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Water Permeability of Isolated Cuticular Membranes: The Effect of Cuticular Waxes on Diffusion of Water

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Summary. The water permeability of astomatous cuticular membranes isolated from *Citrus aurantium* L. leaves, pear (*Pyrus communis* L.) leaves and onion (*Allium cepa* L.) bulb scales was determined before and after extraction of cuticular waxes with lipid solvents. In pear, the permeability coefficients for diffusion of tritiated water across cuticular membranes (CM) prior to extraction [P_d(CM)] decreased by a factor of four during leaf expansion. In all three species investigated P_d(CM) values of cuticular membranes from fully expanded leaves varied between 1 to 2×10^{-7} cm^{-3} s^{-1} · P_d(CM) values were not affected by pH. Extraction of cuticular waxes from the membranes increased their water permeability by a factor of 300 to 500. Permeability coefficients for diffusion of THO across the cutin matrix (MX) after extraction [P_d(MX)] increased with increasing pH. P_d values were not inversely proportional to the thickness of cuticular membranes. By treating the cutin matrix and cuticular waxes as two resistances acting in series it was shown that the water permeability of cuticles is completely determined by the waxes. The lack of the P_d(CM) values to respond to pH appeared to be due to structural effects of waxes in the cutin matrix. Cuticular membranes from the submerse leaves of the aquatic plant *Potamogeton lucens* L. were three orders of magnitude more permeable to water than the cuticular membranes of the terrestrial species investigated.

Introduction

Swelling and pore volume of isolated cuticular membranes devoid of cuticular waxes have recently been shown to depend on pH and the kind of cations (Schönherr, 1976). Swelling increased with increasing pH and was much greater when the cutin matrix was in the Na^+ than in the Ca^{2+} form. The radius of the water filled pores in the cutin matrix was estimated to be 0.46 nm and was independent of pH. With increasing pH the number of pores increased but not their radii. This paper deals with the effect of cuticular waxes on swelling and water permeability of isolated cuticular membranes.

Abbreviations: CM = cuticular membrane; MX = cutin matrix; WAX = waxes

Materials and Methods

Cuticular Membranes

Astomatous cuticles were isolated from the adaxial leaf surfaces of *Citrus aurantium* L. and pear (*Pyrus communis* L. cv. Köstliche aus Charneu), from both surfaces of *Potamogeton lucens* L. leaves and from the inner bulb scales of onion (*Allium cepa* L.).

Small disks (10 to 20 mm in diameter) were punched from the leaves and incubated at 38° C in a mixture of 4% pectinase and 0.4% cellulase (*w/v*) (ICN Pharmaceuticals, Inc., Cleveland, Ohio) adjusted to pH 3.8 (Orgell, 1955). After 48 h the cuticular membranes could be separated from the outer wall of the epidermis. Adhering cellular debris was removed with a jet of water and the cuticular membranes converted into the H^+ form by three successive treatments with 1 N HCl for 10 min each followed by washing with deionized water to remove all sorbed HCl. Cuticular waxes were extracted from selected cuticles by placing them at room temperature in methanol (3 changes), one part methanol and one part chloroform (3 changes), and chloroform (5 changes) for 10 min each. All cuticular membranes were air dried, mounted between silicon rubber gaskets as described elsewhere (Schönherr, 1976) and stored for further use.

As cuticles of *Potamogeton* and onion are rather fragile it was more convenient to mount them prior to isolation. Disks of leaves or epidermis were glued with uncured silicon rubber on silicon rubber gaskets having a center hole of 2 to 4 mm diameter. After isolation and drying a second silicon rubber gasket of identical dimensions was mounted on top of the cuticle, carefully matching the holes in the two gaskets.

Determination of THO Diffusion

Details concerning the transport apparatus, tests for membrane integrity and experimental procedures have previously been de-

scribed (Schönherr, 1976). Briefly, cuticular membranes mounted between silicon rubber gaskets were inserted between the two compartments of a transport apparatus and 10 ml of identical buffer solutions (between pH 3 and 7 0.01 M citric acid – Na_2PO_4, at pH 9 0.01 M disodiumtetraborate adjusted with HCl) were added into each compartment. THO was added to the inner solution (solution facing the morphological inner side of the cuticle) resulting in a specific activity of 7×10^7 dpm ml^{-1}. At zero time and at 15 min intervals a 1 ml sample was withdrawn from the outer solution and the radioactivity determined by scintillation counting. Each time a sample had been taken "cold" buffer was added to the receiver side to maintain constant volume. At least six successive samples were taken and the rate of diffusion (dn/dt in cpm s^{-1}) was determined under steady state conditions by the method of least squares. The permeability coefficient of diffusion (P_d) was determined from the equation

$$P_d = \frac{dn}{dt} \cdot \frac{1}{C^*} \quad (1)$$

where C^* is the concentration of THO (in cpm cm^{-3}) in the donor. For brevity the presence or absence of cuticular waxes in the membranes used for the determination of P_d will be indicated by the suffix (CM) or (MX), respectively. Thus P_d(CM) means that the cuticular membrane still contained cuticular waxes, while P_d(MX) refers to coefficients obtained using membranes from which waxes had been extracted.

Determination of Osmotic Water Permeability and Pore Radius.

The volume flux of water (J_v in cm^3 s^{-1}) caused by a concentration gradient of solute (ΔC_s) to which the cuticular membranes are impermeable was determined using identical buffer solutions in both conpartments of the transport apparatus and a raffinose concentration of 0.25 molal in the outer solution. The increase in volume of the outer solution was read directly from a calibrated capillary connected to the compartment containing the outer solution. The hydrodynamic coefficient of the membranes (L_p) was determined using equation

$$J_v = -L_p RT \Delta C_s \quad (2)$$

where R and T are the gas constant and absolute temperature, respectively.

The radius of the polar pores (r_p) in the cutin matrix was calculated from the equation

$$r_p = -a\left[2a^2 + \frac{8\eta L_p}{A_w/\Delta x}\right]^{1/2} \quad (3)$$

derived by Paganelli and Solomon (1957); here η = viscosity of the solution, a = radius of the water molecules (taken to be 0.197 nm) and $A_w/\Delta x = P_d/D$ (D = self diffusion coefficient of water taken to be 2.44×10^{-5} cm^2 s^{-1}). For details see Schönherr (1976).

Results

Extraction of cuticular waxes from cuticles of fully expanded *Citrus* leaves increased their water permeability 350 to 500 fold. P_d(CM) values were not significantly different at the two pH values used, while P_d(MX) values were higher at pH 9 than at pH 3 (Table 1).

Very similar results were obtained for cuticular membranes of fully expanded pear leaves. The absolute values of the permeability coefficients are similar to those obtained for cuticles from *Citrus* leaves (Table 2).

Cuticular membranes isolated from young, expanding pear leaves exhibited a 4 fold decrease in P_d(CM) during leaf expansion (Fig. 1) while the permeability of the cutin polymer matrix, P_d(MX), was not significantly altered. The thickness of the cuticular

Table 1. The effect of pH and extraction of cuticular waxes on diffusion of THO across cuticular membranes isolated from fully expanded leaves of *Citrus*

pH	P_d(CM) $\times 10^7$ cm^{3}s^{-1}	P_d(MX) $\times 10^5$ cm^{3}s^{-1}	P_d(MX)/P_d(CM)
3.0	1.06 (0.39)	3.73 (0.46)	352
9.0	1.75 (0.76)	8.63 (1.57)	493

Values are averages of one determination on three membranes per leaf and 9 successive leaves of a single shoot. The youngest leaf was just fully expanded. Six membranes were isolated per leaf and 3 selected at random and subjected to extraction. Standard deviation given in parenthesis.

Table 2. The effect of pH and extraction of cuticular waxes on diffusion of THO across cuticular membranes isolated from fully expanded pear leaves

pH	P_d(CM) $\times 10^7$ cm^{3}s^{-1}	P_d(MX) $\times 10^5$ cm^{3}s^{-1}	P_d(MX)/P_d(CM)
3.0	0.78 (0.32)	2.47 (0.36)	317
5.0	0.86 (0.32)	2.64 (0.59)	307
7.0	0.75 (0.39)	3.63 (0.47)	484
9.0	1.01 (0.48)	5.63 (0.75)	557

Values are averages of duplicate determinations on three membranes taken from a fully expanded pear leaf. Standard deviation given in parenthesis.

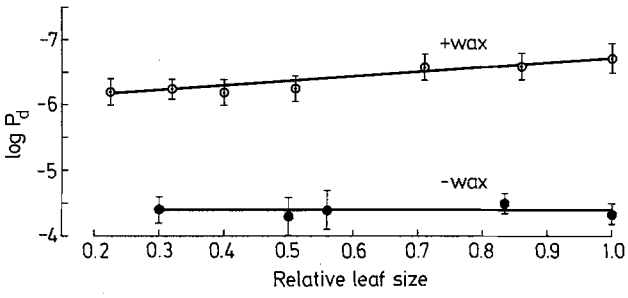

Fig. 1. The effect of relative leaf size and cuticular waxes on THO diffusion across isolated pear leaf cuticular membranes. Each point represents the average of one determination on 3 membranes per leaf. Twelve leaves of two shoots from the same tree were analyzed. Relative leaf size was calculated as the ratio of the area of immature leaf divided by the mean area of the first 3 fully expanded leaves. The vertical bars represent the standard deviation

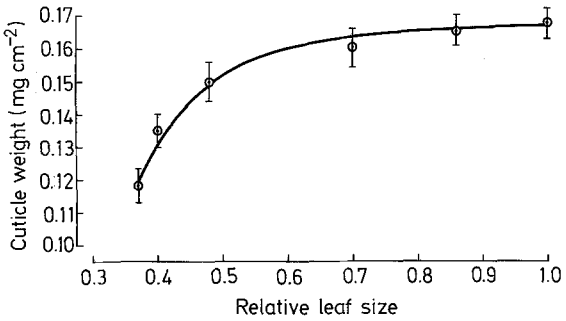

Fig. 2. The effect of relative leaf size on cuticle development. Data taken from the cuticles used in the experiment presented in Fig. 1. The vertical bars represent the standard deviation

Table 3. The effect of extraction of cuticular waxes on THO diffusion across the epidermis and isolated cuticular membranes of onion bulb scales at pH 9

Membrane	P_d(CM) $\times 10^7$ cm^{3}s^{-1}	P_d(MX) $\times 10^4$ cm^{3}s^{-1}	P_d(MX)/P_d(CM)
Epidermis	2.08 (0.99)	1.19 (0.21)	570
Cuticle	2.17 (1.08)	0.98 (0.10)	452

Values are averages of duplicate determinations on 6 membranes taken from the third bulb scale. THO diffusion across the entire epidermis (either before or after extraction of waxes) was determined first. Thereafter the cuticles were isolated from the epidermal cells and outer cell walls by incubation with enzymes for 24 h and the rate of THO diffusion was determined again. Standard deviation given in parenthesis.

membranes (measured as weight per unit area) increased during leaf expansion (Fig. 2).

Cuticular membranes of onion bulb scales had P_d(CM) values similar to that of *Citrus* and pear leaves. Removal of waxes from the cuticular membranes increased the water permeability by approximately 500 fold. There was no significant difference between water permeability of isolated cuticles and the entire epidermis (Table 3).

The water permeability of cuticular membranes from Potamogeton leaves was approximately 3 orders of magnitude higher than that of the terrestrial species investigated. Again, there was no significant effect of pH on permeability (Table 4). The water permeability of the entire leaf (consisting of two cuticles and three layers of cells together 60 µm thick) was lower than that of isolated cuticular membranes. Leaves with viable mesophyll cells had the same permeability as leaves whose mesophyll cells had been killed with NaN_3.

The cutin polymer matrix of *Citrus* and onion cuticular membranes both had pore radii of 0.41 nm even though the polymer matrix of onion cuticles had a water permeability almost six times higher that that

Table 4. The effect of pH on diffusion of THO across isolated *Potamogeton* leaf cuticles and whole leaves

Membrane	pH	Permeability coefficient cm^{3}s^{-1}
Cuticle	3.0	2.29×10^{-4} (0.40×10^{-4})
	7.0	2.55×10^{-4} (0.34×10^{-4})
	9.0	2.76×10^{-4} (0.47×10^{-4})
Leaf[a]	7.0	8.35×10^{-5} (1.33×10^{-5})
Dead leaf[b]	7.0	8.67×10^{-5} (1.63×10^{-5})
Mesophyll[c]	7.0	2.42×10^{-4}

[a] Fresh leaves were mounted between silicon rubber gaskets, inserted between the two compartments of the transport apparatus without alowing them to dry and THO diffusion was determined.
[b] The same leaves as above, but treated for 24 h with NaN_3 (10^{-3} M)
[c] Calculated from the equation $1/P_d(\text{LEAF}) = 1/P_d(\text{CM}) + 1/P_d(\text{MESOPHYLL}) + 1/P_d(\text{CM})$. The thickness of the mesophyll layer was 60 µm
Values are averages of duplicate determinations on 3 cuticular membranes or leaves, respectively. Standard deviation given in parenthesis

Table 5. L_p, P_d and the radii of polar pores in the cutin polymer matrix of isolated cuticular membranes at pH 7

Species	L_p(MX) $\times 10^{13}$ cm^{3}dyn^{-1}s^{-1}	P_d(MX) $\times 10^4$ cm^{3}s^{-1}	r_p nm
Allium	5.76 (0.57)	3.88 (0.48)	0.41 (0.04)
Citrus	0.99 (0.03)	0.67 (0.20)	0.41 (0.07)

Values are averages of duplicate determinations on three membranes. Standard deviation given in parenthesis.

of *Citrus* cuticles (Table 5). Attempts failed to measure the osmotic water permeability of cuticular membranes containing waxes. Water fluxes caused by a gradient of osmotic pressure were too small to be measured with the apparatus available.

Discussion

The water permeability of cuticular membranes of all three terrestrial species was low. A measure for the protection against water loss provided by these cuticles can be obtained by comparing experimental P_d(CM) values with the P_d value of a water layer of equal thickness. Cuticular membranes of *Citrus*, pear and onion were approximately 6 orders of magnitude less permeable than a water layer of equal thickness (Table 6, last column). Differences between species are not significant as the Δx estimates are only rough approximations. Cuticular membranes from the leaves of the aquatic plant *Potamogeton* also restricted diffusion of water, but to a much lower degree.

Table 6. The contributions of the permeabilities of the cutin polymer matrix [P_d(MX)] and the cuticular waxes [P_d(WAX)] to the total permeability [P_d(CM)] of isolated cuticular membranes

Species	Δx[a] cm	P_d(CM) $cm^3 s^{-1}$	P_d(MX) $cm^3 s^{-1}$	P_d(WAX)[b] $cm^3 s^{-1}$	$\frac{P_d(HOH)^c}{P_d(CM)}$
Citrus	2.5×10^{-4}	1.75×10^{-7}	8.63×10^{-5}	1.753×10^{-7}	5.57×10^{5}
Pyrus	2.0×10^{-4}	1.10×10^{-7}	5.63×10^{-5}	1.012×10^{-7}	1.21×10^{6}
Allium	0.5×10^{-4}	2.17×10^{-7}	0.98×10^{-4}	2.175×10^{-7}	2.24×10^{6}
Potamogeton	0.2×10^{-4}	2.76×10^{-4}	–	–	4.42×10^{3}

[a] Thickness of *Citrus* and pear leaf cuticular membranes calculated from the weight per unit area, assuming a specific gravity of 1.1 $g\,cm^{-3}$; Δx of onion cuticle measured from cross sections using a light microscope; thickness of *Potamogeton* assumed, because the cuticle could not be resolved using cross sections and a light microscope

[b] Calculated from Equation (5)

[c] $P_d(HOH) = DA_w/\Delta x$, $= 2.44 \times 10^{-5}\ cm^4 s^{-1}/\Delta x$

The fluxes observed did not conform to the modified Fick equation

$$\frac{dn}{dt} = -\frac{DA_w\vartheta}{\Delta x}\Delta C^* \tag{4}$$

where A_w = area of the membrane permeable to water, Δx = membrane thickness and ϑ = tortuosity factor. Equation (4) predicts that the flux should be inversely proportional to the membrane thickness (assuming ϑ to be fairly constant). Reference to Table 6 shows that P_d(CM) values for the terrestrial species were constant, while Δx varied by a factor of 5. Even though the thickness of the cuticle of *Potamogeton* is not known (its thickness being below the resolution of the light microscope) it is presumably not three orders of magnitude thinner than that of onion bulb scales. Likewise, extraction of cuticular waxes from the membranes did certainly not reduce their thickness by 300 to 500 times (Table 1 to 3). The weights of the cuticular membranes used were approximately 320 (*Citrus*) and 250 $\mu g\,cm^{-2}$ (pear). The wax contents of cuticles of a variety of citrus species were reported to vary between 54 and 99 $\mu g\,cm^{-2}$ (Baker et al., 1975; Baker and Procopiou, 1975). For the adaxial pear leaf cuticle a figure of 109 $\mu g\,cm^{-2}$ was reported (Norris and Bukovac, 1968). Finally, pear leaf cuticular membranes increased in thickness during leaf expansion by a factor of 2, while P_d(CM) decreased by a factor of 4 and P_d(MX) did not show any significant variation at all (Fig. 1).

Clearly, water permeability of isolated cuticular membranes cannot be explained on the basis of thickness of the membranes. By comparing the rates of cuticular transpiration of leaves from a great variety of plant species with the thickness of the cuticles Kamp (1930) had come to the same conclusion.

Plant cuticles are polymer membranes of heterogeneous composition. They consist of a cutin polymer matrix which often contains varying proportions of other non-extractable components such as cellulose, polyuronic acids, proteins and phenolic compounds (Huelin, 1959; Martin and Juniper, 1970; Schönherr and Bukovac, 1973; Schönherr, 1976). Extractable lipids (usually referred to as waxes) are embedded in the cutin matrix and deposited superficially in all cuticular membranes (Norris and Bukovac, 1968; Martin and Juniper, 1970; Kolattukudy and Walton, 1972; Baker et al., 1975; Baker and Procopiou, 1975).

As a first approximation one may therefore consider cuticular membranes to be made up of two components: the extractable waxes and the non-extractable polymer matrix. The contribution of the waxes to the overall water permeability of cuticular membranes can be estimated by treating the polymer matrix and the waxes as two resistences acting in series. From the equation

$$\frac{1}{P_d(CM)} = \frac{1}{P_d(MX)} + \frac{1}{P_d(WAX)}. \tag{5}$$

P_d(WAX) can be calculated as the other two quantities are known from the experiments.

Equation (5) implies that cutin matrix and waxes occur in alternate layers normal to the direction of water transport. Studies with polarized light revealed in cuticles layers of crystalline waxes oriented parallel to the surface (Roelofson, 1952; Sitte and Rennier, 1963; Norris and Bukovac, 1968).

P_d(CM) is completely determined by P_d(WAX), the contribution of the polymer matrix to the overall resistence being negligible (Table 6). This is the reason why the water permeability of cuticular membranes (which consist mainly of polymer matrix) is independent of membrane thickness. The effect of waxes in reducing water permeability of cuticles (Skoss, 1955; Baker and Bukovac, 1971) or artificial membranes

(Grncarevic and Radler, 1967) has been reported before.

The decrease in P_d(CM) during leaf expansion observed with pear leaf cuticular membranes (Fig. 1) is probably due to the incorporation of waxes into the cutin matrix during this period. P_d(CM) values were not significantly affected by pH. This is in contrast to the results obtained using cuticular membranes from which waxes had been extracted with lipid solvents (Table 1 and 2; Schönherr, 1976). P_d(MX) values generally increased 2 to 3 fold between pH 3 and 9. It was previously shown that this increase in permeability is due to a higher water content of the polymer matrix caused by the dissociation of $-$COOH groups fixed to the cutin matrix (Schönherr, 1976). These groups are accessible to water and inorganic cations even in cuticular membranes containing waxes, since cation exchange capacity and isoelectric point were identical before and after extraction of waxes (unpublished results). The absence of an effect of pH on P_d(CM) confirms that Equation (5) is a valid description of the system. The water permeability of a non-ionic wax layer cannot be expected to vary with pH. Since P_d(WAX) completely determines P_d(CM) and since P_d(WAX) should be pH-independent, P_d(CM) should also be pH-independent. This was in fact observed (Tables 1 and 2).

The failure to observe any significant pH effect on P_d(CM) is in contrast to the results of Härtel (1947) showing a pH dependence of cuticular transpiration with a maximum near neutrality. Even though cuticles of species used by Härtel were not included in this study, serious doubts arise that the pH effect Härtel observed is a reflection of pH dependent changes in water permeability of the cuticle. As pointed out by Stålfelt (1956) the use of entire leaves in studying cuticular transpiration is subject to criticism because of the presence of stomata.

The polymer matrix of cuticular membranes from *Citrus* leaves and onion bulb scales contains polar pores of an equivalent radius of 0.41 nm (Table 5). This is in good agreement with a figure of 0.46 nm previously reported for the cutin matrix of *Citrus* leaves (Schönherr, 1976). It appears that the radius of the equivalent pores is independent of the thickness of the membranes. Since both L_p(MX) and P_d(MX) of the cuticle of onion bulb scales are 5.8 times larger than the respective values for *Citrus* leaf cuticles, one might conclude that the onion cuticular membranes have 5.8 time more pores per cm^2 than those of *Citrus* leaves. According to Equation (4) the number of pores can be calculated by dividing P_d by D (assuming that the diffusion coefficient of water in the membrane pores is the same as in bulk liquid, in spite of the fact that the activation energy for water diffusion across cuticles is 3 times higher than for diffusion in bulk liquid (Schönherr, 1976)) and multiplying the result by Δx (assuming $\vartheta = 1$). Assuming the pores to be circular in cross section the number of pores (n) per cm^2 of membrane can be calculated from the equation $A_w = n\pi r_p^2$. D may be different in the membrane pores than in bulk water, but it is probably similar in the pores of cuticular membranes from *Citrus* leaves and onion bulb scales, particularly since the pores have the same dimension. Therefore, differences observed in P_d may not be attributed to differences in D. The assumption $\vartheta = 1$ is most likely in error. The tortuosity factor was shown to depend on the water content of the membrane (ϑ greater the higher the water content) and the size of the permeating molecule (ϑ greater the larger the molecule) (Ginzburg and Katchalsky, 1963). For a dializing membrane (volume fraction of water in the membrane 0.68) a value of 0.34 was reported for water transport (Ginzburg and Katchalsky, 1963), that is, the diffusion path of the water molecules was approximately three times longer, than the thickness of the membrane.

For determination of ϑ the water content of the membranes must by estimated by a method independent of P_d. The water content of cuticular membranes determined from THO diffusion is extremely low (Schönherr, 1976) and all attempts to estimate it by a method independent from P_d failed. A tortuosity factor could therefore not be obtained. It is most likely smaller than 0.34 and some or even all of the variation in P_d(MX) and L_p(MX) shown in Table 5 may be due to a variation in ϑ, while the number of pores varies very little. The problem cannot be solved until the water content of cuticular membranes can be estimated by independent means.

As pointed out to me by Dr. E.A. Baker (personal communication) the same problem arises in the interpretation of the pH effect on P_d(MX). The increase in P_d(MX) with increasing pH cannot be attributed solely to an increase in the number of pores (Schönherr, 1976) as the possibility of an increase in ϑ with pH cannot be precluded.

The cuticular membranes of submerse leaves of *Potamogeton* are very different from those of terrestrial plants. The P_d(CM) values are of the same order of magnitude as the P_d(MX) values of the terrestrial plants. Attempts to extract isolated cuticular membranes with lipid solvents failed (the membranes broke). Therefore, the question whether this high permeability of *Potamogeton* cuticles is due to the absence of cuticular waxes or not cannot be answered yet. That the isolated membranes were in fact cuticles is indicated by the fact that they did not dissolve in a solution of $ZnCl_2$ in concentrated HCl (1.7 g $ZnCl_2$ per ml HCl) (Martin and Juniper, 1970).

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