A new technique for measurement of water permeability of stomatous cuticular membranes isolated from *Hedera helix* leaves

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Abstract

Transpiration of cuticular membranes isolated from the lower stomatous surface of *Hedera helix* (ivy) leaves was measured using a novel approach which allowed a distinction to be made between gas phase diffusion (through stomatal pores) and solid phase diffusion (transport through the polymer matrix membrane and cuticular waxes) of water molecules. This approach is based on the principle that the diffusivity of water vapour in the gas phase can be manipulated by using different gases (helium, nitrogen, or carbon dioxide) while diffusivity of water in the solid phase is not affected. This approach allowed the flow of water across stomatal pores (‘stomatal transpiration’) to be calculated separately from the flow across the cuticle (cuticular transpiration) on the stomatous leaf surface. As expected, water flux across the cuticle isolated from the astomatous leaf surface was not affected by the gas composition since there are no gas-filled pores. Resistance to flux of water through the solid cuticle on the stomatous leaf surface was about 11 times lower than cuticular resistance on the stomatous leaf surface, indicating pronounced differences in barrier properties between cuticles isolated from both leaf surfaces. In order to check whether this difference in resistance was due to different barrier properties of cuticular waxes on both leaf sides, mobility of $^{14}$C-labelled 2,4-dichlorophenoxy-butyric acid ($^{14}$C-2,4-DB) in reconstituted cuticular wax isolated from both leaf surfaces was measured separately. However, mobility of $^{14}$C-2,4-DB in reconstituted wax isolated from the lower leaf surface was 2.6 times lower compared with the upper leaf side. The significantly higher permeability of the ivy cuticle on the lower stomatous leaf surface compared with the astomatous surface might result from lateral heterogeneity in permeability of the cuticle covering normal epidermal cells compared with the cuticle covering the stomatal cell surface.

Key words: Cuticular transpiration, diffusion, helium, Knudsen diffusion, plant cuticle, pores, stomatal transpiration.

Introduction

The plant cuticle forms the outermost layer of leaves and fruits, separating the living cells from the atmosphere. The development of the ‘waterproof’ cuticle was a prerequisite for the colonization of dry land, because the permanent water deficit in the atmosphere causes a constant loss of water from the plant surface into the atmosphere. However, a reduced permeability of the leaf surface for water simultaneously reduced CO$_2$ uptake. This dilemma between carbon uptake and water loss was solved by the evolution of stomata, often located only on the shaded lower (abaxial) leaf side. In hypostomatous leaves the upper (adaxial) surface is covered with a continuous...
cuticle. On leaf surfaces carrying stomata, the cuticle covers the outer epidermal cell walls and periclinal walls of guard cells, and it extends down the ventral walls forming the stomatal pore to the substomatal cavity (Appleby and Davies, 1983a; Wullschleger and Oosterhuis, 1989; Osborn and Taylor, 1990; Pesacreata and Hasenstein, 1999).

Under favourable environmental conditions, 94–99.7% of the gas exchange occurs through stomatal pores (Körner, 1994). However, under drought stress with stomata closed, the survival of plants depends on the barrier properties of the cuticle, which effectively control water loss. Consequently, cuticles of plant species adapted to dry conditions have, by one to two orders of magnitude, lower permeances for water than cuticles of mesophytes from more humid climates (Schreiber and Riederer, 1996). The transport properties of cuticles could also play a significant physiological role in the short-term control of leaf water loss via humidity-sensing stomata (Kerstiens, 1996a, b). The direct response of stomata to air humidity assumes a higher permeance of the cuticle covering the guard cells walls and facing the stomatal pores (Appleby and Davies, 1983a, b).

Present knowledge of the transport properties of cuticles is based exclusively on results obtained with astomatous leaf surfaces (Holmgren et al., 1965; Kerstiens, 1995; Boyer et al., 1997; Kirsch et al., 1997; Kerstiens, 1997) or astomatous cuticular membranes (SchoÈnherr and Lendzian, 1981; Schreiber and Riederer, 1996, 1997). The investigation of cuticular water permeability of stomatous leaf cuticles is difficult, because stomata may contribute to ‘cuticular transpiration’ due to an imperfect stomatal closure (Hygen, 1951; Slavík, 1958; Hoad et al., 1996; Kerstiens, 1996a, b) or due to limited possibilities in elimination or evaluation of diffusion through open stomata (Moreshet, 1970; Šantrůček and Slavík, 1990a, b; Šantrůček, 1991). In both cases, the residual water diffusing through stomata leads to an overestimation of cuticular permeability.

A newly developed approach is presented here for measuring transpiration across isolated stomatous cuticles, by separating quantitatively the diffusion of water across the gas-filled stomatal pores from water diffusion through the solid cuticle polymer. By applying this technique it was possible for the first time to measure the water permeabilities of cuticles from stomatous leaf surfaces and to compare them with water permeabilities of cuticles from astomatous leaf surfaces.

**Material and methods**

**Isolation of cuticular membranes**

Ivy (Hedera helix L.) leaves were collected in the Botanical Garden in Würzburg (Germany) in autumn 1999. Cuticular membranes (CM) were isolated as described in detail by Schönherr and Riederer (1986) by immersing leaf discs in a mixture of 2% (v:v) cellulase (Celluclast, Novo Nordisk, Bagsvaerd, Denmark) and 2% (v:v) pectinase (Trenolín Super DF, Erbshöf, Geisenheim, Germany) dissolved in citric buffer (pH=3.0, adjusted with KOH). 1 mol m⁻³ sodium azide (Na₃N₃, Fluka, Neu-Ulm, Germany) was added to prevent growth of micro-organisms. Isolated cuticles (CM) from upper and lower leaf surfaces were separated, carefully washed with deionized water, air-dried, and stored at room temperature.

**Extraction of cuticular wax and desorption experiments**

Cuticles isolated from the upper and lower sides of 50 ivy leaves were extracted separately in chloroform at 40 °C yielding 99 mg and 76 mg wax, respectively. The bulk solutions in chloroform (Roht, Karlsruhe, Germany) were vacuum evaporated (Büchi B124, B481, B168, Germany) to a final wax concentration of 50 mg ml⁻¹. Waxes extracted from the upper and lower leaf surfaces were used for measuring diffusion coefficients of ¹⁴C-labelled 2,4-dichlorophenoxy-butyric acid (¹⁴C-2,4-DB; specific activity: 3.3 MBq in 1000 ml of Toluol) in reconstituted cuticular wax as described in detail in Schreiber and Schönherr (1993). Desorption of ¹⁴C-2,4-DB into borax buffer (10⁻² mol l⁻¹, pH 9.0) was measured at time intervals of 5, 15, 30, 60, and 90 min. Radioactivity was determined by liquid scintillation counting (Wallac counter, model 1409, Turku, Finland). Diffusion coefficients were obtained from plots of relative amounts of ¹⁴C-2,4-DB desorbed from the wax versus the square root of time (Schreiber and Schönherr, 1993). As it was shown that cuticular permeability was correlated with diffusion in reconstituted wax (Kirsch et al., 1997), this method can also be used for comparing permeability between adaxial and abaxial leaf sides.

**Diffusion through the isolated stomatous cuticles**

Theory: The diffusion of water vapour through nitrogen-filled pores (stomata) of a membrane (stomatous cuticle) is defined as $F_{sN}$ and diffusion through the cuticle itself is defined as $F_c$. The total flux $F_{sN}$ of water through the membrane in a nitrogen atmosphere is given by:

$$F_{sN} = F_{sN} + F_c$$

(1)

Water flux $F_{sH}$ through the same membrane surrounded by helium is given by:

$$F_{sH} = F_{sH} + F_c$$

(2)

provided that the solid membrane and $F_c$ are not affected by the gas species. Steady-state diffusion of water along the two defined pathways depends on the concentration gradient and on the diffusivity of the substance. The diffusion coefficient, $D$, as a measure of diffusivity, is a function of the mean free path of the molecule (here water) in the gas phase. It is affected by the mass of both the diffusing substance and the surrounding molecules, by temperature, and by the pressure in the gas system. Thus, the flux of water vapour (solute) at a constant concentration gradient across pores of invariable geometry will depend only on the molecular characteristics of the gas (solvent). The ratio of water fluxes across the pores filled with helium and with nitrogen is given by the following equation

$$\frac{F_{sH}}{F_{sN}} = \frac{D_{wH}}{D_{wN}} \approx 3.6$$

(3)

where $D_{wH}$ and $D_{wN}$ are binary diffusion coefficients of water in helium and in nitrogen, respectively. The diffusion of water vapour in helium should be 3.6 times faster than that in nitrogen ($D_{wN} = 2.56 \times 10^{-5}$ m² s⁻¹ at 34 °C and $D_{wH} = 9.08 \times 10^{-5}$ m² s⁻¹ at 25 °C; Cusself, 1987). The ratio $D_{wH}/D_{wN}$ will be called $R$ and the parameter $k=R/(R-1)$ can be defined. Substituting $F_{sH}$ in equation (2) by equation (3) and combining equations (1) and (2) the
Fig. 1. Relative flux of water through the solid cuticle, $F_s/F_{tN}$, and through stomata pores, $F_c/F_{tN}$, as a function of the ratio of total diffusive fluxes in helium and nitrogen, $F_{tH}/F_{tN}$. Simulation by using equations 5a and 5b.

The following equation describing the water flux through the solid-phase of the stomatous cuticle is obtained

$$F_s=k	imes F_{tN}-(k-1)	imes F_{tH}$$  (4)

Equation 4 states that when the fluxes measured in helium and in nitrogen have the same values ($F_{tH}=F_{tN}$), then the total flux of water $F_t$ is realised only across the solid cuticle ($F_{tN}=F_c$) and there is no diffusion across pores. This situation is expected in an stomatous cuticle. At the opposite extreme, the flux through the solid cuticle, $F_c$, is zero when the $F_{tH}/F_{tN}$ ratio approaches the $R$ value ($F_{tH}/F_{tN}=k/(k-1)$ when $F_c=0$ in equation 4). This might be the case with flux through aluminium foil into which holes have been punched, for example. The relative proportions of ‘cuticular water loss’, $F_c$, and ‘stomatal water loss’, $F_{tN}$, are

$$\frac{F_c}{F_{tN}} = k-(k-1)\frac{F_{tH}}{F_{tN}}$$  (5a)

and

$$\frac{F_{tN}}{F_{tH}} = (k-1)\left(\frac{F_{tH}}{F_{tN}} - 1\right)$$  (5b)

The lines in Fig. 1 show the complementarity of the relative cuticular and stomatal conductances when the helium/nitrogen water flux ratio varies between its limits 1 and $R$.

The binary diffusion coefficients $D_{tH}$ and $D_{tN}$ and the gas-phase fluxes involved in the above equations require that the water molecules interact predominantly with molecules of the gas phase (helium or nitrogen), but not with boundary structures of the diffusion path. This may not be the case with a substance diffusing through narrow gas-filled pores and the Knudsen diffusion should have been taken into account (Cussler, 1987). However, the diffusion coefficients are employed here as the ratio $R$ only; fluxes are also in the form of ratios. Thus, the effect of Knudsen diffusion is largely eliminated provided it is equal for both water in nitrogen and water in helium. This situation will be discussed later in this paper. Another important point should be mentioned here and will be solved later. Equations 3–5 are valid only for binary diffusion, that is, for a system having two components: one solvent (gas) and one solute (water vapour). When, for example, a radiolabelled probe is used, correction for ‘tracer diffusion’ has to be taken into account (Cussler, 1987).

Transport experiments in helium and nitrogen

Stomatous (abaxial) and astomatous (adaxial) cuticular membranes (CM) isolated from ivy leaves have been used. Wax-free polymer matrix membranes (MX) were prepared by extracting single CM with chloroform (20 ml) for 1 h at room temperature. For permeability measurements in helium and nitrogen, four different types of cuticle samples have been used: stomatous CM (CMs), stomatous MX (MXs), astomatous CM (CMa), and astomatous MX (MXa). Water flux across the membranes was measured using a newly developed transport chamber (Fig. 2) and two different ways of measuring water transport: (i) measurements with radiolabelled water ($^3$H$_2$O; specific activity: 925 MBq g$^{-1}$; Hartmann Analytic, Braunschweig, Germany); and (ii) a gravimetric method.

When using $^3$H$_2$O, membranes were mounted between two compartments, a donor compartment with the $^3$H$_2$O donor solution and a receiver compartment with water-saturated filter paper (Fig. 2). The concentration gradient of $^3$H$_2$O between donor and receiver compartments across the membrane represents the driving force of $^3$H$_2$O diffusion across the membrane. Amounts of radioactivity accumulating in the receiver during a certain period of time are proportional to the water flux, which can be used to calculate the permeance of the membrane (see Appendix for details). High-vacuum silicon grease (Wacker Chemie, Burghausen, Germany) was used to mount and seal the membrane on the top of the donor compartment. Usually, the physiological inner side of CMa or MXa was in contact with the donor solution and the physiological outer side of the membrane was oriented towards an air-filled receiver compartment (Schreiber et al., 2001). The arrangement is modified here. In order to avoid penetration of the liquid donor solution through the pores of CMa or MXa and to allow measurements in a particular gas atmosphere, a chamber as shown in Fig. 2 has been used. The membrane is surrounded by a gas medium on both donor and receiver sides and the receiver compartment can be flushed with the proper gas. The receiver, which is a dish of filter paper, 10 mm in diameter, soaked with 12 µl of deionized water, was fixed to a
Teflon plug on a sealed revolving rod (Fig. 2). This allowed sampling and replacement of the receiver without a substantial contamination of the atmosphere inside the chamber. Nevertheless, after each sampling and before the next measurement of $^3$H$_2$O flux started, the receiver compartment was flushed for 30 s with the appropriate gas (helium or nitrogen with purity better than 99.9%).

Two stainless-steel chambers were used in parallel for the experiments. One was filled with nitrogen and the other with helium. Five samplings at intervals of between 10 and 20 min were carried out with each chamber, then the chambers were reconnected to the other gas, thoroughly flushed (20 min) with the new gas and again sampled five times. The gas cylinders and other equipment were in thermal equilibrium with the laboratory atmosphere ranging from 21–24 °C. At each sampling, the paper disc from the receiver compartment was quickly transferred to a scintillation vial, 2 ml of scintillation cocktail was added, and the amount of radioactivity was counted in a scintillation counter (Wallac Counter, Model 1409, Turku, Finland). In this way, $^3$H$_2$O flux through every CM or MX investigated was measured five times in nitrogen and five times in helium under otherwise identical conditions (geometry, temperature, pressure). In order to estimate the ratio of the diffusion coefficients (R) in different gases, $^3$H$_2$O-diffusion in empty chambers without membranes were measured. Such $R$ was typical for the geometry of this experimental set-up. In addition, estimating diffusion coefficients of $^3$H$_2$O in empty chambers was also necessary to address the problem of tracer diffusion (Cussler, 1987), which is related to the fact that water flux measured using radiolabelled water as a tracer does not necessarily correspond to the water flux measured using non-radiolabelled water. This problem is considered in the Appendix.

Alternatively to radiolabelled water, water fluxes in two different gases (helium and carbon dioxide) were measured by gravimetry. The gravimetric technique is less sensitive than the radioactive technique, but it has an advantage in avoiding the corrections for tracer diffusion (see Appendix). To increase the sensitivity, carbon dioxide has been used instead of nitrogen, because CO$_2$ is a ‘heavier’ gas with a lower diffusion coefficient. Aluminium caps were fixed to the receiver compartments of the revolving rod instead of the Teflon plugs (Fig. 2). Inside the aluminium cap was a paper disc soaked with a saturated solution of water-free lithium chloride dried at 120 °C. During sampling, the aluminium cap was quickly removed and regularly weighed at predetermined time intervals on a microbalance (Mettler MT5, Toledo) with an accuracy of 1 µg. The flux of water was calculated from the kinetic of water accumulation in the receiver using equation A1 (see Appendix). The slope $s$ was proportional to the flux of water and the parameter $a$ was set to 1 in the case of non-labelled water.

**Conductance of pores and the solid phase of cuticle for water**

The diffusive flux of water vapour through a barrier depends on the gradient (or difference $\Delta c$) of water vapour and on the transport properties of the barrier. The conductance for the total water transported in this chamber across CM or MX (reciprocal value of total resistance $r_t$) can be calculated from the simple flux resistance–driving force relationship $1/r_t = F_1/\Delta c_w$. Both, the total flux in nitrogen $F_{1,N}$ and the difference of water vapour pressure between donor and receiver $\Delta c_w$ (driving force) can be assessed. In a similar way, this approach can be used for the solid phase of CM or MX cuticles. As the flux across the solid cuticle is known from equation 4, the relationship $1/r_t = F_1/\Delta c_w$ can be used. However, this simple method is not suitable for the estimation of the resistance of pores ($r_p$). Therefore, the following approach for the estimation of $r_p$ has been used. The resistance of the porous membranes (CM or MX), $r_m$, is composed of two parallel resistances: the resistance of the pores $r_p$ and the resistance of the solid cuticle $r_c$ ($1/r_m = 1/r_p + 1/r_c$).

In addition, the membrane is arranged in series with the boundary layer of the unstirred gas in the receiver compartment with the resistance $r_b$. Therefore, the total resistance for the water diffusion in the chamber is $r_t = r_m + r_b$. The parallel and serial arrangement of these resistances leads to the following equation:

$$r_t = \frac{r_pr_c}{r_p + r_c} + r_b$$

(6)

where the composite term on the right hand side is the resistance $r_m$. Rearranging equation 6 leads to:

$$r_m = \frac{(r_b - r_t)}{r_t - r_b - r_c}$$

(7)

The boundary layer resistance is defined as $r_b = l/D$, where $l$ is the distance between the cuticle and the receiver and $D$ is the diffusion coefficient of water in nitrogen. As the distance between the mounted cuticle and the receiver was 12 mm in experiments with radiolabelled water and 18 mm when measuring water flux gravimetrically, $r_b$ was 510 or 594 s m$^{-1}$, respectively. These values were used for calculating permeances.

**Electron microscopic observations**

The possible structural effects of wax extraction on the stomatal pores and on the cutin matrix were investigated by scanning electron microscopy (JEOL 6300 SEM, Japan). Lengths and widths of antestomatal chambers (the throat between the external ledges) were measured and images of the cuticle surfaces were taken.

**Statistics and units used**

Water transport using radiolabelled water was measured in helium and in nitrogen using 6 CMs and 9 MXs. Using the gravimetric method, 6 CMs and 9 MXs were investigated. As the results obtained by radiometry and gravimetry did not differ significantly, they were pooled. In order to check the premise of the method that the transmembrane fluxes through the solid phase in helium and nitrogen are equal, antostomatous membranes were also measured: water transport in He and N$_2$ through 4 CMs and 3 MXs and four paraffin discs. Due to small fluxes and the lower sensitivity of gravimetry, only radiometry was used for antostomatous membranes. Results are given as means and 95% confidence intervals (CI). Desorption experiments measuring diffusion coefficients of $^{14}$C-2,4-DB in reconstituted cuticular wax were conducted by investigating five replicates of each leaf side. Results are given as means and 95% CI. For 30–60 randomly selected stomata, the length and width of their antestomatal chambers were measured and given as means and 95% CI.

For the sake of clarity, the transport properties of the gas and solid phase are initially presented in terms of fluxes and then as resistances or their reciprocals, conductances, as appropriate.

**Results**

**Water diffusion in chambers without membranes (‘empty’ chambers)**

The kinetics of $^3$H$_2$O accumulation in the receiver of empty chambers filled with nitrogen or with helium were linear, with correlation coefficients greater than 0.99 (Fig. 3a). The average flux of tritiated water was 2.1 times higher in helium than in nitrogen (see $F_{1,N}^{tH}$ and $F_{1,N}^{tH}$ in Table 1). Diffusion coefficients of tritiated water in nitrogen and helium were $D_{w,N}^* = 2.36 \times 10^{-5}$ and $D_{w,H}^* =$...
4.87 × 10⁻⁵ m² s⁻¹, respectively, when calculated from the experimentally estimated fluxes. The relevant handbook-tabulated binary diffusion coefficients of water in nitrogen and helium are \(D_{wN} = 2.42 \times 10^{-5}\) and \(D_{wH} = 8.81 \times 10^{-5}\) m² s⁻¹, respectively. Based on this, all fluxes measured in helium were corrected using the ratio \(D_{wH}/D_{wN} = 1.809\) (see equation A3 in the Appendix).

Diffusion coefficients of non-labelled water in helium and in CO₂ measured by gravimetry were close to those tabulated by Cussler (1987) \(9.08 \times 10^{-5}\) and \(2.02 \times 10^{-5}\) m² s⁻¹, for \(D_{wH}\) at 25 °C and \(D_{wC}\) at 34 °C, respectively. The mean value of \(R\) measured in gravimetry was 3.9, which was 87% of the theoretical value (4.5).

Water diffusion through membranes

Astomatous and stomatous cuticular membranes (CMₐ and CMₛ, respectively), extracted membranes (MXₐ, MXₛ), and parafilm showed linear transport kinetics with correlation coefficients greater than 0.99 (Fig. 3b–d). The difference between the slopes of transport kinetics in nitrogen and helium corresponded with the presence (Fig. 3a) or absence (Fig. 3b) of gas-filled pores between the donor and receiver compartments. The total water flux through CMₛ was 25 times higher than the flux through CMₐ when measured in nitrogen. Wax extraction increased the flux through MXₛ 7.5 times when compared to CMₛ (Fig. 4). The ratios of fluxes in helium and nitrogen were 1.21, 1.14, 1.93, 1.91, and 1.94 for parafilm, CMₐ, CMₛ, MXₐ, and MXₛ, respectively (Table 1). From these ratios the relative fractions of the total flux along the solid cuticle and pores can be calculated using equations 5a and 5b. On average, 35% of water diffused along gaseous pores in CMₛ and this proportion remained the same in MXₛ. A non-significant portion of total flux passed apparent pores in CMₛ and parafilm (5% and 8%) with 95% confidence intervals exceeding 2–3 times these values and thus including zero. However in MXₛ, one-third of the total flux passed through gaseous pores. Wax extraction in CMₛ increased water flux across both the solid cuticle and across pores by a factor of 7.5.

In terms of resistances, the solid phase of CMₛ had an 11.4 times lower resistance than the CMₐ (Table 1). Wax extraction decreased the resistance of the solid stomatous cuticle by a factor of 9. Diffusive resistance of pores in CMₛ was even higher than that of the solid cuticle and dropped 15.5 times after the wax had been extracted. All measurements in terms of conductances \(g\) (inverse values of resistance, also called permeance \(P\)) are summarized in Fig. 5. Conductance of the apparent gas phase in CMₛ and parafilm was several orders of magnitude lower than conductance of the solid phase in these membranes. In each of the other three types of membranes including MXₛ, the conductances of pores and of the solid membrane were in a similar range. The conductance of the solid phase increased by approximately one order of magnitude each time between parafilm, CMₛ, CMₛ, and MXₛ. Conductance of MXₛ was in the same range as conductance of CMₛ.

Scanning electron microscopic investigations

Electron microscopic investigation of stomatal pores in isolated cuticles showed that the internal pore between the cuticles of guard cells was very small (tenths of micrometers), difficult to estimate reliably or even absent (Fig. 6a, b). Wax extraction had no pronounced effect on the opening of the antestomatal chamber (the cross-sectional area of the throat between the external ledges) (Table 2).

Desorption experiments

Desorption kinetics of ¹⁴C-2,4-DB in reconstituted wax isolated from astomatous (adaxial) and stomatous (abaxial)
leaf surfaces were linear, with a correlation coefficient greater than 0.99. The diffusion coefficient of $^{14}$C-2,4-DB was 2.6 times higher in adaxial wax than in wax isolated from the abaxial leaf surface (Table 3).

**Discussion**

It was shown that water flux across isolated stomatous cuticles of *H. helix* was about 25 times higher than water flux across astomatous cuticles (Table 1). This is obviously due to the presence of stomatal pores in isolated stomatous cuticles. However, surprisingly, a substantial part of the water flux (about 65%) passed the solid non-porous phase of the stomatous cuticle (Table 1). Furthermore, the diffusive resistance of the solid cuticle on the stomatous leaf surface was about 11 times lower than the resistance of the astomatous cuticle. These results look strange at first sight and if they are true this would have important physiological implications for the gas exchange of intact leaves. However, before discussing these points any further, three important aspects concerning the applied technique itself and its reliability need to be mentioned. (i) Why is the diffusion coefficient of tritiated water in helium in this system only slightly more than half of that tabulated for non-labelled water in handbooks? (ii) Why is the flux of water through stomatous cuticles measured here distinctly lower than that expected from gas exchange measurements with intact illuminated leaves (open stomata)? (iii) How far is the flux fractionation into solid and gaseous pathways in stomatous cuticles affected by the dimensions and boundaries of the pores?

**Methodological aspects of the flux partitioning technique**

*Tracer diffusion*: The binary diffusion of water in helium is described by the binary diffusion coefficient $D_{\text{w}H}$ which is
independent of molar fractions of water and helium. However, using labelled water as a tracer of water transport, the diffusion coefficient of the tracer, \( D_{wH}^* \), will be lower than \( D_{wH} \) and it will vary with the molar fraction of water. This is due to additional interactions of tritiated water with non-labelled water molecules. This type of diffusion is called ‘tracer diffusion’ (Cussler, 1987). Because the molar fraction of water in water-saturated nitrogen is very low and because the diffusivity of water in nitrogen is also fairly low, the interactions between \(^3\)H-labelled water and non-labelled water have low frequency in nitrogen. Therefore, the tracer diffusion coefficient in nitrogen is very close to the binary diffusion coefficient of water. However, water diffusivity is much higher in helium, and here the tracer diffusion coefficient decreases significantly. Cussler (1987) suggests how the diffusion coefficient of tritiated water in a water vapour-saturated atmosphere of helium can be calculated.

However, for this calculation, data on the diffusivity of tritiated water in water vapour (self-diffusion) and data on the molar fraction of water in helium at different degrees of water saturation are required. As these data were not available, \( D_{wH}^* \) was estimated empirically in this experimental system and it was related to tabulated \( D_{wH} \). This \( D_{wH}^* \) was 55% of \( D_{wH} \). In addition to tracer diffusion, there are probably other factors contributing to the reduction of \( D \) value. The same factors probably caused the 14% drop in \( D_{wH} \) when it was measured gravimetrically. However, when the empirical value of \( D_{wH}^* \) or \( R \) used is constant (geometry, leakage, etc. of the chamber are invariant with the gas species), the flux partitioning is not affected. This is demonstrated by the consistency of the results obtained with tritiated water and by gravimetry (Fig. 4).

**Stomatous water flux in intact leaves compared to isolated stomatous cuticles:** Water flux across stomatous cuticular membranes measured here was in the same range compared to published values obtained with isolated stomatous cuticles of *H. helix* measured at saturated air humidity (Kerstiens, 1996a; Schreiber et al., 2001). Furthermore, the values of the flux measured with isolated stomatous cuticles correspond to the data for isolated stomatous cuticles of *Prunus laurocerasus* L. (Schreiber and Riederer, 1996). However, the flux across isolated stomatous cuticle \( (CM_a) \), on average 0.08 mmol m\(^{-2}\) s\(^{-1}\), was between 1.3–31 times lower than transpiration rates obtained with intact leaves of *H. helix*. Transpiration rates of attached irradiated (1 mmol (photons) m\(^{-2}\)s\(^{-1}\)) leaves ranged from 0.1–2.5 mmol (H\(_2\)O) m\(^{-2}\)s\(^{-1}\) measured in a ventilated LI-6400 leaf chamber. Both leaf sides were...
measured and transpiration was calculated on a projected leaf area basis. Leaf–air vapour pressure differences ranged from 1.5 to 4.1 kPa. Total leaf resistances of intact leaves varied from $2.0 \times 10^4$ sm $^{-1}$ (with stomata closed) to $2.4 \times 10^2$ sm $^{-1}$ (with stomata opened). These figures roughly correspond to conductances ranging from 0.002–0.17 mol m$^{-2}$ s$^{-1}$. The average total resistance of the isolated stomatous CM was $1.8 \times 10^4$ sm $^{-1}$ ($1/(1/r_c+1/r_s)$). This is a surprising result, as one should expect water permeability of isolated stomatous cuticles to be close to that of intact leaves with stomata opened. Two factors could contribute to this discrepancy. First, gas exchange measurements are normally performed in a well-mixed air atmosphere with a thickness of the boundary layer in the order of tenths of millimetres (Nobel, 1991). The boundary layer conductance of 2.8 mol m$^{-2}$ s$^{-1}$ in the ventilated leaf chamber of LI-6400 is equivalent to a resistance of 15 s m$^{-1}$ and this corresponds to a thickness of the boundary layer of 0.36 mm. In this study with isolated cuticles, the thickness of the unstirred air layer between cuticle surface and receiver was about 12 mm, which corresponds to a 33 times thicker boundary layer. However, since the total resistance ($r_c$ and $r_s$ arranged in parallel) of CM mounted in the chamber was $1.8 \times 10^4$ s $^{-1}$, the additional boundary resistance of $5.1 \times 10^2$ s $^{-1}$ only accounts for 2.4% of the total. Thus, the fairly thick boundary layer cannot explain the low flux across stomatous cuticles observed here. Alternatively, it is more reasonable to assume that the aperture of the stomatal pores in isolated stomatous cuticles may be significantly smaller than the aperture of stomatal pores in the intact leaves. This explanation is confirmed by the SEM observations of stomatal pores of isolated cuticles, which all looked fairly closed (Fig. 6). Moreover, the leaf internal cuticle, which remains intact after the enzymatic isolation (Pesacreta and Hasenstein, 1999), seems to occlude the stomatal pores of isolated cuticles.

**Effect of the pore dimension:** In the extreme case of very narrow pores where the collisions between water molecules and the pore wall dominate over the collisions between the gas molecules, the difference between water flux in helium and in nitrogen will be drastically reduced. This situation is called the Knudsen diffusion and it is of significance in porous membranes with pore diameters below the mean free paths of the diffusing molecules (Cussler, 1987). However, provided that the pores reduce free diffusion in helium and in nitrogen to a similar extent, the ratio of diffusion rates (and diffusion coefficients) should not change substantially. Because the ratio of fluxes and ratio of $D$ is considered (equations 5a, b), the helium–nitrogen method of flux partitioning should yield correct numbers even for the pores with diameters well below the mean free path of molecular motion. Nevertheless, there might be a particular pore diameter where water diffusion in helium is affected, but water diffusion in nitrogen much less so. For example, in air at room temperature, the mean free path of water is about 60 nm long, whereas it is 130 nm in helium. Consequently, diffusion through pores with diameters of 0.1 $\mu$m obeys Knudsen $D$ in helium, but ordinary $D$ in nitrogen. This leads to depression of $F_{tH}$ relative to $F_{tN}$. As a result, $F_c$ may be overestimated (equation 5a). However, there is no sharp transition between Knudsen and regular diffusion and the pore

![Fig. 6. SEM micrographs of stomatal pores in cuticular membrane isolated from the abaxial surface of a *H. helix* leaf. The pore viewed from the physiologically outer (a) and inner (b) side of the cuticle.](https://academic.oup.com/jxb/article-abstract/55/401/1411/478981)
diameter probably has a wide distribution. Hence, it is reasonable to assume that \( R \) does not change dramatically with the pore diameter and that the flux through the solid cuticle is not significantly overestimated in these measurements.

In support of the flux partitioning in stomatous cuticles shown here, there is substantial indirect evidence that the cuticle of the stomatous leaf surface is more permeable than the cuticle of the astomatous leaf surface. For example, permeance for 3-(3,4-dichlorophenyl)-1,1-dimethyleura (DCMU) across adaxial and abaxial surfaces of intact \( H. \) helix leaves was measured using chlorophyll fluorescence imaging. DCMU is a photosynthetic herbicide blocking electron transport in photosystem II and, therefore, increasing dissipation of radiation energy in chlorophyll fluorescence. The kinetics of the fluorescence increase can be used for distinguishing between less and more permeable cuticle in intact leaves. The results show a 6.3 times higher permeability of the stomatous leaf surface than the astomatous surface (J Šantrúček and L Schreiber, unpublished results). The corresponding permeability ratio estimated here by the nitrogen-helium method was 11.4. Qualitatively similar differences between cuticular permeabilities of intact leaves have been reported independently several times (Sargent and Blackman, 1962; Schönherr and Bukovac, 1978; Schreiber, 1990). Up to now there are no further data available comparing the permeability of the solid stomatous to the astomatous cuticle, except for the indication of increased water permeance of stomatous guard cells (peristomatal transpiration) shown by Maier-Maercker (1983).

**Physiological significance**

These results show that 35% of water crossed stomatal pores and 65% the solid phase of the CM \(_s\) membrane. Measurement of flux across the astomatous CM (CM\(_s\)) leads to the conclusion that cuticular transpiration of the stomatous leaf surface was about 11 times higher than that of the astomatous leaf surface. There is a long-standing discrepancy between cuticular conductance measured with intact leaves using gas exchange equipment and those measured with isolated cuticles. This difference amounts, on average, to one order of magnitude (Kerstiens, 1996a). It is usually argued that this discrepancy is caused by an incomplete stomatal closure in intact leaves compared with isolated astomatous cuticles. Alternatively, accepting the finding that the permeability of the solid cuticle on the lower (stomatous) leaf surface is significantly higher than the permeability of the astomatous cuticle and keeping in mind that the opposite is true for diffusion in waxes comparing lower and upper leaf surfaces, a lateral non-uniformity of cuticular resistance would be the most likely explanation for this observation. There are indeed some reports showing that the cuticle on guard cells lack the amorphous cuticle proper, that it is thinner than the cuticle on periclinal walls of the other non-stomatal epidermal cells and that, like subsidiary cells, guard cells may have a reticulate cuticle (Osborn and Taylor, 1990). This could indicate a lower resistance of the guard cell cuticle and, at least in part, provide an explanation for the apparently lower resistance of the stomatous cuticle reported here. Water loss from guard and/or subsidiary cells, the so-called ‘peristomatal transpiration’, has been hypothesized as a prerequisite for the direct stomatal response to air humidity (Maier-Maercker, 1983; Kappen and Haeger, 1991), but this is still a matter of controversy.

**Effect of wax extraction on permeability of solid versus gas phase in stomatous cuticles**

From the nitrogen–helium experiments it was found that both stomatal and cuticular fluxes increased by approximately the same factor after wax extraction. This is surprising, since one would expect only cuticular flux to increase after wax extraction. Porous fluxes should not be affected by wax extraction and their relative proportion should, therefore, decrease. While the flux partitioning in CM\(_s\) raised doubts about overestimation of the cuticular flux, the partitioning in MX\(_s\) seems to overestimate the flux through pores.

In the following, it will be judged what the effect of wax extraction on the pores in CM\(_s\) could be. Starting with the equations introduced earlier (see Materials and methods), flux partitioning in CM\(_s\) and MX\(_s\) can be expressed as

\[
F_{1\text{N CM}_s} = F_s \text{N CM}_s + F_c \text{CM}_s \tag{8a}
\]

\[
F_{1\text{H CM}_s} = F_s \text{H CM}_s + F_c \text{CM}_s \tag{9a}
\]

\[
F_{1\text{H MX}_s} = F_s \text{H MX}_s + F_c \text{MX}_s \tag{9b}
\]

It is suggested that the wax extraction did not change dimensions of stomatal pores and thus water fluxes through the pores in the particular gas remain the same in both CM\(_s\) and MX\(_s\). It follows that \( F_s \text{N CM}_s = F_s \text{N MX}_s \) and \( F_s \text{H CM}_s = F_s \text{H MX}_s \). When \( F_s \text{N MX}_s \) is expressed through equation 8b and substituted for \( F_s \text{N CM}_s \) in equation 8a, and the same is done in equations 9a and 9b, stomatal flows from equations 8a and 9a are eliminated. Both equations contain the terms \( F_c \text{MX}_s \) and \( F_c \text{CM}_s \) and can be condensed into:

\[
F_{1\text{H MX}_s} - F_{1\text{H CM}_s} = F_{1\text{N MX}_s} - F_{1\text{N CM}_s} \tag{10}
\]

which is only another expression for the ‘null-hypothesis’ that stomatal pores (and stomatal fluxes) did not change during the wax extraction. However, now the hypothesis is testable as all four terms in equation 10 are estimated directly in the experiments. Equation 10 states that the whole increment in flux caused by wax extraction is due to an enhanced transport in the solid cuticle and, therefore, must be the same in helium and in nitrogen. The average difference (MX\(_s\)–CM\(_s\)) of total fluxes in helium was
0.99 mmol m\(^{-2}\) s\(^{-1}\) and that in nitrogen was 0.49 mmol m\(^{-2}\) s\(^{-1}\) (see Table 1 for stomatous CM and stomatous MX) and, therefore, the null- hypothesis must be rejected. In conclusion, the gas-filled pores and flux across the pores changed due to the wax extraction. From this it is not possible to say whether wax extraction resulted in enlarged stomatal pores or if it created new pores.

However, the presence of pores in the matrix, whether they are an artefact of wax extraction or naturally present and filled with wax in the intact cuticle, was demonstrated in astomatous MX\(_a\) as well (Fig. 5; Table 1). While this seems rather surprising it corresponds to previous observations that MX\(_a\) of several plant species were easily penetrated by dead bacteria (Knoll, 1998), indicating the presence of pores in the range between 0.5–1 \(\mu\)m. CM\(_a\) were not penetrated by dead bacteria. Additional experiments using special transpiration chambers with 30 cm long glass capillaries fixed gas-tight to an opening at the rear of the transpiration chambers strongly confirmed these conclusions. These chambers were turned upside down and a pressure gradient across the cuticle was induced by placing a 1 cm column of mercury at the top of the capillary. With CM\(_a\) \((n=8)\) the mercury column did not move downwards at all within 1 h, indicating the absence of gas-filled pores (only with two CM\(_a\) the mercury did move downwards within 1 h by 1–2 mm). However, when MX\(_a\) \((n=10)\) was used, the mercury column rapidly moved downwards (on average 2–4 cm min\(^{-1}\)), which is direct proof of continuous gas-filled pores traversing right through the entire wax-free MX\(_a\). Thus, the pores that formed in MX\(_a\) membranes upon wax extraction must have been occluded by cuticular wax in CM\(_a\) membranes. A mechanistic model of plant cuticles containing wax-filled pores was also described in studies analysing the optical properties of cuticles (Meyer, 1938; Roelofsen, 1952). The nitrogen–helium technique proved the absence of significant free diffusion in gas-filled pores in CM\(_a\) (Fig. 5). An attempt was made to check the presence of pores in MX\(_a\) by scanning electron microscopy. There were no open pores or cracks visible on the upper side of MX\(_a\), but narrow cracks were observed on the physiologically inner side of MX\(_a\) (Fig. 7b).

Conclusions

Data are presented indicating that the solid phase of the abaxial stomatous cuticular membrane (CM\(_b\)) has a lower resistance to the diffusion of water than the adaxial astomatous CM (CM\(_a\)) isolated from the same leaf of Hedera helix. A new method allowing the estimation of transport resistance of the solid cuticle in the presence of pores was employed. An attempt to explain the difference in resistances by a difference in transport properties of wax isolated from opposite leaf surfaces surprisingly showed that the diffusion coefficient for 2,4-DB C\(^{14}\) in adaxial wax was 2–3 times higher than that in abaxial wax. It is possible to explain the discrepancy by lateral heterogeneity of cuticular conductance, for example, by differences in transport properties of epidermal, subsidiary, and guard cell cuticle. Finally, there were pores allowing gas-phase diffusion in astomatous wax-free matrix (MX\(_a\)), but not in intact CM\(_a\).

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Appendix

Measurements of water diffusion in a gas using \(^3\text{H}_2\text{O}\) as a tracer

By sequential sampling and scintillation counting of the receiver, a cumulative kinetic of \(^3\text{H}\) activity in the receiver is obtained. The kinetic yields a linear increase with the slope \(s\) proportional to the total \(^3\text{H}_2\text{O}\) flux across the membrane at the particular gas composition (\(F_{\text{HI}}\) for helium and \(F_{\text{NI}}\) for nitrogen). The examples of the kinetic are shown in Fig. 3. The activity \(a\) of the donor solution (dpm \(\mu\text{L}^{-1}\)) was adjusted for CM, MX, and for empty chambers, in order to obtain a good final resolution. The apparent flux of non-labelled water in mmol \((\text{H}_2\text{O})\) m\(^{-2}\) s\(^{-1}\) was calculated as

\[
F_i = \frac{s \times 10,000}{a \times A \times 60 \times 18} \quad (A1)
\]

where \(s\) is the slope of the kinetic of \(^3\text{H}_2\text{O}\) activity in the receiver in dpm, \(a\) is specific activity of donor solution in dpm \(\mu\text{L}^{-1}\), and \(A\) is area of exposed cuticle in cm\(^2\); in the chambers used here (12 mm diameter) it was 1.13 cm\(^2\). (Note that there is no net flux of non-labelled water in this radiometric system with the gas at the donor and receiver saturated with water. The accumulation of activity in the receiver shows the net flux of labelled water, which is constant with time if the receiver activity is negligibly small when compared with the donor activity. This is why the flux of non-labelled water calculated from equation A1 is called an ‘apparent’ flux of water.)

While this formula yields sufficiently accurate results for the apparent flux of water vapour in nitrogen, it fails for water flux in helium. This is because \(^3\text{H}_2\text{O}\) does not truly trace water vapour in the presence of non-labelled water in helium. \(^3\text{H}_2\text{O}\) molecules are significantly slower than \(^2\text{H}_2\text{O}\) in helium (but not in nitrogen) due to the phenomenon called tracer diffusion (Cussler, 1987). A system is considered as having three physically different substances even if two of them are chemically identical: \(^2\text{H}_2\text{O}\), \(^3\text{H}_2\text{O}\), and \(\text{He or N}_2\). In this case the diffusion coefficient of \(^2\text{H}_2\text{O}\) can be substantially different from the binary diffusion coefficient of water especially at the exceptional mobility of helium (Cussler, 1987). A tracer, which can be, for example, a radiolabelled substance or an optical isomer added in small quantity to a chemically identical substance, collides with the identical counterpart molecules in addition to its collisions with the solvent (gas medium). Diffusion coefficient (and flux) of the tracer \((D^*\)\) is lower than those of the other non-labelled molecules \((D)\) which still behave like a substance in a binary system. While \(D\) does not depend on concentration of the substance in question, \(D^*\) changes with its molar fraction. For small molar fractions and dense media like for fraction of water in water-saturated nitrogen or air (close to 0.03) and nitrogen, respectively, the difference between \(D\) and \(D^*\) is small. However, when diffusion of \(^3\text{H}_2\text{O}\) in helium saturated with water vapour is considered, \(D_{\text{wH}}^*\) can be significantly smaller than \(D_{\text{wH}}\) (see Cussler, 1987, for the calculation of \(D^*\)).

Therefore, the apparent flux of non-labelled water calculated from the accumulation of tracer activity, \(F_{\text{IH}}\), will be smaller than the flux of non-labelled water in helium measured, for example, by a tracer-independent gravimetric method, \(F_{\text{IH}}\). The correction factor is \(D_{\text{wH}}^* / D_{\text{wH}}\):

\[
F_{\text{IH}} = F_{\text{IH}} \times \frac{D_{\text{wH}}}{D_{\text{wH}}^*} \quad (A2)
\]

This correction is valid only when all water is transported in continuous helium (for example, water diffusing in the chamber filled with helium without a membrane septum). With a porous membrane, the \(D_{\text{wH}} / D_{\text{wH}}^*\) factor has to be applied only to the flux of tritiated water passing the helium-filled pores, \(F_{\text{is H}}^*\), not to the whole \(F_{\text{IH}}^*\). How can the \(F_{\text{is H}}^*\) flux be assessed? It was assumed in equations 1 and 2 that \(F_{\text{IH}}^* = F_{\text{IC}} = F_{\text{LC}}\). Then, when subtracting equations 2 and 1 written for labelled water (\(F_{\text{IH}}^* = F_{\text{IN}} + F_{\text{IN}}^*\)) provided \(F_{\text{IN}}^* = F_{\text{IN}}\). The fluxes across the pores filled with nitrogen and helium can be related as \(F_{\text{IN}} = F_{\text{IN}}^* / R^*\). The parameter \(R^*\) is the ratio of fluxes of labelled water in an empty chamber filled with helium and nitrogen, which is the same as \(D_{\text{wH}}^* / D_{\text{wN}}\) (see equation 3 written for labelled water). Substitution for \(F_{\text{IN}}^*\) into the (equation 2–equation 1) relation given above yields \(F_{\text{IH}}^* = F_{\text{IH}}^* \times (F_{\text{IN}}^* / R^* – 1)\). \(F_{\text{IN}}^*\) is the portion of the total flux which has to be removed (subtracted) first, then multiplied with the \(D_{\text{wH}}^* / D_{\text{wN}}\) factor, and added again to obtain the flux of non-labelled water: \(F_{\text{IH}}^* = F_{\text{IH}}^* \times (F_{\text{IN}}^* / R^* – 1)\) for labelled water.

Appendix

Estimation of diffusion coefficient of \(^3\text{H}_2\text{O}\) water in helium saturated with water, \(D_{\text{wH}}^*\)

As explained above, diffusion coefficients for labelled water vapour in nitrogen, \(D_{\text{wN}}^*\), and for labelled water in helium, \(D_{\text{wH}}^*\), were experimentally estimated. The measurements were carried out with the chambers shown in Fig. 2, but without membranes between the donor and receiver compartments. The distance \(l\) in the gas phase between the donor surface and receiver was 16 mm. The gas at the surface of the donor solution was saturated with water containing significant amounts of \(^3\text{H}_2\text{O}\) molecules; the receiver had a concentration of \(^3\text{H}_2\text{O}\) molecules close to zero. Thus, the donor–receiver concentration difference of \(^3\text{H}_2\text{O}\) was equivalent to a water concentration difference of 0.961 mol m\(^{-2}\) at 20 °C (Nobel, 1991). Under these conditions, it was estimated that the average flow of water vapour in nitrogen was 1.416 mmol m\(^{-2}\) s\(^{-1}\). If the diffusion coefficient is calculated as flux multiplied by length \((16 \text{ mm})\) and divided by the concentration difference (all in the appropriate units), it is obtained for \(D_{\text{wN}}^* = 2.36 \times 10^{-5} \text{ m}^2 \text{ s}^{-1}\). Average flux of tritiated water in helium was 2.922 mmol m\(^{-2}\) s\(^{-1}\) which from a similar calculation yields \(D_{\text{wH}}^* = 4.87 \times 10^{-5} \text{ m}^2 \text{ s}^{-1}\). The value of \(D_{\text{wH}}^*\) at 20 °C is 2.42 \times 10^{-5} \text{ m}^2 \text{ s}^{-1} (Nobel, 1991) which is close to the estimated value of \(D_{\text{wN}}^* = 2.36 \times 10^{-5} \text{ m}^2 \text{ s}^{-1}\) and, for nitrogen, it is assumed that \(D_{\text{wN}}^* = D_{\text{wN}}\). So, in nitrogen, the tracer (tritiated water) directly measures transport of non-labelled water. For helium, however, the tabulated value of the binary diffusion of water is \(D_{\text{wH}}^* = 9.08 \times 10^{-5} \text{ m}^2 \text{ s}^{-1}\) at 25 °C (Cussler, 1987) and, with \(3\%\) correction for a temperature difference of 5 °C, \(D_{\text{wH}}^* = 8.81\) at 20 °C. The estimate of the tracer diffusion coefficient here is \(D_{\text{wH}}^* = 4.87 \times 10^{-5} \text{ m}^2 \text{ s}^{-1}\). Therefore, to obtain the real flux of water in equation A3, the factors \(D_{\text{wH}}^* / D_{\text{wN}}^* = 1.809\) and \(R^* = D_{\text{wH}}^* / D_{\text{wN}} = 2.064\) have been used. Now, the ratio can be evaluated as \(D_{\text{wH}}^* / D_{\text{wN}} = 0.881 / 0.236 = 3.733\). This \(R^*\) value was used in equations 5a, and 5b for all calculations of relative \(F_{\text{IC}}\) and \(F_{\text{LC}}\).
References


