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Transport of the anti-cancer drug doxorubicin across cytoplasmic membranes and membranes composed of phospholipids derived from *Escherichia coli* occurs via a similar mechanism

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Abstract

An assay was developed to measure and directly compare transport of doxorubicin across right-side-out cytoplasmic membrane vesicles (ROV) and across model membranes (LUVET) composed of pure phospholipids, isolated from the corresponding cells. *Escherichia coli* was used as a model organism, since mutants are available which differ in phospholipid composition. Both in LUVET and ROV only passive diffusion across the bilayer is involved, because effects of drug concentration, pH, divalent cations, the phospholipid composition, and the active transport inhibitor verapamil were comparable. Permeability coefficients were about 2–3-times higher in ROV compared to LUVET. Furthermore, in LUVET an average activation energy of 87 kJ/mol and in ROV of 50 kJ/mol was observed. These differences are suggested to result from differences in membrane order between LUVET and ROV and differences in the temperature dependence of membrane order in LUVET and ROV, respectively. Because no background carrier-facilitated doxorubicin transport seems to be present, ROV are an excellent model system to study the effect of phospholipid composition on drug transport after expression of a multidrug resistance-conferring protein. Furthermore, data of passive diffusion of doxorubicin obtained with LUVET are representative for more complex, biologically relevant membrane systems.

Keywords: Permeability; Activation energy; Doxorubicin; Phospholipid; Diffusion; LUVET

1. Introduction

Doxorubicin is a potent and widely applied anti-cancer drug exhibiting effects on DNA and DNA associated enzymes [1,2] as well as on membranes (for a review, see [3]). The main route of entry into a cancer cell is passive diffusion across the plasma membrane of the uncharged form of the drug which is a weak base with a pK_a of 8.3[4–7]. In order to improve drug uptake it is therefore important to gain insight about the parameters that govern the rate of passive diffusion, such as the phospholipid composition and drug binding and insertion into the membranes [8].

Until now studies including passive diffusion of doxorubicin involved either model systems composed of large unilamellar phospholipid vesicles [5,7,9–11] or intact cells [4,12–16] and only few studies have been performed with plasma membrane vesicles [17,18]. The large unilamellar vesicles (LUVET) system allows for detailed characterization of the mechanism and kinetics of uptake of doxorubicin, whereas results obtained from mechanistic and kinetic studies in intact cells are much more difficult to interpret, because of the presence of many intracellular membranes and metabolism. To what extent the data obtained with the use of model membranes are representative

Abbreviations: CL, cardiolipin; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid; IPTG, isopropylthiogalactoside; LUVET, large unilamellar vesicles prepared by extrusion; mdr, multidrug resistance; *P*, permeabilty coefficient doxorubicin; P^0, permeability coefficient for uncharged doxorubicin; PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; P_i, inorganic phosphate; Pipes, 1,4-piperazinediethanesulfonic acid; ROV, right-side-out cytoplasmic membrane vesicles; TLE, total lipid extract.

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to the in vivo situation is not clear, since a direct comparison between doxorubicin transport into cytoplasmic membrane vesicles and the transport across the model membranes composed of the corresponding phospholipids has not yet been made.

We therefore compared transport of doxorubicin in right-side-out cytoplasmic membrane vesicles (ROV) with transport of this drug across membranes of large unilamellar vesicles (LUVET) composed of phospholipids isolated from the corresponding cells. The bacterium *Escherichia coli* was used as a model organism for the following reasons: (i) The availability of different mutants allows for extensive in vivo manipulation of the phospholipid composition, whereas such mutants are not available for cancer cells. (ii) Comparative binding studies of doxorubicin to membranes of LUVET and ROV derived from *E. coli* have already been performed [19]. We developed an assay to measure doxorubicin diffusion in ROV based on a method developed for LUVET [8]. Transport of doxorubicin across membranes of ROV and LUVET derived from several *E. coli* strains differing in phospholipid composition was directly compared.

2. Materials and methods

2.1. Growth of Escherichia coli strains

The following *Escherichia coli* strains were employed: *E. coli* MRE600 [20] 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). *E. coli* HDL11 is a strain in which the gene encoding phosphatidylglycerolphosphate synthase, necessary for the synthesis of phosphatidylglycerol (PG) and cardiolipin (CL), is under control of the lac promoter [21]. In the presence of the inducer isopropylthiogalactoside (IPTG) this strain has a phospholipid composition comparable to the wild type (about 25% negatively charged phospholipids). In the absence of IPTG the level of negatively charged phospholipids is strongly reduced [21]. This strain was grown in LB in the absence or in the presence of 100 μM IPTG. The precultures of strain HDL11 were supplemented with kanamycin (50 μg/ml), tetracycline (10 μg/ml) and chloramphenicol (20 μg/ml).

2.2. Preparation of right-side-out cytoplasmic membrane vesicles

E. coli cells were grown at 37°C until late logarithmic phase (Absorbance at 660 nm 0.6–0.8) as described above, chilled on ice, and harvested by low-speed centrifugation (30 min, 6400 × *g*, 4°C). Right-side-out cytoplasmic membrane vesicles (ROV) were prepared and isolated as described [22]. Cytoplasmic membrane vesicles were washed in Pipes buffer (10 mM Pipes, 100 mM NaCl, 1 mM EGTA, pH 7.4), frozen and stored in liquid nitrogen.

2.3. Isolation and determination of the composition of Escherichia coli lipids

E. coli strain MRE600, and HDL11 were grown as described above. *E. coli* HDL11 was grown either in the absence or in the presence of 100 μM IPTG. Total lipid extracts were prepared from washed *E. coli* cells [23] by extracting the lipids [24] and removing proteins and neutral lipids on a silica column. Lipids were separated by two dimensional high-performance thin-layer chromatography (Kieselgel 60, Merck, Germany) with chloroform/methanol/ammonia/water (68:28:2:2, v/v) in the first dimension and chloroform/methanol/acetic acid (65:25:10, v/v) in the second dimension. Spots were visualized with I_2 and excised for P_i quantification [25]. The lipid composition of ROV was determined after a Bligh and Dyer extraction [24].

2.4. Preparation of right-side-out cytoplasmic membrane vesicles (ROV) and large unilamellar vesicles (LUVET) with or without enclosed DNA for transport experiments

Preparation of DNA to be enclosed in LUVET and ROV was as described [8]. LUVET with or without enclosed DNA were prepared by the method described by Speelmans et al. [8]. In order to enclose DNA in ROV, the vesicles were freeze-thawed five times in the presence of sonicated and extruded DNA. Freezing was performed quickly in liquid nitrogen and thawing was performed slowly at room temperature. The concentration of protein was about 15 mg/ml and of DNA 30 mM (on P_i basis) during the freeze-thaw steps. Subsequently, the ROV were washed three times in Hepes buffer (10 mM Hepes, 100 mM NaCl, 1 mM EGTA, pH 7.4) (40 000 × *g*, 8 min, 4°C) and treated with DNase (5 mg in a total volume of 1 ml) in the presence of 5 mM $MgSO_4$ for 15 min at 37°C. The vesicles were washed three times in Pipes buffer and resuspended in Pipes buffer. Control vesicles without DNA were washed three times in Hepes buffer and after the DNase treatment, the sample was freeze-thawed three times. Additional DNase was added and after two freeze-thaw steps ROV were incubated for 15 min at 37°C in order to remove internal, intrinsic DNA. Subsequently, the control ROV were freeze-thawed, washed and resuspended in Pipes buffer. The amount of DNA-P_i is 6–9% of total P_i and the amount of 2-keto-3-deoxyoctonate 0.01–0.03 mol/mol total P_i. The ratio of protein to phospholipid (gram protein/mmol lipid-P_i) was determined to be 1.3 for MRE600, and 1.4 for HDL11 grown in the presence or absence of IPTG.

2.5. Transport experiments and calculation of the permeability coefficient

Transport of doxorubicin across model membranes in which DNA was enclosed, was determined from the rate of

fluorescence decrease (Exc. 490 nm, Em. 594 nm) in an Aminco fluorimeter as described [8]. The rate constant, k, was calculated as:

$$k = (\Delta F/\Delta t)/\left[(Q_{out} - Q_{in}) \cdot F^0 \cdot f\right] \ (s^{-1})$$

where $\Delta F/\Delta t$ is the initial rate of fluorescence quenching, Q_{out} is the quantum yield of the total drug (free + bound) outside the vesicles, Q_{in} is the quantum yield of the total drug in the lumen of the DNA-containing vesicles, and F^0 is the original fluorescence of the drug before addition of the vesicles. f is the fraction of external free drug after addition of the vesicles. These parameters were determined as described [8]. F^0 and ΔF were corrected for inner filtering, light scatter and dilution. The permeability coefficient was calculated with the formula:

$$P = k/([L_{tot}] \cdot a \cdot N \cdot 1000) \ (m/s)$$

where $[L_{tot}]$ is the lipid concentration, a is the area per lipid molecule, and N is Avogadro's number ($6.022 \cdot 10^{23}$). An area, a, of $3.15 \cdot 10^{-19}$ m^2 per lipid molecule for two opposing phospholipid molecules (in a bilayer) was assumed [26], except when CL was present. In that case an area of $6.0 \cdot 10^{-19}$ m^2 per opposing CL was assumed.

Permeability coefficients specific for uncharged drug, P^0, were calculated using the formula:

$$P^0 = P(1 + 10^{(pK_a - pH)}) \ (m/s)$$

Typically, the transport experiments were performed with 200 μM LUVET-P_i or 40 μg ROV protein/ml, pH 7.4, at 37°C, unless stated otherwise.

2.6. Other methods

P_i was assayed by the method of Rouser [25]. Protein was determined as described [27] using bovine serum albumin as a standard.

2.7. Materials

Doxorubicin (Pharmachemie, Haarlem, The Netherlands) was shown to be pure by high-performance thin-layer liquid chromatography and dissolved just before use. DNA (Type I from calf thymus), DNase I (type DN-25 from bovine pancreas), and RNase were obtained from Sigma, St. Louis, USA. Lysozyme was obtained from Boehringer, Mannheim, Germany.

3. Results

3.1. Transport of doxorubicin into large unilamellar vesicles (LUVET) composed of phospholipids and into right-side-out cytoplasmic membrane vesicles (ROV) derived from Escherichia coli

Large unilamellar vesicles (LUVET) of *E. coli* phospholipids of a wild-type composition were prepared with or without enclosed DNA. Upon addition of DNA-containing LUVET a slow decrease in fluorescence of doxorubicin was observed, which represents transport across the membrane (Fig. 1A). Upon addition of Triton X-100, fluorescence decreased rapidly to the equilibrium level, indicating that internal DNA is immediately available for doxorubicin binding. When LUVET without enclosed DNA were added a small increase in fluorescence is observed, which is relieved upon addition of Triton X-100 (Fig. 1A). This reflects the localization of doxorubicin in the hydrophobic environment of the membrane bilayer [10]. The

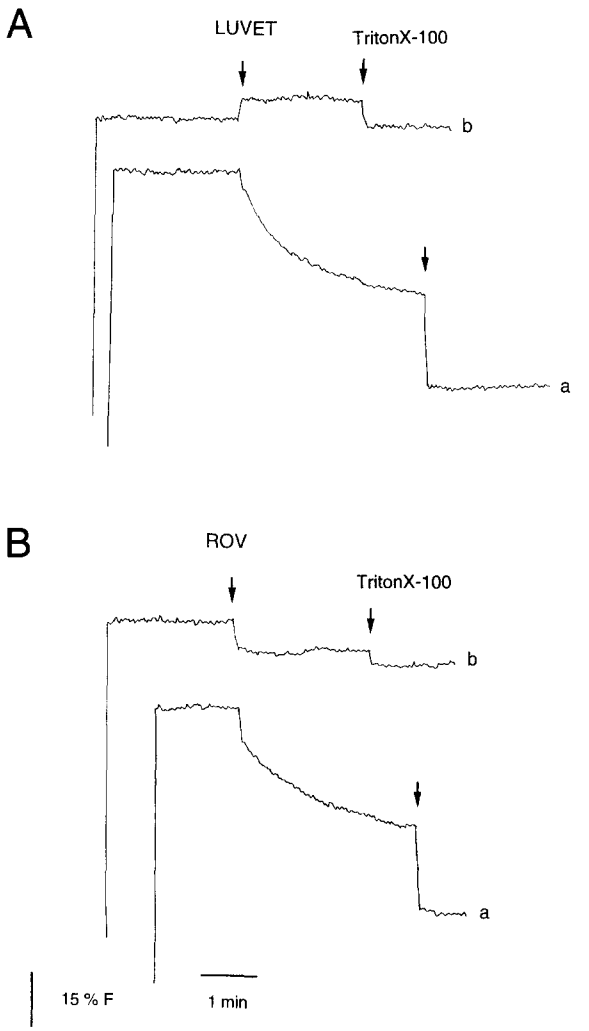

Fig. 1. Doxorubicin transport into LUVET and ROV. Fluorescence traces of 1 μM doxorubicin are shown against time. Upon addition of doxorubicin to Pipes buffer (pH 7.4) an immediate fluorescence is observed (excitation 490 nm, emission 594 nm). (A) As indicated by the first arrow, LUVET (200 μM lipid-P_i), composed of phospholipids isolated from *E. coli* HDL11 grown in the presence of 100 μM IPTG, are added which contained DNA (trace a) or which did not contain DNA (trace b). (B) As indicated by the first arrow, ROV (40 μg protein/ml, representing 28.6 μM lipid-P_i), derived from *E. coli* HDL11 grown in the presence of IPTG, are added which contained DNA (trace a) or which did not contain DNA (trace b). Triton X-100 is added to a final concentration of 0.05% (v/v) as indicated by the second arrows, in order to permeabilize the membranes. The temperature was 37°C. F is expressed as percentage fluorescence of 1 μM free doxorubicin.

absence of this small fluorescence increase in DNA-containing LUVET is due to small amounts of external DNA which could not be removed by the DNase treatment. From these traces transport parameters were measured and permeability coefficients (P^0) were calculated (Table 1).

Although some intrinsic DNA remains inside the ROV after the isolation procedure, differences between external and internal quantum yield are small and permeability coefficients can be calculated less accurately. In order to measure transport of doxorubicin across membranes of right-side-out cytoplasmic membrane vesicles (ROV) with a similar assay as for LUVET, a method had to be developed for the incorporation of high concentrations of exogenous DNA in ROV. Enclosure of exogenous DNA was achieved if DNA, fragmented by sonication and extrusion, was present during freeze-thawing the ROV. After 3 to 5 freeze-thaw steps maximal incorporation was obtained. Most efficient entrapment was observed if the freezing was performed quickly (liquid N_2) and thawing slowly (room temperature or on ice). Assuming an internal volume for ROV of 3 μl/mg protein [28], enclosure efficiencies ranged from 50 to 95%. In control ROV intrinsic DNA had to be removed in order to obtain fluorescence traces without a background decrease, and this was achieved by enclosing DNase in ROV by freeze-thawing (see Section 2). In Fig. 1B traces representative for transport of doxorubicin across membranes of ROV with a wild-type phospholipid composition are shown. A small and immediate decrease in fluorescence of doxorubicin is observed in control ROV and in ROV with enclosed DNA. However, only in ROV with enclosed DNA a slow fluorescence decrease is observed, which represents transport of doxorubicin across the membrane. Upon addition of Triton X-100, internal DNA is immediately available for doxorubicin binding. This assay was used to obtain doxorubicin transport and parameters, such as internal and external quantum yield, necessary to calculate the P^0 values. These parameters were comparable for ROV and LUVET (Table 1), except that the fraction of free drug was higher with ROV, due to the lower amount of lipids present during transport experiments (Table 1); ROV were added in lower concentrations to minimize the effect of light scatter and inner filtering. The permeability coefficients in ROV were 2–3-times higher than the corresponding permeability coefficients in LUVET.

Table 1
Transport parameters for passive diffusion of doxorubicin across membranes of LUVET and ROV derived from *E. coli* HDL11 grown in the presence of 100 μM IPTG

Model system	LUVET	ROV
P^0 (μm/s) [a]	1.5 ± 0.1	4.2 ± 0.6
Q_{out} [b]	1.02	1.06
Q_{in} [c]	0.07	0.16
f [d]	0.76	0.89

[a] The permeability coefficient for uncharged doxorubicin at 37°C. n = 6–12.
[b] Quantum yield of doxorubicin outside and at the outer leaflet of the LUVET or ROV.
[c] Quantum yield of doxorubicin inside and at the inner leaflet of the LUVET and ROV with enclosed DNA.
[d] Fraction of free drug during transport experiments.

3.2. Effect of drug concentration, verapamil, Mg^{2+}, and pH on transport of doxorubicin across membranes of LUVET and ROV

It was investigated whether doxorubicin transport in ROV involves only passive diffusion across the phospholipid bilayer or whether carrier facilitated diffusion is also present. First the effect of verapamil, a specific inhibitor (in the μM range) of P-glycoprotein mediated transport of doxorubicin, but also an inhibitor of bacterial drug efflux systems [29–31], was examined. Verapamil did not inhibit transport up to concentrations of 10 μM in both LUVET and ROV (data not shown). Higher concentrations were not used, since effects on passive diffusion in LUVET and ROV may occur [10].

Subsequently, the influence of the concentration of doxorubicin on transport rate was determined for LUVET and ROV in a range from 0.5 to 4 μM. The effect of doxorubicin concentration on initial transport rate was comparable in LUVET and ROV, yielding apparent K_m values of 5 ± 2 and 4 ± 2 μM, respectively. Saturation of drug transport might become more clear at high drug concentrations, but the fluorescence assay does not allow the use of high drug concentrations. The permeability coefficients decreased with increasing drug/phospholipid ratio as well as for LUVET as for ROV (data not shown). This apparent saturation could be due to self-aggregation of doxorubicin at higher drug concentrations or to the effect of a higher membrane disturbance when more drug is inserted into the membrane, which inhibits passive diffusion [8].

Divalent cations such as Mg^{2+} increase the order of lipid bilayers. P^0 decreases with increasing Mg^{2+} concentrations in all membrane preparations tested (data not shown). Addition of 5 mM Mg^{2+} decreased the permeability coefficient about 45 ± 4% in LUVET and about 60 ± 3% in ROV.

Finally, passive diffusion across the bilayer is supposed to involve the uncharged form of doxorubicin only, whereas enzyme-mediated transport most likely involves positively charged doxorubicin, because the substrate spectrum of drug efflux systems includes permanently positively charged drugs [29–31]. Transport experiments were performed at different pH values and permeability coefficients were calculated for total doxorubicin, charged plus uncharged (pK_a of doxorubicin 8.3). The results show that the pH dependency of doxorubicin diffusion across membranes of LUVET and ROV is comparable, indicating that

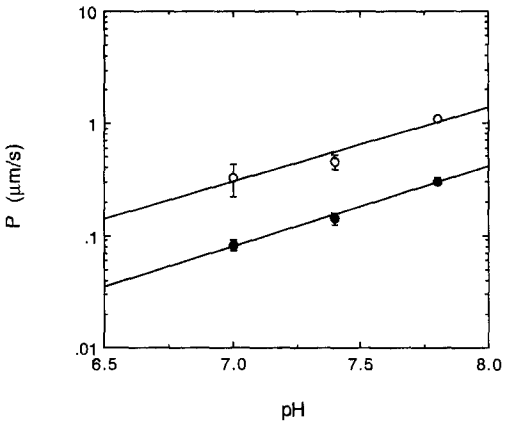

Fig. 2. Effect of pH on transport of doxorubicin into LUVET and ROV. The external pH was varied and permeability coefficients were calculated for the total amount of doxorubicin (charged plus uncharged). (●) LUVET composed of phospholipids isolated from *E. coli* HDL11 grown in the presence of IPTG. (○) ROV derived from *E. coli* HDL11 grown in the presence of IPTG. $n = 4–6$.

mainly passive diffusion of neutral doxorubicin across the phospholipid bilayer is involved (Fig. 2).

3.3. Effect of phospholipid composition on passive diffusion of doxorubicin across membranes of LUVET and ROV

Phospholipids were extracted from *E. coli* MRE600 and HDL11, grown in the absence or in the presence of 100 μM IPTG. The headgroup composition of the phospholipid extracts was determined and found to be similar to those described in literature [19]. The amount of negatively charged phospholipids (sum of CL, PG and PA) of

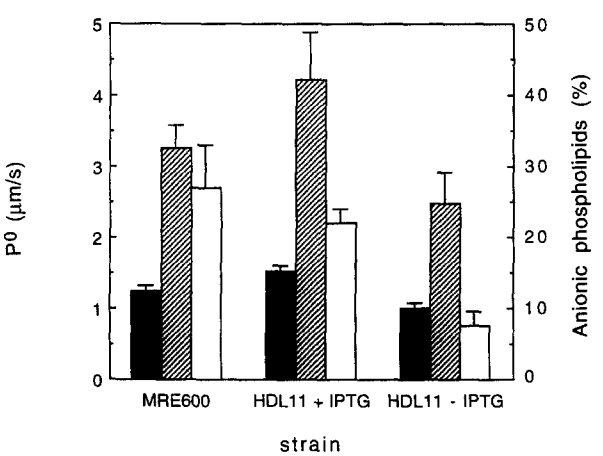

Fig. 3. Permeability coefficients at 37°C of doxorubicin across model membrane systems. Membranes were composed of LUVET (black) or ROV (arced bars) derived from *E. coli* strain MRE600, and HDL11 grown in the presence or absence of IPTG, $n = 6–12$. Permeability coefficients were determined as described in Section 2. White bars: The percentage of anionic phospholipids in the membranes for ROV and LUVET. The phospholipid composition of ROV and LUVET preparations was the same, with a standard deviation of less than 0.05.

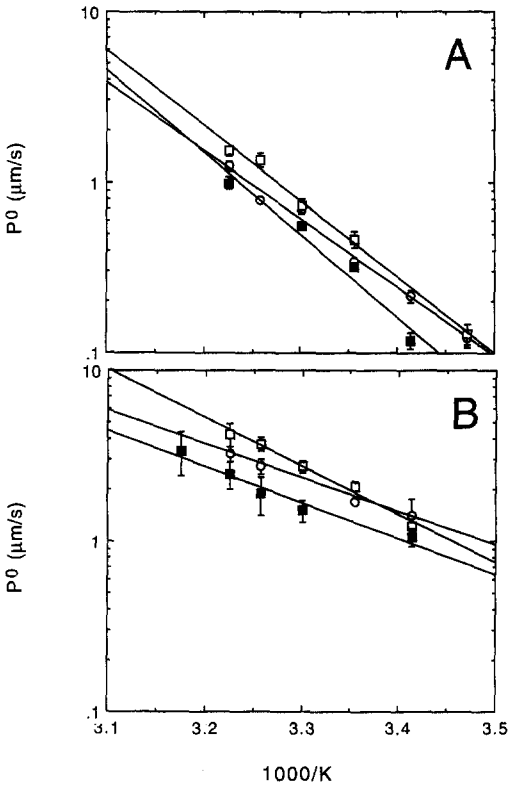

Fig. 4. Arrhenius plot of passive diffusion of doxorubicin. Permeability coefficients were determined as described in Section 2. A: Permeability coefficients of doxorubicin transport into LUVET. B: Permeability coefficients of doxorubicin transport into ROV. (○) *E. coli* MRE600, (□) *E. coli* HDL11 grown in the presence of IPTG, (■) *E. coli* HDL11 grown in the absence of IPTG. $n = 6–12$.

the strains is shown in Fig. 3. The fraction of bound drug in transport experiments increased with increasing amount of negatively charged phospholipids (data not shown). The permeability coefficient for LUVET composed of phospholipids from the wild-type MRE600, and HDL11 grown in the presence of IPTG is similar. Permeability is somewhat lower in LUVET composed of lipids of HDL11 grown in the absence of IPTG (Fig. 3).

ROV were isolated from the *E. coli* strains and the amount of negatively charged phospholipids was similar to the phospholipid composition of the corresponding LUVET. Permeability coefficients of doxorubicin transport in ROV were 2–3-times higher than those obtained for LUVET, but the effect of phospholipid composition was the same (with a standard deviation of less than 0.05) as observed for LUVET. P^0 obtained was highest for strain HDL11 grown in the presence of IPTG, and for MRE600, and lowest in HDL11 grown in the absence of IPTG (Fig. 3).

3.4. Activation energies of passive diffusion of doxorubicin across LUVET and ROV

The temperature dependence of passive diffusion of doxorubicin was determined in LUVET and ROV in the

range from 15 to 37°C for LUVET and from 20 to 42°C for ROV. Arrhenius plots are shown in Fig. 4. The activation energies were corrected for temperature dependent changes in buffer pH and pK_a of doxorubicin [9]. For all LUVET preparations the activation energy is comparable; 79 kJ/mol for MRE600, 88 kJ/mol for HDL11 grown with IPTG, and 94 kJ/mol for HDL11 grown without IPTG (Fig. 4A). Similar values for activation energies of doxorubicin transport were observed in LUVET composed of synthetic lipids, such as DOPC and DOPG/DOPC mixtures (data not shown). The interaction of doxorubicin with negatively charged phospholipids, i.e. binding of positively charged doxorubicin to the negatively charged phospholipid headgroups and penetration into the hydrophobic part of the membrane, has therefore no effect on the mechanism of passive diffusion, or at least on the most temperature dependent step of this process.

The activation energy of doxorubicin diffusion is almost 2-fold lower in ROV; 45 kJ/mol for HDL11 grown without IPTG, 50 kJ/mol for MRE600, and 56 kJ/mol for HDL11 grown with IPTG (Fig. 4B). Also in ROV no effect of the phospholipid headgroup composition on the activation energy was observed.

4. Discussion

Mechanistic and kinetic studies of passive diffusion of doxorubicin across the lipid bilayer have either been performed in vesicles composed of pure (phospho)lipids or in intact cells. In order to directly compare passive diffusion across membranes composed of pure phospholipids and vesicles prepared from biomembranes a transport assay was developed, based on the decrease of doxorubicin fluorescence upon binding to DNA enclosed in the vesicles. DNA was enclosed in large unilamellar vesicles (LUVET) by preparing the vesicles in the presence of DNA and in right-side-out cytoplasmic membrane vesicles (ROV) by applying freeze-thaw steps in the presence of DNA. The membrane systems employed were derived from *Escherichia coli*.

The most likely mechanism of doxorubicin transport present in LUVET and ROV is passive diffusion across the phospholipid bilayer, because (i) transport rate increased with increasing pH to the same extent in LUVET and ROV, indicating that mainly uncharged doxorubicin was transported, (ii) verapamil, an inhibitor of enzymes mediating doxorubicin and daunorubicin transport [29–31], did not have an inhibitory effect in ROV, and (iii) a comparable effect of increasing drug concentration on transport velocity was observed in ROV and LUVET. Furthermore, after loading of EDTA treated *E. coli* cells with doxorubicin, doxorubicin efflux was not observed upon addition of glucose (unpublished). Therefore, we conclude that for passive diffusion the results obtained in model membranes, such as LUVET, are representative for more biologically relevant system, such as cytoplasmic membranes, despite the absence of proteins and the absence of membrane asymmetry.

The effect of phospholipid composition on the permeability coefficient P^0 of doxorubicin was the same in LUVET as in ROV. However, a comparison in doxorubicin diffusion in LUVET and ROV prepared from lipids extracted from *E. coli* strains differing in phospholipid composition did not show the relationship between the amount of negatively charged lipids and passive diffusion observed previously with LUVET composed of synthetic dioleoyl lipids representative for the eukaryotic plasma membrane [8,10]. In doxorubicin transport across bilayers of *E. coli* phospholipids the membrane order probably plays a more important role than binding and insertion of doxorubicin. Membranes composed of pure phosphatidylethanolamine (PE) isolated from *E. coli* have a higher membrane order than wild-type phospholipids, containing 75% PE and 25% negatively charged phospholipids [23] and this is in accordance with the lower P^0 observed in LUVET and ROV from HDL11 grown without IPTG, because these membranes contain a higher percentage PE and lower percentage negatively charged phospholipids. Membrane order also plays a role in the comparable effects of Mg^{2+} on P^0 in ROV and LUVET; in the presence of this divalent cation membrane order is increased [32], resulting in a decreased P^0. Mg^{2+} has an effects on the surface charge resulting in a lower binding of doxorubicin to the membranes (data not shown), but these effects are taken into account in the calculation of the P^0 and are small compared by the effect on membrane order.

The permeability coefficients P^0 for doxorubicin observed with ROV were 2–3-fold higher than those observed with LUVET. Again, this can be explained by an effect on membrane order: In *E. coli* spheroplasts the quadrupolar splitting in the ^{2}H-NMR spectrum of [11,11-$^2H_2$]oleoyl-labeled phospholipids is lower than in LUVET composed of the isolated phospholipids, namely 7.2 and 10.9 kHz at 20°C, respectively [33]. This demonstrates that in protein containing biomembranes the acyl chains are less ordered than in the protein-free system. From the previously observed inverse relationship between the quadrupolar splitting and the permeability coefficient P^0 of doxorubicin a factor 2.3 difference in P^0 at these two values of quadrupolar splitting would be expected [8] which is close to the experimentally determined value.

A difference in temperature dependence of doxorubicin permeation in LUVET and ROV exists. In LUVET the average activation energy is about 87 kJ/mol, which corresponds to values reported in literature, 117 kJ/mol [5] and 92 kJ/mol, calculated from [9]. The activation energy for doxorubicin diffusion in ROV is about 2-times lower than in LUVET. Such a result could be interpreted as a difference in transport mechanism. However, facilitated diffusion in the ROV model system is not very likely

(see above). In ROV doxorubicin diffusion could occur via the protein/lipid interface, which might take place with a different activation energy. An alternative explanation is that the membrane order increases much less with decreasing temperature in the presence of membrane proteins. Indeed, in spheroplasts of *E. coli* the membrane order seems to increase about 1.5–2-times less with decreasing temperature than the membrane order of pure *E. coli* phospholipid extracts [33]. Since the rate of passive diffusion is directly influenced by the membrane order [8] this observation can explain the difference in temperature dependence of the same process, i.e. passive doxorubicin diffusion, in LUVET and ROV. Another example of differences in activation energy in doxorubicin diffusion occurring via the same mechanism is described by Harrigan et al. [5]. Diffusion of doxorubicin in LUVET containing cholesterol exhibited an activation energy of 160 kJ/mol, whereas for LUVET without cholesterol an activation energy of 117 kJ/mol was observed [5].

The assay developed for doxorubicin diffusion and the absence of intrinsic carrier-mediated doxorubicin transport makes the use of cytoplasmic membrane vesicles *E. coli* an excellent model system. Many drug efflux systems can be expressed in this organism, including bacterial [34] and the mammalian multi-drug resistance system [35]. Drug–phospholipid interactions may play a crucial role in the mechanism of these transport systems [36–38]. Various strains differing in phospholipid composition can therefore be employed to study the influence of in vivo manipulated phospholipid composition and doxorubicin binding on active drug transport after (over)expression of an appropriate doxorubicin efflux protein.

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