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Research Papers

Partitioning of an homologous series of alkyl *p*-aminobenzoates into multilamellar liposomes: effect of liposome composition

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Summary

The partitioning of the *n*-alkyl-*p*-aminobenzoates into the lipid bilayer is dependent not only on their physicochemical properties, but also on temperature and bilayer composition. Partitioning studies with this homologous series demonstrate the effect of alkyl chain length on partitioning in pure DPPC liposomes at 23 and 50 °C and in DPPC : Chol liposomes at 23 °C. A general trend is observed wherein increasing alkyl chain length increases partitioning of the drug into the bilayer. It is also shown that inclusion of cholesterol in the bilayer or increasing the temperature increases the partitioning of the *n*-alkyl *p*-aminobenzoates into the bilayer. The distribution coefficients are strongly influenced by the physical structure/state of the membrane. Therefore, factors which increase the fluidity of the bilayer will also increase solute partitioning into the bilayer. Compounds that are highly lipid soluble, such as butyl *p*-aminobenzoate, will preferentially partition into the bilayer, and are relatively insensitive to bilayer composition or to structural changes within the bilayer.

Introduction

Liposomes have great potential as carriers for drug delivery. They are relatively nontoxic, biodegradable and capable of entrapping solute within their aqueous compartments and lipid bilayers. A few important factors that need to be considered when designing a liposome drug delivery system are drug type and size, the relative solubilities of the drug in the aqueous and lipid phases, and liposome type and composition. A physicochemical approach is therefore necessary for optimization of drug entrapment within liposomes.

High encapsulation efficiencies are difficult to achieve with neutral water soluble drugs. Since polar drugs distribute into the internal aqueous compartment of liposomes, the extent of encapsulation of these compounds is a function of the ratio of the internal aqueous volume of the liposome to the volume of the external medium. The entrapment efficiency for nonpolar drugs, which are sequestered in the bilayer structure, is gener-

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ally much higher than that of polar drugs. Therefore, one approach that is used to increase the entrapment of a water soluble drug in a liposome is to synthesize lipophilic derivatives, pro-drugs, with structures which are readily degraded to the parent compound in vivo (Knight, 1981; Sasaki et al., 1985). Such an approach was utilized in this study to increase the entrapment of *p*-aminobenzoic acid (p-aba) in multilamellar liposomes. The pro-drugs of *p*-aminobenzoic acid, methyl to butyl *p*-aminobenzoate (methyl p-aba to butyl p-aba), were used to study homolog partitioning effects in multilamellar liposomes. These particular pro-drugs were also chosen because they possess intermediate octanol/water partition coefficients, hence, they will not be exclusively located in either the aqueous or lipid compartments of the liposome.

The composition of the bilayer can also be modified to increase the entrapment of hydrophilic compounds within the liposomes. Phospholipid chain length, degree of saturation and inclusion of charged phospholipids or cholesterol have been shown to affect the extent of drug entrapment within the liposome (Kimelberg and Mayhew, 1978; Rahman et al., 1985; Luxnat and Galla, 1986; DeYoung and Dill, 1988).

Inclusion of cholesterol in liposomal formulations has been used to reduce the permeability of bilayers and thus the leakage of water soluble molecules from the aqueous compartments of liposomes. The orientation of the cholesterol molecule in the membrane induces a wide variety of effects on the physical properties of the phospholipid bilayers. Alterations of the physical properties of the bilayer, such as surface density, affects solute partitioning into the bilayer (DeYoung and Dill, 1988). Cholesterol increases the surface density of the phospholipid bilayer in the liquid crystalline state resulting in an ordering effect on the bilayer membrane. This effect occurs irrespective of the phospholipid composition and causes a decrease of the (bilayer/water) partition coefficient of the solute in comparison to its partition coefficient in the absence of cholesterol (Simon et al., 1977; Luxnat and Galla, 1986; Antunes-Madeira and Madeira, 1987; DeYoung and Dill, 1988). On the other hand, cholesterol, disrupts the organizational packing of the bilayer when the phospholipids are in the gel crystalline state (Papahadjopoulos and Kimelberg, 1973). Little work has been carried out to determine the effect of cholesterol on solute partitioning into gel crystalline bilayers. It would seem reasonable that inclusion of cholesterol in gel crystalline phospholipid bilayers should increase drug entrapment in the liposome by increasing solute incorporation into the bilayer when compared to a pure phospholipid bilayer system.

The purpose of this study was to investigate the effect of liposome composition on drug distribution between the phospholipid bilayer and aqueous compartments of the liposome. The effect of the solute's chemical structure on partitioning between bilayers and aqueous compartments was also examined. The (bilayer/water) distribution coefficients of a homologous series of compounds, the *n*-alkyl *p*-aminobenzoates, were determined in dipalmitoylphosphatidylcholine : cholesterol (2 : 1 molar ratio) multilamellar liposomes at 23° C. This particular temperature was chosen to study the effects of cholesterol on solute partitioning in a gel-crystalline bilayer and because it represents realistic storage conditions. These distribution coefficients were compared to distribution coefficients obtained for the same alkyl *p*-aminobenzoates in pure dipalmitoylphosphatidylcholine multilamellar liposomes.

Materials and Methods

Materials

Dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification. Cholesterol (Chol) obtained from Sigma Chemical Co. (St. Louis, MO) was recrystallized from ethanol solution three times prior to use. Methyl *p*-aminobenzoate was obtained from Aldrich Chemical Co. (Milwaukee, WI) while ethyl and butyl *p*-aminobenzoate were obtained from Sigma Chemical Co. (St. Louis, MO). Propyl *p*-aminobenzoate was purchased from ICN Biomedicals, Inc., K&K Labs (Costa Mesa, CA). Hepes was purchased from Sigma Chemical Co. (St. Louis,

MO). L-α-[2-*palmitoyl*-9,10-^{3}H(N)]Phosphatidylcholine, obtained from New England Nuclear (spec. act. 58 Ci/mmol), was diluted with a 1 : 1 (v/v) ratio of ethanol : toluene to a specific activity of 2.9 Ci/mmol. [^{14}C]Inulin (total activity 250 μCi), diluted with water to a specific activity of approx. 3 μCi/ml, and Ecolite +® scintillation solvent were both obtained from ICN Radiochemicals. Absolute ethanol (200 proof) was purchased from Aaper Alcohol and Chemical Co. (Louisville, KY). All chemicals were used without further purification.

Preparation of multilamellar vesicles

Multilamellar liposomes were prepared by a slight modification of the method described by Bangham et al. (1965). DPPC, with or without cholesterol, and the alkyl *p*-aminobenzoate ester of interest were first dissolved in chloroform. [^{3}H]DPPC was used as the lipid marker; the ratio of DPPC to [^{3}H]DPPC used in all experiments was $1 : 1 \times 10^{-8}$ M. All liposome preparations that employed cholesterol were prepared using a DPPC : Chol molar ratio of 2 : 1. This lipid mixture was then deposited as a uniform thin film on the sides of a round bottom flask by removal of the organic solvent, chloroform, at 50 ° C in vacuo in a Buchi rotary evaporator (Buchi Technical Laboratories, Switzerland). Residual chloroform was removed by placing the flask under vacuum for at least 12 h. The dried film was then hydrated with 0.05 M Hepes buffer, pH 7.4 containing trace amounts of [*carboxyl*-^{14}C]inulin (0.015 μCi/ml buffer) which served as the aqueous marker. In all systems, the dried lipid film was initially hydrated with buffer for 15 min at 50 ° C and the resulting phospholipid concentration was 68 μmol/ml. Further incubation of the multilamellar liposome dispersion continued for 24, 48 or 72 h at 23 ° C. The hydration of the lipid film was aided by gentle mechanical agitation. Experiments with pure DPPC multilamellar liposomes were also carried out at 50 ° C and the temperature of the dispersion was always maintained at 50 ° C. In order to carry out these studies at roughly equivalent thermodynamic activities, the amount of each of the alkyl esters added was equivalent to 15% saturation in the aqueous phase (Table 1).

Separation of free and entrapped solutes

Liposomes containing encapsulated [^{14}C]inulin and the alkyl *p*-aminobenzoate of interest were separated from unentrapped [^{14}C]inulin and alkyl *p*-aminobenzoate by ultracentrifugation. A 175 μl sample of the liposomal dispersion was placed in a Beckman Airfuge (Spinco Division, Palo Alto, CA) and spun at $160\,000 \times g$ at 23 ° C for a period of 1 h. For $T > T_c$ experiments, the temperature in the airfuge was maintained at 50 ± 1 ° C using inlet air preheated with a copper coil. These systems were spun at $160\,000 \times g$ for 1 h at 50 ° C. At the end of 1 h, all supernatants were optically clear and all pellets were compact. The weights of the pellet and supernatant were determined immediately after centrifugation.

Determination of internal (captured) volume

[^{14}C]Inulin was used to determine the internal aqueous volume of the liposomes used in the calculation of distribution coefficients. The assumption is made that inulin will only distribute into water and will not associate with the bilayer. It is also assumed that the concentration of free inulin is the same as its concentration in the

TABLE 1
Drug concentration in liposome dispersion prior to separation procedure [a]

Liposome composition	Incubation temperature (° C)	[Methyl p-aba] (mM)	[Ethyl p-aba] (mM)	[Propyl p-aba] (mM)	[Butyl p-aba] (mM)
DPPC	23	0.93 (9)	0.67 (11)	0.38 (14)	0.16 (17)
DPPC : Chol (2 : 1)	23	1.3 (13)	0.61 (10)	0.41 (15)	0.19 (20)
DPPC	50	1.4 (6)	0.44 (4)	0.27 (6)	0.14 (8)

[a] Percent saturation in aqueous phase is listed in parentheses.

internal water compartment of the liposome. Entrapped liposomal inulin was separated from free inulin as described above. The amount of lipid present in the dispersion was determined by using tritiated DPPC in a $1:1\times10^{-8}$ molar ratio to non-tritiated DPPC. After ultracentrifugation, the supernatant and pellet were analyzed for [^{14}C]inulin and [^{3}H]DPPC radioactivity using a dual-labelled ^{3}H and ^{14}C counting program and a Beckman LS5000TD scintillation counter (Palo Alto, CA). Internal aqueous volumes were calculated and expressed as μl of trapped volume per μmol of phospholipid.

Determination of drug concentration

The amount of alkyl *p*-aminobenzoate entrapped by the liposomes was determined by separating free from entrapped drug by ultracentrifugation. The pellet and supernatant were analyzed for presence of ester by using a Perkin-Elmer 7 UV/Vis spectrophotometer. The supernatant and pellet were dissolved in known amounts of ethanol and the ultraviolet absorbance of the supernatant and dissolved pellet were then measured at the wavelength of maximum absorbance of the alkyl *p*-aminobenzoate. Blank liposomes dissolved in ethanol were used as reference standards in order to correct for any turbidity effects. Standard absorbance curves of the esters in ethanolic solution were constructed. The concentration of solute in both phases was determined from the standard curves in the linear region of the Beer-Lambert plot.

Calculation of bilayer / aqueous compartment distribution coefficient

The distribution coefficient, DC, of a drug between the aqueous compartments and lipid bilayers can be expressed by the following general relationship:

$$\mathrm{DC} = \frac{C_{\mathrm{bilayer}}}{C_{\mathrm{aqueous}}} = \frac{A_{\mathrm{sb}}}{W_{\mathrm{b}}}\frac{W_{\mathrm{aq}}}{A_{\mathrm{sw}}} \qquad (1)$$

where C_{bilayer} and C_{aqueous} are the concentration of the drug (expressed as g of solute per g of phase) in the bilayer and aqueous phase, respectively. W_{b} and W_{aq} are the weights of each phase, and A_{sb} and A_{sw} are the amount of the drug in each phase. A standard state of an infinitely dilute drug solution is postulated so that activity coefficients are equal to unity. This is an experimentally convenient standard state, since concentration can then be used in place of activity.

In actuality, the amount of drug in the bilayer cannot be directly determined experimentally without altering the original liposome system. However, ultracentrifugation of a liposome dispersion will yield a liposome pellet (containing both the bilayer and internal aqueous phase) and a supernatant. Hence, Eqn 1 must be modified to account for the amount of water entrapped within the pellet and the amount of solute associated with the trapped water. The amount of drug in the bilayer, A_{sb}, can be determined by subtracting the amount of drug present in the pellet, A_{sp} from the amount of drug associated with the trapped water in the liposome pellet, A_{st}. The amount of drug associated with trapped water, A_{st}, is independently determined by assuming that, at equilibrium, the trapped water inside the liposomes has the same physical properties as bulk water and that the concentration of drug in the entrapped water is the same as that in bulk water. Eqn 1 can therefore be rewritten as:

$$\mathrm{DC} = \frac{A_{\mathrm{sp}}W_{\mathrm{o}} - W_{\mathrm{t}}A_{\mathrm{saq}}}{A_{\mathrm{saq}}W_{\mathrm{b}}} \qquad (2)$$

where W_{o}, W_{b} and W_{t} represent the weights of the supernatant, lipid bilayer, and weight of trapped water, respectively. W_{b} and W_{t} are directly determined by using [^{3}H]DPPC as the lipid phase marker and [^{14}C]inulin as the aqueous phase marker. A_{saq} and A_{sp} represent the amount of solute in the supernatant and liposome pellet, respectively. The above calculation for the distribution coefficient of the *n*-alkyl *p*-aminobenzoates in multilamellar liposomes is similar to that proposed by Katz and Diamond (1974).

Free energy of partitioning

The free energy of partitioning of drug from the aqueous phase to the lipid bilayer can be

calculated from the distribution coefficient, DC, using the equation:

$$\Delta G^\circ = -2.3RT \log DC \quad (3)$$

where ΔG is expressed in units of cal mol^{-1}. This represents the change in free energy upon transferring one mole of solute from the aqueous phase to the lipid bilayer.

Contribution of cholesterol to the free energy of partitioning

The contribution of cholesterol to the free energy of partitioning of the *n*-alkyl *p*-aminobenzoates into the lipid bilayer was determined from the difference between the free energy of partitioning in DPPC:Chol multilamellar liposomes and in pure DPPC multilamellar liposomes;

$$\Delta(\Delta G^\circ)_{Chol} = (\Delta G^\circ)_{DPPC:Chol} - (\Delta G^\circ)_{DPPC} \quad (4)$$

where all free energies are expressed in units of cal mol^{-1}.

Contribution of temperature to the free energy of partitioning

The contribution of temperature to the free energy of partitioning of the *n*-alkyl *p*-aminobenzoates into the lipid bilayer was determined from the difference between the free energy of partitioning in pure DPPC multilamellar liposomes in their liquid-crystalline state (50 °C) and in their gel-crystalline state (23 °C);

$$\Delta(\Delta G^\circ)_{Temp} = (\Delta G^\circ)_{DPPC,50\,^\circ C} - (\Delta G^\circ)_{DPPC,23\,^\circ C} \quad (5)$$

where all free energies are expressed in units of cal mol^{-1}.

Results and Discussion

Local anesthetics, similar in structure or more lipophilic than the *n*-alkyl *p*-aminobenzoates, have been shown to perturb bilayer fluidity (Hill, 1974; Jain et al., 1975; Papahadjopoulos et al., 1975; Ueda et al., 1977) when very high concentrations of these anesthetics are incorporated into the bilayer. Therefore, to only investigate the effects of temperature and cholesterol on drug partitioning between the lipid bilayers and aqueous phase, the relatively less hydrophobic alkyl *p*-aminobenzoates were studied (methyl to butyl p-aba) and the amount of each alkyl ester added was equivalent to approx. 15% of its saturation solubility in the aqueous phase. The effects that the drug concentrations used in the study would have on the phase transition behavior of DPPC MLVs was studied by differential scanning calorimetry. The results showed that the alkyl *p*-aminobenzoates, at the concentrations used, did not alter bilayer fluidity (unpublished data).

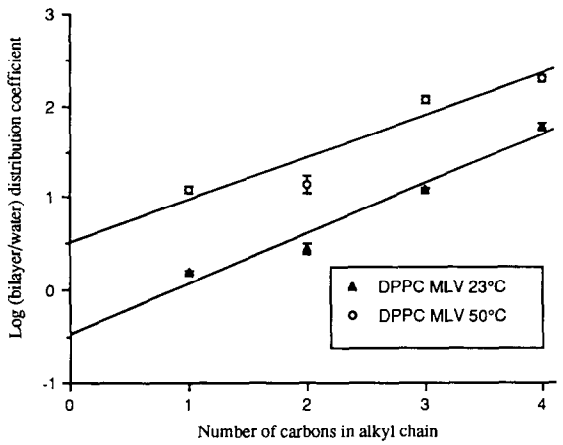

Fig. 1. The dependence of (bilayer/water) distribution coefficient of alkyl *p*-aminobenzoates in DPPC MLV at 23 °C and 50 °C on alkyl chain length of the drug. Drug concentrations used in the study are shown in Table 1. Points represent the average of three independent determinations ± SE.

The partitioning of the alkyl *p*-aminobenzoates in DPPC liposomes at 23 °C was compared to the partitioning behavior of the same compounds at 50 °C (Fig. 1). At both temperatures, increasing the lipophilicity of *p*-aminobenzoic acid by the addition of methylene groups resulted in an increase in bilayer partitioning. It is also apparent from Fig. 1 that partitioning of these compounds into the bilayer is favored at the higher temperature (50 °C), where the DPPC bilayer is in the liquid-crystalline state.

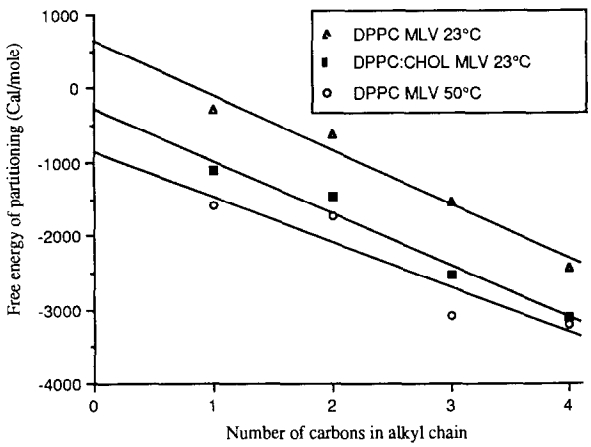

Fig. 2. The free energy of partitioning of alkyl *p*-aminobenzoates in DPPC MLV at 23°C and 50°C and in DPPC : Chol MLV at 23°C.

A comparison of the free energies of partitioning of the alkyl esters in pure DPPC liposomes at 23 and 50°C is presented in Fig. 2. The free energy of partitioning into the bilayer decreases with increasing chain length and solute partitioning into the bilayer is favored when the phospholipid bilayer is in the fluid state. The free energy of partitioning of butyl *p*-aminobenzoate from the aqueous phase to the lipid bilayer is similar for both the gel and liquid-crystalline state. This similarity in partitioning suggests the possibility that at both temperatures butyl *p*-aminobenzoate is intercalated between the acyl chains of the lipid bilayer. The more lipophilic the compound, the more readily it will be accommodated into the bilayer, regardless of its physical state (Pope and Dubro, 1986).

The difference between the free energies of partitioning of the alkyl *p*-aminobenzoates from the aqueous phase into the pure DPPC bilayer when the distribution coefficient is determined at 50°C and into the pure DPPC bilayer when the distribution coefficient is determined at 23°C yields the net decrease in free energy caused by an increase in temperature. The net decrease in the free energy of partitioning due to temperature is more pronounced with the lower chain homologs of *p*-aminobenzoic acid than it is with the butyl homolog. The net decrease in free energy caused by temperature for the methyl, ethyl and propyl esters was about 1400 cal/mol. This temperature induced decrease in free energy for the butyl ester was 850 cal/mol, approx. 600 cal/mol lower than that for the other esters. These results suggest that the effect of temperature on butyl ester partitioning is less than that seen with the lower homologs of *p*-aminobenzoic acid.

The above findings are consistent with temperature induced structural changes within the phospholipid bilayers. Increasing temperature reduces the ordering of the hydrocarbon chains (Seelig and Seelig, 1974). The acyl chains of the phospholipid go from the highly ordered extended trans conformation to the highly disordered gauche conformation. The disordering of the chains is accompanied by a decrease in the surface density of the bilayer (Seelig and Seelig, 1974; DeYoung and Dill, 1988). Above the phase transition, the surface density of the bilayer decreases and solute partitioning is favored (Simon et al., 1977; Luxnat and Galla, 1986; Antunes-Madeira and Madeira, 1987; DeYoung and Dill, 1988). The average area of a phospholipid headgroup, determined by X-ray diffraction methods, for pure DPPC multilamellar liposomes increases from 50 $Å^2$ at 25°C to 68 $Å^2$ at 50°C (Janiak et al., 1976, 1979). This change in structure would more readily permit accommodation of solute in the bilayer. Similar trends were also noted by Betageri and Rodgers (1989) when studying the partitioning behavior of the nitroimidazoles at 30°C in DPPC MLV (gel state) and in DMPC MLV (fluid state). At 30°C, the DMPC MLV exhibited higher partition coefficients for each of the nitroimidazoles when compared to the DPPC MLV.

A plot of the log (bilayer/water) distribution coefficient against alkyl chain length yields two parallel lines and clearly illustrates that the alkyl *p*-aminobenzoates studied exhibit higher (bilayer/water) distribution coefficients at 23°C in DPPC : Chol multilamellar liposomes than in pure DPPC liposomes (Fig. 3). The results indicate that the inclusion of cholesterol in the membrane of DPPC liposomes does not change the solvent properties of the bilayers, but rather alters the structural organization of the bilayer. The solvent characteristics of the bilayer can be estimated by comparing the contribution of a methyl-

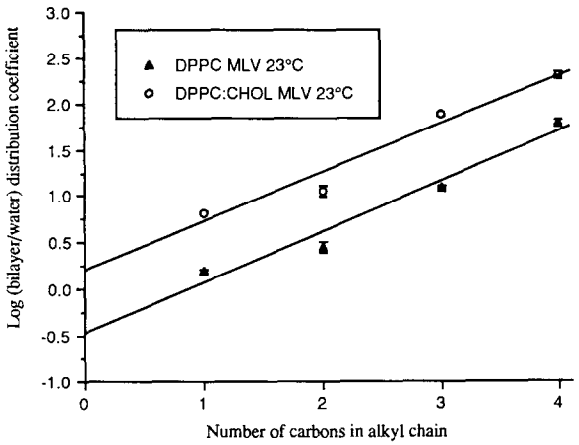

Fig. 3. Comparison of (bilayer/water) distribution coefficients of alkyl *p*-aminobenzoates in DPPC MLV and in DPPC : Chol MLV at 23° C as a function of alkyl chain length of the drug. Drug concentrations used in the study are shown in Table 1. Points represent the average of three independent determinations ± SE.

ene unit to partitioning in the pure DPPC and DPPC : Chol liposome systems. Each methylene group that is added to the side chain of *p*-aminobenzoic acid causes a fixed change in the distribution coefficient, determined at 23° C, in both DPPC and DPPC : Chol liposome systems (Fig. 3). The slopes determined from Fig. 3, by linear regression, reflect changes in the activity coefficient of *p*-aminobenzoic acid in the aqueous compartment and lipid bilayer of the liposome as the length of its alkyl chain is varied. For DPPC and DPPC : Chol liposomes the slopes were 0.54 ($r^2 = 0.97$) and 0.53 ($r^2 = 0.96$), respectively. The regression slope should be different for a given homologous series partitioning between different hydrophobic solvents and water. Thus, if inclusion of cholesterol changed the DPPC bilayer into a different 'organic solvent', the slope would have been different from that obtained with the pure DPPC bilayer. Since the slopes were essentially identical it is reasonable to conclude that cholesterol does not change the bilayer solvent property. The contribution of a methylene unit to partitioning in pure DPPC liposomes was also determined by linear regression at 50° C and was found to be 0.46. This value was found to be similar to the contribution of a methylene group to partitioning in DPPC and DPPC : Chol liposomes at 23° C. These results indicate that the solvent properties of the DPPC bilayer are not affected by the presence of cholesterol and the physical state of the bilayer.

The presence of cholesterol in the bilayer causes a structural change within the membrane. When ^{2}H-NMR studies were used to study the degree of motional freedom within the lipid bilayer, order parameter profiles of the pure DPPC bilayer, conducted at temperatures below its phase transition, showed a highly ordered plateau region with the first 9 to 10 carbons from the polar head group of the phospholipid (Seelig and Seelig, 1974; Pope et al., 1989). The phospholipid headgroups are closely spaced together, with the average area of a phospholipid head group of approx. 49 Å^2 (Janiak et al., 1976, 1979). This area has been shown to be entropically less favorable to the inclusion of molecules (McIntosh et al, 1980; DeYoung and Dill, 1988; Pope et al., 1989). The acyl chains of the phospholipids are in the extended trans conformation, and tend to be less ordered near the center of the bilayer (Levine and Wilkins, 1971). DPPC order parameter profiles are altered by the presence of solute, temperature and cholesterol (Oldfield and Chapman, 1971; Seelig and Seelig, 1974; DeYoung and Dill, 1988; Pope et al., 1989).

Cholesterol exerts an ordering effect on the bilayer when it is in the liquid crystalline state and thereby condenses the bilayer. Order parameter profiles of the DPPC bilayer show that the motional freedom of the plateau region decreases with increasing cholesterol concentration (Bush et al., 1980; Pink et al., 1981). Solute partitioning and solubility in the bilayer decreases with this increase in chain order and surface density caused by cholesterol (Korten et al., 1980; Luxnat and Galla, 1986; Antunes-Madeira and Madeira, 1987; DeYoung and Dill, 1988; Pope et al., 1989). The opposite effect occurs when the DPPC : Chol bilayer is in the gel-crystalline state. Inclusion of cholesterol partially disrupts the highly ordered crystalline packing of the bilayer and increases the distance between the phospholipid headgroups (Papahadjopoulos and Kimelberg, 1973). The general effect of cholesterol on bilayers in the gel state is to increase the fluidity of the hydrocarbon

chains (Oldfield and Chapman, 1971). These structural changes caused by cholesterol allow the alkyl esters of *p*-aminobenzoic acid to be readily accommodated into the bilayer. This is exemplified by the higher distribution coefficients of the esters into DPPC : Chol bilayers and by the lower free energy of partitioning values (Figs 2 and 3).

Fig. 2 compares the free energies of partitioning in DPPC and DPPC : Chol MLVs at 23° C. The free energy of partitioning of the esters into liposome bilayers was found to decrease linearly with their alkyl chain length and the presence of cholesterol also favors solute partitioning into the bilayer. The difference between the free energies of partitioning of the alkyl *p*-aminobenzoates from the aqueous phase into a DPPC : Chol bilayer and into the pure DPPC bilayer is a reflection of the net decrease in free energy of partitioning caused by cholesterol. The net decrease in free energy caused by cholesterol for the methyl to propyl homologs of *p*-aminobenzoic acid was about 1000 cal/mol. For *n*-butyl *p*-aminobenzoate, however, this decrease was about 700 cal/mol, almost 30% less than that for the other esters. These results indicate that the presence of cholesterol in the bilayer affects the C_1–C_3 alkyl esters more significantly than it does the butyl ester. The smaller effect of cholesterol on the lowering of free energy of partitioning for the butyl ester as compared to the three other lower homologs of *p*-aminobenzoic acid may be due to the inherent solubility of the butyl ester in lipid bilayers. Thus, it is not as sensitive to bilayer organization changes induced by cholesterol.

The partitioning of the alkyl *p*-aminobenzoates in DPPC : Chol liposomes at 23° C was compared to partitioning of the same compounds in DPPC liposomes at 50° C. The (bilayer/water) distribution coefficients in the DPPC : Chol MLV at 23° C were found to be lower than the (bilayer/water) distribution coefficient in DPPC MLVs at 50° C (Fig. 4). The net decrease in free energy for the methyl to propyl homologs of *p*-aminobenzoic acid is also slightly greater with temperature than it is with cholesterol (Fig. 2). These results are not unexpected, since the degree of motional freedom in the bilayer at 50° C will most likely be greater

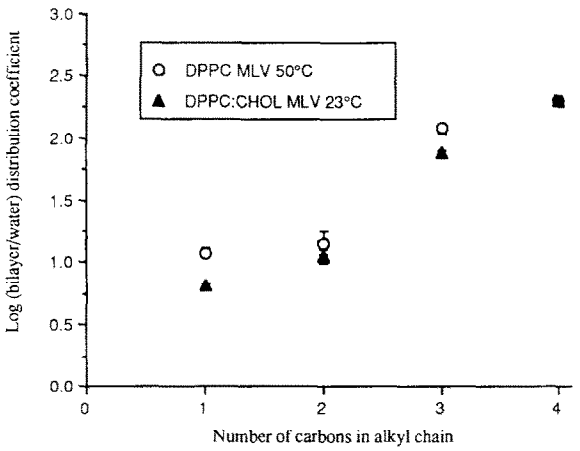

Fig. 4. The dependence of the (bilayer/water) distribution coefficient of alkyl *p*-aminobenzoates in DPPC MLV at 50° C and in DPPC : Chol MLV at 23° C on the alkyl chain length of the drug. Drug concentrations used in the study are shown in Table 1. Points represent the average of three independent determinations ± SE.

than that induced by the presence of cholesterol in the bilayer at 23° C. Temperature influences the mobility and ordering of all portions of the fatty acyl chains of DPPC, whereas cholesterol, due to structural constraints, exerts its effects primarily near the phospholipid headgroups (Papahadjopoulos and Kimelberg, 1973; Seelig and Seelig, 1974). Cholesterol, because of its short hydrophobic tail, does not interfere with the packing or fluidity of the hydrocarbon chains of DPPC that are near the center of the bilayer (Bush et al., 1980; Pink et al., 1981).

The distribution coefficients obtained for the *n*-alkyl *p*-aminobenzoates were found to be highly dependent on their aqueous and lipid solubilities, liposome composition and temperature. This is most readily seen with methyl *p*-aminobenzoate. In the absence of cholesterol, at 23° C, methyl *p*-aminobenzoate has a (bilayer/water) distribution coefficient of unity, indicating that it has no particular preference for the bilayer or aqueous phase. The free energy of partitioning of this solute into the bilayer is significantly lower in the DPPC : Chol (23° C) and DPPC (50° C) liposome systems than in the DPPC (23° C) liposome system (Fig. 2). The distribution coefficient of methyl *p*-aminobenzoate increases 7-fold upon the inclu-

sion of cholesterol in the bilayer and increases 12-fold when the temperature is raised to 50 °C. These results indicate that partitioning methyl *p*-aminobenzoate into bilayers is highly influenced by structural changes in the bilayer, such as those caused by the addition of cholesterol. Cholesterol or increasing temperature lowers the surface density of the bilayer and increases the spacing between the phospholipid headgroups. These structural changes readily permit incorporation of methyl *p*-aminobenzoate into the bilayer.

In conclusion, the results of this study suggest that the distribution coefficients of the *n*-alkyl *p*-aminobenzoates are strongly influenced by the physical structure/state of the bilayers. Factors that affect bilayer fluidity, such as temperature and cholesterol, would therefore affect the partitioning behavior of solutes. The extent to which fluidity changes affect solute partitioning depends on the hydrophobicity of the solute.

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