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Interactions between model membranes and lignin-related compounds studied by immobilized liposome chromatography

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Abstract

In order to elucidate the modes of interaction between lignin precursors and membranes, we have studied the influence of temperature, lipid composition and buffer composition on the partitioning of monolignol and dilignol model substances into phospholipid bilayers. The partitioning was determined by immobilized liposome chromatography, which is an established method for studies of pharmaceutical drugs but a new approach in studies of lignin synthesis. The temperature dependence of the retention and the effect of a high ammonium sulfate concentration in the mobile phase demonstrated that the interaction involved both hydrophobic effects and polar interactions. There was also a good correlation between the partitioning and the estimated hydrophobicity, in terms of octanol/water partitioning. The partitioning behavior of the model substances suggests that passive diffusion over the cell membrane is a possible transport route for lignin precursors. This conclusion is strengthened by comparison of the present results with the partitioning of pharmaceutical drugs that are known to pass cell membranes by diffusion.

Keywords: Immobilized-liposome chromatography; Lignin; Liposomes; Monolignol; Octanol/water partitioning; Phospholipid bilayers

1. Introduction

Although the function of the plant cell wall is well established, the mechanism of its biosynthesis is not [1]. The cell wall consists largely of randomly packed cellulose fibers in a matrix of hemicellulose and pectin. In parts of many terrestrial plants, lignin is a further support to the cell wall, and it is also important for water and nutrient conduction by lining the vessels/tracheids to make them impermeable [1,2]. The lignin also has a role in the defense against microbial attack [2]. Lignin is a polymer of the unstable aromatic monomers coniferyl alcohol, *p*-coumaryl alcohol and sinapyl alcohol, so-called monolignols, that bind to each other via radical coupling to form a three-dimensional lignin web of varying size [1,3]. Other phenolic components might also be incorporated into the lignin molecule [4]. The formation of radicals is accomplished by the action of H_2O_2-dependent peroxidases or O_2-requiring laccases [1,5]. However, there seems to be something more to the mechanism of lignin synthesis. One theory is that a protein, termed dirigent, helps in the actual coupling of the radicals [6]. However, there are several contradictions to this theory [2]. The formation of lignin from the monomers synthesized within the cell presumably occurs in the cell wall [1,6] or in the cell wall/plasma membrane interface [5]. A crucial step here, which needs to be elucidated, is obviously the transport of the monomers through the cell membrane. Some alternatives are possible: exocytosis, specific transport systems or partition-dependent diffusion. A crucial requirement for the diffusion alternative is a pronounced partitioning of a molecule into the membrane [7]. Therefore, a convenient first step is to estimate the partitioning of the monomers into relevant membrane model systems.

The most common phospholipids forming the membrane bilayers in plant cells are the zwitterionic phosphatidylcholine (PC) and phosphatidylethanolamine, and the sterols are mainly stigmasterol, sitosterol and campesterol [8,9]. Because of its hydrophobic core the bilayer is selectively

Abbreviations: PC, phosphatidylcholine; ILC, immobilized liposome chromatography; PS, phosphatidylserine; DSTAP, 1,2-distearoyl-3-methylammoniumpropane; VG, veratryl glycol; MSA, α-methylsyringyl alcohol; TMBA, 3,4,5-trimethoxybenzyl alcohol; ADMPA, 4-acetyl-2,6-dimethoxyphenyl acetate; APA, 4-acetoxy-3,5-dimethoxy-α-(2,6-dimethoxyphenoxy) acetophenone; APH, 4′-hydroxy-3′,5′-dimethoxy-2-(o-methoxyphenoxy) acetophenone; HMPA, 1-(4-hydroxy-3-methoxyphenyl)-2-(2,6-dimethoxyphenoxy) acetophenone; PP, 3-hydroxy-3′,4′-dimethoxy-2-(o-methoxyphenoxy) propiophenone

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permeable, so that hydrophobic molecules can partition into the lipid bilayer and, thereby, diffuse freely from one side to the other, whereas most hydrophilic solutes are excluded and require more specific mechanisms to pass the membrane.

Immobilized liposome chromatography (ILC) [10–13] has been documented as a tool to study the partitioning of various substances, e.g., pharmaceutical drugs, into biomembranes. Liposomes or proteoliposomes entrapped in gel beads serve as model membranes. The partitioning is calculated [12,14] as a capacity factor from the retention volume and provides information about the interaction between the lipids and the substances studied. ILC is a convenient method, since the membranes are stable for months and the analysis is quite rapid, which enables extensive substance and parameter screening. The reproducibility and precision of the data is also high [e.g., 12–14] and competes favorably with other methods used for membrane interaction studies.

A classical method in cell membrane interaction studies is octanol/water partitioning, where the octanol phase is assumed to mimic the hydrophobic part of the phospholipid bilayer [15]. The partitioning into the octanol phase has been shown to correlate with the partitioning into cell membranes in the case of neutral substances [16,17]. However, it does not include polar interactions, which are chiefly dependent on the hydrophilic phospholipid head group region [16,17] and may be significant for the partitioning into biomembranes. Moreover, it is a tedious method due to dissolution problems, slow equilibration and slow phase separation.

In this work, we have used ILC to determine the partitioning of stable lignin precursor model substances into liposomes in order to estimate their ability to diffuse through the bilayer. The liposomes were constituted from the zwitterionic PC alone or immobilized together with either the negatively charged phospholipid phosphatidylserine (PS) or the positively charged artificial lipid 1,2-distearoyl-3-trimethylammoniumpropane (DSTAP). The retention data at a series of temperatures allowed an estimate of the entropy and enthalpy values for the water–lipid transfer. ILC is an established method for different kinds of membrane studies, but here we present it as a new approach in plant physiological studies. In addition we employed octanol/water partitioning to estimate the hydrophobicity of the model substances and relate this to the liposomal partitioning.

2. Experimental procedures

2.1. Materials and buffers

2.1.1. Materials

We purchased glass columns (HR 5, i.d. 5 mm) and Superdex 200 prep grade gel from GE Healthcare (Uppsala, Sweden), DSTAP (>99%) and PC (hen's egg, >99%) from Avanti Polar Lipids (Alabaster, AL, USA), and PS (bovine brain, approx. 98%) from Sigma (St. Louis, MO, USA). Veratryl glycol (VG) was prepared from bromo-acetoveratrone by acetylation and reduction of the acetate with lithium aluminium hydride [3] (Fig. 1). The other model substances: α-methylsyringyl alcohol (MSA), 3,4,5-trimethoxybenzyl alcohol (TMBA), 4-acetyl-2,6-dimethoxyphenyl acetate (ADMPA), 4-acetoxy-3,5-dimethoxy-α-(2,6-dimethoxyphenoxy) acetophenone (APA), 4′-hydroxy-3′,5′-dimethoxy-2-(o-methoxyphenoxy) acetophenone (APH), 1-(4-hydroxy-3-methoxyphenyl)-2-(2,6-dimethoxyphenoxy) acetophenone (HMPA) and 3-hydroxy-3′,4′-dimethoxy-2-(o-methoxyphenoxy) propiophenone (PP) (Fig. 1), were gifts from Gunnar Henriksson and Anders Holmgren (Royal Institute of Technology, Stockholm, Sweden). All other chemicals used were of analytical grade.

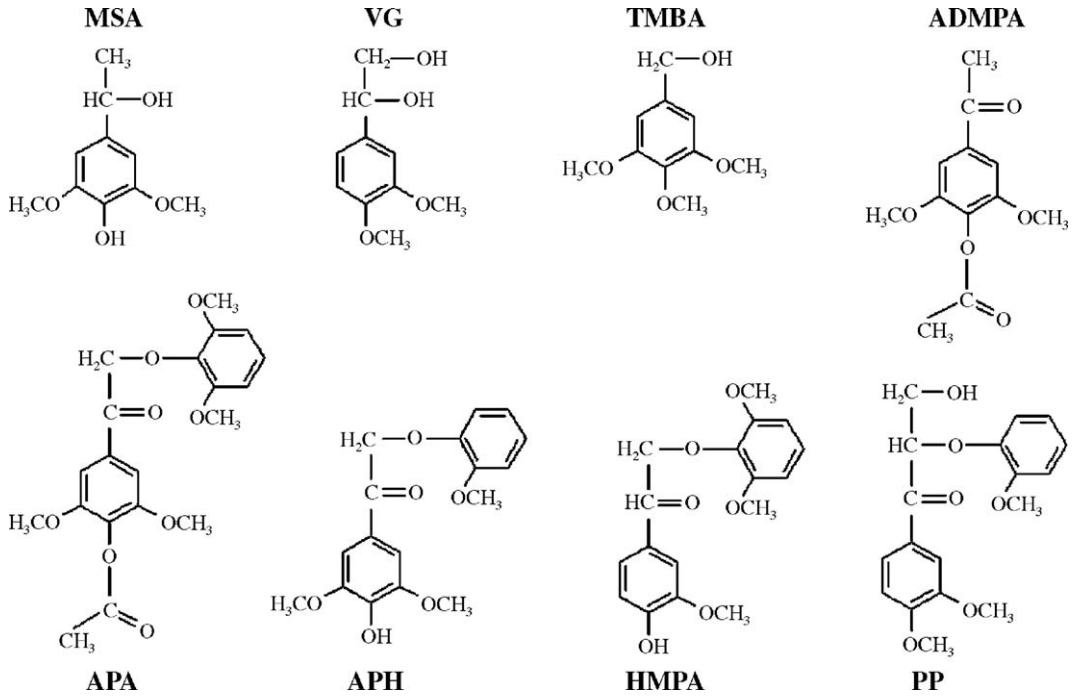

Fig. 1. Structures of the model substances used: α-methylsyringyl alcohol (MSA); veratryl glycol (VG); 3,4,5-trimethoxybenzyl alcohol (TMBA); 4-acetyl-2,6-dimethoxyphenyl acetate (ADMPA); 4-acetoxy-3,5-dimethoxy-α-(2,6-dimethoxyphenoxy) acetophenone (APA); 4′-hydroxy-3′,5′-dimethoxy-2-(o-methoxyphenoxy) acetophenone (APH); 1-(4-hydroxy-3-methoxyphenyl)-2-(2,6-dimethoxyphenoxy) acetophenone (HMPA), and 3-hydroxy-3′,4′-dimethoxy-2-(o-methoxyphenoxy) propiophenone (PP).

2.1.2. Buffers

Buffer A consisted of 10 mM Tris, 150 mM NaCl and 1 mM Na_2EDTA adjusted to pH 7.4 with HCl at 22 °C. Buffer B consisted of 10 mM Tris, 150 mM NaCl, 1 mM Na_2EDTA and 1 M $(NH_4)_2SO_4$ with an pH value of 7.6 at 22 °C.

2.2. Immobilized-liposome chromatography

Liposomes were immobilized in gel beads according to [11,13]. Briefly, a lipid film consisting of PC alone or together with either PS (20 wt.%) or DSTAP (32 wt.%) was rehydrated with buffer A to form multilamellar liposomes in suspension. The percentages of charged lipids in the mixtures were chosen to give maximal effect without impairing liposome stability. The suspension was mixed with dried Superdex 200 gel beads, followed by swelling and freeze–thawing cycles. Finally, the gel suspension was washed by centrifugation, packed into a HR glass column and equilibrated with buffer A or B. Triplicates of the substacnces (0.1 mg/ml in dH_2O and ≤10 vol.% ethanol, injection volume 10 or 20 μl) were run at 0.1 or 1 ml/min at 10–50 °C and detected at 270 nm on an automatic HPLC system, LaChrom Elite, from Merck Hitachi (Darmstadt, Germany).

The interaction between analytes and liposomes was expressed as a partition coefficient, K_S (M^{-1}) (Eq. (1)):

$$K_S = \frac{V_E - V_0 - V_G}{A} \quad (1)$$

where V_E is the elution volume of the analyte, V_0 is the void volume, represented by the elution volume of $Cr_2O_7^{2-}$, which does not interact with the liposomes or the gel, and V_G is the retention volume of the analyte on a liposome-free gel bed, which is included to compensate for the analyte-gel interactions [13,14]. The phospholipid amount A in the gel bed was determined by phosphorus analysis [18] after the experiment series. The weight fraction of DSTAP in the PC/DSTAP mixture was included in the A values in Eq. (1).

2.3. Octanol/water partitioning

In the octanol/water partitioning studies 1-octanol and buffer A were equilibrated before use. The substances were dissolved in the octanol to stock solutions of up to 1.2 mM. Standard curves were made from duplicate absorbance measurements at four different concentrations of each substance on an UV-Visible spectrophotometer, UV-1601, from Shimadzu (Kyoto, Japan) at its absorbance maximum. The same procedure was used for standard curves in the aqueous buffer. However, only low concentrations of the substances could be dissolved in buffer A after several hours in a sonication bath and, unfortunately, APH and PP were impossible to dissolve, hence, they are not included in this study.

The partitioning studies were modified from [19]. Briefly, 4 ml of substance in octanol and 20 ml of buffer A were mixed and then rotated for 24 h at room temperature (23 ± 0.5 °C). The sample was shaken vigorously 3 times during this period. The phases were allowed to separate over night before transfer into separate tubes, where they again were allowed to stand over night until the solutions were clear and their absorbances were measured. For each substance two partitioning studies were made, except for VG due to lack of raw material, and the absorbance of each phase was measured 3–6 times.

The concentrations were calculated from the standard curves and the partitioning was calculated according to Eq. (2):

$$P = \frac{C_O}{C_W} \quad (2)$$

where P is the partitioning, C_O is the equilibrium concentration of the substance in the octanol phase and C_W is the equilibrium concentration in the water phase.

3. Results

Generally, the retention data and the peak shapes were virtually identical for flow rates 0.1 and 1 ml/min, indicating adequate local equilibrium in the ILC studies.

3.1. Effects of ammonium sulfate on the partitioning

All of the model substances showed larger retentions when 1 M ammonium sulfate was included in the buffer. Regardless of the temperature, the retention increased most in the PC/DSTAP columns and least in the PC/PS columns, whereas the changes in the retention of the substances in the PC columns were intermediate in virtually all cases. The changes in elution volume between the buffer A system and the buffer B system are exemplified by MSA and PP data (Fig. 2).

3.2. Relation between estimated hydrophobicity and partitioning

The partitioning of the model substances into the liposomes correlated well with the hydrophobicity, as judged by the

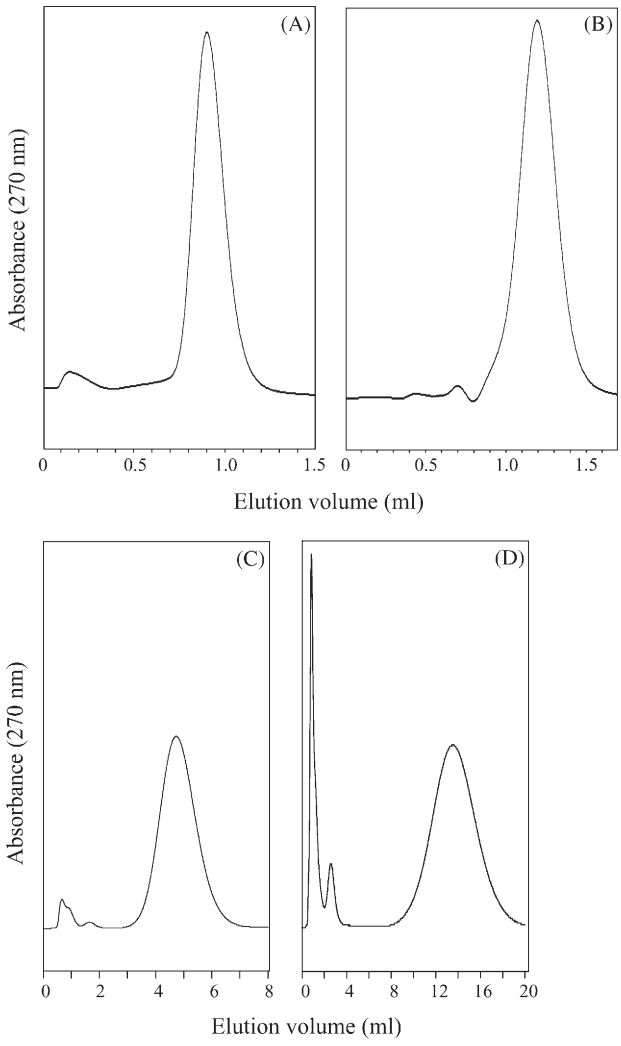

Fig. 2. Effect of 1 M ammonium sulfate in the buffer. Chromatograms of α-methylsyringyl alcohol (MSA) run on the phosphatidylcholine (PC) column with (A) buffer A (V_0 = 0.72) and (B) buffer B (V_0 = 0.76) at 30 °C and 1 ml/min, and chromatograms of 3-hydroxy-3′,4′-dimethoxy-2-(o-methoxyphenoxy) propiophenone (PP) run on the PC/phosphatidylserine (PS) column with (C) buffer A (V_0 = 0.70) and (D) buffer B (V_0 = 0.73) at 10 °C and 1 ml/min. The amount of lipids (A) was 14.9 μmol in the PC column and 15.1 μmol in the PC/PS column.

relation between the logK_S values at 20 °C and the octanol/water partitioning, log$P$, at room temperature (23±0.5 °C) (Fig. 3). The regression analysis gave correlation (R) values of 0.87, 0.88 and 0.91 for PC, PC/PS and PC/DSTAP liposomes, respectively.

3.3. Effects of temperature on the partitioning

The temperature had an effect on the partitioning of the substances, although not conclusive in all cases. Overall, the retention decreased or remained virtually constant as the temperature was increased from 10 °C to 50 °C in both buffer systems and for all three lipid-phases. The van't Hoff plots of the data are shown in Fig. 4.

3.4. Determination of the enthalpy and the entropy of transfer

The changes in enthalpy and entropy [20] for transfer of the model substances were calculated from Eqs. (3)–(5):

$$\Delta G = \Delta H - \Delta S * T \tag{3}$$

$$RT * \ln K_S = -\Delta G = T * \Delta S - \Delta H \tag{4}$$

$$\ln K_S = \frac{\Delta S}{R} - \frac{\Delta H}{R} * \frac{1}{T} \tag{5}$$

where ΔG is the Gibbs free energy change, ΔH is the enthalpy change, ΔS is the entropy change, R is the gas constant and T is the absolute temperature.

The temperature dependence of the partitioning suggests a certain enthalpy contribution to the interaction energy. The PC liposomes gave the smallest ΔH- and ΔS-values, whereas the PC/DSTAP liposomes gave the largest, in most cases (Table 1).

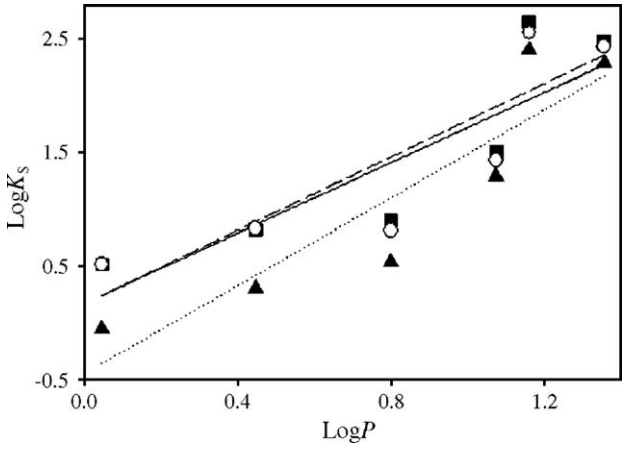

Fig. 3. Correlation between the partitioning into liposomes and the substance hydrophobicity. LogK_S in the buffer A system at 20 °C and 1 ml/min is plotted versus log$P$ at room temperature (23±0.5 °C). The liposomes consisted of phosphatidylcholine (PC) (–○–) (correlation coefficient, R, 0.87), PC/phosphatidylserine (–––■–––) (R, 0.88) and PC/1,2-distearoyl-3-trimethylammoniumpropane (···▲···) (R, 0.91). The average SD was 0.014 for the logK_S values and 0.050 for the log$P$ values.

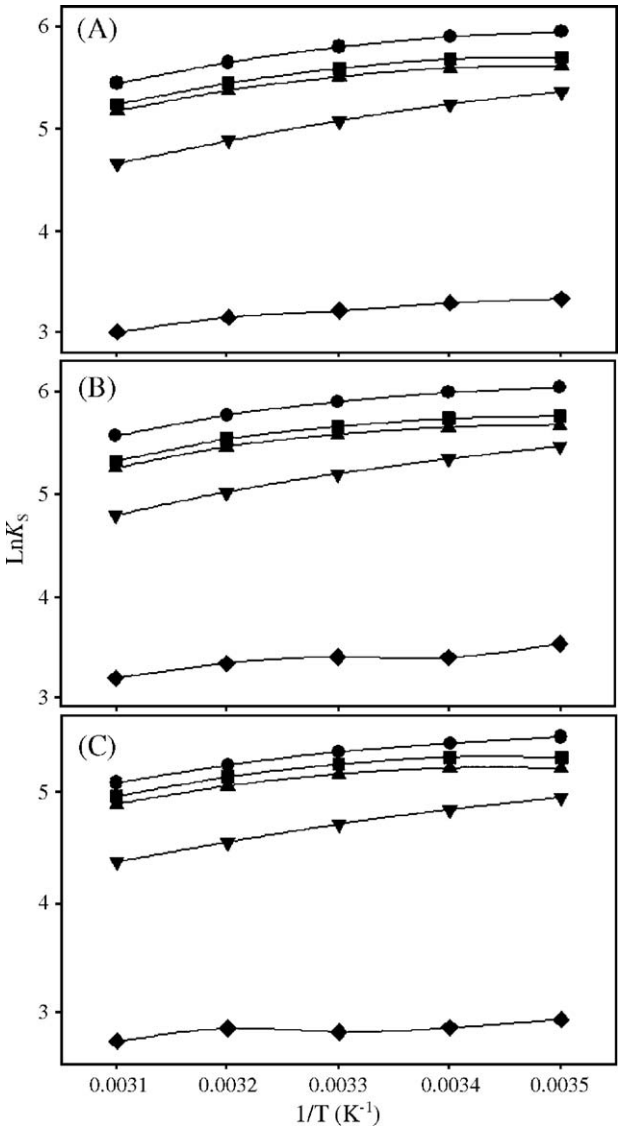

Fig. 4. Effect of the temperature on the partitioning of substances into liposomes as determined by immobilized liposome chromatography. LnK_S is plotted versus $1/T$ (K^{-1}), for (A) phosphatidylcholine (PC) liposomes, (B) PC/phosphatidylserine liposomes, and (C) PC/1,2-distearoyl-3-trimethylammoniumpropane liposomes run with buffer A at flow 0.1 ml/min. The substances shown are APA (●), APH (■), HMPA (▲), PP (▼) and ADMPA (♦) (for full names and structures see Fig. 1). The average SD of the lnK_S values of these substances was 0.006.

4. Discussion

Addition of ammonium sulfate to the buffer enhances hydrophobic interactions but decreases polar interactions [21,22]. Since the partitioning of the model substances increased in the presence of 1 M ammonium sulfate (Fig. 2), the hydrophobic interactions between the model substances and the lipid bilayers exceeded the polar ones, which was expected due to their hydrophobic nature (Fig. 1). However, since the partitioning into charged liposomes (PC/PS (80/20 wt.%) and

Table 1
The change in enthalpy and entropy for three model substances interacting with three different lipid bilayers

Liposomes	Substance	Buffer A			Buffer B		
		ΔH (kJ/mol)	ΔS (J/K*mol)	ΔG [a] (kJ/mol)	ΔH (kJ/mol)	ΔS (J/K*mol)	ΔG [a] (kJ/mol)
PC [b]	ADMPA [b]	−5.02	10.11	−7.88	−6.29	14.73	−10.46
	HMPA	−7.07	21.62	−13.19	−4.61	37.76	−15.30
	PP	−11.50	3.98	−12.63	−15.20	2.47	−15.90
PC/PS	ADMPA	−5.04	11.67	−8.35	−5.23	19.22	−10.67
	HMPA	−6.82	24.10	−13.64	−1.35	48.63	−15.12
	PP	−11.55	5.51	−13.11	−11.05	16.01	−15.58
PC/DSTAP	ADMPA	−1.53	19.50	−7.05	−5.54	16.14	−10.11
	HMPA	−4.67	27.58	−12.48	−0.60	49.62	−14.65
	PP	−9.45	8.38	−11.82	−10.95	14.70	−15.12

[a] ΔG at 10 °C.
[b] For full names of lipids and substances, see Materials.

PC/DSTAP (68/32 wt.%)) differed from the partitioning into neutral liposomes (PC), the polar interactions are also significant.

The lipid arrangement in the various liposomes was not determined, however, since only phospholipids are included in the liposomes we assume that microdomains do not exist. These so called lipid rafts require cholesterol and usually sphingolipids to form [23,24]. We also assume that the phospholipids PS and DSTAP are evenly distributed in their respective environment since electrostatic repulsion between those charged lipids should force them apart as far as possible.

A good correlation (Fig. 3, $R \geq 0.9$) was seen between the partitioning and the hydrophobicity of the substance, which corresponds well to the expected behavior of solute-diffusion over membranes. The hydrophobicity is interpreted as the partitioning of the substances between 1-octanol and buffer A (Eq. (2)). The deviation from a 1:1 slope and the spread around the regression lines (Fig. 3) is most likely due to polar interactions with the lipid head groups, which are not present in the octanol/water partitioning. For the substances chosen, there is a rather parallel variation of the number of polar and non polar groups. As a consequence, the increase in hydrophobic interaction is accompanied by a parallel increase in interaction with the head groups of a lipid bilayer that, thus, actually enhances the partitioning into liposomes, whereas the same polar groups rather counteract the partitioning into octanol. Hence, a lipid bilayer should be more accurate to use than octanol/water partitioning when studying membrane interactions. There were indeed also some differences in partitioning for the different compositions of bilayer studied. It should be noted that all types of substances might interact with a membrane, although neutral substances diffuse more easily into the bilayer, whereas charged solutes and other molecules with larger polar structures partition mostly within the polar lipid head-group region. As a result, some molecules that combine a strong interaction between hydrophobic parts of the molecule and the bilayer phase with a similarly strong interaction between polar parts of the molecule and the polar lipid head region can display a pronounced partitioning into the membrane but a poor ability to diffuse through it. It is reported, however, that up to three hydroxyl groups can be tolerated before the ability to diffuse through the membrane is seriously impaired [7].

We have also compared the partitioning of the model substances with the partitioning of pharmaceutical drugs from previous work [14,25] (Fig. 5). Structure similarities of the model substances and the drugs are the hydrophobic cores and the small hydrophilic parts spread around them. Parallels could be drawn between the dilignol models, i.e., APA, APH, HMPA and PP, and the neutral drugs of similar size used in the above mentioned studies. The partitioning not only ended up in the same logK_S-range for PC liposomes in all cases (Fig. 5), but also in a similar log$P$ range (Fig. 3 and [16]). It should be noted that in [14] 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, and not PC, was used and the buffer (F) is slightly different from Buffer A by having only 5 mM, instead of 10 mM Tris, however, this is not expected to affect the results significantly. Furthermore, there seems to be a fair correlation between the partitioning of the dilignol models and of the drugs into liposomes with PS and DSTAP. However, the data set is too small [25] to allow definitive conclusions. The glucocorticoids corticosterone, cortisone, dexamethasone and hydrocortisone all pass cell membranes by simple diffusion [26]. Metolazone is well adsorbed by the gastrointestinal tract, as are the benzodiazepines desmethyldiazepam and oxazepam [26]. All these drugs, thus, easily pass cell membranes, and since their partitioning correlates with the partitioning of APA, APH, HMPA and PP (Fig. 5), we conclude that diffusion is a possible transport route for these model substances, even though actual diffusion cannot be proven by ILC. The smaller models, MSA, VG, TMBA and ADMPA, showed a less pronounced partitioning into the liposomes, although they probably interact both with the hydrophilic head groups as well as the hydrophobic tails, as can be concluded from the data using different lipid phases and buffers. However, since the monolignol models showed a clear

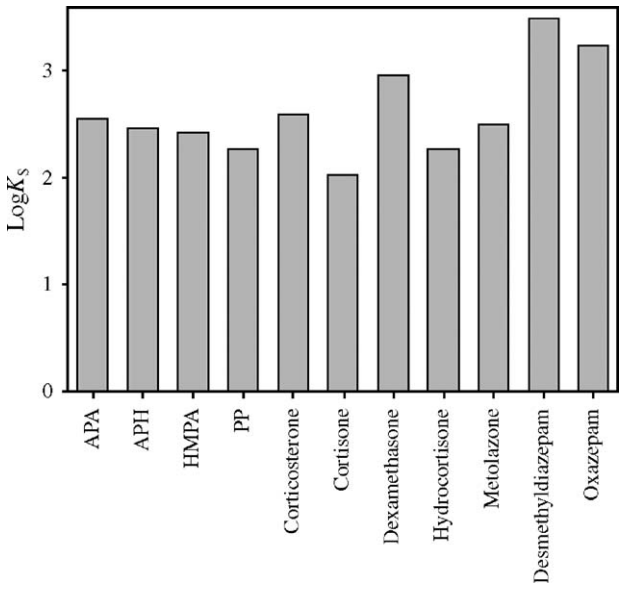

Fig. 5. Comparison between the logK_S values of the dilignol models (for full names and structures see Fig. 1) in phosphatidylcholine (PC) liposomes, buffer A system and 20 °C, and the logK_S values of seven neutral pharmaceutical drugs in PC or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine liposomes, buffer A or F (see Discussion) system and room temperature.

partitioning between octanol and water both hydrophobic and polar interactions do exist and the models are most probably able to cross a lipid bilayer.

The partitioning of the model substances decreased as the temperature increased. The slight curvature (Fig. 4) of the van't Hoff plots indicates that the enthalpy and entropy of transfer are changing over the temperature interval studied. The transition temperature of the bilayer may be in this temperature region, possibly in the lower part of it. However, the transition temperature is normally quite low (10±5 °C for PC [27]), so we believe that the bilayer is in its liquid–crystalline phase throughout this study. Upon increasing the temperature the lipid bilayers becomes less ordered due to higher fluidity [28], which might be an explanation for the gradual changes in enthalpy and entropy. As expected, the entropy contribution to the partition energy is prominent (Table 1). Furthermore, the effects of bilayer composition indicate that there are considerable polar interactions in addition to the hydrophobic interactions. A somewhat deviating behavior is observed for ADMPA on the positive PC/DSTAP liposomes. This can possibly be explained by the structure (Fig. 1) where no free hydroxyl groups are present, thus altering the hydrogen bonding capabilities.

5. Conclusions

The pronounced partitioning into the model membranes suggests diffusion as a possible membrane passage mechanism for lignin precursors, since the number of hydroxyl groups appear to be within the allowed limits [7] and all substances partitioned well into the octanol phase. This conclusion is further strengthened by the fact that the partitioning of the dilignols is similar to the partitioning of pharmaceutical drugs known to diffuse over cell membranes.

The hydrophobicity of the substances correlates with the partitioning into the lipid bilayers and appears to be of major importance for the interaction. The temperature dependence indicates the presence of enthalpy effects in addition to the strong hydrophobic interactions between model substances and model membranes. Moreover, the differences in partitioning observed when the lipid compositions were varied emphasize the effect of polar interactions.

Acknowledgements

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