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Characterization of aqueous pores in plant cuticles and permeation of ionic solutes

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Abstract

Plant cuticles are lipid membranes with separate diffusion paths for lipophilic non-electrolytes and hydrated ionic compounds. Ions are lipid insoluble and require an aqueous pathway across cuticles. Based on experimental data, the aqueous pathway in cuticles has been characterized. Aqueous pores arise by hydration of permanent dipoles and ionic functional groups. They can be localized using ionic fluorescent dyes, silver nitrate, and mercuric chloride. Aqueous pores preferentially occur in cuticular ledges, at the base of trichomes, and in cuticles over anticlinal walls. Average pore radii ranged from 0.45 to 1.18 nm. Penetration of ions was a first order process as the fraction of the salt remaining on the cuticle surface decreased exponentially with time. Permeability of cuticles to ions depended on humidity and was highest at 100% humidity. Wetting agents increased rate constants by factors of up to 12, which indicates that the pore openings are surrounded by waxes. The pores in cuticular ledges of *Helxine soleirolii* allowed passage of berberine sulphate, which has a molecular weight of 769 g mol^{-1}. Increasing the molecular weight of solutes from 100 to 500 g mol^{-1} decreased the rate constants of penetration by factors of 7 (*Vicia faba*) and 13 (*Populus canescens*), respectively. Half-times of penetration of inorganic salts and organic ions across *Populus* cuticles and *Vicia* leaf surfaces varied between 1 and 12 h. This shows that penetration of ionic compounds can be fairly rapid, and ions with molecular weights of up to 800 g mol^{-1} can penetrate cuticles that possess aqueous pores.

Key words: Aqueous pores, chelates, ectodesmata, elicitors, foliar nutrition, gene expression, glyphosate, ion permeability, lipophilic pathway, pesticides, polar pathway, pore size, promoters, stomata, transpiration, trichomes, *Vicia faba*, water permeability.

Introduction

Aerial primary organs of higher plants are covered by cuticles. They form the interface between plant and environment, and protect plants from adverse environmental stresses. Barrier properties of cuticles are vital for the survival of terrestrial plants. They have been studied by scientists from diverse fields. Botanists and ecologists are interested in water stress and have studied transpiration and water permeability of plant cuticles (Schönherr, 1982; Kerstiens, 1996*a*, *b*; Schreiber and Riederer, 1996). Phytopathologists have dealt with various aspects of cuticles as infection barriers for viruses, bacteria, and fungi (Mendgen, 1996). Cuticles have been studied as habitats for epiphyllic micro-organisms (Schönherr and Baur, 1996; Schreiber *et al.*, 2005*b*). Environmental chemists focused on the accumulation of xenobiotics in cuticles and plants from the environment as this is the prime route of entrance of highly lipophilic chemicals into the food chain of humans and animals (Riederer, 1995). Cuticular penetration of effectors that bind to promoters which regulate gene expression has great relevance to molecular biologists (Gatz and Lenk, 1998; Jepson *et al.*, 1998). More recently, molecular and developmental biologists observed that signals from the cuticle affect epidermal cell differentiation, such as initiation, development, and density of stomata and

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Abbreviations: APG, alkylpolyglycosides; BS, berberine sulphate; *C*, concentration; CM, cuticular membrane; *D*, diffusion coefficient; 2,4-D, 2,4-dichlorophenoxyacetic acid; E_a, energy of activation; *J*, flux; *K*, partition coefficient; *k*, rate constant of penetration; *M*, amount; MX, polymer matrix; *P*, permeance or permeability coefficient; p/p_o, partial pressure; POD, point of deliquescence; SOFU, simulation of foliar uptake; $t_{1/2}$, half-time.

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trichomes (Bird and Grey, 2003) in the epidermis of leaves and fruits.

For decades, researchers have tried to improve the efficacy of pesticides and to minimize residues in crop plants by enhancing cuticular penetration of pesticides and other agrochemicals (Roberts *et al.*, 1948; Currier and Dybing, 1959; Crafts and Foy, 1962; Hartley and Graham-Bryce, 1980; Schönherr and Baur, 1994; Baur, 1998; Kirkwood, 1999). Most studies dealing with penetration across plant cuticles were purely descriptive rather than analytical, and this is one of the reasons why water and solute penetration are still not fully understood. Another reason is the complexity of the chemistry and structure of plant cuticles. Plant cuticles are permeable to ions and other polar compounds, and it has been suggested that two parallel pathways in cuticles are responsible for the transport of lipophilic and hydrophilic substances, respectively (Roberts *et al.*, 1948; Currier and Dybing, 1959; Crafts and Foy, 1962; Foy, 1964). While the lipophilic path was attributed to cutin and waxes, the nature of the polar, hydrophilic, or aqueous pathways remained obscure. Characterization of polar pathways in plant cuticles and their role in plant growth regulation, plant protection, foliar nutrition, and interactions between terrestrial plants and their environments will be the topic of this review.

Classification of polar solutes and polar functional groups in cuticles

The concept of two parallel pathways in plant cuticles implies chemical and structural heterogeneity. Before this aspect can be addressed, a look at the physico-chemical properties of polar solutes and polar constituents in cuticles should be helpful.

Solutes are classified according to their solubility in water and in organic solvents. Differential solubility in the membrane matrix and in water is expressed as the partition coefficient (K). K is the ratio of the molal concentrations (C) of a solute in a lipid phase and in water:

$$K = \frac{C_{\text{lipid}}}{C_{\text{water}}} \quad (1)$$

Lipophilic solutes are more soluble in lipid than in water, hence $K > 1$. The octanol/water partition coefficient (K_{ow}) or the cuticle/water partition coefficient (K_{cw}) have been used to characterize differential solubility. K_{ow} is more popular since for a large number of solutes K_{ow} has been determined experimentally. If experimental values are not available, K_{ow} or K_{cw} can be estimated using various methods (Kerler and Schönherr, 1988*a*, *b*; Brudenell *et al*., 1995; Sangster, 1997). At low concentrations, K_{ow} and K_{cw} are very similar but, when concentrations in cuticles are extremely high, the partition coefficient decreases and may approach unity because sorption sites in cuticles are limited (Riederer and Schönherr, 1986).

Most polar molecules are non-electrolytes and carry no net charge, but many possess an electric dipole. For instance, in water, the oxygen atom is strongly electronegative and tends to draw electrons away from the hydrogen atoms. This imparts to the oxygen a partial net negative charge and to the hydrogens a partial net positive charge. In nearby water molecules, these positively charged hydrogen atoms are electrostatically attracted to negatively charged oxygen atoms. This leads to hydrogen bonding between molecules with a bond energy of $\sim$20 kJ mol^{-1}. Molecules having permanent dipoles are called polar molecules. Dipole–dipole interactions occur between solutes and functional groups of cuticles with permanent dipoles, but their dipole moments depend on the environment. Permanent dipoles occur only in asymmetric molecules. Hence, the dipole moments of alkanes, benzene, and CO_2 are zero. Water has a permanent dipole of 1.85, while the dipole moments of methanol, ethanol, hexanol, octanol, and acetic acid are 1.7 Debye units. Permanent dipoles lead to extensive hydrogen bonding between water molecules but also with other polar molecules. Characteristic dipole moments can also be assigned to bonds and groups. The bond moments for C-H$^+$ are 0.4, for O-H$^+$ 1.51, and for C=O$^+$ 2.3–2.7 Debye units. Examples for group moments are C-$^+$OH (1.65), C-$^+$NH$_2$ (1.2–1.5), C-$^+$CH$_3$ (0.4), C-$^+$COOH (1.7), and C$^+$- NO$_2$ (3.1–3.8) (Israelachvili, 1992). All of these bonds and groups occur in cuticles. The magnitude of hydration is related to the dipole moment of these groups. However, the dipole–dipole interaction is usually not strong enough to lead to any strong mutual alignment of polar molecules in the liquid state.

The second type of electrostatic interaction is that between a charged ion and a polar molecule, for instance between Na$^+$ and water. The ion–dipole interaction is much stronger than the dipole–dipole interaction; it is equal to or stronger than the product of the Boltzmann constant and the temperature (kT), which amounts to 96.5 kJ mol^{-1} at 25 °C. This high energy has a strong aligning effect on water molecules surrounding an ion. The number of water molecules orientationally bound to an ion is called the hydration number. The hydration number increases with valency of the cations, and is generally much larger for cations than anions. Hydration numbers for monovalent cations (Na$^+$, K$^+$) range from 3 to 5, while six water molecules are bound to Ca^{2+} and Mg^{2+}. NO_3^- and N(CH$_3$)$^{4+}$ are not hydrated, while the hydration number for Cl$^-$ is only 1. Hydration increases the effective radius of an ion in water (Israelachvili, 1992).

Water molecules bound to ions are not completely immobilized; they exchange with the bulk water. At room temperature, water molecules tumble about with a mean reorientation time or rotational correlation time of $\sim 10^{-11}$ s^{-1}. For monovalent cations, the residence times of water molecules in the primary hydration shells are $\sim 10^{-9}$ s^{-1}, while with Ca^{2+} and Mg^{2+} rotational correlation times are 10^{-8} and 10^{-6} s^{-1}, respectively. With trivalent

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cations (Al^{3+}, La^{3+}, and Cr^{3+}), rotational correlation times range from seconds to hours. In these cases, binding is so strong that an ion–water complex of fixed stoichiometry is formed.

Chemical and structural heterogeneity of plant cuticles

Plant cuticles are solid-state lipid membranes made up of cutin, cutan, waxes, and some polysaccharides. Cutin is a polymer composed of cross-linked hydroxy fatty acids. It is insoluble in organic solvents (Holloway, 1982*a*, *b*). Waxes are complex mixtures of aliphatic alkanes, alcohols, fatty acids, and esters with 16–50 carbon atoms (Baker, 1982). In addition, they often contain triterpenes and other cyclic lipophilic constituents. Their melting points are usually above 60 °C (Sitte and Rennier, 1963) and they are soluble in organic solvents such as chloroform. Cuticles always contain small amounts of cellulose and pectins, but these polar constituents are confined to the inner cuticular layer, which is in contact with the epidermal wall. The outer part of the cuticle facing the air is called the cuticle proper, and it is believed to be free of polar polysaccharides derived from the cell wall. This view is backed by numerous studies using polarized light (Sitte and Rennier, 1963) and electron microscopy (Holloway, 1982*a*; Jeffree, 1996). The cuticle proper is a pure lipid phase composed of cutin and waxes, while the cuticular layer underneath contains increasing amounts of polar materials that reach a maximum at the cell wall–cuticle interface. In cross-sections of cuticles, this transversal heterogeneity is seen as a gradient in staining with osmium tetroxide, uranyl acetate, or lead citrate (Holloway, 1982*a*; Jeffree, 1996).

When isolated cuticles containing sorbed 2,4-dichlorophenoxyacetic acid (2,4-D) are simultaneously desorbed from the outer and inner surfaces of the cuticle, the bulk of 2,4-D is desorbed very rapidly from the inner surface while only a few per cent are desorbed from the outer surface. This is another manifestation of transversal heterogeneity showing that the outer layer of the cuticle represents the major resistance to diffusion. This part of the cuticle has been termed limiting skin or limiting layer, and in the cuticles studied it amounted to ~10% of the total mass of the cuticle (Schönherr and Riederer, 1988). The remainder of the cuticle (cuticular layer), in which the mobility of 2,4-D was much greater than in the limiting skin, is called the sorption compartment (Schönherr and Riederer, 1988; Buchholz, 2006).

The sorption compartment can be loaded with radiolabelled solutes, which are subsequently desorbed unilaterally from the outer surface. In this way, the mobility of solutes in the limiting skin can be quantified (Bauer and Schönherr, 1992; Buchholz, 2006). The method is called unilateral desorption from the outer surface (UDOS) and it has been used extensively to study effects of solute size, temperature, and adjuvants on solute mobility (Bauer and Schönherr, 1992, 1995; Schönherr and Baur, 1994; Buchholz *et al*., 1998; Buchholz and Schönherr, 2000). Unfortunately, the method is limited to astomatous isolated cuticular membranes (CMs) and to relatively lipophilic solutes.

Most leaves have various kinds of trichomes on both surfaces, and stomata occur at least on the abaxial surface of the leaves (Bird and Gray, 2003; Glover and Martin, 2003). As specialized epidermal cells, trichomes are covered by a cuticle while the cuticle is perforated between guard cells. When the guard mother cell divides to form a pair of guard cells, the procuticle in between them is dissolved. Trichomes and stomata are interspersed between ordinary epidermal cells (pavement cells) and impart to the epidermis and cuticle a lateral heterogeneity. There is ample evidence that the cuticle over trichomes, guard cells, accessory cells, and anticlinal cell walls differs in structure and permeability from the cuticle covering the bulk of the leaf surface.

Membrane models

Membranes separate two compartments, and selective permeability is their most interesting property. Selective permeability means that membranes are not equally permeable to all solutes. The relationship between chemistry and structure of membranes on the one hand and properties of solutes (polarity, electric charge, and molar volume) on the other has been studied for decades, for both synthetic and natural membranes.

With homogeneous membranes, permeability (quantified as permeance or permeability coefficient, P) is proportional to K and to the diffusion coefficient (D), and inversely proportional to membrane thickness (λ):

$$P = \frac{D \times K}{\lambda} \qquad (2)$$

The diffusion coefficient (D) is a measure of solute mobility in the membrane matrix and is related to the number of jumps per unit time and jump distance (Crank and Park, 1968). Cuticles are heterogeneous membranes, and the composition as well as solute mobility vary with position in the membrane. Due to structural heterogeneity, permeability is not inversely proportional to membrane thickness, and a tortuosity coefficient must be included in the denominator of equation 2 to account for the total length of the diffusion path (Baur *et al*., 1999; Buchholz and Schönherr, 2000). This aspect is dealt with in more detail by Buchholz (2006) and Schreiber (2006). Apart from this complication caused by structural heterogeneity of cuticles, equation 2 is a good descriptor of diffusion of lipophilic solutes in cuticles. Permeability increases with increasing K, i.e. with increasing solubility in cutin and amorphous waxes.

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The above considerations do not imply that fluxes (J) across cuticles increase with K, because the flux is proportional to P and to the driving force. With non-electrolytes, the concentration difference across the membrane (ΔC), between the donor and receiver compartments, is often used as the driving force instead of the chemical potential gradient:

$$J = P\Delta C \quad (3)$$

In the steady state, ΔC is constant and $C_{receiver}$ is practically zero. Under this condition, the driving force is C_{donor}, which is equal to KC_{water}. With an aqueous donor, the maximum possible concentration (i.e. the concentration of a saturated solution) in the aqueous donor decreases as water solubility decreases with increasing K (Sangster, 1997). Lipophilic pesticides have a K_{ow} of up to 10^4 and their solubility in water is in the range of a few milligrams per litre. Hence the positive effect of K on P (equation 2) is offset by the negative effect of low aqueous solubility of lipophilic solutes on the driving force (ΔC_{water}). On the other hand, with polar or ionic solutes, aqueous solubility may be as high as 1 kg l^{-1} and with these compounds the driving force can be larger by a factor of 10^6 compared with sparingly soluble lipophilic solutes. Hence, rates of foliar penetration of relatively polar solutes need not necessarily be smaller than those of very lipophilic ones (Baur, 1998).

In the majority of studies concerned with foliar penetration, the rate of disappearance from the leaf surface or the rate of appearance inside the leaves was measured. Usually, the amount or percentage of solute that penetrated in an arbitrary time interval has been published. Such data cannot explain how the rates of penetration depend on the properties of solutes, cuticles, and environmental factors. For elucidating the relationships between chemistry and structure of membranes, properties of solutes, the nature of the driving force, and membrane permeability, a number of quantitative membrane models are in use.

Common models used for analysing permeability of polymer membranes, cell membranes, and bilayers are the solubility membrane and the porous membrane (Helfferich, 1962; Stein, 1967; Crank and Park, 1968; Kesting, 1971; Brock 1983). Figure 1 represents a schematic drawing of a solubility membrane traversed by an aqueous pore. Cations and anions are restricted to the aqueous phase inside the pore, while lipophilic non-electrolytes dissolve in the membrane lipids.

The solubility membrane

Diffusion across synthetic polymer membranes, fluid membranes, and cell membranes (Schatzberg, 1965; Stein, 1967; Crank and Park, 1968; Finkelstein, 1976) can often be accounted for by a solubility mechanism. Water and solutes dissolve in the polymer or the bilayer and cross it by diffusional jumps. In solid membranes, water molecules and solutes jump into voids or defects that arise due to molecular motion by the polymer segments. The model is based on the transition state theory (Glasstone *et al*., 1941). Such a mechanism has been successfully applied to diffusion of lipophilic non-electrolytes across cuticles (Baur and Schönherr, 1995; Buchholz *et al.*, 1998; Buchholz and Schönherr, 2000; Buchholz, 2006) and waxes (Schreiber, 2006).

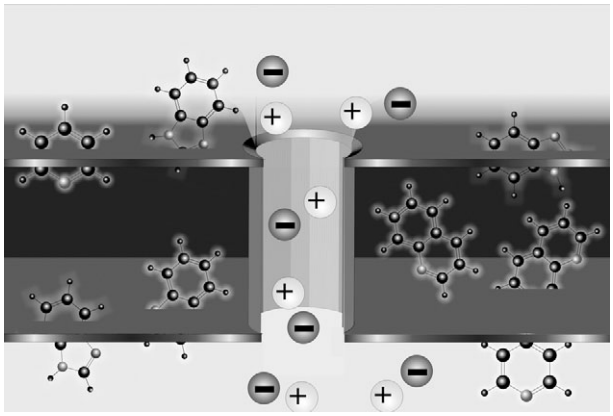

Fig. 1. Schematic drawing (not to scale) of a solubility membrane traversed by an aqueous pore. Non-electrolytes dissolve in the membrane matrix, while ions are restricted to the aqueous pore. To maintain electroneutrality, cations and anions must penetrate in equivalent amounts.

Cuticular waxes embedded in cutin are responsible for the barrier properties of cuticles. Extracting waxes increases permeability to water and lipophilic solutes by 2–4 orders of magnitude, even though waxes amount to only 5–20% of the total mass of the cuticles (Schönherr and Riederer, 1989; Riederer and Schreiber, 1995). Crystalline waxes are not accessible to water and solutes; they are responsible for increasing the tortuosity of the diffusion path (Buchholz, 2006; Schreiber, 2006). Diffusion of water and solutes is limited to the amorphous wax fraction. In *Citrus aurantium* and *Fagus sylvatica*, amorphous leaf waxes amount to about 80 and 70 %, respectively. In barley leaf wax (*Hordeum vulgare*), only ~48% of the wax is in the amorphous state at 25 °C (Reynhardt and Riederer, 1994). The amorphous wax phase must be continuous if it is to function as a lipophilic pathway across cuticles.

Waxes are very complex mixtures comprising a wide range of functional groups and chain lengths, but they are mainly composed of methylene groups. Leaf wax of *C. aurantium* consists of 93.6% methylene groups, 3.9% methyl groups, and only 2.5% polar (hydroxyl, carboxyl, and ester) groups (Riederer and Schneider, 1990). Hence, water and solute molecules crossing the *Citrus* cuticle encounter mainly methylene groups. Thermal motion creates temporary voids in the CH_2 network, and both water and solutes move in this environment by jumping from void to void. As temperature increases, voids appear and disappear more frequently, and rates of diffusion in cuticles greatly increase. Activation energies of diffusion

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of lipophilic solutes are very high and range from 75 to 200 kJ mol^{-1} (Baur *et al.*, 1997). A linear free energy relationship between entropy and enthalpy for diffusion of various lipophilic solutes across cuticles from 14 plant species was observed (Buchholz, 2006). This indicates that in all cuticles studied, all lipophilic solute molecules diffused in the same microenvironment predominantly composed of methylene groups (Baur *et al.*, 1997; Buchholz and Schönherr, 2000; Buchholz, 2006).

The porous membrane

Cell membranes are lipid membranes in which membrane proteins enable and regulate active and passive transport of ions and water (Steudle and Henzler, 1995; Steudle and Frensch, 1996; Steudle, 2002). These transport proteins are essential as the lipid double layer is practically insoluble to hydrated ions. The concept of polar or aqueous pores in cuticles was originally introduced to account for the empirical observation that ionic pesticides were biologically active and penetrated the lipophilic cuticle. However, the concept remained vague and the question of the chemical and structural basis for polar pores in cuticles was never approached directly.

Cuticles are permeable to water, but this does not require the presence of aqueous pores. Water dissolves in hydrocarbon liquids or in hydrophobic polymers such as polyethylene (Table 1), albeit in small amounts. However, these water molecules are isolated within the methylene groups. They are not linked by hydrogen bridges. These water molecules are mobile and can diffuse in lipophilic phases (Yasuda and Stannett, 1962; Barrie, 1968). They diffuse as individual molecules and not in bulk.

Polymers containing large amounts of polar functional groups sorb water by hydrogen bonding to permanent dipoles of the membrane matrix. Depending on the distribution of the polar groups within the lipophilic polymer, isolated aqueous clusters may develop or a continuous aqueous phase could form if the polar groups are spread out accordingly (Barrie, 1968). Such structures are called aqueous pores. They are dynamic structures that develop only in the presence of water. Ion exchange, dialysis, and desalination membranes are typical examples of porous membranes (Kesting, 1972; Brock, 1983).

These must not be confused with permanent and macroscopic pores such as in technical filter membranes used for particle removal from air or liquids (Brock, 1983). Viscous flow of air is a good criterion for the presence of permanent pores in dry membranes. At room temperature and 50% humidity, astomatous CM of pear, *Citrus*, *Stephanotis*, and *Populus* leaves and tomato fruits proved essentially impermeable to air under a pressure head of 30 mm mercury (equivalent to 40 hPa). Hence, these dry astomatous cuticular membranes did not contain permanent macroscopic pores, unless they had been damaged during the isolation procedure (unpublished data).

It is much more difficult to test for the presence of aqueous pores in cuticles, to characterize them chemically, and localize them. The water content of a swollen (i.e. hydrated) membrane is an insufficient criterion, since all or part of the water may be contained in isolated clusters as in ethyl cellulose (Yasuda and Stannett, 1962). As already mentioned, waxes contain only 2.5% carboxyl, hydroxyl, and ester groups, while methylene and methyl groups amount to 97.5% of chain elements (Riederer and Schneider, 1990). Table 1 shows that polymers lacking polar functional groups (e.g. polyethylene) and a liquid C_{30} hydrocarbon sorb extremely small amounts of water; they all have linear sorption isotherms (i.e. dependence of water content on partial pressure of water). Natural and artificial polymers which contain polar functions sorb much more water, and their sorption isotherms are usually of type II in the Brunauer, Emmett, and Teller (B.E.T.) classification (Barrie, 1968). That means partitioning increases with partial pressure, and at 100% humidity they contain much more than twice the amount of water compared with 50% humidity. As was shown for ethyl cellulose, this alone is an insufficient criterion for the presence of continuous aqueous pores. These membranes exhibited a type II sorption

Table 1. *Sorption of water in polymers and hydrocarbon liquids*

Lipid phase	p/p_o[a] (T, °C)	Water concentration (g H_2O in 100 g lipid)	Reference
C_{30} hydrocarbon	1.0 (25)	0.0055	Schatzberg (1965)
Polyethylene	1.0 (25)	0.0062	Barrie (1968)
Octanol	1.0 (25)	0.06	Chiou and Block (1986)
Cotton	1.0 (30)	0.23	Chiou and Block (1986)
Cellulose acetate	1.0 (30)	0.18	Chiou and Block (1986)
Ethyl cellulose	1.0 (25)	0.16	Yasuda and Stannett (1962)
Pyrus leaf MX	0.91	5.45	Chamel *et al.* (1991)
Citrus leaf MX	0.89	5.66	Chamel *et al.* (1991)
Ficus leaf MX	0.88	5.12	Chamel *et al.* (1991)
Capsicum fruit MX	0.83	4.57	Chamel *et al.* (1991)
Lycopersicon fruit MX	0.93	4.85	Chamel *et al.* (1991)
Lycopersicon fruit cutin	0.93	1.80	Chamel *et al.* (1991)

[a] p/p_o is the actual partial pressure of water vapour divided by the saturation partial pressure.

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isotherm, but water permeability did not increase with partial pressure of water and water content of the membranes, as would be expected if aqueous pores were present (Yasuda and Stannett, 1962).

Cotton and secondary cellulose acetate sorb ~40 times more water than polyethylene because of numerous hydroxyl groups of the glucose monomers (Table 1). Sorption of water to polar functional groups is due to hydrogen bonding, and the affinity for water of these groups differs. For carboxyl and hydroxyl groups and peptide bonds in polymers, the number of moles of water bound per polar group was near 1. The sorptive capacity of many polymers is reduced by crystallinity and by interchain hydrogen bonding. For instance, in crystalline cellulose, only 32% of the hydroxyl groups are accessible to water (Barrie, 1968). It follows that the number and types of polar groups in a polymer reveal little about their capacity to sorb water and to form continuous aqueous pores.

The polymer matrix (MX) derived from isolated plant cuticles by extracting cuticular waxes sorbs ~5% water (Table 1). Plant cuticles contain fixed ionic charges (Schönherr and Bukovac, 1973; Schönherr and Huber, 1977) as well as permanent dipoles contributed by cutin and polysaccharides (cellulosic and pectic materials). When polysaccharides were removed by acid hydrolysis from tomato fruit, MX water sorption was reduced by about two-thirds (Table 1). The polymer remaining after acid hydrolysis is cutin (plus cutan in some types of cuticles), possibly modified since some of the polar constituents (flavonoids) are covalently bound to cutin (Luque *et al.*, 1995). Recently, Graça *et al.* (2002) found significant amounts (1–15% by weight) of glycerol esterified to fatty acids in the polymer matrix of all 14 plant species investigated. This had been overlooked in all previous studies of cutin composition.

Plant MX membranes sorb ~25 times more water than ethyl cellulose, secondary cellulose, or cotton (Table 1). This is astounding since the hydrolysable fraction (pectins, celluloses, and polypeptides) range only from 15–22% by weight (Schreiber and Schönherr, 1990; Chamel *et al.*, 1991; Luque *et al.*, 1995). Cutin did sorb some water, but it is clear that the polysaccharides, polypeptides, and phenols present in MX membranes (Schönherr and Bukovac, 1973; Schönherr and Huber, 1977) were responsible for most of the water sorbed. MX membranes are weakly acid ion exchangers. Exchange capacity increased with pH. Carboxyl groups (pH <9) accounted for ~0.55 meq g^{-1} and phenolic hydroxyl groups (pH >9) for another 0.5 meq g^{-1}. These groups sorb water even when not ionized, but sorption will be greatly enhanced upon ionization (Helfferich, 1962). Sorption of water to hydroxyl groups from polysaccharides and cutin is independent of pH.

Plant cuticles contain considerable amounts of polar groups, and the bulk of them are contributed by the polymer matrix, not by the waxes. Whether a continuum of polar groups extends through the cuticle proper cannot be decided on the basis of these data. However, the amount of permanent dipoles and ionizable groups in cuticles is small, and a random distribution of polar groups in cuticles is not likely to give rise to polar pores. Continuous polar pores could arise only when these polar functions are clustered. Attempts to localize such polar pores have been made and will be summarized later.

Diffusion paths of solutes and water in membranes

In membranes with two parallel pathways, solutes and water can move along both, at least theoretically. Water can be sorbed as individual molecules even in hydrocarbon liquids and non-polar polymers, and this water is mobile (Schatzberg, 1965). Water sorbed to permanent dipoles in a membrane is also mobile and, when dipoles are numerous, closely spaced, and interconnected, water clusters or narrow aqueous pores can arise in a lipophilic membrane matrix. Similar arguments apply to polar non-ionic solutes (alcohols, sugars, glycols, etc.). Both pathways are available to them but the relative importance of the two pathways depends on water and lipid solubility of the solutes and their mobilities in the two phases. Lipophilic solutes will prefer the lipophilic path because their solubility in lipids is high while solubility in water is low. Since the total amount of water sorbed by cuticles amounts to only a few per cent of the weight of the cuticle (Chamel *et al.*, 1991), the volume of the lipophilic path is much larger than that of the aqueous path.

The hydration shell renders ions essentially insoluble in organic solvents such as hexane or olive oil. However, finite K_{ow} values for ionic solutes have been published (Sangster, 1997) and for amino acids they range from 0.09 (tryptophan) to 4.7×10^{-4} (serine). For zwitterionic agrochemicals, K_{ow} values of 0.01 (maleic hydrazide) and 1.6×10^{-4} (glyphosate) have been published (Brudenell *et al.*, 1995). The significance of these figures is doubtful. Even though octanol and water are usually considered immiscible, the concentration of water in octanol at 25 °C is 0.6 g l^{-1} (Chiou and Block, 1986), i.e. octanol contains significant amounts of water, and clusters of water may form micelles with organic ions.

Cuticular waxes have very few functional groups which can be hydrated. A liquid paraffin or olive oil would be a better model solvent for partitioning in cuticular waxes. This is also evident from the fact that the cuticle–water partition coefficients (similar to K_{ow}, see above) are up to 10 times larger than the wax–water partition coefficients (Schreiber, 2006). Furthermore, when partition coefficients are very high or very low, their accuracy is doubtful since extremely low concentrations must be detected either in water or in octanol, and small amounts of impurities can introduce large errors. Under physiological conditions,

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inorganic and organic ions are not likely to lose their hydration shell when dissolved in water and their concentration in cuticular waxes can safely be taken to be practically zero. Hence, they are restricted to an aqueous environment.

It should be stressed that this conclusion applies only to ions of strong acids or bases. Ionization of weak electrolytes depends on pH, ionic strength, and type of solvent. When the pH is two units below the pK_a, 99% of the carboxyl groups are non-ionized. High ionic strength reduces ionization, and dissolving a weak acid in an organic solvent having a lower dielectric constant than water lowers the apparent pK_a, reduces the degree of ionization, and increases the partition coefficient (Kerler and Schönherr, 1988*a*). The consequence of this is that weak electrolytes can occur as non-ionized species that can be quite lipophilic and highly soluble in cuticles (Kerler and Schönherr, 1988*a*; Brudenell *et al.*, 1995).

In laboratory studies, astomatous plant cuticles have been shown to be permeable to inorganic salts (Yamada *et al.*, 1964; McFarlane and Berry, 1974; Schönherr and Huber, 1977; Tyree *et al.*, 1990; Schönherr, 2000, 2001; Schönherr and Luber, 2001; Schönherr and Schreiber, 2004) and to ionic organic solutes such as glyphosate (Schönherr, 2002) and paraquat (Schönherr and Baur, 1996). Numerous systemic herbicides such as asulam, bentazone, diquat, glyphosate, phenoxyacetic acids, and quinclorac are ionic when formulated as salts (Tomlin, 1997). For biological effectiveness, they must penetrate the cuticle and, since their ionization cannot be suppressed, they can penetrate cuticles only through aqueous pores. The arguments put forward above show that their penetration is excellent evidence that these cuticles have aqueous pores. The localization and properties of these aqueous pores will be discussed next.

Localization of aqueous pores

Attempts to localize aqueous pores were based either on tracing the flow of water with ionic fluorescent dyes or by localizing metal precipitates in cuticles or epidermis following application of aqueous salt solutions to leaf surfaces.

Ionic fluorescent dyes as tracers

Strugger (1939) used the cationic dye berberine sulphate (BS) to trace the water in the xylem and the leaf apoplast. BS is a salt consisting of two berberine cations and one sulphate group. The formula weight of this salt is 768.8 g mol^{-1}. BS shows only weak fluorescence when dissolved in water, but when bound to the cell wall very intense fluorescence is observed. The cytoplasm, nuclei, and vacuoles do not exhibit fluorescence. The mechanism of binding has not been studied but, since BS is a fairly strong base, ionic interaction between positively charged berberine and negatively charged carboxyl groups of cutin and pectins is probably involved.

When small cut branches of *Helxine soleirolii* were placed in aqueous BS (1 g l^{-1}), the ascent in the xylem vessels was rapid and took only a few minutes, especially when stomata were open and transpiration was taking place. The dye spread from the veins to interveinal regions of the leaves and accumulated in cuticular ledges of guard cells, the basal cells of trichomes, and in anticlinal walls. Accumulation of BS in the upper epidermis, which lacks stomata, proceeded more slowly, but the pattern of distribution in epidermal cells and trichomes was similar while fluorescence was not quite as intense. Fluorescence was more intense in younger than in older leaves. When transpiration was inhibited by submerging leaves in water, the extrafascicular spread of BS was eliminated. In the dark, when stomata were closed or when transpiration was reduced by very high humidity, BS spread much more slowly and fluorescence intensity in guard cells was reduced. Strugger (1939) suggested that BS accumulated at sites where cuticular and stomatal transpiration occurred, and fluorescence intensity was proportional to the amount of BS deposited at these sites. The possibility that the density of carboxyl groups was higher at the sites of exit of water was not considered as a factor involved in determining the intensity of fluorescence.

Similar results were obtained when the tips of the twigs were submerged in BS solution and transpiration took place from the leaves at the base of the stem exposed to air. In the transpiring leaves, cuticular ledges of guard cells showed fluorescence within minutes, and later anticlinal walls, trichomes, and veins started to fluoresce. Not all stomata took up the dye, and Strugger pointed out the patchiness of BS distribution. Fluorescence developed more slowly when stomata were closed. Uptake was faster with younger leaves. These results were interpreted as evidence for uptake of water and BS by leaves.

Additional experiments were conducted by Strugger (1939) to test if sites exhibiting fluorescence were permeable for BS. Cut stems were placed in BS solution and, after ~30 min of transpiration in light, all leaves were uniformly stained with BS. A solution consisting of 50 g l^{-1} gelatine, 0.9 mol l^{-1} glucose, and 0.1 mol l^{-1} potassium rhodanide (KCNS) was prepared, cooled, and immediately before its solidification some leaves were covered with this mixture, which then quickly solidified. In this way, a strong osmotic gradient was generated which induced water flux into the gelatine. With KCNS, BS forms insoluble crystals with intense fluorescence. After just 10 min fluorescing, crystals of berberine rhodanide could be observed in the gelatine over cuticular ledges and at the bases of glandular trichomes. With young leaves, some fluorescent crystals were seen over anticlinal walls. These results clearly demonstrate that cuticular ledges of guard cells and basal cells of

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glandular trichomes of *Helxine* have aqueous pores large enough to allow passage of ionic BS.

Similar results were obtained by Bauer (1953) with *Rhoeo discolor* and *Hydrocleys nymphoides*. Rapid accumulation of BS in cuticular ledges of open transpiring stomata was observed, while closed stomata accumulated only little dye. *Vicia faba* and *Commelina communis* also accumulated BS in cuticular ledges (Maier-Maercker, 1979). The ingenious KCNS method invented by Strugger was not used by Bauer (1953) and Maier-Maercker (1979), and for this reason the data simply indicate selective permeability of cuticular ledges to water and accumulation of BS.

As it was not possible to reproduce pictures from the original publications in satisfactory quality, some of Strugger's experiments were repeated following his methods (Strugger, 1939). Leaves of *H. soleirolii*, *V. faba*, and *Phaseolus vulgaris* were floated in aqueous berberine chloride solutions (1 g l^{-1}, pH 4.2) for 2–3 h in the dim light of the laboratory. The dye penetrated the cuticle and produced intense fluorescence in cuticular ledges and anticlinal walls (Fig. 2). In Fig. 2A, the anticlinal walls

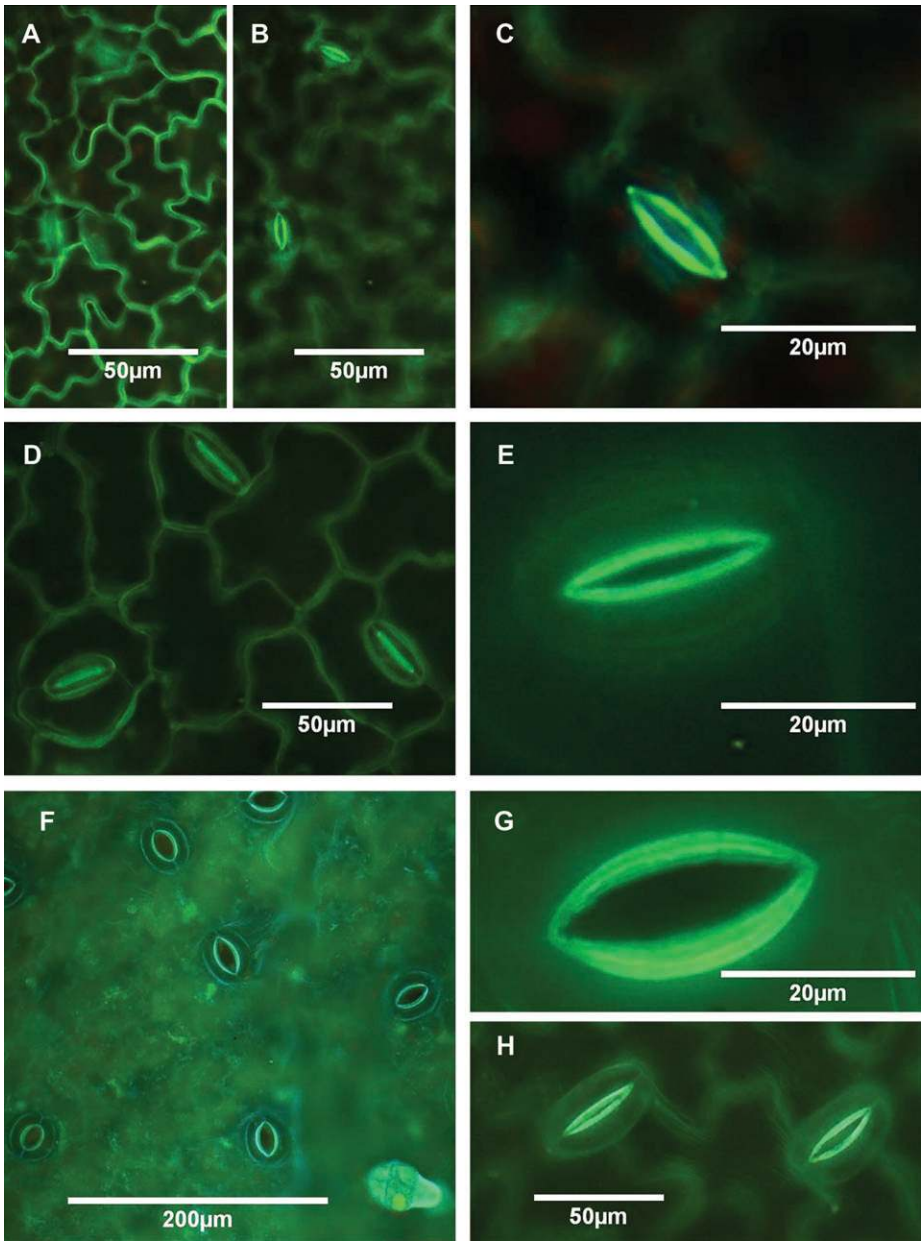

Fig. 2. Fluorescence micrographs of stomatous leaf surfaces of *Helxine soleirolii* (A–C), *Phaseolus vulgaris* (D, E), and *Vicia faba* (F–H) treated with berberine chloride (1 g l^{-1}) for 2–3 h in light. The photographs were taken using a Zeiss Axioplan2 microscope equipped with a Zeiss AxioCam digital camera and AxioVision 3.1 software (Zeiss, Germany). The Zeiss filter set No. 02 (excitation: G 365 nm; beam splitter FT 395 nm, excitation: LP 420 nm) was used.

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are in focus and fluorescence can be seen clearly. Figure 2B shows the same sample with the focus on cuticular ledges. In all three species, glandular trichomes also fluoresced intensely (Fig. 2F). No surfactant was added to the dye solutions.

Precipitation of silver

Precipitates formed in the epidermis following application of aqueous silver nitrate have been used to indicate the sites of selective permeability of cuticles to ions and salts. Glass cylinders were glued to leaf surfaces (primary leaves of *P. vulgaris*) with silicon rubber, and penetration of silver nitrate (0.01 mol l^{-1}) from aqueous solutions (200 µl covering ~0.5 cm^2) was followed for 1 h. The effects of illumination and surfactants were studied (Schönherr, 1969).

Bright field microscopy revealed silver precipitates in cuticular ledges of guard cells (Fig. 3). More silver accumulated in light (6 klx) (Fig. 3B, D) than in the dark (Fig. 3A, C). The surfactant Tween-20 (1 g l^{-1}) increased the number of precipitates in cuticular ledges, stomata (including chloroplasts), and anticlinal walls (Fig. 3D). In the dark and in the absence of a wetting agent, silver precipitates were found in the wall between guard cell and accessory cell (Fig. 3A), while in the light silver was located in cuticular ledges (Fig. 3B, D). Even in the presence of a wetting agent, silver was never found in the stomatal pore or in the antechamber. Glandular trichomes accumulated silver in the dark (Fig. 4A) and in the light

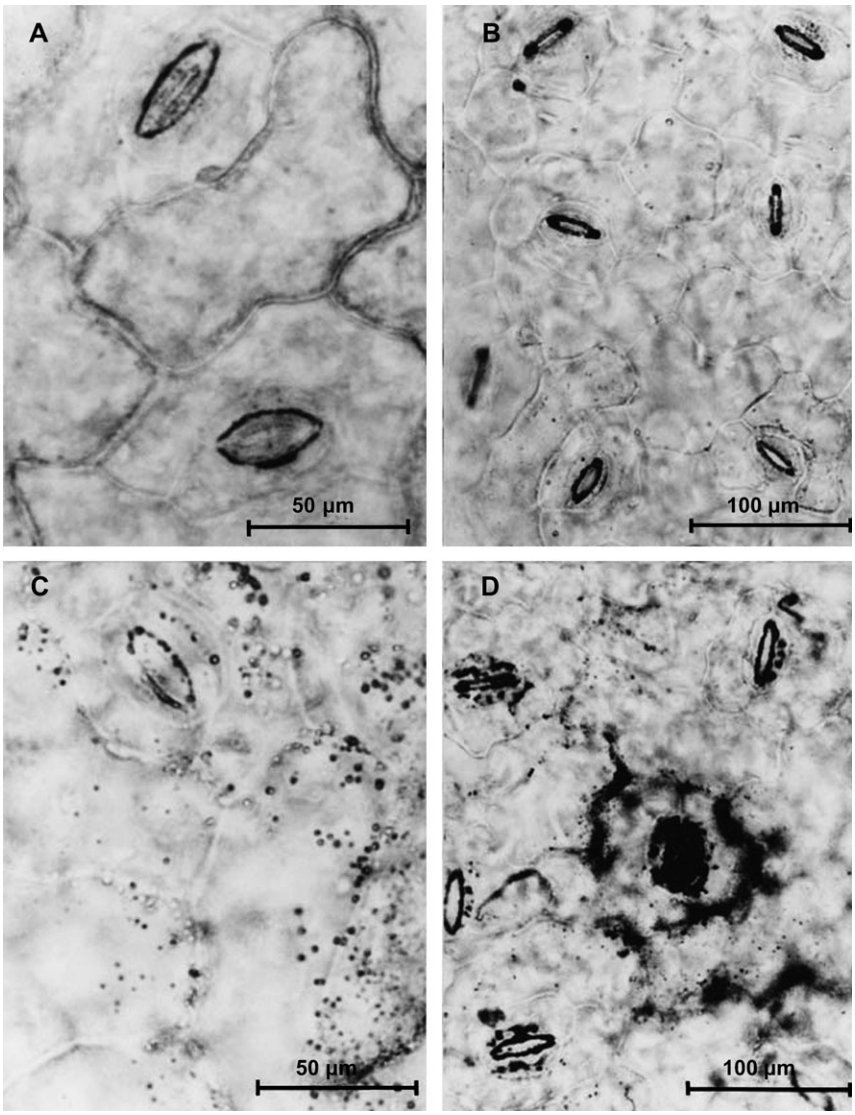

Fig. 3. Bright field micrographs showing silver precipitates in cuticular ledges of stomata of *Phaseolus vulgaris* leaves treated with silver nitrate (0.01 mol l^{-1}) for 1 h in the dark (A, C) or in light (B, D). Tween-20 (1 g l^{-1}) was added as wetting agent to the leaves shown in (B) and (D) (taken from Schönherr, 1969).

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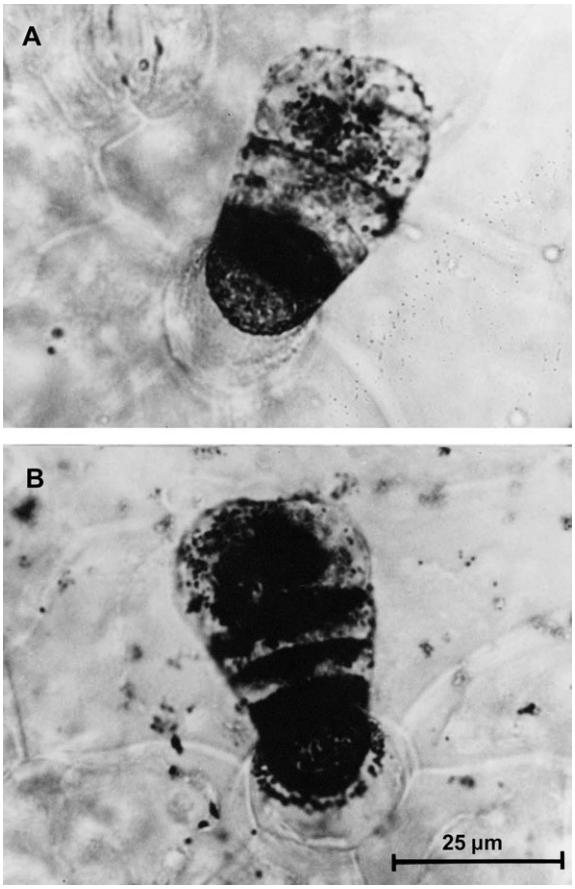

Fig. 4. Bright field micrographs showing silver precipitates in glandular trichomes of *Phaseolus vulgaris* leaves treated with silver nitrate (0.01 mol l^{-1}) for 1 h in the dark (A) or in light (B). Both treatments contained Tween-20 (1 g l^{-1}) as wetting agent (taken from Schönherr, 1969).

(Fig. 4B), but in light the basal cell exhibited a ring of silver precipitates. Wavelength-dispersive X-ray analysis revealed preferential accumulation in cuticular ledges, in guard cells, in the vicinity of guard cells, and in glandular trichomes. Single-celled hooked trichomes did not accumulate silver. Cross-sections showed blackening of cuticular ledges, chloroplasts, and anticlinal walls, and the absence of silver in palisade cells (Schönherr, 1969). Silver precipitates were found in the antechamber and in palisade cells only after vacuum infiltration with silver nitrate solution.

Similar experiments were conducted using adaxial pear (*Pyrus communis* L.) (Green and Bukovac, 1974) and apple (*Malus domestica* Borkh.) leaves (Lord *et al*., 1979). Again, in the absence of a surfactant, infiltration of stomata was never observed, while with surfactants intense blackening of a few stomata was observed after just 2 min of exposure. This was taken as evidence for stomatal infiltration. The fraction of stomata blackened ranged from 0.5% (Tween-20) and 2.0% (X-77) to 4.4% (Vatsol OT). The surface tensions of these solutions decreased in the same order from <40 to <30 mN m^{-1} (Green and Bukovac, 1974). In the presence of a wetting agent, stomatal ledges blackened within 2 min. After 30 min, silver was found in anticlinal walls and inside guard cells.

Vicia faba leaves treated in light with silver nitrate solutions plus surfactant accumulated black silver precipitates in guard cells and glandular trichomes. Energy-dispersive X-ray analysis (EDX) revealed that silver had precipitated as silver chloride (Schlegel *et al*., 2005). Schreiber (2005) observed silver precipitates in astomatous cuticles from *Populus canescens* leaves. Isolated cuticles were subjected to penetration of $AgNO_3$ solution from the morphological outer surface and NaCl solution from the inner surface. AgCl precipitates (EDX) formed in and on the outer surface of the cuticles. Treatment with Ag^+ reduced water permeability by a factor of 2.8 (Schreiber *et al*., 2005*a*).

The above data indicate that stomatal ledges are preferential sites of entry for ionic substances, and permeability seems to be higher in light, when stomata are open, than in the dark. This is in good agreement with the results of Strugger (1939) even though he did not use surfactants in his studies. There is also agreement that entrance of aqueous dye solutions by mass flow into open stomata did not occur as long as no surfactants were employed.

Penetration of ionic compounds was also studied by Eichert *et al.* (1998) and Eichert and Burkhardt (2001). They interpreted their data as evidence showing that even in the absence of surfactants, infiltration of stomatal pores can happen, at least with a few stomata. They suggested that thin water films containing dissolved solutes extend from the surface of the epidermis through the stomatal pore into the substomatal chamber.

Ectodesmata

Ectodesmata were first viewed as protoplasmatic strands in the outer epidermal wall, analogous to plasmodesmata which connect neighbouring cells (Schumacher and Lambertz, 1956; Franke, 1960). Later Franke (1961, 1967) favoured the idea that ectodesmata were not plasmatic strands, but well-defined structures in the epidermal wall rich in reducing substances. Ignoring the fact that ectodesmata are structures in the epidermal wall, not in the cuticle, Franke (1967) later suggested that ectodesmata were involved in foliar penetration of polar substances. At that time, it was well accepted that the lipophilic cuticle is the limiting barrier in foliar penetration of polar compounds, rather than the aqueous cell wall (van Overbeek, 1956; Currier and Dybing, 1959; Crafts and Foy, 1962).

Ectodesmata were demonstrated in epidermal walls of mesophytic plant species following fixation with Gilson solution. The Gilson solution is a mixture of 40 ml of 30% ethanol, 10 ml of formic acid, 5 ml of formalin, and 2 g of oxalic acid that is saturated with mercuric chloride ($HgCl_2$).

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Whole leaves or large leaf segments were fixed for 12 h at 38 °C. After fixation, the epidermis was stripped off (indicating hydrolysis of pectins in the cells wall), washed in 30% ethanol to remove $HgCl_2$, treated with potassium iodide (16%) for 5–10 min, and then stained with pyoktanin (6 g l^{-1} in 10% sulphuric acid). The tissue was then washed in water and mounted on glass slides for microscopic inspection in glycerine.

After this procedure, ectodesmata extend as dark bands from the cuticle towards the protoplast of the epidermal cells. If treatment with potassium iodide and pyoktanin is omitted, ectodesmata appear crystalline and birefringent in polarized light (Schönherr and Bukovac, 1970*a*, *b*). These crystals are mercurous chloride (HgCl), which is practically insoluble in water and ethanol. Treatment of these HgCl crystals with KI leads to disproportionation, i.e. half of the Hg(I) atoms are reduced to metallic mercury while the other half are oxidized to divalent mercury. The black precipitates formed in the epidermal wall after KI treatments represent metallic mercury, while soluble $HgCl_2$ is washed out during tissue preparation (Schönherr and Bukovac, 1970*b*).

Often ectodesmata are arranged in rows in anticlinal walls, such as in onion leaves or adaxial surfaces of *Convallaria* leaves. On the abaxial leaf surface, ectodesmata are randomly distributed. Guard cells are always filled with black mercury precipitates. In cross-sections, it can be seen that mercury precipitates originate at the inner surface of the cuticle and extend into the epidermal walls. In cell walls of onion leaves, ectodesmata resemble icicles, while in *Convallaria* leaves they are more hemispherical. Light brushing of the leaf surface destroyed the typical pattern over anticlinal walls and new rows of ectodesmata appeared along the tracks of the bristles. Rinsing the leaf surfaces with chloroform also destroyed the typical pattern of ectodesmata over epidermal cells, but stomata were still black. Copious amounts of mercury precipitates formed in cells and cell walls of subepidermal cells (Schönherr and Bukovac, 1970*a*). These results show that it is selective permeability of cuticles that causes the characteristic pattern of distribution of ectodesmata rather than hypothetical pores in the aqueous cell wall. This was substantiated by the following experiments. When cuticles were isolated enzymatically from onion leaves or onion bulb scales and mounted on gelatine or agar containing ascorbic acid as reducing agent, the pattern of 'ectodesmata' found there was the same as in epidermal strips. Clearly, ectodesmata are formed in the cell wall or in the agar at sites where the cuticle is selectively permeable to $HgCl_2$ provided the underlying matrix contains a reducing agent, which is needed for the formation of insoluble HgCl.

Franke argued that formation of mercury precipitates (ectodesmata) often occurred at the same sites which strongly fluoresce with BS, i.e. cuticular ledges, guard cells, anticlinal walls, and glandular trichomes (Franke, 1960, 1967). As pointed out already, the pattern of distribution of ectodesmata is a property of cuticles and not of epidermal walls. However, it can be questioned if the Gilson fixative is suitable to localize aqueous pores in cuticles. The fixative is not really an aqueous solution and it is highly phytotoxic. $HgCl_2$ solutions in water have an extremely low electrical conductivity, as mercury and chlorine are connected by divalent rather than ionic bonds. It dissolves in water, alcohols, ether, benzene, and other organic solvents (Falbe and Regitz, 1992). When cuticles are penetrated by non-ionic $HgCl_2$, it is not surrounded by a hydration shell. It is lipid soluble and does not depend on aqueous pores like BS or silver nitrate. This raises the question as to the nature of sites in cuticles selectively permeable to $HgCl_2$. The Gilson solution is very acidic (pH 1.0), and carboxyl groups or phenolic hydroxyl groups are certainly not ionized at this pH value, while amino groups would be. Polypeptides occur in cuticles, albeit only in small amounts (Schönherr and Bukovac, 1973; Schönherr and Huber, 1977). Possibly, Hg precipitates marked sites with non-ionic functional groups such as hydroxyl, aldehyde, phenolic hydroxyl, and ether or ester bonds. Carboxyl groups would be undissociated at these low pH values and may also be present along the $HgCl_2$ diffusion paths.

Cuticles over anticlinal walls form plenty of ectodesmata, while they are rarely found over periclinal walls. The effect of removing waxes on the distribution pattern of ectodesmata indicates that waxy domains are impermeable to $HgCl_2$ even though it dissolves in benzene. Perhaps crystalline wax domains are impermeable to $HgCl_2$ while amorphous lipids are not. This would imply that ectodesmata form in the cell wall wherever cuticular waxes are in the amorphous state and crystalline waxes are scarce.

Karabourniotis *et al*. (2001) observed blue fluorescence in/over guard cells and at the base cells of trichomes when treated with KOH. They examined 25 species and all showed this type of fluorescence, albeit to different degrees. It was suggested that the method could be used to estimate stomatal density. Fluorescence intensity decreased after immersion of leaves in chloroform for 2 s, indicating that fluorescence was due in part to fluorescent constituents of cuticular wax. It was suggested that ferulic acid was one of the fluorochromes, and its concentration was much higher on stomatous abaxial than on astomatous adaxial leaf surfaces of *Prunus persica* and *Olea europaea* (Liakopoulos *et al*., 2001). These results show differences in the chemical composition of the polymer matrix and soluble lipids over stomata, trichomes, and periclinal walls. Phenolics are known to be constituents of the cutin matrix and of cuticular waxes (Schönherr and Bukovac, 1973; Luque *et al*., 1995), but their possibly unequal distribution went unnoticed because extraction of leaves or large pieces of cuticles and their depolymerization yields only average values for those relatively large areas. Analysis of chemistry

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and structure with spatial resolution in the micrometre range is needed to establish the chemical basis and actual involvement in selective permeability of ionizable groups and permanent dipoles.

Permeability of porous cuticular membranes

Measurements of water and solute permeability allow prediction of fluxes under various conditions, and the effects of pH, humidity, and temperature on membrane permeability have been studied. Most of these studies used isolated CMs, and there is ample evidence that enzymatic isolation (Schönherr and Riederer, 1986) does not change the permeability of cuticles (Kirsch *et al*., 1997). Working with isolated CMs has many advantages. Sampling is non-destructive and fluxes are measured by following the disappearance from the donor or appearance in the receiver volumes. CMs can be used repeatedly, which allows the study of the effects of a number of variables using the same set of CMs. Permeability of an individual CM can differ by an order of magnitude or more. Using the same CM or set of CMs (method of paired comparisons) to study the effects of humidity, temperature, solutes, or adjuvants renders these studies very sensitive since large variability in the permeability of individual CMs is eliminated and small effects can be detected.

Estimating the size of aqueous pores from water permeability of cuticles

Water may diffuse as individual molecules by a solubility mechanism or it may diffuse in pores lined with dipoles and/or fixed ionic charges. When aqueous pores are present, water could use either one, while ionic solutes are restricted to aqueous pores lined with fixed charges. Dipoles and/or fixed charges in a cuticle would be surrounded by water molecules; the water content of the CM increases with humidity, and the CM swells. If dipoles and fixed charges are distributed as a continuum throughout the cuticle, aqueous pores extend through the entire CM and water permeability should increase with increasing humidity. This has been tested using CM and MX membranes. MX membranes are obtained by extracting waxes from CMs with chloroform. The water permeability of MX membranes is higher by 2–4 orders of magnitude (Schönherr, 1982). There is no correlation between the effect of wax extraction on water permeability and amounts or composition of extracted waxes.

Astomatous MX membranes from *C. aurantium* leaves exhibited swelling, and water permeability increased 2.5-fold when the pH increased from 3 to 9, provided the carboxyl groups in the membranes were neutralized with Na^+ (Schönherr, 1974, 1976*a*). For tomato fruit MX, the shape of a plot of permeability versus pH resembled that of a plot of ion exchange capacity versus pH (Schönherr and Bukovac, 1973). Below pH 9, ion exchange capacity is due to -COOH groups of pectins and cutin (Schönherr and Bukovac, 1973; Schönherr and Huber, 1977). In ripe tomato, flavonoids (naringenin, chalconaringenin) contribute to ion exchange capacity (Luque *et al*., 1995). Carboxyl groups have a very high selectivity for Ca^{2+} and even in the presence of high concentrations of Na^+ the -COOH groups are neutralized by Ca^{2+}. MX membranes in the Ca^{2+} form exhibited very little swelling, and water permeability increased only slightly with pH. These results are good evidence that at least part of the water diffused through the MX in aqueous pores lined with fixed ionic charges.

The size of the pores in MX membranes was estimated from the ratio of water permeability measured under an osmotic gradient (P_f, filtration or viscous flux) to water permeability (P_d) measured with tritiated water (diffusion). P_f also increased with pH and, between pH 3 and 9 the ratio P_f/P_d was $\sim$ 2.6. The fact that $P_f > P_d$ is evidence for aqueous pores (Schönherr, 1976*a*). Since diffusion is proportional to the square of the pore radius (r^2) while filtration is proportional to r^4, the radius of the aqueous pores can be estimated from P_f/P_d. The average pore radius of 0.44–0.47 nm did not depend on pH but the permeability increased, which indicates that the number of pores per unit area of MX increased with pH. Between pH 3 and 9, the number of pores per cm^2 of MX membrane increased from 5.1×10^{10} to 15.8×10^{10} and the total pore area per cm^2 membrane increased from 4.4×10^{-4} to 10.5×10^{-4} cm^2. These figures apply to astomatous *Citrus* MX in Na^+ form (Schönherr, 1976*a*). No attempt was made to localize these aqueous pores. These membranes were practically impermeable to saccharose (342 g mol^{-1}) and raffinose (505 g mol^{-1}) which have molecular radii of 0.56 and 0.65 nm, respectively. These are polar non-ionic solutes, which were excluded from the aqueous pores by virtue of their size. They could have diffused across the lipophilic matrix by a solubility mechanism, but a possible contribution of the lipophilic pathway was not detectable. These pores are smaller than those in cuticular ledges of *Helxine*, which were permeable to BS having a molecular weight of 768.8 g mol^{-1} (Strugger, 1939).

Water permeability of the CM is 2–4 orders of magnitude smaller than that of the MX, and this prevented measuring water flow in the CM under an osmotic gradient. Hence, estimates of the size of aqueous pores in CMs are not available. Recourse was made to studying the effect of humidity at the outer surface of the CM on water permeability (Schönherr, 1976*b*, 1982; Schönherr and Schmidt, 1979). The inner surface of the CM was in equilibrium with water, while air of constant humidity was blown over the outer surface. Water permeability of the CM of all plant species increased by up to a factor of 2 when humidity was raised from 20% to 100%. The effect of humidity on water permeability was taken as evidence for swelling or deswelling of aqueous pores

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(Schönherr, 1982). The CM from onion bulb scales was the only exception, as its water permeability increased by only 8% between 20% and 100% humidity (Schönherr, 1982). MX membranes from onion bulb scales are porous, with an average pore radius of 0.41 nm (Schönherr and Mérida, 1981). Water permeability of onion MX membranes increased with increasing humidity, but this depended on the position of the bulb scale. Swelling was least with the outer two bulb scales, while with the 3rd and 4th bulb scales water permeability increased by a factor of ~2 when humidity increased from 20% to 100% (Schönherr and Mérida, 1981). It appears that most of the aqueous pores in the CM were blocked with wax. CMs from onion bulb scales are extremely thin (<1 μm); they contain very little wax, but wax extraction increased water permeability by factors of 1352–2053.

Recently, Beyer *et al.* (2005) published additional values for P_f and P_d measured with isolated cuticular membranes. Using the approach of Schönherr (1976*a*), radii of aqueous pores in tomato fruits and pepper fruits of 1.18 and 0.87 nm can be calculated from their data. They also estimated P_f and P_d for sweet cherry fruit cuticles with non-functional stomata. Estimates of aqueous pore radii (they prefer the non-committal term aqueous continuum) are 1.00 and 0.70 nm for the varieties 'Hedelfinger' and 'Sam', respectively. Pore radii in these cuticular membranes are ~1.5–2.5 times larger than those in the *Citrus* polymer matrix.

Rates of water uptake into ripe sweet cherry fruits were reduced when aqueous donor solutions contained $AgNO_3$, $CuCl_2$, $HgCl_2$, or $AlCl_3$ (Weichert *et al*., 2004). The effect could not be accounted for by the reduction in the difference of water potential between solution and cherry mesophyll. Furthermore, specific cation effects were observed, and the authors suggest involvement of deswelling of cuticles by these cations.

Most astomatous CMs from other plant species exhibited swelling, as their water permeability increased with humidity by factors of up to three (Schönherr, 1982; Schreiber *et al*., 2001). When the outer surfaces of astomatous isolated CMs were treated with silver nitrate and the inner surfaces were in contact with aqueous NaCl, water permeability decreased by a factor of up to three and the effect of $AgNO_3$ was greater with CMs having high initial water permeability (Schreiber *et al*., 2005*a*). The authors suggested that precipitation of AgCl in aqueous pores plugged them up. Among 15 plant species, 13 responded with reduced water permeability, *Hedera helix* and *Nerium oleander* being the only exceptions. The water permeability was measured with 0.01 mol l^{-1} NaCl on the inner side of the CM and 3% humidity on the outer side. Hence, minimum water permeabilities were measured, and it would be interesting to measure the effect at maximum swelling of the CM.

Luque *et al*. (1995) used X-ray diffraction to study the structure of green and ripe tomato fruit cuticles. They detected two peaks and assigned one spacing (d_0) (0.43–0.46 nm) to the average distance between hydrocarbon chains of the cutin polymer. The d_1 spacing (1.10–1.20 nm) varied with the ionic form and was attributed to flavonoids present only in ripe tomato fruits. This d_1 spacing is about the same as the pore radius estimated from viscous and diffusional flow of water across fully swollen tomato fruit cuticles (Beyer *et al.*, 2005). As X-ray diffraction was measured at 60% humidity, CMs were not fully swollen.

The above data suggest that water penetrates cuticles by two parallel pathways. The bulk of the mass of cuticles are lipids, and water diffuses in lipids (Yasuda and Stannett, 1962; Schatzberg, 1965; Barrie, 1968). However, waxes present a formidable barrier for water, as can be seen from the fact that water permeability of cuticles often increases by several orders of magnitude when waxes are extracted. At very low humidity when cuticles should be barely swollen, the bulk of the water most probably diffuses along the lipophilic pathway. When cuticles are fully swollen, diffusion and viscous flow across aqueous pores contribute to the total water flux. Quantitative separation of the contributions of the two pathways is difficult and has not been accomplished (Kerstiens, 2006). However, there is excellent evidence showing that cuticles are traversed by polar pores: (i) cuticles swell and this depends on humidity, pH, and the nature of cations associated with the cuticle's carboxyl groups; (ii) P_f is larger than P_d, and the pore radius estimates ranging from 0.45 to 1.2 nm appear reasonable and are compatible with the solute permeabilities reported; and (iii) the activation energy for viscous water flow in aqueous pores was nearly zero (Beyer *et al*., 2005) while for diffusion of water it ranged from 50 to 70 kJ mol^{-1} (Schönherr *et al*., 1979; Schönherr and Mérida, 1981; Beyer *et al*., 2005). These values are in the same range as energies of activation for diffusion of water in non-porous polyethylene (50 kJ mol^{-1}) and polypropylene (69 kJ mol^{-1}) (Yasuda and Stannett, 1962).

The presence of aqueous pores in cuticles is ecologically meaningful. When the air is very dry, pores shrink or disappear and water permeability decreases. This helps to retain water. During fog and rain, cuticles swell, and leaves and fruits can take up water (Grammatikopoulos and Maneta, 1994; Yates and Hutley, 1995).

Permeability to ionic solutes

Mechanistic studies of the penetration of electrolytes were conducted with astomatous CMs isolated from *P. communis* and *P. canescens* leaves and with leaf discs from *V. faba*, *P. communis*, and *M. domestica*. A method called simulation of foliar uptake (SOFU) was used, which allows the study of permeation under controlled conditions (Buchholz, 2006). In particular, humidity over the salt residue can be controlled. Isolated CMs or leaf discs were mounted in transport chambers depicted in Fig. 5. The morphological

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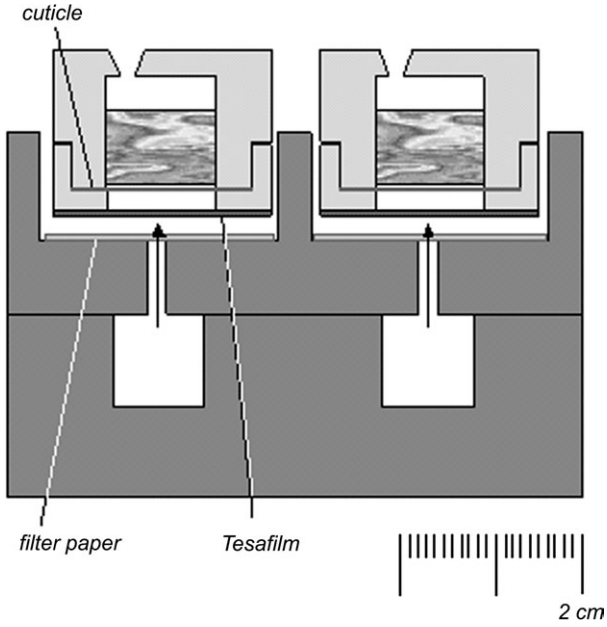

Fig. 5. Schematic drawing of transport chambers (to scale) used in SOFU experiments with isolated cuticular membranes. The membranes are inserted between donor and receiver (top) compartments, and the chambers are positioned in a thermostated aluminium block. Air of constant humidity is blown over the donor residues (arrows) on the outer surface of the membranes. When working at 100% humidity, the donor compartment was usually sealed with clear adhesive tape (Tesafilm). When humidity was <100%, the Tesafilm was omitted.

outer surface of the CM faced the air while the receiver was in contact with the morphological inner surface of the CM. When leaf discs were used, the mesophyll served as the receiver. Small droplets (5 µl) of radiolabelled solutes were applied to the outer surface of the CM. After droplet drying, air of constant humidity was blown over the salt residues and the rate of appearance of radiolabel in the receiver was monitored by periodically exchanging the receiver solution and counting its radioactivity. This method is not destructive, and a set of CMs can be used repeatedly for many experiments. When working with leaf discs, penetration was estimated either by measuring rates of disappearance from the leaf surface or by analysing radioactivity inside the leaves. This method is destructive, and different discs must be used for each data point.

Penetration data can be presented in various ways. Figure 6A shows the traditional method of plotting percentage penetrated versus time. With zwitterionic proline and other ionic solutes, the salt is, for all practical purposes, either on the cuticle (donor) or in the receiver, as only minute amounts are sorbed in the CM. On the right ordinate, the percentage of proline remaining on the surface of the CM is shown. The slopes of the plots depend on the humidity over the salt residue. The slopes (amount penetrated per unit time) are the rates of penetration, but they decrease with time because the driving force diminishes and they are not very useful.

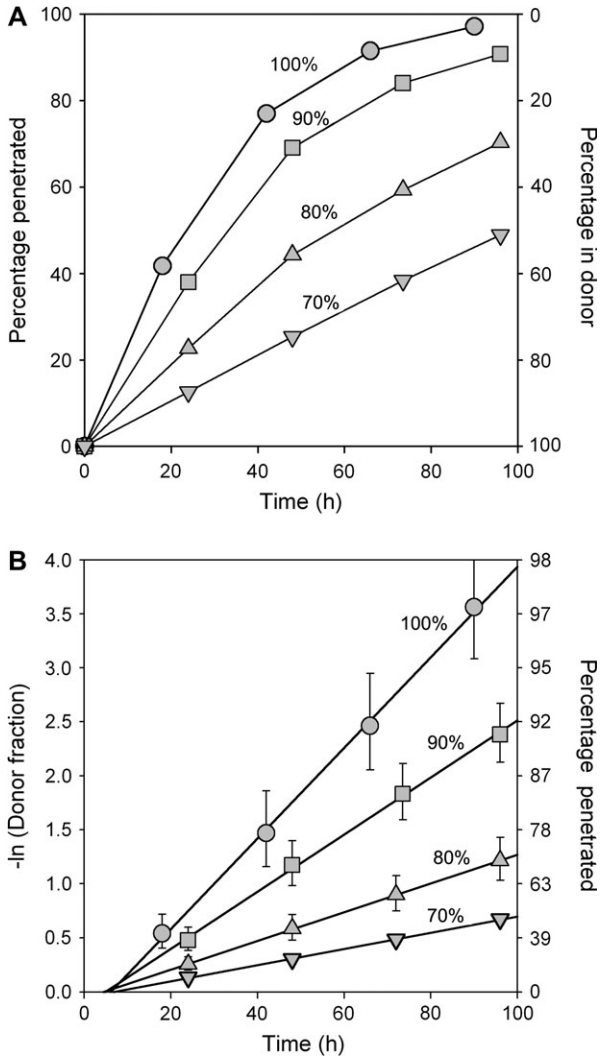

Fig. 6. Penetration of proline (5 g l^{-1}) across astomatous *Pyrus communis* leaf cuticular membranes at 20 °C. The donor solutions contained 0.2 g l^{-1} Glucopone 215 CSUP as wetting agent. Humidity over the proline residues is given in the graphs.

By plotting the natural logarithm of the fraction of solute remaining on the CM (percentage in donor/100), straight lines were obtained at all humdities (Fig. 6B). This was the case with practically all salts tested and with all cuticles and leaf discs studied. It indicates that cuticular penetration of salts is a first order process. The fraction of salt left in the donor falls exponentially with time, and this process (like radioactive decay) can be described with a rate constant (k in h^{-1}) which is equivalent to the slope of the plot. Penetration is quantitatively accounted for by the equation:

$$\frac{M_t}{M_0} = e^{-kt} \quad (4)$$

where M_t is the amount that has penetrated at time t and M_0 is the initial amount applied at t=0. Once k has been determined, the fractional penetration can be calculated for any time interval. By solving equation 4 for t and letting

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M_t/M_0 equal 0.5 (50% penetration), the half-time of penetration $t_{1/2}$ can be obtained. For instance, at 100% humidity, the rate constant for penetration of proline across the pear leaf CM was 0.0419 h^{-1} and the $t_{1/2}$ was 16.5 h. The $t_{1/2}$s at the other humidities were 26.2 h (90%), 52.1 h (80%), and 92.8 h (70%). Half-times of penetration are a very convenient way of expressing permeability and predicting amounts penetrated. After two half-times, 75% of the dose has penetrated and for three half-times, total penetration amounts to 87.5% of the dose applied. Unfortunately, in most published studies dealing with cuticular penetration, only one time interval was used.

Dependence of rate constants on humidity is a general feature observed with all salts and cuticles studied so far (Schönherr, 2000, 2001, 2002; Schönherr and Luber, 2001). Some additional unpublished data are shown in Fig. 7. Permeability of astomatous poplar CM for the different salts varied considerably and rate constants decreased with humidity by factors ranging from 3 ($CaCl_2$, betaine) to 7 (glyphosate) (Fig. 7). Rate constants for putrescine were nearly zero at 70% humidity because the donor was crystalline. When humidity was >70%, putrescine dissolved and penetrated rapidly.

An essential prerequisite for cuticular penetration is dissolution of the salt. Salts and ions penetrate only when dissolved in water. Crystals dissolve depending on their hygroscopicity. A quantitative measure of hygroscopicity is the point of deliquescence (POD), which is the humidity at equilibrium over a saturated salt solution containing some undissolved crystals. If humidity is higher than the POD, water is sorbed by the solution, and salt crystals dissolve. At humidity below the POD, water evaporates and new salt crystals form. Fortunately, POD does not greatly depend on temperature. POD values have been published (Kolthoff *et al*., 1969; Tomlin, 1997), but they are easily determined, not only for pure salts but also for mixtures.

For potassium carbonate and potassium nitrate, POD is 33% and 95% humidity, respectively. Hence, KNO_3 penetrated only when humidity was >90% (Fig. 8). A small amount of penetration was detected at 90% and below. This is due to unstirred layers of air over the salt residue and some dissolution at sites of contact between the salt and the cuticle due to water penetrating from the aqueous receiver. When nutrients or other ionic compounds are sprayed on leaves, these stagnant air layers can result in significant penetration rates even at humidities below the POD, especially at low wind velocity. Many salts, including Fe chelates, have the POD at ~100% humidity (Schönherr *et al*., 2005), and foliar penetration of these compounds will occur mainly during the night when wind speed is low and dew develops. POD of potassium carbonate is 33%, and high rate constants were measured at 50% (Schönherr and Luber, 2001) or 70% humidity (Fig. 8). When humidity is higher than the POD, salts dissolve, penetration takes place, and rate constants increase with humidity. This humidity effect on penetration above the POD was attributed to swelling of cuticles (Schönherr, 2000, 2001, 2002). The suggestion that swelling is involved in the humidity dependence of salt penetration is supported by the reduction of salt and water permeability by Ag^+,

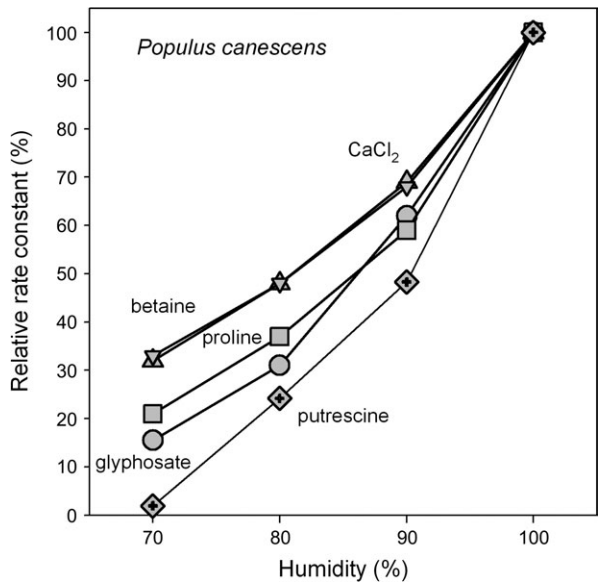

Fig. 7. Penetration of selected ionic solutes across *Populus canescens* cuticular membranes at 20 °C and various humidities. Solute concentrations were 5 g l^{-1} and the donors contained 0.2 g l^{-1} Glucopone 215 CSUP. Maximum rate constants at 100% humidity were 0.99 h^{-1} (putrescine), 0.66 h^{-1} ($CaCl_2$, proline, betaine), and 0.31 h^{-1} (IPA-glyphosate), respectively.

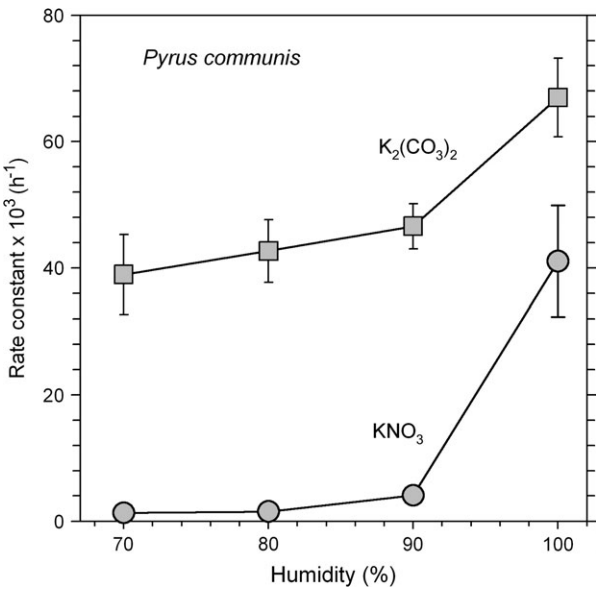

Fig. 8. Dependence of rate constants of penetration of K_2CO_3 and KNO_3 across *Pyrus communis* cuticular membranes on humidity over the salt residue. Solute concentrations were 5 g l^{-1} and the donors contained 0.2 g l^{-1} Glucopone 215 CSUP.

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Cu^{2+}, Fe^{3+}, and Al^{3+} ions (Weichert *et al*., 2004; Schreiber *et al.*, 2005*a*; Schönherr *et al*., 2005).

The addition of small amounts of surfactants to the donor solutions increased the rate constants greatly. In most studies, alkylpolyglycoside (APG) surfactants at only 0.2 g l^{-1} were used, and this increased the rate constant for $CaCl_2$ penetration across pear leaf CMs by factors between 9.6 (Schönherr, 2000) and 12 (Schönherr, 2001). The APG surfactant Glucopon 215 CSUP increased k for IPA glyphosate by ~300% (Schönherr, 2002). These surfactants reduced surface tension to ~29 mN m^{-1} (Schlegel and Schönherr, 2004), and this indicates that improved wetting is involved in the surfactant effect on salt penetration. It was suggested that perfect wetting of cuticles is needed to establish good contact between salt solutions and aqueous pores in cuticles (Schönherr, 2000, 2001, 2002). This is plausible and agrees with data on silver nitrate penetration (Figs 2, 3), but direct evidence is lacking.

Some surfactants and other adjuvants increase the fluidity of waxes, and this increases rates of diffusion across cuticles (Buchholz, 2006). Such adjuvants have been termed accelerator adjuvants (Schönherr, 1993*a*, *b*). Ionic solutes are hydrated and this hydration shell excludes them from any lipophilic phase, whether it is liquid or solid. Thus, accelerator adjuvants should not affect penetration rates of ionic solutes, as long as they do not modify aqueous pores. In fact, the accelerator adjuvant Genapol C-100 did not increase rate constants of penetration of $CaCl_2$, and the accelerators Genapol C-100, diethylsuberate, and Ethomeen T 25 had no effect on rates of glyphosate penetration across astomatous poplar cuticles (Schönherr, 2002).

When estimating pore sizes of astomatous cuticles from the ratio P_f/P_d, no surfactants were used (Schönherr, 1976*a*; Beyer *et al*., 2005). With penetration of salt solutions into leaves or fruits, entrance of solutions into open stomata or lenticels is a possibility (Schlegel and Schönherr, 2002). Schönherr and Bukovac (1972) studied this phenomenon using *Zebrina* leaves. They concluded that in the absence of hydrostatic pressure, surface tensions below 30 mN m^{-1} are needed for mass flow into open stomata. This is in agreement with reports showing stomatal infiltration by absolute ethanol and other organic solvents that perfectly wet cuticles, provided stomata are open. With these solvents, it can be tested whether stomata are open, but the degree of opening cannot be estimated (Schorn, 1929). Certain organosilicone surfactants reduce surface tensions to 25 mN m^{-1} or below, and they have been shown to induce stomatal infiltration (Knoche, 1994). With other conventional surfactants, stomatal penetration amounted to a few per cent of the dose (Green and Bukovac, 1974; Eichert *et al*., 1998; Eichert and Burkhardt, 2001; Schlegel *et al*., 2005). There is some controversy about the practical significance of stomatal infiltration, but the surfactant effect on salt penetration across astomatous cuticles is certainly not related to infiltration of stomatal pores in cuticles.

Diffusion of non-electrolytes in cuticles greatly increases with temperature, and energies of activation (E_a) range from 75 to 200 kJ mol^{-1} (Baur *et al*., 1997; Buchholz, 2006). E_a for diffusion of cations in cross-linked ion exchange resins range from 21 to 42 kJ mol^{-1}, and they were higher in more highly cross-linked resins (Meares, 1968). However, in the range of 15–30 °C, the rate constant of penetration (k) of inorganic salts [$CaCl_2$, $K_2(CO_3)$] across pear leaf CMs was not affected by temperature (Schönherr, 2000; Schönherr and Luber, 2001). Rate constants of penetration are related to permeability coefficients (equation 2), which are mixed quantities that depend on solute mobility (diffusion coefficient) and solute sorption (partition coefficient). The temperature dependence of partition coefficients for 4-nitrophenol in *Ficus* leaf and tomato fruit cuticles has been studied (Riederer and Schönherr, 1986) and turned out to be rather complex. Sorption was a spontaneous process, i.e. the free energy change ΔG was negative. Sorption (K_{cw}) was greater in tomato fruit than in *Ficus* leaf cuticles, and extracting cuticular waxes increased partition coefficients. K_{cw} decreased with increasing temperature and solute concentration. In addition, increasing temperature increased the number of sorption sites in cutin of tomato fruits but not of *Ficus* leaves. No such sorption data are available for ions or water in aqueous pores of cuticles, and it is not known how concentrations of salt and swelling of cuticles are affected by temperature. Possibly, the salt concentration in pores decreased (due to the exothermic nature of sorption) while ion mobility increased when the temperature was increased, such that both processes cancelled each other out.

Rate constants of penetration of organic salts (proline, glyphosate) increased with increasing temperature (Fig. 9). Absolute values of k were larger with pear than with poplar cuticles, and E_a were 68 and 42 kJ mol^{-1}, respectively. Possibly, a differential effect of temperature on sorption of organic and inorganic ions in aqueous pores may have caused this, but data are not available.

Size selectivities of aqueous pores in astomatous cuticles (*P. canescens*) and stomatous leaf discs (*V. faba*) at maximum swelling have been studied (Schönherr and Schreiber, 2004; Schlegel *et al*., 2005). As test compounds, calcium salts with molecular weights ranging from 111 ($CaCl_2$) to 755 g mol^{-1} (Ca-lactobionate) were used. Concentrations were 0.04 mol l^{-1}, and the pH of donor solutions varied only a little (6.3–8.3) such that pH effects on swelling were small (Schönherr and Schreiber, 2004). Measurements were limited to 100% humidity over the salt residue because most of the compounds had their POD near 100%.

Both isolated non-stomatous CMs and stomatous leaf discs were permeable to all compounds tested, but rate constants were higher with stomatous broad bean leaves

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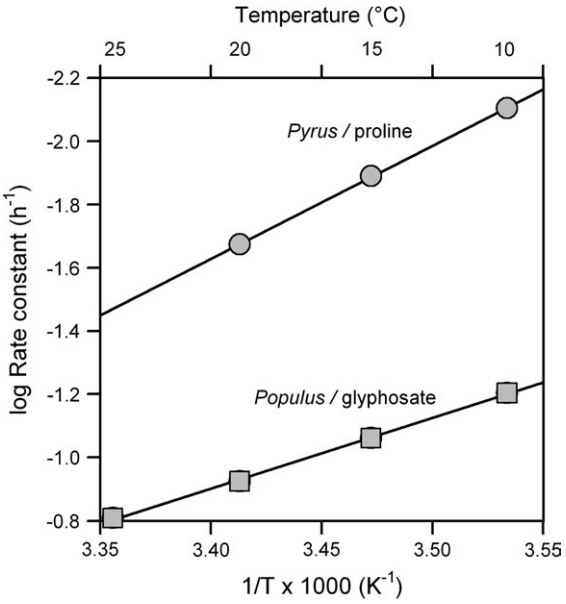

Fig. 9. Arrhenius graph showing the effect of temperature on rate constants of penetration of proline across *Pyrus communis* cuticular membranes and glyphosate across *Populus communis* cuticular membranes at 90% humidity. Solute concentration was 5 g l^{-1} and the donor contained 0.2 g l^{-1} Glucopone 215 CSUP.

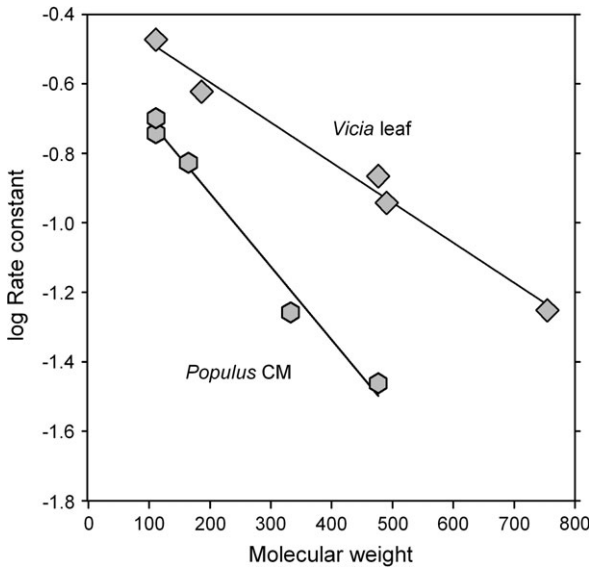

Fig. 10. The effect of molecular weight of selected calcium salts on rate constants of penetration across astomatous *Populus canescens* cuticular membranes and the upper leaf cuticles of *Vicia faba* leaf discs. Humidity of the salt residues was 100%, temperature was 20 °C, and solute concentrations were 0.04 mol l^{-1}. The donor contained 0.2 g l^{-1} Glucopone 215 CSUP. The slopes of the plots are -2.11×10^{-3} mol g^{-1} and -1.15×10^{-3} mol g^{-1} for *Populus* and *Vicia*, respectively.

than with astomatous poplar CMs (Fig. 10), and the dependence on molecular weight was greater with poplar (-2.11×10^{-3} mol g^{-1}) than with *Vicia* (-1.15×10^{-3} mol g^{-1}). An increase in molecular weight from 100 to 500 g mol^{-1} resulted in a decrease in rate constants by factors of 7 (*Vicia*) and 13 (*Populus*), respectively. This indicates that cuticular ledges of *Vicia* stomata are more permeable and less size selective than aqueous pores in poplar cuticles. *Vicia* leaves have glandular trichomes, but their contribution to fluxes and size selectivity was not studied. With *Vicia* leaves, permeability was about twice as high in light than in the dark, but size selectivity was not affected (Schlegel *et al*., 2005). Higher permeability of stomatous leaf surfaces in the light has been reported previously, for both inorganic and organic ions (Schönherr and Bukovac, 1978; Schlegel and Schönherr, 2002).

For the same range of molecular weights, selectivity for neutral lipophilic solutes was much larger; their mobility decreased by >3 orders of magnitude (Buchholz *et al*., 1998; Buchholz, 2006) in CMs of all species tested. Hence, size discrimination of large ionic solutes in aqueous pores is much less severe than for neutral molecules diffusing in the lipophilic pathway (cutin and amorphous waxes). Formulating weak acids or bases might be advantageous if cuticular penetration of large compounds is necessary for biological activity.

Based on the data discussed so far, it appears that permeabilities of isolated cuticles (pear and poplar) and stomatous leaf surfaces (*V. faba*, *Zea mays*, *M. domestica*, and *P. communis*) to inorganic ionic solutes and organic solutes all follow a similar pattern. The main exception is the temperature effect on rate constants for organic and inorganic ions. However, permeation of Fe(III) chelates differs. Penetration was a first order process as well, and at 100% humidity the Fe(III) chelates disappeared exponentially with time from the surface of the CM. At 90% humidity or below, penetration was negligible. Rate constants of penetration were much smaller than expected from the data obtained with Ca salts (Fig. 10). Permeability decreased with increasing Fe chelate concentration. At 100% humidity, half-times measured with poplar CM were 11 h (2 mmol l^{-1}), 17 h (10 mmol l^{-1}), and 36 h (20 mmol l^{-1}), respectively. In the presence of FeEDTA, penetration of $CaCl_2$ was slowed down greatly. Half-times for penetration of $CaCl_2$ were 1.9 h in the absence of FeEDTA but rose to 3.1 h in the presence of an equimolar concentration of EDTA and to 13.3 h when the FeEDTA concentration was doubled. Hence Fe chelates reduced the permeability of CMs to $CaCl_2$ and to the Fe chelates themselves. It was suggested that Fe chelates reduced the size of aqueous pores. This view is supported by the fact that rate constants for calcium salts were ~5 times higher than for Fe chelates with the same molecular weights. Water permeability of cherry fruit cuticles was reduced by $FeCl_3$ (Weichert *et al*., 2004), and AgCl reduced water permeability of poplar CMs (Schreiber *et al*., 2005*a*).

Rate constants of penetration of Fe chelates across poplar CMs were independent of molecular weight in the range

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254–507 g mol^{-1}. This is another difference compared with calcium salts for which a good correlation between log (rate constant) and anhydrous molecular weight was observed (Fig. 10). Ca^{2+} ions carry hydration shells, while anions are not or only weakly hydrated (Israelachvili, 1991). Residence times of water molecules in the primary hydration shell of Ca^{2+} are of the order of 10^{-8} s^{-1}, and it has been argued that hydration water oscillates between calcium ions and dipoles lining the pore walls (Schönherr *et al.*, 2005). With trivalent cations such as Fe^{3+}, rotational correlation times are much longer and range from seconds to hours. In narrow hydrated pores, this may lead to a competition for water between Fe^{3+} and ionized carboxyl groups, leading to partial dehydration of the pore. As already mentioned, trivalent ions may bind water very strongly, and ion–water complexes of fixed stoichiometry can be formed. If this happens in the narrow aqueous pores, it could explain the lack of correlation between rate constants and molecular weights. Furthermore, hydrogen bonds might be formed between the $Fe(III)(HOH)_n$ complexes and dipoles of the pore wall. This could slow diffusion of Fe chelates in these pores, as was in fact observed (Schönherr *et al.*, 2005).

It has been argued that penetration of ionic solutes is evidence for aqueous pores in cuticles, and it may be asked to what extent these pores contribute to penetration of polar non-electrolytes. They are expected to diffuse in aqueous pores as their water solubility is much higher than their lipid solubility. However, since they interact with water only via hydrogen bonds, they can also diffuse in the lipophilic pathway by a solubility mechanism. The question arises of which fraction of polar non-electrolytes uses the aqueous and lipophilic pathways. This was investigated with pear and *Stephanotis* leaf CMs, $CaCl_2$, and methyl glucose (log K_{ow}=–3.0). Using the method of paired comparisons, the rate constants of penetration of ionic $CaCl_2$ and non-ionic methyl glucose were measured (Shi *et al.*, 2005). With a fraction of the CM sample population, equal fluxes of $CaCl_2$ and methyl glucose were measured, but with most CMs the rate constants for methyl glucose were somewhat higher. From the data, it was concluded that on average 60% of the methyl glucose diffused across aqueous pores, while 40% used an alternative pathway. Interestingly, the permeability of cuticles for the hydrophilic methyl glucose was not increased by accelerators which increase solute mobility in the lipophilic pathway (Baur, 1998; Buchholz and Schönherr, 2000; Buchholz, 2006; Schreiber, 2006). Since the solubility of methyl glucose in wax is extremely low and accelerators did not increase the permeability for methyl glucose, it is not likely that the lipophilic pathway served as an alternative pathway. The permeability of this alternative pathway was increased only by ethoxylated dodecanols with six or eight ethylene oxide groups. It was speculated that $C_{12}E_6$ and $C_{12}E_8$ sorbed in cuticles might have generated an additional polar pathway for methyl glucose (Shi *et al.*, 2005).

Conclusions

The evidence presented supports the concept of two separate and parallel diffusion pathways in plant cuticles. The bulk of cuticles consist of methylene and methyl groups, and they constitute the lipophilic pathway. Properties of the lipophilic pathway have been characterized, and the contributions to fluxes of solute mobility, solute sorption, and driving forces been studied directly (Buchholz, 2006; Schreiber, 2006). Evidence in support of the aqueous pathway is largely indirect, and characterization of aqueous pores has to rely on interpretations of fluxes of water and electrolytes, respectively. In some cases, permeability coefficients or rate constants have been obtained, but the contributions of sorption and solute mobility to fluxes is unknown. Likewise, the chemistry and structure of the pore walls have not been studied directly.

The best evidence in support of the occurrence of aqueous pores in certain parts of cuticles is the fact that they are permeable to hydrated ionic species. Ions are surrounded by water molecules, which form hydration shells. These ion–dipole interactions are very strong (bond energy >100 kJ mol^{-1}) and, therefore, under physiological conditions, ions are hydrated. This renders them insoluble in liquid and solid lipid phases, and the lipophilic pathway becomes inaccessible to them. Cuticles also contain permanent dipoles such as hydroxyl, amino, and carboxyl groups. These permanent dipoles sorb water, and cuticles swell depending on water activity or partial pressure of water vapour. If permanent dipoles (dipole–dipole interactions) and ionizable groups (ion-dipole interactions) are continuous across cuticles, sorbed water can form an aqueous phase in cuticles, i.e. the aqueous pores. These aqueous pores form only in the presence of water and have been localized using ionic fluorescent dyes, silver nitrate, and mercuric chloride. Aqueous pores preferentially occur in cuticular ledges, at the base of trichomes, and in cuticles over anticlinal cell walls. The biological function of aqueous pores in cuticles is subject to speculation. Aqueous pores are crucial for foliar penetration of agricultural chemicals. They can be important in plant research because they permit the administration of large ionic molecules to leaves, stems, and flowers without injury.

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