower than the binding levels of daunomycin in the corresponding sensitive P388 cells, at comparable free drug concentrations [data calculated from the results shown in Escriba et al. (1990)].

Implications of Membrane Binding for the Distribution of Doxorubicin and Other Anthracyclines in the Cell: Extrapolation to Eukaryotic Cells. By extrapolating these binding data, we estimate that in most cell types and under most conditions the fraction of free doxorubicin in the cytoplasm will be very small and most of the drug will be bound to membranes or DNA. In eukaryotic cells, this is partly the result of the large amount of membranes present in the cytoplasm. Consider first a hypothetical spherical cell with a diameter of 10 μM, surrounded by a plasma membrane, but containing no membranes at all in the cytoplasm. If the phospholipids on the cytoplasmic side of the membrane would account for 50% of the cytoplasmic membrane surface, the internal phospholipid concentration would be about 0.8 mM P_i, assuming that each lipid molecule covers about 0.65 nm^2 (Demel et al., 1975; De Wolf et al., 1991b). According to the

present binding data, 70–95% of the total internal doxorubicin would be bound to the cytoplasmic side of the membrane in this hypothetical cell (10^{-4}–10^{-1} mol of drug bound/mol of lipid–P_i) if the internal free drug concentration would be in the range of 10^{-8}–10^{-5} M (70% is an underestimate derived from the binding isotherm of synthetic mixed membranes consisting of 10% DOPG and 90% DOPC; plasma membranes bind relatively more drug). In the cytoplasm of a real eukaryotic cell, the total membrane surface available for drug binding is much higher, relative to the volume of the cytoplasm, due to the presence of many organelles and vesicles. Thus, on the basis of our data obtained with model systems, we expect that in most cell types and under most conditions almost all the doxorubicin present in the cytoplasm will be bound to membranes. The fraction of cytoplasmic drug bound to the intracellular membranes will be even larger in the case of more hydrophobic anthracycline derivatives (Israel et al., 1987; Constantinides et al., 1989).

Of course, the high affinity of doxorubicin for DNA (Chaires et al., 1982; Graves & Krug, 1983) and the amount of DNA in the cell imply that the overall distribution of the total intracellular drug is to a significant extent determined by the DNA. Part of the drug could also be bound to RNA and ribosomes (Nicolay et al., 1984; Simpkins et al., 1984). The occurrence of drug resistance in cancer cells is sometimes accompanied by an intracellular redistribution of the drug from the nucleus (N) toward the cytoplasm (C) (Hindenburg et al., 1989; Schuurhuis et al., 1989, 1991; Lankelma et al., 1991). The large density of membranes in the cytoplasm makes it difficult to discern membrane-bound drug from free drug, for example by fluorescence microscopy [cf. Lankelma et al. (1991)]. In this respect, membrane binding is a potentially important factor that has not yet been sufficiently studied and could, for example, influence the N/C ratio and the degree of drug resistance under conditions of restricted drug uptake (transient drug administration).

In situations where anthracyclines are actively translocated from one compartment to another, for example, out of the cell (Frézard & Garnier-Suillerot, 1991; Spoelstra et al., 1992), the efficient partitioning of the drug into the membrane implies that it may be more efficient to pump the bound drug out of the membrane than to pump the free drug out of the cytosol, even if free drug and bound drug are in fast equilibrium. At a free drug concentration of 0.7–5 μM, the binding stoichiometry is about 7×10^{-4} to 0.2 mol of doxorubicin/mol of lipid–P_i, implying that the local drug concentration in the membrane is some 1000–10000-fold higher than the concentration of the free drug. Indeed, the multidrug resistance-conferring P-glycoprotein has been proposed to pump drugs out of the membrane (Raviv et al., 1990; Safa, 1992; Higgins & Gottesman, 1992). On the basis of our present data, we expect that anionic phospholipids play an important role in all these processes.

As it appears, *E. coli* plasma membranes are attractive systems for future studies on the mechanism of drug pumping by resistance-conferring proteins of mammalian or prokaryotic origin (*E. coli*), in relation to the presence of anionic phospholipids.

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REFERENCES

Anraku, Y. (1967) *J. Biol. Chem. 242*, 793–800.
Arancia, G., Molinari, A., Crateri, P., Calcabrini, A., Silvestri, L., & Isacchi, G. (1988) *Eur. J. Cell Biol. 47*, 379–387.
Asai, Y., Katayose, Y., Hikita, C., Ohta, A., & Shibuya, I. (1989) *J. Bacteriol. 171*, 6867–6869.
Bachur, N. R., Yu, F., Johnson, R., Hickey, R., Wu, Y., & Malkas, L. (1992) *Mol. Pharmacol. 41*, 993–998.
Bligh, E. G., & Dyer, W. L. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Burke, T. G., & Tritton, T. R. (1985) *Biochemistry 24*, 1768–1776.
Burke, T. G., Morin, M. J., Sartorelli, A. C., Lane, P., & Tritton, T. R. (1987) *Mol. Pharmacol. 31*, 552–556.
Burke, T. G., Sartorelli, A. C., & Tritton, T. R. (1988) *Cancer Chemother. Pharmacol. 21*, 274–280.
Burke, T. G., Israel, M., Seshadri, R., & Doroshow, J. H. (1990) *Cancer Biochem. Biophys. 11*, 177–185.
Burns, C. P., & North, J. A. (1986) *Biochim. Biophys Acta 888*, 10–17.
Burns, C. P., North, J. A., Petersen, E. S., & Ingraham, L. M. (1988) *Proc. Soc. Exp. Biol. Med. 188*, 455–460.
Cammack, K. A., & Wade, H. E. (1965) *Biochem. J. 96*, 671–680.
Capranico, G., Kohn, K. W., & Pommier, Y. (1990) *Nucleic Acids Res. 18*, 6611–6619.
Chaires, J. B., Dattagupta, N., & Crothers, D. M. (1982) *Biochemistry 21*, 3933–3940.
Cheneval, D., Müller, M., Toni, R., Ruetz, S., & Carafoli, E. (1985) *J. Biol. Chem. 260*, 13003–13007.
Chupin, V., Killian, J. A., & de Kruijff, B. (1987) *Biophys. J. 51*, 395–405.
Comfurius, P., & Zwaal, R. F. A. (1977) *Biochim. Biophys. Acta 488*, 36–42.
Constantinides, P. P., Ghosaini, L., Inouchi, N., Kitamura, S., Seshadri, R., Israel, M., Sartorelli, A., & Sturtevant, J. M. (1989) *Chem. Phys. Lipids 51*, 105–118.
Dalmark, M., & Storm, H. H. (1981) *J. Gen. Physiol. 78*, 349–364.
De Chavigny, A., Heacock, P. N., & Dowhan, W. (1991) *J. Biol. Chem. 266*, 5323–5332.
De Gier, J. (1992) *J. Electroanal. Chem. 342*, 1–10.
Demel, R. A., Geurts van Kessel, W. S. M., Zwaal, R. F. A., Roelofsen, B., & van Deenen, L. L. M. (1975) *Biochim Biophys. Acta 406*, 97–107.
Devaux, P. F. (1991) *Biochemistry 30*, 1163–1173.
De Vrije, T., Tommassen, J., & De Kruijff, B. (1987) *Biochim. Biophys. Acta 900*, 63–72.
De Wolf, F. A. (1991) *Biosci. Rep. 11*, 275–284.
De Wolf, F. A., Maliepaard, M., van Dorsten, F., Berghuis, I., Nicolay, K., & de Kruijff, B. (1991a) *Biochim. Biophys. Acta 1096*, 67–80.
De Wolf, F. A., Demel, R. A., Bets, D., van Kats, C., & de Kruijff, B. (1991b) *FEBS Lett. 288*, 237–240.
De Wolf, F. A., Nicolay, K., & de Kruijff, B. (1992) *Biochemistry 31*, 2952–2962.
Dordal, M. S., Winter, J. N., & Atkinson, A. J., Jr. (1992) *J. Pharmacol. Exp. Ther. 263*, 762–766.
Dupou-Cézanne, L., Sautereau, A.-M., & Tocanne, J. F. (1989) *Eur. J. Biochem. 181*, 695–702.
Eilers, M., Endo, T., & Schatz, G. (1989) *J. Biol. Chem. 264*, 2945–2950.
Escriba, P. V., Ferrer-Montiel, A. V., Ferragut, J. A., & Gonzalez-Ros, J. M. (1990) *Biochemistry 29*, 7275–7282.
Farren, S. B., Sommerman, E., & Cullis, P. R. (1984) *Chem. Phys. Lipids 34*, 279–286.
Foglesong, P. D., Reckord, C., & Swink, S. (1992) *Cancer Chemother. Pharmacol. 30*, 123–125.
Folch, J., Lees, M., & Sloanne Stanley, G. H. (1957) *J. Biol. Chem. 226*, 497–509.

Frézard, F., & Garnier-Suillerot, A. (1991) *Eur. J. Biochem. 196*, 483–491.
Garnier-Suillerot, A., & Gattegno, L. (1988) *Biochim. Biophys. Acta 936*, 50–60.
Gasset, M., Killian, J. A., Tournois, H., & De Kruijff, B. (1988) *Biochim. Biophys. Acta 939*, 79–88.
Goldman, R., Facchinetti, T., Bach, D., Raz, A., & Shinitzky, M. (1978) *Biochim. Biophys Acta 512*, 254–269.
Goormaghtigh, E., Chatelain, P., Caspers, J., & Ruysschaert, J.-M. (1980) *Biochim. Biophys. Acta 597*, 1–14.
Graves, D. E., & Krugh, T. R. (1983) *Biochemistry 22*, 3941–3947.
Griffin, E. A., Vanderkooi, J. M., Maniara, G., & Erecinska, M. (1986) *Biochemistry 25*, 7875–7880.
Heacock, P. N., & Dowhan, W. (1989) *J. Biol. Chem. 264*, 14972–14977.
Henry, N., Fantine, E. O., Bolard, J., & Garnier-Suillerot, A. (1985) *Biochemistry 24*, 7085–7092.
Higgins, C. F., & Gottesman, M. M. (1992) *Trends Biochem. Sci. 17*, 18–21.
Hindenburg, A. A., Gervasoni , J. E., Jr., Krishna, S., Steward, V. J., Rosado, M., Lutzky, J., Bhalla, K., Baker, M. A., & Taub, R. N. (1989) *Cancer Res. 49*, 4607–4614.
Hope, M. J., Bally, M. B., Webb, G., & Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55–65.
Israel, M., Seshadri, R., Koseki, Y., Sweatman, T. W., & Idriss, J. M. (1987) *Cancer Treat. Rev. 14*, 163–167.
Kaback, H. R. (1971) *Methods Enzymol. 22*, 99–120.
Karkhanis, Y. D., Zeltner, J. Y., Jackson, J. J., & Carlo, D. (1978) *Anal. Biochem. 85*, 595–601.
Killian, J. A., Fabrie, C. H. J. P., Baart, W., Morein, S., & De Kruijff, B. (1992) *Biochim. Biophys. Acta 1105*, 253–262.
Kusters, R., Dowhan, W., & De Kruijff, B. (1991) *J. Biol. Chem. 266*, 8659–8662.
Lankelma, J., Mulder, H.-S., Van Mourik, F., Wong Fong Sang, H. W., Kraayenhof, R., & Van Grondelle, R. (1991) *Biochim. Biophys. Acta 1093*, 1147–1152.
Lowry, O. H., Rosebrough, N. V., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 103*, 265–275.
Mikkelsen, R. B., Lin, P.-S., & Wallach, D. F. H. (1977) *J. Mol. Med. 2*, 33–40.
Müller, M., & Blobel, G. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7421–7425.
Nicolay, K., & de Kruijff, B. (1987) *Biochim. Biophys. Acta 892*, 320–330.
Nicolay, K., Timmers, R. J. M., Spoelstra, E., van der Neut, R., Fok, J. J., Huigen, Y., Verkleij, A., & de Kruijff, B. (1984) *Biochim. Biophys. Acta 778*, 359–371.
Nicolay, K., Fok, J. J., Voorhout, W., Post, J. A., & De Kruijff, B. (1986) *Biochim. Biophys. Acta 887*, 35–41.
Nicolay, K., Sautereau, A.-M., Tocanne, J. F., Brasseur, R., Huart, P., Ruysschaert, J. M., & De Kruijff, B. (1988) *Biochim. Biophys. Acta 940*, 197–208.
Oakes, S. G., Schlager, J. J., Santone, K. S., Abraham., R. T., & Powis, G. (1990) *J. Pharmacol. Exp. Ther. 252*, 979–983.
Op den Kamp, J. A. F. (1979) *Annu. Rev. Biochem. 48*, 47–71.
Overath, P., Schairer, H. U., & Stoffel, W. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 606–612.
Posada, J., Vichi, P., & Tritton, T. R. (1989) *Cancer Res. 49*, 6634–6639.
Raetz, C. R. H. (1978) *Microbiol. Rev. 42*, 614–659.
Raetz, C. R. H., & Dowhan, W. (1990) *J. Biol. Chem. 265*, 1235–1238.
Ramu, A., Pollard, H. B., & Rosario, L. M. (1989) *Int. J. Cancer 44*, 539–547.
Raviv, Y., Pollard, H. B., Bruggeman, E. P., Pastan, I., & Gottesman, M. M. (1990) *J. Biol. Chem. 265*, 3975–3980.
Rouser, G., Fleischer, S., & Yamamoto, A. (1970) *Lipids 5*, 494–496.
Safa, A. R. (1992) *Cancer Invest. 10*, 295–305.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) in *Molecular Cloning—A Laboratory Manual*, pp E3–E4, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Schuurhuis, G. J., Broxterman, H. J., Cervant s, A., van Heijningen, T. H. M., de Lange, J. H. M., Baak, J. P. A., Pinedo, H. M., & Lankelma, J. (1989) *J. Natl. Cancer Inst. 81*, 1887–1892.
Schuurhuis, G. J., Broxterman, H. J., de Lange, J. H. M., Pinedo, H. M., van Heijningen, T. H. M., Kuiper, C. M., Scheffer, G. L., Scheper, R. J., van Kalken, C. K., Baak, J. P. A., & Lankelma, J. (1991) *Br. J. Cancer 64*, 857–861.
Seelig, J. (1977) *Q. Rev. Biophys. 10*, 353–418.
Seelig, J., & Seelig, A. (1980) *Q. Rev. Biophys. 13*, 19–61.
Simpkins, H., Pearlman, L. F., & Thompson, L. M. (1984) *Cancer Res. 44*, 613–618.
Smaal, E. B., Romijn, D., Geurts van Kessel, W. S. M., de Kruijff, B., & de Gier, J. (1985) *J. Lipid Res. 26*, 634–637.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem. 150*, 76–85.
Spoelstra, E. C., Westerhoff, H. V., Dekker, H., & Lankelma, J. (1992) *Eur. J. Biochem. 207*, 567–579.
Sun, I. L., Sun, E. E., Crane, F. L., Morré, D. J., & Faulk, W. P. (1992) *Biochim. Biophys. Acta 1105*, 84–88.
Tarasiuk, J., & Garnier-Suillerot, A. (1992) *Eur. J. Biochem. 204*, 693–698.
Thompson, M. G., Chawala, S. B., & Hickman, J. A. (1987) *Cancer Res. 47*, 2799–2803.
Tritton, T. R. (1991) *Pharmacol. Ther. 49*, 293–309.
Tritton, T. R., & Yee, G. (1982) *Science 217*, 248–250.
Van Deenen, L. L. M., & de Haas, G. H. (1964) *Adv. Lipid Res. 2*, 168–229.
Vichi, P., & Tritton, T. R. (1992) *Cancer Res. 52*, 4135–4138.
Vichi, P., Robison, S., & Tritton, T R. (1989) *Cancer Res. 49*, 5575–5580.
Voelker, D. R. (1991) *J. Biol. Chem. 266*, 12185–12188.