Characterization of hydrophilic and lipophilic pathways of *Hedera helix* L. cuticular membranes: permeation of water and uncharged organic compounds

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Abstract

The permeability of astomatous leaf cuticular membranes of *Hedera helix* L. was measured for uncharged hydrophilic (octanol/water partition coefficient \(K_{OW} \leq 0\)) and lipophilic compounds (\(K_{OW} > 0\)). The set of compounds included lipophilic plant protection agents, hydrophilic carbohydrates, and the volatile compounds water and ethanol. Plotting the mobility of the model compounds versus the molar volume resulted in a clear differentiation between a lipophilic and a hydrophilic pathway. The size selectivity of the lipophilic pathway was described by the free volume theory. The pronounced tortuosity of the diffusional path was caused by cuticular waxes, leading to an increase in permeance for the lipophilic compounds after wax extraction. The size selectivity of the hydrophilic pathway was described by hindered diffusion in narrow pores of molecular dimensions. A distinct increase in size selectivity was observed for hydrophilic compounds with a molar volume higher than 110 cm\(^3\) mol\(^{-1}\). Correspondingly, the size distribution of passable hydrophilic pathways was interpreted as a normal distribution with a mean pore radius of 0.3 nm and a standard deviation of 0.02 nm. The increased permeance of the hydrophilic compounds by the removal of cuticular waxes was attributed to an increase in the porosity, a decrease in the tortuosity, and a widening of the pore size distribution. Cuticular transpiration resulted from the permeation of water across the hydrophilic pathway. The far-reaching implications of two parallel pathways for the establishment of correlations between cuticular structure, chemistry, and function are discussed.

Key words: Carbohydrates, partition coefficient, permeability, permeance, plant cuticle, water.

Introduction

The plant cuticle constitutes the interface between the primary parts of higher plants and the atmosphere. The cuticular membrane is composed of the biopolymer cutin, constituting the matrix for embedded intracuticular waxes and forming the basis for the deposition of epicuticular waxes (Jeffree, 1996). Cuticular waxes have been identified as the main transport barrier of cuticular membranes that reduce uncontrolled water loss (Kerstiens, 1996; Riederer and Schreiber, 2001), minimize leaching of plant metabolites from the leaf interior (Tukey, 1970), and limit the entrance of xenobiotics such as plant protection agents and environmental pollutants (Schönherr and Riederer, 1989). Cuticular membranes are of a heterogeneous structure with the transport-limiting barrier located towards the physiological outer side (Riederer and Schreiber, 1995). Although cuticular membranes are mainly considered as a lipid barrier, hydrophilic structures are also present. Cutin contains non-esterified hydroxyl- and carboxyl-groups (Schönherr and Huber, 1977). In addition, polysaccharides, such as pectin and cellulose, have been identified as components of cuticular membranes in considerable amounts (Jeffree, 1996; Marga et al., 2001) and water sorption to cuticular membranes can be attributed to a polysaccharide fraction with a high hydration capacity (Domínguez and Heredia, 1999; Luque et al., 1995).

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For lipophilic compounds, cuticular permeances can be predicted by the simple parameters of an octanol/water partition coefficient and molar volume (Schönherr and Riederer, 1989; Schönherr and Baur, 1994; Buchholz et al., 1998). When applying these prediction models to the permeation of uncharged hydrophilic plant metabolites such as carbohydrates, it seems very unlikely that these compounds can permeate across cuticular membranes (Schönherr and Baur, 1996). This is in contrast to the experimentally determined permeabilities of isolated cuticular membranes of Prunus laurocerasus for glucose, fructose, and sucrose (Stammitti et al., 1995), which were in the same order of magnitude as the permeability observed for lipophilic compounds (Kirsch et al., 1997). This comparison is based on data derived from different references. There are no other data of sufficient quality available in the literature that allow the analysis of the transport pathways of uncharged hydrophilic compounds compared with lipophilic compounds. However, primary metabolites such as carbohydrates are frequently found on plant surfaces. They are believed to be leached out of the leaf interior through cuticular membranes (Fiala et al., 1990). The measurement of the cuticular uptake of charged compounds such as calcium salts (Schönherr, 2000; Schönherr and Schreiber, 2004) and glyphosate salts (Schönherr, 2002) has been taken as a direct evidence for the existence of aqueous polar pores. By contrast, the permeation of uncharged, hydrophilic organic compounds has not been examined systematically. Therefore, the present study aims to characterize the transport properties of hydrophilic and lipophilic pathways of cuticular membranes for the permeation of uncharged organic compounds. Measurement of cuticular transpiration was included, since the mechanisms of water movement across cuticular membranes are still a matter of debate (Riederer and Schreiber, 2001; Schreiber et al., 2001; Schreiber, 2002).

Materials and methods

Cuticular membranes

Cuticular membranes (CM) were obtained from the upper, amentous leaf surfaces of Hedera helix L. plants growing in the Botanical Garden of the University of Würzburg. Enzymatic cuticle isolation was carried out as described previously (Schönherr and Riederer, 1986). Polymer matrix membranes (MX) were obtained by extracting the cuticular waxes with chloroform (Roth, Karlsruhe, Germany) for 12 h at room temperature. The resulting wax extract was used in order to produce samples of reconstituted waxes on aluminium discs as described previously (Schreiber and Schönherr, 1992).

Compounds

Model compounds were selected in order to obtain a broad spectrum with regard to molecular size and lipophilicity (Table 1). Octanol/water partition coefficients and aqueous solubilities were estimated by using the modelling program EPIWIN v3.11 (freely available from the US Environmental Protection Agency, http://www.epa.gov). Molar volumes were calculated according to McGowan and Sowada (1993). Carbohydrates were assumed to be preferentially in the cyclic form (Angyal, 1987).

<table>
<thead>
<tr>
<th>Compound</th>
<th>MW (g mol⁻¹)</th>
<th>MV (cm³ mol⁻¹)</th>
<th>log K_{O/W}</th>
<th>log WS (mol kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>18</td>
<td>17</td>
<td>-1.38</td>
<td>1.74</td>
</tr>
<tr>
<td>Ethanol</td>
<td>46</td>
<td>45</td>
<td>-0.14</td>
<td>1.24</td>
</tr>
<tr>
<td>Urea</td>
<td>60</td>
<td>47</td>
<td>-1.56</td>
<td>0.85</td>
</tr>
<tr>
<td>Erythrose</td>
<td>120</td>
<td>80</td>
<td>-1.52</td>
<td>0.92</td>
</tr>
<tr>
<td>Xylose</td>
<td>150</td>
<td>100</td>
<td>-1.98</td>
<td>0.82</td>
</tr>
<tr>
<td>Glucose</td>
<td>181</td>
<td>120</td>
<td>-2.89</td>
<td>0.74</td>
</tr>
<tr>
<td>Maltose</td>
<td>342</td>
<td>223</td>
<td>-5.03</td>
<td>0.47</td>
</tr>
<tr>
<td>Maltotriose</td>
<td>504</td>
<td>326</td>
<td>-7.36</td>
<td>0.30</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>122</td>
<td>93</td>
<td>1.87</td>
<td>-1.69</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>138</td>
<td>99</td>
<td>2.24</td>
<td>-1.56</td>
</tr>
<tr>
<td>2,4-D</td>
<td>221</td>
<td>138</td>
<td>2.62</td>
<td>-2.82</td>
</tr>
<tr>
<td>Metribuzin</td>
<td>214</td>
<td>162</td>
<td>1.49</td>
<td>-2.22</td>
</tr>
<tr>
<td>Cloquintocet-mexyl</td>
<td>336</td>
<td>257</td>
<td>5.28</td>
<td>-5.94</td>
</tr>
<tr>
<td>Bitertanol</td>
<td>337</td>
<td>267</td>
<td>4.07</td>
<td>-4.70</td>
</tr>
</tbody>
</table>

Table 1. Molecular weight (MW), molar volume (MV), octanol/water partition coefficient (K_{O/W}), and water solubility (WS) of the hydrophilic and the lipophilic compounds.

a MW values were estimated using EPIWIN software.

b Sources: 2, 4, 5, 6, 7, 8, Sigma-Aldrich, Taufkirchen, Germany; 9, 11, 13, Syngenta Crop Protection, Bracknell, Berkshire, UK; 12, 14, Bayer, Leverkusen, Germany; 3, 10, Du Pont de Nemours, Dreieich, Germany. Values were estimated using EPIWIN software.

The weak acids benzoic acid (pKₐ=4.19), salicylic acid (pKₐ=2.97), and 2,4-D (pKₐ=2.85) were measured at pH 2 and the weak base cloquintocet-mexyl (pKₐ=3.03) at pH 6 in order to ensure that the compounds were predominately non-ionized (99% for benzoic acid, 90% for salicylic acid, 88% for 2,4-D, and 99.9% for cloquintocet-mexyl). In all calculations only the concentration of the non-dissociated species of these compounds was used.

Carbohydrates were quantified using an oxidase–peroxidase colour reagent (sample:colour reagent=1:3 v/v) (Siemens and Mitchell-Olids, 1998). Maltose and maltotriose were digested to their glucose monomers using α-glucosidase (Sigma-Aldrich, Taufkirchen, Germany) before the colour reagent was added. The amounts of carbohydrates were assayed with a spectrophotometer (Multiskan, Thermo Labsystems, Vantaa, Finland) at 490 nm. The calibration curves were added for each microplate (r² ≥0.98). Urea was quantified using ninhydrin which forms a purple dye. To obtain a stable colour product a Ninhydrin-Reagent-Set (Fluka, Neu-Ulm, Germany) was used (volume ratio sampling probe:colour reagent=1:1). The amounts of urea were also assayed with a spectrophotometer at 570 nm. The volatile compounds ethanol and water were detected gravimetrically using a micro-balance (±1 µg; Sartorius, Göttingen, Germany). All other compounds were ¹⁴C-labelled. The amounts of radioactivity were determined by liquid scintillation counting (Tri Carb 2500, Canberra Packard, Frankfurt, Germany) after the addition of scintillation cocktail (Ultima Gold XR, Canberra Packard, Dreieich, Germany) to the samples.
Partition coefficients

The cuticle/water partition coefficient \( K_{C/W} \) is defined as the ratio between the equilibrium concentration in the cuticle \( c_{\text{cuticle}} \) and the equilibrium concentration in the aqueous phase \( c_{\text{water}} \):

\[
K_{C/W} = \frac{c_{\text{cuticle}}}{c_{\text{water}}} \tag{1}
\]

Cuticular membranes were added to aqueous solutions of the respective compound. Equilibrium was achieved by rotating the probes for 24 h at 25 °C. The cuticular membranes were then removed and the concentration in each phase was determined.

Sorption of water and ethanol to cuticular membranes was measured from a permanently saturated atmosphere of the volatile compound within the enclosed space of the balance \( c_{\text{water}}=23.07 \text{ g m}^{-3} \) for water and \( c_{\text{water}}=146.8 \text{ g m}^{-3} \) for ethanol) yielding the cuticle/air partition coefficient \( K_{C/A} \):

\[
K_{C/A} = \frac{c_{\text{cuticle}}}{c_{\text{air}}} \tag{2}
\]

Cuticle/air water partition coefficients can be converted into cuticle/water partition coefficients according to (Merk and Riederer, 1997):

\[
K_{C/W} = K_{C/A} \times K_{A/W} \tag{3}
\]

The air/water partition coefficient \( K_{A/W} \) is identical to the dimensionless Henry constant \( K_{A/W}=2.3 \times 10^{-5} \text{ for water and } K_{A/W}=2.0 \times 10^{-7} \text{ for ethanol} \).

Correspondingly, matrix membrane/water partition coefficients \( K_{M/W} \) and wax/water partition coefficients \( K_{W/W} \) were measured with the exception that matrix membranes or reconstituted wax samples were used instead of cuticular membranes.

Permeances

Permeance experiments were carried out with transport chambers made out of stainless steel (Schreiber et al., 1995). The cuticles were mounted between a donor and a receiver compartment with the physiological outer side pointing to the donor compartment. Experiments were initiated by the addition of an aqueous solution of the respective compound to the donor chamber. The amounts of molecules permeated were detected in regular time intervals in the receiver chamber. The latter was filled with a phospholipid suspension (1 g l⁻¹, soybean lecithin, Roth, Karlsruhe, Germany) in the case of the lipophilic compounds and with deionized water for the hydrophilic compounds. Between the sampling points, the transport chambers were rotated at 25 °C. Linear transport kinetics were obtained in all cases and permeances \( P \) were calculated from the flow \( F \), the exposed area \( A \) and the concentration difference between the donor and the receiver chamber \( \Delta c \):

\[
P = \frac{F}{A \times \Delta c} \tag{4}
\]

Permeances of the volatile compounds were measured with modified transport chambers without a receiver compartment. The flow was determined by measuring the weight loss of the chambers as a function of time, resulting from the volatilization of the compounds by permeation through the cuticular membrane into the surrounding air. The transport chambers were stored in a glass cuvette. Relative humidity (60% at 25 °C) was adjusted by a cold trap (Walz, Effeltrich, Germany) and boundary layer effects were avoided by the application of a high flow rate of the carrier gas (1.0 l air min⁻¹) directly over the cuticle surfaces. Permeances of volatiles can be expressed either on the basis of the liquid state or on the basis of the vapour state. The conversion factor is given by the ratio of the corresponding densities. The factors were calculated to be 43 400 for water and 5500 for ethanol at 25 °C. Permeances referring to the concentration in the liquid state as the driving force can easily be converted by multiplication with the corresponding conversion factors.

The permeance is a composite quantity consisting of the diffusion coefficient \( D \), the partition coefficient \( K \), the membrane thickness \( l \), and the tortuosity of the diffusional path length \( \tau \):

\[
P = \frac{D \times K}{\tau \times l} \tag{5}
\]

The quotients of the permeance and the partition coefficient can be regarded as a mobility parameter \( m \):

\[
m = \frac{P}{K} = \frac{D}{\tau \times l} \tag{6}
\]

Within one plant species the variability of the mobility can be attributed directly to changes in the diffusion coefficient. Plotting the logarithm of the mobility versus the molar volume \( MV \) yields the relationship (Schönherr and Baur, 1994):

\[
\log m = \beta' \times MV - \log m_0 \tag{7}
\]

The slope of the regression line \( (\beta') \) represents the size selectivity of the intercept \( (m_0) \) is equal to the mobility of a molecule of zero molar volume which is a measure for the tortuosity of the diffusion path.

Cuticular permeances of benzoic acid, water, and xylose were measured in the temperature range from 15–35 °C. The effect of temperature was analyzed by Arrhenius plots (natural logarithm of the permeance versus the inverse of the absolute temperature) and the activation energy was obtained from the slope of the regression line by multiplication with the gas constant. For water, the driving force was calculated using the vapour-based water densities in order to describe exclusively the temperature effect on membrane permeability and to exclude the influence of the latent heat of vaporization on the permeance (Kerstiens, 1996).

Non-ionic surfactants act as accelerators of diffusion in the cuticular wax barrier (Schreiber et al., 1996). The effect of the alcohol ethoxylate triethylene glycol monododecylether \( (C_{12}E_3, \text{ Fluka, Neu-Ulm, Germany}) \) on the permeance of xylose and benzoic acid was measured by the application of the surfactant (0.1 g l⁻¹) in the receiver compartment of the transport chambers.

Sample size and statistics

All sorption and permeation experiments were based on at least 12 replications. Results are given as means with 95% confidence intervals. The partition coefficients and permeances were tested for normal distribution by the Kolmogorov–Smirnov test which validated the use of parametric statistics in all cases.

Results

Cuticle/water partition coefficients, matrix membrane/water partition coefficients, and wax/water partition coefficients

The measured cuticle/water partition coefficients ranged from 26 (metribuzin) to 53 560 (cloquintocet-mexyl) for the lipophilic compounds and a good correlation with octanol/water partition coefficients \( K_{O/W} \) was found \((r^2=0.98)\):

\[
\log K_{C/W} = 0.95(\pm 0.18) \times \log K_{O/W} - 0.15(\pm 0.58) \tag{8}
\]
Cuticle/water partition coefficients ranged from 0.03 (water) to 99 (erythrose) (Fig. 1) for the hydrophilic compounds and were not correlated with octanol/water partition coefficients ($r^2=0.08$).

Matrix membrane/water partition coefficients ($K_{MW/w}$) were, on average, 1.4-fold higher than cuticle/water partition coefficients ($r^2=0.97$):

$$\log K_{MW/w} = 1.01 (\pm 0.13) \times \log K_{C/W} + 0.12 (\pm 0.30)$$

The wax/water partition coefficients ($K_{Wax/w}$) of the lipophilic compounds were about one order of magnitude lower than the cuticle/water partition coefficients and were also related to the octanol/water partition coefficients ($r^2=0.99$):

$$\log K_{Wax/w} = 1.03 (\pm 0.07) \times \log K_{C/W} - 0.92 (\pm 0.23)$$

Sorption of the hydrophilic compounds to reconstituted cuticular waxes was not detectable.

**Cuticular permeances**

The cuticular permeances ($P_{CM}$) of the lipophilic compounds ranged from $1.75 \times 10^{-11}$ (metribuzin) to $5.81 \times 10^{-9}$ m s$^{-1}$ (cloquintocet-mexyl) and were correlated with the corresponding cuticle/water partition coefficients ($K_{C/W}$) ($r^2=0.86$):

$$\log P_{CM} = 0.57 (\pm 0.32) \times \log K_{C/W} - 11.2 (\pm 0.93)$$

The permeances of the hydrophilic compounds ranged from $2.74 \times 10^{-15}$ (maltotriose) to $4.70 \times 10^{-8}$ m s$^{-1}$ (erythrose). Variation of permeances could only partially be described for five compounds as a function of the cuticle/water partition coefficient ($r^2=0.85$):

$$\log P_{CM} = 0.70 (\pm 0.53) \times \log K_{C/W} - 9.07 (\pm 0.69)$$

Glucose, maltose, and maltotriose were not included for the calculation of the regression equation (Fig. 1).

Analysis of regression by plotting the solute mobility in cuticular membranes ($m_{CM}$) versus the molar volume ($MV$) yielded the regression equation ($r^2=0.98$):

$$\log m_{CM} = -0.0088 (\pm 0.0015) \times MV - 10.8 (\pm 0.29)$$

for the lipophilic compounds and the regression equation ($r^2=0.88$):

$$\log m_{CM} = -0.017 (\pm 0.012) \times MV - 8.05 (\pm 0.78)$$

for the hydrophilic compounds of smaller size ($MV < 110$ cm$^3$ mol$^{-1}$). The hydrophilic compounds of larger size deviated significantly from this regression line (Fig. 2).

**Permeances of polymer matrix membranes**

The effect of wax extraction (Fig. 3), expressed as the quotient of the permeance of the polymer matrix membrane and the cuticular permeance ($P_{MX}P_{CM}^{-1}$), ranged from 222 (cloquintocet-mexyl) to 418 (metribuzin) for the lipophilic compounds and effects were correlated with the cuticular permeance ($r^2=0.97$):

$$\log P_{MX}P_{CM}^{-1} = -0.11 (\pm 0.04) \times \log P_{CM} + 1.46 (\pm 0.35)$$
Removal of waxes enhanced permeances of the hydrophilic compounds by factors between 1.8 (erythrose) and 993 (maltose) and is described by the regression equation ($r^2=0.92$):

$$
\log P_{MX}P_{CM}^{-1} = -0.45(\pm 0.18) \times \log P_{CM} - 3.02(\pm 1.85)
$$

Plotting the mobility in matrix membranes ($m_{MX}$) versus the molar volume for the lipophilic compounds ($MV$) yielded the regression equation given below ($r^2=0.99$):

$$
\log m_{MX} = -0.0090(\pm 0.0032) \times MV - 8.36(\pm 0.57)
$$

The hydrophilic compounds (Fig. 4) can be described by the following regression equation ($r^2=0.89$):

$$
\log m_{MX} = -0.023(\pm 0.011) \times MV - 6.68(\pm 1.35)
$$

**Temperature and surfactant effects on cuticular permeance**

Cuticular permeances of the uncharged species of benzoic acid increased in the temperature range from 15–35 °C by a factor of 75. The activation energy of permeation obtained from the Arrhenius plot amounted to 164 (±26) kJ mol$^{-1}$. Cuticular water permeability increased by a factor of 1.7 yielding an activation energy of 23 (±3) kJ mol$^{-1}$. No significant effect of temperature on the permeance of xylose was detected. Consequently no activation energy could be calculated.

The non-ionic surfactant C$_{12}$E$_3$ enhanced the permeance of benzoic acid by a factor of 4.0 (±1.7) while no significant effect on the permeance of xylose (1.6±0.7) was observed.

**Discussion**

**Partition coefficients**

Organic compounds can be classified as lipophilic when log $K_{O/W}>0$ and as hydrophilic when log $K_{O/W} \ll 0$. The data of the present study confirm that, for lipophilic compounds, octanol/water partition coefficients can be used successfully as predictors for cuticle/water partition coefficients (Kerler and Schönher, 1988). However, the sorption of the hydrophilic compounds into cuticular membranes (Fig. 1) is significantly higher than expected from octanol/water partition coefficients (Table 1). Although this deviation may partly arise by the inaccuracy of the fragmental method used for the calculation of octanol/water partition coefficients by the modelling program EPIWIN, the comparable high cuticle/water partition coefficients prove that sorption of the hydrophilic compounds does not take place in the lipophilic compartments of cutin and the cuticular waxes. It is more likely that polysaccharides, which have been found in substantial amounts in cuticular membranes (Marga et al., 2001; Dominguez and Heredia, 1999), are the potential sorption sites for these compounds.

For lipophilic compounds, it was shown that cuticular waxes form the transport-limiting barrier of cuticular membranes (Riederer and Schreiber, 1995). Sorption of lipophilic compounds to cuticular waxes was up to one order of magnitude lower than sorption to cuticular membranes while sorption of the hydrophilic compounds was not measurable. This implies that for hydrophilic compounds the transport across the cuticular wax barrier is not relevant. It has been suggested that wax/water partition coefficients should be used instead of cuticle/water partition coefficients in order to describe the permeation of
lipophilic compounds through the transport-limiting barrier of cuticular membranes (Schönherr and Baur, 1994). However, cuticular membranes are of a heterogeneous structure combining lipophilic and hydrophilic properties arising from the components of cutin, cuticular waxes, and polysaccharides. Cuticular permeances are related to transport across the whole cuticular membrane. Therefore, in the present work cuticle/water partition coefficients are used for the analysis of cuticular transport in order to ensure a consistent treatment of the hydrophilic and lipophilic compounds.

**Sorption-diffusion model of cuticular permeation**

Cuticular permeation can be described in the simplest way as a diffusion process from an aqueous donor compartment across the cuticular membrane into an aqueous receiver compartment (Schönherr and Riederer, 1989). This implies that the permeating compound has to be dissolved in the corresponding pathway of the cuticular membrane. Accordingly, cuticular permeances should increase with increasing cuticle/water partition coefficients (equation 5). If permeances are plotted versus cuticle/water partition coefficients (Fig. 1), it becomes obvious that the hydrophilic and the lipophilic compounds cannot be analyzed in a single modelling approach. A good correlation is found for all lipophilic compounds. The successful application of this approach without considering the diffusion coefficient is probably founded in the great variation of the partition coefficient. It changes by a maximum factor of 2040 between metribuzin and cloquintocet-mexyl (Fig. 1). At the same time the molar volume, which is closely related to the diffusion coefficient, varies only by a maximum factor of 2.9 between benzoic acid and bitertanol (Table 1).

For the hydrophilic compounds a satisfactory correlation between sorption and permeation can only partially be established only (Fig. 1). For five compounds the cuticle/water partition coefficient is the predominating factor of the permeance. For these compounds the partition coefficients varied at most by a factor of 3960 between water and erythrose. The deviation of glucose, maltose, and maltotriose from the regression line implies that the contribution of the diffusion coefficient cannot be disregarded and the drastic decrease of the permeance with increasing molar volume indicates a pronounced size dependence of diffusion. Even though the partition coefficients were in the same order of magnitude, permeances varied by a factor of 3940 (Fig. 1).

**Size selectivity of the lipophilic pathway**

The slope of the regression line obtained from the plot of the mobility (permeance divided by the partition coefficient) versus the molar volume represents the size selectivity of diffusion (Fig. 2). With an alternative experimental approach that measured the rate constants of desorption of solutes across the transport-limiting barrier of cuticular membranes it was found that the size selectivity varied only a little between plant species and an average value of 0.0095 mol cm\(^{-3}\) was obtained (Buchholz et al., 1998). This value fits quite well with the size selectivity found in the present study (0.0088 mol cm\(^{-3}\)). The size selectivity of the lipophilic cuticular pathway can be interpreted by the free volume theory claiming an exponential distribution of the free volume (hole or vacancy) size. A mean free volume \((V_f)\) of 49 cm\(^3\) mol\(^{-1}\) can be obtained directly from the size selectivity (\(\beta\)):

\[
V_f = \frac{1}{2.303 \times \beta'}
\]  

Accordingly, diffusion in the lipophilic pathway takes place in holes which temporarily emerge by the segmental motion of the aliphatic alkyl chains of the amorphous cutin and wax fractions.

The effect of temperature on the permeance of the uncharged species of benzoic acid can be attributed to an increase of the mean free volume available for diffusion due to increased thermal chain motion with rising temperature. The activation energy represents the energy required to produce free volumes which are of sufficient size and frequency for the passage of the respective compound. The value for benzoic acid is in the typical range of activation energies found for lipophilic compounds (Baur et al., 1997).

The enhancement of the mobility of active ingredients in the transport-limiting barrier of cuticular membranes by accelerator adjuvants like alcohol ethoxylate surfactants has been attributed to a plasticizing action leading to an increase of the mean free volume by fluidization of the amorphous wax fractions (Schreiber et al., 1996). Accordingly, the accelerating effect of C\(_{12}\)E\(_3\) on the permeance of benzoic acid can be explained by a reduction of the size selectivity of the lipophilic pathway in the presence of the surfactant.

**Size selectivity of the hydrophilic pathway**

Sorption of the hydrophilic compounds to reconstituted cuticular waxes were not measurable, indicating that permeation occurs not across the lipophilic cutin–wax route. It has been found that cuticular membranes are, on average, made up of 21% non-lipid materials (Riederer and Schönherr, 1984). For the leaf cuticle of Cirsium horridulum a 2:1 ratio of carbohydrates to lipids was reported by Marga et al. (2001). Water sorption to cuticular membranes has been attributed mainly to a polysaccharide fraction with a high hydration capacity (Dominguez and Heredia, 1999). Therefore, the hydrophilic pathway probably consists of a reticulum of polysaccharide microfibrils ramifying and stretching through the cuticular membrane (Jeffree, 1996). The permeation of hydrated ionic calcium salts, which cannot shed their hydration shell, has been taken as direct
evidence for the existence of aqueous pores in cuticular membranes (Schönherr, 2000, 2002). Up to now a distinction between hydrophilic diffusion pathways and water-filled pores building up an aqueous continuum across the membrane has not been possible.

The pronounced size selectivity of diffusion of the hydrophilic compounds indicates that diffusion is hindered by narrow pores. If diffusion coefficients of hydrophilic uncharged compounds of comparable size in an aqueous solution (Mitragotri, 2003) are plotted according to equation 7, the size selectivity of diffusion would amount to 0.003 mol cm$^{-3}$s$^{-1}$. For the diffusion in aqueous pores of molecular dimensions, steric restriction at the pore entrance and friction at the pore wall is taken into account by the hindrance factor ($H$) relating the diffusion coefficients in the pore ($D_p$) and in aqueous solution ($D_w$):

$$D_p = H(\lambda) \times D_w \quad (20)$$

The hindrance factor is a function of the ratio of the radius of the diffusing molecule ($r_m$), which is available from the molar volume assuming a spherical shape, and the pore radius ($r_p$):

$$\lambda = \frac{r_m}{r_p} \quad (21)$$

For small molecules the hindrance factor is given by (Mitragotri, 2003):

$$H(\lambda) = (1 - \lambda)^4 \quad (22)$$

Applying this approach to the smaller hydrophilic compounds ($MV < 110$ cm$^3$ mol$^{-1}$) in order to explain the discrepancy between the size selectivities in an aqueous solution (0.003 mol cm$^{-3}$) and the hydrophilic cuticular pathway (0.017 mol cm$^{-3}$) yields an average pore radius of 0.50 nm. Accordingly, a molecule with a molar volume of 310 cm$^3$ mol$^{-1}$ would fit these pore dimensions. This value seems to underestimate the real pore dimensions slightly, since permeation of maltotriose ($r_m=0.506$ nm) through cuticular membranes was still detectable.

A distinct increase in the size selectivity can be observed for the larger hydrophilic compounds ($MV > 110$ cm$^3$ mol$^{-1}$) (Fig. 2). At first sight a more tortuous pathway would be a reasonable explanation. Alternatively, it seems very likely that pore size distribution limits the permeation of the large hydrophilic compounds. The probability ($p$) of a diffusing molecule with the radius $r_m$ to find a passable pathway is equal to the cumulative frequency ($f$) of pores with the radius $r_p$ of equal or larger size:

$$p(r_m) = \int_{r_m}^{\infty} f(r_p)dr_p \quad (23)$$

For $r_m \rightarrow 0$ there is no limitation by pore size distribution and the cumulative frequency of passable pores amounts to $f(r_p)=1$. In contrast to that, for $r_m \rightarrow \infty$, suitable pores are not available and the cumulative frequency of passable pores reaches $f(r_p)=0$. Qualitatively this can be illustrated by assuming a normal distribution of the pore radius (Fig. 5): the frequency of passable pores decreases dramatically with increasing molar volume of the diffusing molecule.

The most accurate description of the cuticular permeance of hydrophilic compounds is as follows:

$$P_{CM} = K_{C/W} \times \frac{e}{\varepsilon \times l} \times D_w \times \int_{r_m}^{\infty} f(r_p) \times H(\lambda)dr_p \quad (24)$$

This relationship is equal to equation 5 with the exception that the diffusion coefficient in hydrophilic cuticular pores is expressed as the product of the porosity ($\varepsilon$: ratio of pore area and total area), the diffusion coefficient in an aqueous solution ($D_w$), the frequency of passable pores ($f$) and the corresponding hindrance factor ($H$). Unfortunately, this relationship cannot be solved quantitatively, since adequate assumptions on porosity, tortuosity and pore size distribution are not available. However, for water as the smallest compound it can be assumed that the relative frequency of passable pores is close to $f(r_p)=1$. If the permeance ($P_{CM} = 7.2 \times 10^{-11}$ m s$^{-1}$, Fig. 1) is multiplied by the membrane thickness ($l=43 \mu$m, Becker et al., 1986) and divided by the cuticle/water partition coefficient ($K_{C/W} = 0.03$, Fig. 1), the diffusion coefficient in an aqueous medium ($D_w = 1.4 \times 10^{-9}$ m$^2$ s$^{-1}$, Mitragotri, 2003) and the hindrance factor ($H=0.15$, equation 22 assuming a pore radius of 0.50 nm) a value of $5 \times 10^{-5}$ is obtained as the ratio of porosity and tortuosity. Assuming that the hydrophilic pathway takes a straight course through the cuticular membrane (tortuosity factor $\tau=1$) the porosity would amount to 0.005%. Due to this low value of the ratio of pore area to total area it would appear that permeation of

![Fig. 5](https://example.com/image.jpg)

**Fig. 5.** Size distribution of passable hydrophilic pores as a function of the molar volume of the diffusing compound (MV) assuming a normal distribution with a mean pore radius of 0.3 nm and a standard deviation of 0.02 nm.
hydrophilic compounds occurs via imperfections or cracks in the cuticular wax barrier. In spite of the limited pore area the cuticular permeability for hydrophilic compounds is comparably high (Fig. 1), since permeation across the hydrophilic pathway is favoured by a low tortuosity and high diffusion coefficients in a more or less aqueous environment.

The permeance of xylose is enhanced neither by increasing temperature nor by the accelerator surfactant C12E3. Comparable results have been found for the penetration of glyphosate salts and can be taken as evidence for the passage through aqueous pores (Schönherr, 2002), since pronounced temperature and accelerator effects on cuticular permeability of organic compounds are typical for the lipophilic pathway due to a decrease of the viscosity of the amorphous cuticular wax fraction.

The activation energy for the permeation of water across membranes allows conclusions about the corresponding transport pathway. The transport across a lipid membrane in a hydrophobic environment by a solubility-diffusion mechanism is characterized by a comparably high activation energy (\(E_a > 42 \text{ kJ mol}^{-1}\)). Water transport mediated by polar pores in a hydrophilic environment is indicated by a comparably low activation energy (\(E_a < 25 \text{ kJ mol}^{-1}\)). The activation energy of self-diffusion of water amounts to \(19 \text{ kJ mol}^{-1}\) (Elmoazzen et al., 2002). Correspondingly, the activation energy found for cuticular membranes suggests that permeation of water occurs across the hydrophilic pathway.

**Effect of wax extraction**

Cuticular waxes form the transport-limiting barrier of cuticular membranes (Riederer and Schreiber, 1995), since permeances increase considerably after removal of waxes (Fig. 3). Extraction of cuticular waxes did not affect the size selectivity of the lipophilic pathway and effects are solely attributed to an increase in the mobility of a molecule of zero molar volume (Figs 2, 4). This value can be regarded as a measure for the tortuosity of the diffusion path (equation 5) due to crystalline wax regions which are not accessible for diffusing molecules (Riederer and Schreiber, 1995). The contribution of cuticular waxes to the tortuosity of the lipophilic pathway is given by the difference between matrix membranes and cuticular membranes. Accordingly, in *H. helix*, cuticular waxes cause an extension of the diffusional pathway by a factor of 273. Since the size selectivity of the lipophilic pathway differs only slightly between plant species (Buchholz et al., 1998), tortuosity of the diffusional path length can be considered as the main reason for the species-specific variability of cuticular permeances (Baur et al., 1999).

For the hydrophilic compounds, the mobility of a molecule of zero molar volume in matrix membranes is higher by a factor of 24 than in cuticular membranes (Figs 2, 4). According to equation 24 this can be attributed to an increase of the ratio of porosity to tortuosity. This implies that cuticular waxes block potential polar pathways and the removal of waxes increases the pore area and/or decreases the tortuosity of the hydrophilic pathway. Further, the pronounced decrease of the mobility in cuticular membranes for comparably large compounds, suggesting a sigmoidal shape of the curve (Fig. 2), shifts to a more linear shape for matrix membranes (Fig. 4). This phenomenon can be explained by a broadening of the pore size distribution after wax removal with the effect that pores of larger dimensions are more frequent in matrix membranes than in cuticular membranes. Correspondingly, the effect of wax extraction is higher for larger hydrophilic compounds exhibiting a lower cuticular permeance (Fig. 3).

**Cuticular water permeability**

Various attempts have been undertaken in the past in order to find a correlation between cuticular water permeability, wax chemistry, and the physical properties of cuticular waxes (Riederer and Schneider, 1990; Schreiber and Riederer, 1996). None of them has been successful. The reason for this is obvious as the present study strongly suggests that water permeates mainly across the hydrophilic pathway. The narrow pore dimensions of the hydrophilic pathway agree with the observation that *Hedera helix* as an evergreen species is well protected against uncontrolled water loss (Riederer and Schreiber, 2001) with a cuticular permeance amounting to only \(7.2 \times 10^{-11} \text{ m s}^{-1}\) (Fig. 1). The size selectivity of polar cuticular pores of *Populus canescens* amounted to 0.002 mol g\(^{-1}\) using molecular weight as an indicator for the size of the permeating compounds (Schönherr and Schreiber, 2004). Characterizing the hydrophilic cuticular pathway of *Hedera helix* in this manner would yield a size selectivity of 0.01 mol g\(^{-1}\). *Populus canescens* is a deciduous species and the natural habitat is characterized by sufficient availability of water from the soil. Accordingly, the cuticular permeance for water amounts to \(2 \times 10^{-9} \text{ m s}^{-1}\) (Krüger, 1999). Therefore, in future investigations, the permeability of polar non-charged compounds should preferably be interpreted in terms of the properties of the polar pathway as given by equation 24 and not by correlating permeabilities with the chemistry of the cuticular waxes.

**Conclusions**

The permeation of hydrophilic compounds through cuticular membranes (Fig. 1) demonstrated in this study provides a mechanism by which polar and apolar organic metabolites can be lost from the leaf interior when water is present on the leaf surface, for example, as droplets of rain or dew. Leaf surface carbohydrates as found in leaf washings (Leveau, 2004) can be interpreted as leachates originating from this source. Below a critical threshold
value of the molar volume amounting to 110 cm$^3$ mol$^{-1}$ diffusion across the hydrophilic pathway is even faster than diffusion across the lipophilic pathway (Fig. 2). This finding may also be relevant for the application and formulation of hydrophilic active ingredients in plant protection agents (Ramsey et al., 2005). The radius of polar pores in cuticular membranes is specific for plant species. For polymer membrane filters of Citrus aurantium an average pore radius of 0.46 nm was reported (Schönhherr, 1976). From penetration studies of calcium salts through cuticular membranes of Populus canescens (Schönherr and Schreiber, 2004) the size selectivity of diffusion implies an even larger pore radius and a faster permeation across the hydrophilic pathway for compounds with molar volumes larger than 110 cm$^3$ mol$^{-1}$. Further studies are necessary to clarify whether both ionic and non-charged hydrophilic compounds penetrate across the same pathway.

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