Urea Penetration of Isolated Tomato Fruit Cuticles

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Abstract. ¹⁴C-urea penetration of isolated tomato (*Lycopersicon esculentum* Mill. cv. 'Pik Red') fruit cuticular membranes (CM) was studied as a function of concentration and temperature. There was no significant effect of cuticular wax on urea penetration at 25C, permeances for the CM being 8.4×10^{-10} and dewaxed CM (DCM) $11.1 \times 10^{-10} \text{ m} \cdot \text{s}^{-1}$. Time lags were near zero for both CM and DCM. Steady-state diffusion analysis suggests that the relatively low cuticular permeance of urea is due to low partitioning that offsets high diffusivity. Urea flux through the CM and DCM showed ≈ 1.5 - and 1.9 -fold increases, respectively, for each 10C increase between 5 and 45C. Urea flux across CM and DCM increased linearly with concentration (10 μM to 1 M) and, thus, was a first-order process.

Urea is frequently used as a fast-acting, inexpensive, foliar-applied nitrogen fertilizer and is often included in spray solutions to improve the effectiveness of other foliar-applied chemicals (Bowman and Paul, 1992; Kannan, 1980; Weinbaum, 1988). Despite the widespread use of urea, little is known about its penetration through the cuticle.

The cuticle is a heterogeneous, lipophilic, polymeric membrane that covers the aerial parts of terrestrial plants (Cutler et al., 1982) and acts as the primary barrier to the penetration of foliar-applied chemicals (Bukovac et al., 1981). Given its prominent role in foliar penetration, there is a fundamental benefit to using isolated plant cuticles as model systems for elucidating the kinetics and mechanism of penetration (Bukovac and Petracek, 1993). The infinite-dose technique, commonly used in polymer permeability studies, has been particularly useful (Crank and Park, 1968; Schönherr and Riederer, 1989). Transport characteristics such as flux, permeance, diffusion, and partition coefficients can be readily determined with this approach by measuring the transfer rate of the solute from a donor to receiver cell solution through an interfacing cuticular membrane (CM).

Previous studies on urea diffusion through isolated tomato fruit cuticles differed from that of other solutes in two ways. First, the relationship between urea concentration and flux through the cuticle was nonlinear (Yamada et al., 1965). Since the concentration gradient and flux across the membrane are related linearly according to Fick's first law of diffusion in an ideal system (Comyn, 1985; Crank and Park, 1968; Nobel, 1983), this observation suggests that urea may facilitate its own diffusion, as well as that of other chemicals, by altering cuticular permeability. Second, cuticular waxes did not markedly affect urea penetration of tomato fruit cuticles (Shafer and Schönherr, 1983), which is in contrast to earlier studies showing that waxes reduced cuticular penetration of a wide range of solutes by several fold (Schönherr and Riederer, 1989). Therefore, we examined in greater detail urea penetration through isolated tomato fruit cuticles as affected by concentration and temperature to better understand urea-cuticle interactions.

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Materials and Methods

Cuticle isolation. Disks (20 mm in diameter) free of visible defects were excised by corkbore from locally field-grown mature tomato fruit (*Lycopersicon esculentum* Mill. cv. 'Pik Red') and greenhouse-grown ficus (*Ficus elastica* Roxb. ex Hornem) leaves. The excised tissue was incubated in a mixture of cellulase (0.2% w/v, Sigma, St. Louis), pectinase (4% w/v, ICN Nutritional Biochemicals, Costa Mesa, Calif.), and NaN_3 (1 mM to prevent fungal and bacterial growth) in sodium citrate buffer (50 mM, pH 4.0) prepared in distilled water (Shafer and Bukovac, 1987; Yamada et al., 1964). Enzyme solution was changed several times over a 2-week period. The CM released from the tissue were rinsed repeatedly in distilled water, air-dried, and stored at room temperature. Dewaxed cuticular membranes (DCM) were prepared by batch extracting the soluble cuticular lipids with 10 exchanges of 1 chloroform : 1 methanol at 45C over 3 days.

Chemicals. Buffer solution for diffusion studies was prepared with citric acid (20 mM, pH 3.0) in deionized, distilled water with 1 mM NaN_3 included to prevent microbial growth. Donor solution consisted of ¹⁴C-labeled urea (specific activity 1.94 GBq·mmol⁻¹, 99% radiochemical purity, Amersham Corp., Arlington Heights, Ill.) prepared in buffer solution. Receiver cell solution consisted of buffer solution only.

Initial time-course study. The diffusion apparatus and procedure for these studies were similar to that described by Kerler et al. (1984). Briefly, cuticle disks were weighed and mounted between plexiglass holders and the holders were fixed between glass half-cells by vacuum grease. Cuticles mounted in diffusion cells were exposed to buffer with a slight hydrostatic pressure applied to the outer morphological surface (donor cell side) for a minimum of 12 h to hydrate the cuticle and identify cuticles with holes. Cuticles and diffusion units used for the initial time-course study were reutilized in the temperature and concentration experiments.

Diffusion cells were positioned in a thermostated water bath on a multistirring plate and equilibrated to 25C. Time-course experiments were initiated by replacing the receiver cell buffer solution with 5 ml fresh buffer and donor cell solution with 5 ml ¹⁴C-labeled urea (10 μM) donor solution. Donor and receiver cell solutions were stirred vigorously by spin fins throughout the experiment to minimize boundary layer effects. Samples (1 ml) were removed from the receiver cell at 0, 0.25, 0.5, 2, 3, 4, 5, 6, and 7 h after initiation of the experiment and replaced with buffer solution. The samples were taken up in 10 ml scintillation cocktail (Safety-

Fig. 1. Time-course of diffusion of urea ($10 \mu\text{M}$ in 20 mM citrate buffer solution, pH 3.0, 25°C) through isolated tomato fruit CM and DCM. Bars represent SE ($n = 10$ for CM; $n = 8$ for DCM). Regressed lines depict steady-state flows. The x-intercept of the regressed lines represents time lags.

Solve, Research Products International Corp., Mount Prospect, Ill.) and radioassayed by liquid scintillation spectrometry (LSC model 1211, LKB-Wallac, Gaithersburg, Md.). A regression line was fitted through the linear portion of the plot of amount penetrated vs. time (Fig. 1). The slope and x-axis intercept of the extrapolated regression line corresponded to the steady-state flow rate (F) and time lag (t_e), respectively. Steady-state flux (J) and permeance coefficient (P) were determined from the steady-state flow (Nobel, 1983):

$$J = F/A \quad \text{Eq. [1]}$$

and

$$P = F/A \cdot \Delta C \quad \text{Eq. [2]}$$

where A is the cross-sectional area of the cuticle in contact with solution (113 mm^2) and ΔC is the concentration gradient across the membrane. The diffusion coefficient (D) was calculated from the time lag (Crank, 1975):

$$D = l^2/6 \cdot t_e \quad \text{Eq. [3]}$$

where l is the thickness of the cuticle estimated gravimetrically from a specific density of 1.20 (Petraček, 1991). Average estimated thickness was 12 ± 3 and $11 \pm 3 \mu\text{m}$ for CM and DCM, respectively. The partition coefficient (K) was calculated from P and D (Crank, 1975):

$$K = P/D \cdot l \quad \text{Eq. [4]}$$

Temperature study. Following the initial time-course study, the system was equilibrated to 5°C . After steady-state flux was re-established ($<24 \text{ h}$), a minimum of six samples per cell were taken over time and flux was calculated. The temperature was then increased to 45°C and then decreased to 5°C in increments of 10°C with steady-state flux determined at each temperature. Fluxes at a given temperature were similar for the ascending and descending temperature analyses and, therefore, were averaged. Activation energies (E_a) and pre-exponential factors (A) of permeance were calculated from the Arrhenius equation:

$$\ln P = \ln A - E_a/R \cdot T \quad \text{Eq. [5]}$$

Table 1. Diffusion of urea ($10 \mu\text{M}$ in 20 mM citrate buffer solution at pH 3.0, 25°C) through isolated tomato fruit cuticular membranes (CM) and dewaxed cuticular membranes (DCM). Values are the means ($\pm \text{SE}$) of ten and eight replications of cuticular membranes (CM) and dewaxed cuticular membranes (DCM), respectively. Fluxes were not statistically different ($P = 0.05$) by analysis of variance.

Cuticle	Time lag (h)	Flux ($\text{pmol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$)	Permeance ($10^{-10} \text{ m} \cdot \text{s}^{-1}$)
CM	0.18 ± 0.09	8.7 ± 2.5	8.4 ± 2.4
DCM	-0.01 ± 0.09	11.6 ± 4.0	11.1 ± 3.8

where R is the gas constant (Crank and Park, 1968).

Concentration study. Urea concentration in the donor solution was increased by 10-fold increments from $10 \mu\text{M}$ to 1 M by adding nonlabeled urea. Steady-state flux at 25°C was determined for each concentration by taking a minimum of six samples per cell at 1-h intervals.

Statistics. This series of experiments was performed on two sets of cuticles of ten and eight replications for CM and DCM, respectively, except for the concentration study where five replications were used for CM and DCM. Time lags, fluxes, activation energies, and permeance, diffusion and partition coefficients were calculated for each cuticle. Data were subjected to analysis of variance where appropriate.

Results and Discussion

The time-course of urea penetration through isolated tomato fruit cuticles at 25°C was characterized by short time lags followed by a linear phase of steady-state flux (Fig. 1 and Table 1). Since time lags represent the amount of time needed to fill one-sixth of the total sorption capacity of a membrane (Riederer and Schönherr, 1985), it is interesting to compare the results obtained herein (i.e., ≈ 10 -min time lag for CM) with other cuticles and compounds. For instance, time lags for urea with *Ficus* and *Citrus* leaf and pepper fruit CM were ≈ 320 , 8, and 36 min, respectively (Shafer, unpublished). In terms of other chemicals, phenol, 2-nitrophenol, 4-nitrophenol, and 2,4-D all had longer time lags (≈ 2 - to 5-fold increases) in tomato CM than urea (Riederer and Schönherr, 1985; Shafer and Schönherr, 1985). An even greater extreme is represented by benzyladenine, which was found to have a time lag in tomato CM of $\approx 300 \text{ min}$ (Petraček and Bukovac, unpublished).

In this system, permeance was not significantly affected by dewaxing of the cuticle (8.4×10^{-10} and $11.1 \times 10^{-10} \text{ m} \cdot \text{s}^{-1}$ for CM and DCM, respectively). In contrast, urea penetration of isolated *Ficus* leaf DCM was nearly 30 times greater than that of CM (permeance coefficients of 4.7×10^{-10} and $121 \times 10^{-10} \text{ m} \cdot \text{s}^{-1}$, respectively). Although somewhat surprising, given the general trend of increased permeance upon dewaxing (see review by Schönherr and Riederer, 1989), these results confirm previous observations of wax effects on urea flux through isolated tomato fruit and *Ficus* leaf cuticles (Shafer and Schönherr, 1983).

Steady-state analysis of diffusion Eq. [1] suggested that relatively short time lags resulted from large diffusion coefficients. Further, small permeances coupled with large diffusion coefficients indicated that the effective partition coefficient of diffusion was also small Eq. [2]. Since urea is a relatively small and polar (0.025 octanol/water partition coefficient) molecule, it should be a highly diffusive molecule that partitions poorly into the nonpolar cuticle. Example calculated diffusion and partition coefficients for CM penetration by urea are $4.6 \times 10^{-14} \text{ m}^2 \cdot \text{s}^{-1}$ and 1.0, respectively.

Thus, these expectations are consistent with the steady-state diffusion data for urea.

Cuticular permeances of urea and water should be similar because of their structural similarities. However, urea penetration may be somewhat more restricted because it is slightly larger than water (molecular radii of 0.26 vs. 0.20 nm). Schönherr (1976a) suggested that citrus leaf cuticle penetration by polar compounds may be size-limited by the radii of water-filled pores (≈ 0.40 nm). Polar molecules that are larger than these "polar pathways" must solubilize into the nonpolar cuticle if they are to penetrate. This nonpolar route or "solubilization pathway" does not readily accommodate polar compounds such as urea and would result in low effective partition coefficients. Further, Schönherr and Riederer (1989) suggested that wax has a greater effect on penetration through polar pathways because these routes can be more completely blocked by wax. This hypothesis that wax blocks polar pathways was supported by the significant reduction of urea penetration caused by waxes in *Ficus* leaf cuticles. However, lack of a wax effect on urea penetration through tomato fruit CM suggested that (1) urea penetration of polar pathways was not obstructed by tomato fruit CM wax or (2) the polar pathways themselves obstructed urea penetration and that urea penetration is entirely by solubilization in the cuticle.

Alternatively, urea may affect its own penetration by changing water/cuticle interactions. The effect of urea as a protein denaturant has been attributed to its action as a hydrogen bond breaker and that of a hydrophobic interaction enhancer (Creighton, 1984). Since water acts as a plasticizer of the cuticle (Petracek and Bukovac, 1991), urea may alter this effect by either depressing plasticization of the DCM or enhancing plasticization of the CM, thereby resulting in unexpectedly low DCM or high CM permeances. Confirmation of these effects requires further study.

The effect of temperature was examined to gain an understanding of the mechanism of penetration. Urea flux showed 1.5- and 1.9-fold increases for CM and DCM, respectively, with a 10°C increase in temperature (Fig. 2). The Arrhenius equations for CM and DCM were significantly different ($P = 0.05$), and activation energies (26.4 and 45.0 $\text{kJ}\cdot\text{mol}^{-1}$, respectively, Table 2) were in a range similar to those determined for other diffusants of the cuticle (e.g., 53 $\text{kJ}\cdot\text{mol}^{-1}$ for water, Schönherr, 1976a).

Fig. 2. Effect of temperature on the urea flux ($10\ \mu\text{M}$ in 20 mM citrate buffer solution, pH 3.0) through isolated tomato fruit CM (●) and DCM (○). Bars represent SE ($n = 10$ for CM; $n = 8$ for DCM). The inset represents the Arrhenius relationship of permeance.

Table 2. Effect of temperature on urea permeance ($10\ \mu\text{M}$ in 20 mM citrate buffer solution at pH 3.0) of isolated tomato fruit cuticular membranes (CM) and dewaxed cuticular membranes (DCM). Values are the means ($\pm$ SE) of ten and eight replications of CM and DCM, respectively. The Arrhenius equations for CM and DCM were significantly different ($P = 0.05$) by analysis of variance.

Cuticle	Activation energy ($\text{kJ}\cdot\text{mol}^{-1}$)	Ln pre-exponential factor ($\text{m}\cdot\text{s}^{-1}$)
CM	26.4 ± 5.1	-10.6
DCM	45.0 ± 4.6	-2.6

Fig. 3. The relationship between urea donor solution concentration (initial concentrations of 10^{-2} to 10^3 mM in 20 mM citrate buffer solution, pH 3.0, 25°C) and flux through isolated tomato fruit CM (●) and DCM (○). Inset illustrates the effect of urea donor solution concentration on the permeance coefficient. Bars represent SE ($n = 5$).

The activation energy of permeation is the sum of the activation energy of diffusion (energy required to create free volume for molecular diffusion) and the change in enthalpy of sorption (energy required for or released during sorption, Rogers, 1985). Thus, the lower activation energy of CM permeance suggested either that wax improves mobility of the relevant moieties in the cuticle or that wax makes sorption energetically less favorable (more positive ΔH). Since rheological studies indicated that waxes act as a filler (Petracek and Bukovac, 1991) and, thus, reduce polymer chain movement in the cuticle, the increased activation energy of permeance may be attributed to a weaker interaction of urea with the cuticle in the presence of wax.

Urea concentration was related linearly with flux (Fig. 3) and, thus, permeance was constant (inset). This was indicated by the log-log relationship between donor concentration and flux with a slope of unity. Because the donor concentration represents the concentration gradient, a slope of unity, based on Fick's first law of diffusion, indicates that the process of urea penetration is first order (i.e., 0.6 calculated from Yamada et al., 1965). Therefore, urea diffusion and partition coefficients for the tomato fruit cuticle were apparently not concentration-dependent unless their dependencies are compensatory. This relationship between concentration and flux was not affected by the removal of waxes (CM, $\log \text{flux} = 1.007 \cdot \log \text{donor concentration} - 0.31$; DCM, $\log \text{flux} = 1.003 \cdot \log \text{donor concentration} - 0.23$).

One mechanism by which urea could affect uptake of other compounds is by altering cuticular permeability. However, our data using an infinite dose diffusion system did not show any

apparent urea-induced increase in tomato fruit CM permeability to urea or other alteration of the cuticle. Thus, unlike Yamada et al. (1965), we found: (1) no oil-like substance was extracted by urea, (2) no prolonged lag phase, and (3) no self-enhanced penetration as indicated by the donor concentration and flux relationship (Fig. 3). Reasons for these differences may include the following:

(1) The previous system was prone to boundary layer formations and low sensitivity of GM-window counting.

(2) The cuticles used in this study were hydrated, whereas there was no prehydration in the earlier study. This may also explain the lack of steady-state flux in the first 24 h of the experiment.

(3) The cuticles used in this study may have different characteristics due to differences in genetic composition and/or growing conditions.

In addition to yielding information on penetration mechanisms, infinite-dose diffusion studies provide an effective technique for evaluating factors that affect cuticular penetration and ensuing uptake of foliar-applied chemicals. The effects of temperature and concentration on penetration are of particular interest not only because they are often substantial, but because changes in temperature and concentration occur during spray application and urea uptake. Thus, the coefficients determined from these studies may be useful in developing time-dependent functions that model and predict the complex events of penetration/diffusion from spray droplets.

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