Quantification of stomatal uptake of ionic solutes using a new model system

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Abstract

Evidence for stomatal uptake of solutes by leaves without the application of surfactants or pressure has recently been provided (Eichert et al., 1998). In the present study, experimental conditions were refined in that the water potential was held at \( <0 \) on the physiologically inner side of the epidermal strips (ES) by use of a ceramic plate or the proper mesophyll of the plant. The penetrated substances were immobilized on the inner side by ion exchange membranes. The influence of humidity, light, stomatal density, and re-wetting on the uptake of anions (fluorescein) and cations (Fe\(^{3+}\)) was investigated, using leaves of Allium porrum, Commelina communis and Sedum telephium. Uptake increased with humidity, stomatal aperture and stomatal density. It was restricted to stomatal areas, and was especially high below the rims of drying droplets. Again, penetration of stomatal pores was observed. Uptake was strongly correlated with the number of penetrated stomata, although usually less than 10\% of the stomata contributed to uptake. The number of stomata that had been penetrated was highly variable, increasing extremely significantly with the number of repeated drying/wetting cycles. These results indicate that stomatal uptake can be a major pathway for the foliar uptake of ionic solutes. It is a dynamic process, depending on environmental conditions and history of the residues on the leaf, aspects that had been neglected in previous concepts.

Key words: Cuticle, epidermis, foliar uptake, leaf surface, stomata.

Introduction

In the past few decades there has been considerable interest in processes that regulate foliar uptake and in the release of substances that has mainly been motivated by agricultural or environmental questions such as pesticide uptake, foliar fertilization or forest decline. Concepts of the processes involved changed repeatedly, focusing on the existence and role of ectodesmata (Franke, 1967), stomatal uptake (Middleton and Sanderson, 1965; Eichert et al., 1998), and the relevance of the peristomatal regions (Maier-Maercker, 1979; Schönherr and Bukovac, 1978). The former two were rejected after a longer debate in the 1960s and early 1970s and only cuticular transport was retained. Most of the experimental studies were conducted using atomatous isolated cuticles, others used epidermal strips (ES; Eichert et al., 1998), leaf discs (Schönherr and Bukovac, 1978; Hoad et al., 1997; Chamel et al., 1997) or entire leaves (Dybing and Currier, 1961). Whereas uptake into attached leaves represents the most realistic conditions, quantification of the fluxes is difficult due to concurrent processes such as translocation of substances or strong adsorption of the applied substances to the leaf surface, thus impeding the quantification of the effective uptake. The use of epidermal strips provides more experimental control and gave both a qualitative and a quantitative proof of stomatal uptake of substances into leaves (Eichert et al., 1998), contrary to the general belief of exclusive cuticular uptake. The experiments using ES differed, however, from real leaf conditions in that they used a receiving solution on the physiological inner side of the epidermis. Stomata are protected against penetration of liquids from the outer leaf side (Schönherr and Bukovac, 1972), but it is not known if penetration of liquids from the inner to the outer side of the epidermis is also hindered. Hence, model

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Abbreviations: AEM, anion exchange membrane; CEM, cation exchange membrane; ES, epidermal strip; IEM, ion exchange membrane (AEM or CEM); PPFD, photosynthetic active photon flux density; RH, relative humidity.

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systems for the study of foliar uptake that utilize a liquid as a receiving medium for penetrated substances, which is common in experiments with stomatal isolated cuticles, may not be appropriate when stomatal epidermal strips are used. The measured uptake rates could then be artificially high due to liquid connections that are caused by penetration of stomata from the interior to the outer leaf side.

In the present paper, the influence of humidity, stomatal area and number of wetting-cycles on stomatal uptake of uranine (Na-fluorescein) is investigated by a new experimental system with the following important features: (i) the avoidance of liquid water underneath the epidermal strip, (ii) the conservation of uptake patterns for a qualitative investigation of uptake, and (iii) the quantification of uptake rates after desorption of the ions.

Materials and methods

Table 1 gives a brief overview of the experiments presented in this paper.

Plant material

Plants of Allium porrum L. (cv. Tropita) and Convolvulina communis L. were grown from seeds. Schedum telephium L. was propagated from leaf cuttings. The plants were grown in a commercial peat-clay mixture (Einheitserde Typ T) in a greenhouse, watered daily and fertilized once a week with a commercial liquid fertilizer (Wuxal Super, Bayer) applied to the soil.

Experiment I

The objective of this experiment was to provide additional evidence that uptake via stomata is a process that is relevant for intact plants. This experiment was performed with intact C. communis and S. telephium plants. 10 μl droplets of a 1 mM aqueous uranine solution were applied to the adaxial (stomatal) leaf surfaces. During this experiment the plants were illuminated with a PPFD of 200 μmol m⁻² s⁻¹ without any further control of climatic conditions. Upon drying of the droplets the treated leaves were excised and examined with an epifluorescence microscope.

The model system

In experiments II to VI foliar uptake was studied with a model system composed of an epidermal strip (ES) and an ion exchange membrane (IEM). The IEM was sandwiched between the ES and a water storing medium that should balance the evaporative water losses from the ES during the experiment. In experiments II, III and VI with A. porrum ES, this medium was a ceramic plate hydraulically connected to a water reservoir 80 cm below the level of the ES (Fig. 1A) providing a negative water potential in the IEM. In experiments with S. telephium a different approach was chosen for water supply (Fig. 1B, C). As the epidermis of S. telephium is rather soft and flexible the ES tended to curl and contract after peeling. To ensure that the ES remained in the original shape and dimension after peeling, the epidermis was not cut off from the mesophyll but remained still attached at one end. After gently blotting the inner side of the epidermis and the mesophyll with dry filter paper, a 1 × 5 cm section of IEM was inserted and the ES was folded back into the original position. The incisions were sealed with adhesive tape (Tesa film, Beiersdorf) which fixed the entire leaf section on a microscope slide. The succulent mesophyll tissue provided enough water to prevent the ES from desiccation.

An anion exchange membrane (‘AEM’, NA 45, Schleicher & Schuell) was used in experiments II to V where foliar uptake was studied with the anionic fluorescent dye Na-fluorescein (uranine). Uptake studies with Fe-salts (experiment VI) were performed with a cation exchange membrane (‘CEM’, NA 49, Schleicher & Schuell).

Experiment II

The objective of this preliminary experiment was to test the effect of re-wetting of dried residues on uptake rates of uranine

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Parameter examined</th>
<th>Plant speciesa</th>
<th>Type of IEMb</th>
<th>Water supplyc</th>
<th>Compound tested</th>
<th>Type of evaluationd</th>
<th>n per treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>–</td>
<td>S, C</td>
<td>–</td>
<td>Intact plant</td>
<td>Uranine</td>
<td>Qual.</td>
<td>5</td>
</tr>
<tr>
<td>II</td>
<td>Interaction re-wetting time</td>
<td>A (ES)</td>
<td>AEM</td>
<td>Plate</td>
<td>Uranine</td>
<td>Quant.</td>
<td>10</td>
</tr>
<tr>
<td>III</td>
<td>Re-wetting</td>
<td>A (ES)</td>
<td>AEM</td>
<td>Plate</td>
<td>Uranine</td>
<td>Qual. and quant.</td>
<td>15</td>
</tr>
<tr>
<td>IV</td>
<td>Density of stomata</td>
<td>S (ES)</td>
<td>AEM</td>
<td>Mesophyll</td>
<td>Uranine</td>
<td>Quant.</td>
<td>45:44e 29:25f</td>
</tr>
<tr>
<td>V</td>
<td>Relative humidity, stomatal aperture</td>
<td>S (ES)</td>
<td>AEM</td>
<td>Mesophyll</td>
<td>Uranine</td>
<td>Quant.</td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>–</td>
<td>A (ES)</td>
<td>CEM</td>
<td>Plate</td>
<td>Fe(III)-salts</td>
<td>Qual.</td>
<td></td>
</tr>
</tbody>
</table>

a: S. Schedum telephium, C: Convolvulina communis, A: Allium porrum. In experiment I intact leaves were used, the other experiments were conducted with epidermal strips (ES).
c: Plate: moist ceramic plate, mesophyll: proper leaf mesophyll.
d: Quant: quantitative, Qual: qualitative.
e: RH 40%, stomata open/closed, respectively.
f: RH 76%, stomata open/closed, respectively.
and to exclude any interference of the duration of the experiment, as the ES might become more permeable due to decomposition processes or structural changes in the case of experiments lasting for several hours or days. The experiment was performed using 10 sections of ES peeled from the adaxial sides of *Allium porrum* leaves. These sections (approximately 1.5 × 1.5 cm) were cut from the ES, then gently blotted on the physiological inner side with filter paper and transferred to the AEMs. After the application of a 10 µl droplet of a 1 mM aqueous uranine solution to each ES the droplets dried within 90 min at RH of 76 ± 1% (treatment 1). Subsequently, the ES were transferred to a second ceramic plate with fresh sections of AEMs and separated into two groups. The residues of the first group were re-wetted and dried again (treatment 2). After 280 min the ES of both groups were transferred to fresh AEMs and the residues of the first group were re-wetted and dried a second time (treatment 3). This wetting/drying cycle lasted 1020 min (17 h). Then two additional wetting/drying cycles were conducted with the second group of ES with the same intervals, i.e. 280 min after the first re-wetting (treatment 4) and 1020 min after the second re-wetting (treatment 5), whereas the residues of first group remained unwetted. After each of the five drying cycles the amount of uranine adsorbed by the AEMs was quantified as described below.

**Experiment III**

This second re-wetting experiment was performed similarly to experiment II, but no groups were separated. After the first drying of the droplets within 2 h (treatment 1) all residues were re-wetted three times every 2 h (treatments 2, 3, 4). The further experimental procedure was as described in experiment II. Uptake after each drying period was examined qualitatively and quantitatively as outlined below. Density of stomata was determined under the microscope using a eyepiece graticule.

**Experiment IV**

The objective of this experiment was to compare the uptake of uranine solution through the adaxial and abaxial surfaces of *S. telephium* leaves, which differ in the density of stomata. Three leaves were detached from the plant and cut along the mid vein into two halves. Prior to stripping of the epidermis one half was illuminated on the adaxial leaf side and the corresponding section on the abaxial side with a PPFD of 250 µmol m⁻² s⁻¹ for 4 h. During illumination the leaf sections were placed in a closed glass chamber. The cut edges of the leaf sections were immersed in tap water, while a support underneath each leaf section prevented the major proportion of the leaf blades from immersion. Subsequently, the ES were peeled and the AEMs were inserted between mesophyll and epidermis as described above. Five droplets of 10 µl of 1 mM uranine solution were applied to each ES with a distance of 1 cm between the droplets. The leaf sections were kept at 76 ± 1% RH. After 17 h the uptake experiment was terminated, the AEMs were removed and cut into five 1 × 1 cm sections and uranine penetration was determined.

Stomatal densities of both leaf sides were determined under the microscope with an eye-piece graticule. In order to characterize the wettability of both leaf surfaces static contact angles with droplets of uranine solution and pure water were determined as previously described (Eichert et al., 1998). Furthermore, the areas of dry residues of uranine solution were measured on both leaf sides with a microscope.

**Experiment V**

In this 2-factorial experiment the influence of stomatal aperture and ambient RH on foliar uptake of uranine into *S. telephium* leaves was examined. Fifteen excised leaves were cut into two halves along the mid-vein. One section of each leaf was illuminated for 5 h from the adaxial side as described in experiment III. The corresponding section was kept in darkness. ES were peeled from the adaxial leaf side, AEMs were inserted and uranine solution was applied as described above. Six leaves were randomly chosen for treatment with air of 76 ± 1% RH, 9 leaves were treated with air with a RH of 40 ± 5%. The uptake experiment lasted 18 h. The ES were removed from the leaf sections and uranine penetration was quantified for each droplet as described below.

**Experiment VI**

This experiment was designed to test the applicability of uranine as a model substance for foliar applied ions. Fe(III) was applied as a Cl⁻ or NO₃⁻ salt. One 10 µl droplet containing either 10 µM
Fe(NO₃)₃ or 10 μM FeCl₃ was applied to sections of ES. RH of the air was 40 ± 5%. Residues of the Fe-salts were re-wetted after 2 h and 4 h. After 17 h the ES were removed and the CEMs were examined qualitatively under a stereo microscope.

**Qualitative analysis of uptake**
Following the uptake experiments, leaves were examined with epifluorescence microscopy (Olympus BHS, filter combination: ex. B (410–490 nm)/em. B (> 520 nm)). IEMs were examined under a stereo microscope in normal light. If appropriate, image editing was performed (iPhotoExpress 1.0, Ulead Systems) in order to improve colour contrasts.

**Quantitative analysis of uptake**
Uraniode adsorbed to the AEMs was quantitatively removed using three sequential washes with 2 M KCl solution that was buffered with NaOH/boric acid (pH 10). Each 1 x 1 cm section of AEM was transferred into a 1.5 ml plastic tube, 1 ml of desorbing solution was added and the tubes were shaken for 15 min (100 min⁻¹). The solutions obtained in the three successive desorption steps were collected in a plastic cuvette and uranine concentration was determined with a fluorometer (SpeX Fluoromax, excitation wavelength 491 nm, emission wavelength 515 nm). Recovery of uranine was tested for a range of doses (50–3000 pmol) and was determined to be 96.0 ± 0.8% (n = 36).

**Statistics**
Uptake data are given as medians and standard errors of the medians. U-tests were performed with the penetration data from experiment II and IV. Data from experiment III and V were log(x + 1)-transformed in order to achieve normality and homogeneity of variances and analyses of variance were performed. Wettability of *S. telephium* leaf surfaces, areas of residues and densities of stomata (experiment IV) were compared with t-tests.

**Results**
Figure 2A shows detail of a uranine residue (red) on the surface of an intact *S. telephium* leaf in white light. In Fig. 2B the same detail is shown as viewed with fluorescent light. An isolated spot of yellow fluorescence can be identified. The centre of this spot is located at the margin of the uranine residue. After removal of the epidermis the fluorescent spot was still present (Fig. 2C), indicating that this proportion of the dye was taken up into the leaf. In Fig. 2E the mesophyll of a *C. communis* leaf is shown. The central region is outshone by the bright fluorescence of a high quantity of uranine taken up into the leaf. In the adjacent area of lower uptake, the less intensive fluorescence surrounds the darker substomatal cavities. This indicates that uptake was associated with the stomatal apparatus. In Fig. 2F a *S. telephium* epidermis is shown viewed from the physiological inner side. A conglomeration of concentrated uranine can be seen among the guard cells.

In the experiments using the model system uranine/ES/AEM uranine that penetrated through the ES was not homogeneously distributed on the AEMs but accumulated in patches (Fig. 2D, G, H, I). The patches showed a characteristic pattern of colour: Each patch consisted of a red spot in the centre, indicating a high uranine concentration, surrounded by a yellow rim indicating low uranine concentrations. Occasionally, an orange transient zone between the central spot and the yellow margin was detectable, indicating an intermediate uranine concentration. The central red spots differed in size and form. Small spots (approximately 30 μm length) with elliptic shape (Fig. 2I) were frequently found in experiment III (when penetration rates were low), which correlates perfectly with the dimensions of *A. porrum* stomata. Mostly the red spots were larger (Fig. 2D, G, H). In experiment III, three exceptionally large red spots were found with diameters up to 400 μm (not shown).

Occasionally, annular residues were formed during the drying of the uranine droplets. In those cases most of the penetration took place underneath this annulus (Fig. 2H). Leaves of *A. porrum* are covered with epicuticular waxes that are inhomogeneously distributed and form linear structures on the leaf surface. Penetration of
uranine preferentially took place in leaf regions with low density of epicuticular waxes (Fig. 2D). When penetration of Fe-salts was studied, the patterns on the IEMs were the same as those found in the uranine experiments (Fig. 2K).

The results of experiment II are given in Table 2. Uptake is expressed as a percentage of median uptake after the first application of uranine (treatment 1).

Re-wetting of one half of the residues increased uptake more than 9-fold (treatment 2) and more than 11-fold (treatment 3) whereas the penetration was only 5% for the unwetted residues of the same leaf. When the previously untreated residues were re-wetted in treatments 4 and 5, uptake increased more than 8-fold compared to treatment 1, whereas uptake from the now untreated group was only 12% and 184%, respectively.
These differences were not significant, however. There were no indications for increasing penetration rates due to the duration of the experiment.

During the first period of droplet drying in experiment III $0.10 \pm 0.6\%$ of the applied uranine penetrated through the ES (treatment 1). The first re-wetting of uranine residues and the following second drying period (treatment 2) did not increase uptake ($0.08 \pm 4.2\%$). During the third and forth drying period (treatments 3 and 4) uptake increased to $1.5 \pm 2.6\%$ and $12.4 \pm 3.7\%$, respectively. The number of spots increased with each rewetting from 0.5 to 12.5 (medians; Table 3). In Fig. 3 the correlation between overall uptake and the corresponding number of red spots of uranine on the AEM is shown. Three extremely large spots deviate from the linear regression that was derived from the smaller spots. The density of stomata was determined to be $79.4 \pm 7.1 \text{ mm}^{-2} (n = 11)$.

No differences were found in the contact angles or overall area of residues on both sides of $S$. telephium leaves (Table 4). Median penetration of uranine through ES from the abaxial side was $3223 \text{ pmol}$ which is more than twice as high (ratio 2.4) as penetration through the adaxial side ($1367 \text{ pmol}$). This coincides with a similar ratio (2.1) of stomatal density, being $84.1 \text{ mm}^{-2}$ on the abaxial and $39.2 \text{ mm}^{-2}$ on the adaxial side (Fig. 4A).

The illumination of $S$. telephium leaf sections resulted in considerable opening of stomata. Storing the leaf sections in the dark promoted stomatal closure. Both states of stomatal apertures were maintained after peeling of the epidermis and during the experiment as confirmed under the microscope. Both open stomata and high RH promoted uptake of uranine. Uptake was greater at the higher humidity irrespective of stomatal aperture (Fig. 4B). At the high RH and with

![Figure 3](image)

**Table 2.** Effect of re-wetting of residues on penetration of uranine through *Allium porrum* epidermal strips (ES; experiment II): 10 µl droplets of 1 mM solution were applied to the ES and exposed to a RH of 76% (treatment 1)

In treatments 2 and 3, uranine residues were either re-wetted with 10 µl of distilled water (subgroup 1) or remained unchanged (subgroup 2). In treatments 4 and 5, the previously re-wetted residues remained unchanged and the previously unchanged residues were re-wetted. The uptake time in treatments 1 to 5 was 90, 280, 1020, 280 and 1020 min, respectively. Penetration is expressed as medians ± standard error of median. Medians of treatment 1 (first application of uranine) are set to 100. Differences are not significant.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subgroup 1</td>
<td>100±562</td>
<td>957±1278</td>
<td>1171±3060</td>
<td>12±64</td>
<td>184±92</td>
</tr>
<tr>
<td>Subgroup 2</td>
<td>100±93</td>
<td>5±43</td>
<td>5±68</td>
<td>865±1281</td>
<td>819±464</td>
</tr>
</tbody>
</table>

**Table 3.** Penetration of uranine through epidermal strips of *Allium porrum* as a percentage of the applied dose (experiment III) and corresponding numbers of spots of concentrated uranine on the underlying anion-exchange membranes used as a receiving medium during the experiment

Uranine was applied as 10 µl droplets of 1 mM solution and exposed to a RH of 76%. After evaporation of the droplets (treatment 1) the residues were re-wetted every 2 h in treatments 2 to 4. Data are presented as medians ± standard errors of medians. Figures followed by the same letter or without letter are not significantly different (Tukey-HSD test, $\alpha = 0.05$, log $(x+1)$-transformation).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Penetration (%)</th>
<th>Number of spots</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.10±0.06a</td>
<td>0.5±0.3</td>
</tr>
<tr>
<td>2</td>
<td>0.08±0.42ab</td>
<td>0.5±0.6</td>
</tr>
<tr>
<td>3</td>
<td>1.48±2.44ab</td>
<td>5.0±1.7</td>
</tr>
<tr>
<td>4</td>
<td>12.43±3.51b</td>
<td>12.5±4.3</td>
</tr>
</tbody>
</table>
open stomata uptake was 247 pmol or 2.5% of the applied dose of uranine. On the other hand, with closed stomata and at the low RH uptake was only 5 pmol or 0.05%.

The penetration rates varied widely within and between the experiments. For example, the median penetration through adaxial ES obtained from previously illuminated S. telephium leaves was 13.6% in experiment IV but only 2.5% in experiment V under identical ambient conditions. In the case of A. porrum in particular, the variation within an experiment was considerably high. For example, during the last drying period uptake ranged from 1 to more than 4200 pmol.

### Table 4. Static contact angles and areas of residues of 10 μl droplets on both sides of Sedum telephium leaves

<table>
<thead>
<tr>
<th>Leaf side</th>
<th>Adaxial</th>
<th>Abaxial</th>
<th>n.s., n = 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contact angle water (°)</td>
<td>133.7 ± 7.6</td>
<td>135.0 ± 7.2</td>
<td></td>
</tr>
<tr>
<td>Contact angle uranine solution (°)</td>
<td>134.1 ± 6.2</td>
<td>135.7 ± 8.3</td>
<td></td>
</tr>
<tr>
<td>Area of residue (mm²)</td>
<td>4.2 ± 1.2</td>
<td>3.7 ± 2.0</td>
<td>n.s., n = 19</td>
</tr>
</tbody>
</table>

### Discussion

The repeated experiments using intact leaves with whole plants demonstrate that foliar uptake of uranine is not homogeneous. However, only the finding of uranine accumulation within the stomatal pore of S. telephium (Fig. 2F) is a direct proof of stomatal penetration. This complements previously published results with A. porrum (Eichert et al., 1998), where stomatal uptake of uranine was demonstrated for both intact leaves and epidermal peels. The other results provide rather circumstantial evidence for a stomatal role in the uptake process, as they are inconsistent with cuticular penetration, but in line with stomatal penetration. In Fig. 2A–C it was shown that uranine penetration took place at the margin of the applied droplet (also found in the model experiments with A. porrum). At first sight the size of the uranine spot found in the mesophyll (Fig. 2C) seems to point to uptake through a relatively large cuticular lesion rather than through a much smaller stoma. However, it is very likely that uranine is highly mobile within the plant tissue (Stadelmann and Kinzel, 1972) and spreads quickly from the point of entry. It therefore follows that the hole through which the dye penetrated into the leaf must have been smaller than the fluorescent spot. From the circular shape of this spot, it can be deduced that the corresponding hole was either also circular, or of an arbitrary shape but much smaller than the fluorescent spot, so that it served as a quasi-point source of entry. Comparable
patterns of uranine penetration into *S. telephium* leaves were repeatedly found and damage was never observed under the microscope. The most likely explanation is therefore that penetration of the dye was through stomata.

In the central area of Fig. 2E it can be seen that the spreading of uranine within the mesophyll aggravated the identification of uptake pathways into *C. communis* leaves as well. In areas where uptake was lower, however, the characteristic arrangement of uranine around the substomatal cavities clearly demonstrated that the uptake was associated with the stomatal apparatus.

Both the inhomogeneous (patchy) distribution of applied substances on the IEMs and the matching dimensions of the smallest spots with the shape (*A. porrum*) and size of the stomata are consistent with the findings with intact leaves. Sometimes the development of larger spots was observed. This has to be attributed to the limited exchange capacity of the IEMs leading to lateral diffusion of the compounds after stomatal penetration, which is supported by the observation of a radial concentration gradient within these spots. The lateral movement of uranine after penetration might also have been promoted by the slightly acidic conditions in the epidermal apoplast which could have caused a shift in uranine speciation towards uncharged molecules (Martin and Lindqvist, 1975) and thus might have hindered the uranine adsorption by the AEM. Simultaneous penetration of proximate stomata, in conjunction with high penetration rates, may have caused the large spots that are outside the linear regression calculated from the smaller spots (Fig. 3). The intercept of the regression close to the origin indicates that cuticular penetration of uranine was very low. The maximum number of spots was 35, which under the given conditions (area of the residues about 4 mm\(^2\), stomatal density 80 mm\(^{-2}\)) represents approximately 11% of stomata, indicating only a small proportion of stomata to be involved in the uptake process.

The preferential uptake of externally applied substances by stomatal guard cells has been reported (Franke, 1964), but this is not a conclusive explanation for these results: The average penetration of 56 pmol uranine through one stoma within 2 h, as derived from the slope of the linear regression, would be equivalent to a transport rate of 14 pmol cell\(^{-1}\) h\(^{-1}\) through each guard cell. Maximum rates of glucose uptake by *Pisum sativum* L. guard cell protoplasts, for example, were found to be 3 orders of magnitude lower (Ritte *et al.*, 1999). Considering that uranine is an anion and that uptake through the guard cells would not only have required the passage through the cell membrane, but concomitantly the penetration of the external and internal cuticle layers and the cell wall, it is conceivable that uptake through the guard cells can be discounted in the present study.

Repeated wetting and drying of uranine residues (experiment II and III) strongly increased uptake rates. Linear regression revealed this to be mostly caused by an increase in the number of penetrated stomata. There have been several studies indicating a beneficial effect of repeated drying-wetting cycles on foliar uptake of applied solutions. In experiments investigating the uptake of salts from intermittent sprinkler irrigation the formation of concentrated films on the leaf surface was supposed to increase the penetration rates (Eaton and Harding, 1959; Maas *et al.*, 1982; Maas, 1985). However, sprinkler irrigation leads to additional salt inputs within each re-wetting cycle, causing a successive increase in salt concentration. This differs from the situation reported here, where residues were re-wetted with distilled water and thus concentrations rather decreased. Bukovac suggested the increased penetration of 3-chlorophenoxy-propionic acid through astomatous *Prunus persica* L. cuticles caused by re-wetting was related to the swelling of the cuticle, which increased their permeability (Bukovac, 1965). He reported that two or five re-wetting-drying cycles increased overall absorption by 17% and 47%, respectively. The comparison of these figures with the data presented here confirms that re-wetting of residues can be most effective in the promotion of stomatal penetration. However, it will obviously always depend on the physicochemical properties of the specific compound.

Hydration of the cuticle will not only improve cuticular penetration of hydrophilic compounds (Kirkwood, 1999), but may also reduce the hydrophobic properties of the surface, as is known from polymer surface chemistry (Garbassi *et al.*, 1994). Cuticle hydration may have diminished the extremely hydrophobic behaviour of the *A. porrum* leaf surface, and may thereby have increased the effective contact area between the applied solution and the surface, usually being smaller than the area occupied by the droplet (Bukovac *et al.*, 1995). Consequently a higher proportion of stomata could have come into contact with the solution, which is the first prerequisite for penetration. An example for this interaction between leaf surface wettability and penetration is given in Fig. 2D. The pattern of stomatal penetration was controlled by the distribution of epicuticular waxes. The drying behaviour of the applied droplet affected the pattern of penetration as well, as demonstrated for the annular uranine residues (Fig. 2H). This might be related to the accumulation of uranine inside the annulus causing higher concentrations which may build up a higher driving force for penetration.

As both leaf sides of *S. telephium* showed no differences in wettability, the differences in uptake rates that were observed in experiment IV can not be explained with differences in contact area or dose of uranine per unit leaf area. The close relationship between uptake rates and stomatal density that was observed is in line with
various reports (Sargent and Blackman, 1962; Schönherr and Bukovac, 1978) and was sometimes interpreted as proof of stomatal penetration (Sands and Bachelard, 1973). In other studies, stomatal penetration was discounted and differences in uptake rates were ascribed to putative differences in permeability of the cuticle, especially of the peristomatal region above the guard cells (Schönherr and Bukovac, 1978). Both the experiments with intact leaves and with epidermal strips indicated that penetration of uranine through the cuticle apart from the stomatal region can be rejected. Peristomatal cuticular penetration is a hypothesis that was developed in order to explain the obvious effect of stomata on uptake rates when stomatal penetration is denied, but has never been verified. According to Schönherr and Bukovac ‘the permeability of the cuticular membrane over guard cells of open stomata must be greater than closed stomata’ (Schönherr and Bukovac, 1978). This statement would have to become even more complicated in order to explain these results of this study, as only the cuticle over a small part of open stomata guard cells would be affected.

Uptake through S. telephium ES was significantly lower at 40% RH than at 76% RH. Since the deliquescence point of uranine is in the order of 75% RH (Eichert et al., 1998), the droplets dried completely at the low RH whereas at the higher RH the uranine residues probably remained in a highly concentrated mobile status allowing uptake throughout the experiment. This finding is in line with observations of both autostomous and stomatous leaves (Middleton and Sanderson, 1965; Foster and Maun, 1980).

Uptake rates were higher when stomata had been opened by light prior to application of the uranine solution. Several previous studies have indicated that illumination increases uptake rates (Pallas, 1960; Dybing and Currier, 1961; Middleton and Sanderson, 1965; Eddings and Brown, 1967; Schönherr and Bukovac, 1978). It was mainly proposed that this was due to metabolic processes induced by light and preserving a steep concentration gradient. In contrast, accumulation of the compound in the dark should reduce penetration rates (Moorby, 1964; Sargent and Blackman, 1965). This process, however, does not apply in the present study as uranine was retained and accumulated directly underneath the ES independent of pre-illumination. One mechanism by which open stomata increase uptake rates may be related to the surplus of water vapour emerging from open stomata. This water may keep the foliar-applied compound mobile for a prolonged time by interaction with the droplets or their residues on the leaf surface (Burkhardt et al., 1999). However, this mechanism should not have been decisive at the high humidity when the droplets did not dry completely and immobilization was thus not a limiting factor. As uptake rates increased although the humidity was high it must be concluded that open stomata are generally more penetrable than closed stomata. Either the higher degree of aperture increases the probability for penetration or the higher sectional area permits higher transport rates through the stoma or both.

The number and distribution of penetrated stomata and the penetration rates exhibited high variability. This might be due to intrinsic properties of the epidermis, like wax coverage or composition and stomatal performance. However, this supposedly only played a minor role as in the case of A. porrum the individual sections of ES were obtained from the same leaf and originated from proximate locations. The fact that re-wetting of residues strongly increased the number of permeated stomata suggests the degree of penetration to depend more on ambient conditions than to represent an inherent feature of every stoma. This is also corroborated by the remarkably low proportion of penetrable stomata (usually by far less than 10%). This low proportion indicates that the limitations outlined previously (Schönherr and Bukovac, 1972) were effective in preventing stomatal penetration in the majority of cases. These limitations are based on the interaction between the usually high surface tension of the applied solution, the geometry and the wettability of the stomatal pore. There seem to be no indications that one of these three parameters occurred heterogeneously during the experiments: The surface tension of the uranine solution was not determined, but the high contact angles measured with S. telephium and A. porrum (Eichert et al., 1998) indicate surface tensions too high to permit infiltration of stomata. Evaporation of the solution increased uranine concentrations, but even concentrated uranine solution forms contact angles higher than 100° with A. porrum or a PTFE surface (Eichert et al., 1998). Moreover, there seems to be no reason why the surface tension of the applied solution could exhibit such a high variability, with the result that some stomata may be infiltrated while proximate stomata may not. The contribution of the geometry of stomata to the protection against infiltration is based on the divergent portions of the pore. It is conceivable that some guard cells might deviate from the norm, forming stomata lacking the divergence of the pores. However, it seems impossible that the rate of malformed guard cells is as high as 10%. Furthermore, this rate is expected to be fairly constant and not dependent on environmental conditions. The last factor, the intrinsic wettability of the pore surface, is expected not to be so changeable (for example, by the hydration processes), that a liquid initially forming contact angles higher than 100° might penetrate (according to Schönherr and Bukovac, 1972, a contact angle lower than 20° is usually required for penetration). Consequently, it is most unlikely that one of the three factors was responsible for the penetration of stomata demonstrated here.
This argument (Schönherr and Bukovac, 1972) does not consider that the surface properties of the stomatal pore are not solely determined by the chemical composition of cuticle and epicuticular waxes. The wettability of the plant surface can be altered by epiphytic microorganisms (Schreiber, 1996) or the deposition of hygroscopic aerosols (Burkhardt and Eiden, 1994) enabling (capillary) condensation at low RH and the formation of continuous salt crusts (Eiden et al., 1994; Burkhardt et al., 1999). If hygroscopic substances are present inside a stomatal pore, the almost saturated conditions underneath an applied droplet are likely to promote condensation of water on the walls of the pore. Nutrient ions like Ca$^{2+}$ or SO$_4^{2-}$ which are transported to the evaporation sites within the stomata by the water flow from the roots may form salt residues which contribute to early condensation. The tendency of highly concentrated salt solutions to ‘creep’ could provide additional elements for the formation of a continuous salt bridge between the substomatal cavity and the outer leaf surface. These formation processes would explain why circumstantial events might govern the ‘activation’ of stomata for liquid transport and why the variability of penetration rates within and between the experiments was so high. Once a connection between the leaf surface and the substomatal cavity is established, the specific stoma should be active as an uptake pathway as long as the externally applied compound is mobile and as long as there is a driving force. The increasing number of stomata that was observed after re-wetting of the uranine residues supports this argument. Even if, for example, temporarily low RH immobilized the residues on the leaf surface, uptake probably continues as soon as RH rises again. Only the complete closure of a stoma would block the connection permanently, cutting off the continuum between the leaf surface and the interior. In experiment V, penetration of closed stomata was demonstrated. Although closure was verified under the microscope, it cannot be ruled out that at least some stomata exhibited anomalous behaviour and remained partially open. However, low stomatal apertures are difficult to distinguish from complete closure and it seems to be a matter of definition whether stomata ever close ‘completely’ (Kerstiens, 1996).

Stomata may significantly contribute to the overall uptake of ions once they are ‘activated’ for solute transport. These processes depend on a variety of environmental and physiological factors which strongly modify the conditions, compared to the situation of a statically resting droplet on a smooth clean leaf (as treated in the paper by Schönherr and Bukovac, 1972). The model system presented here is suited to the study of uptake under these changing conditions. It was demonstrated that uptake via stomata is a pathway not only for large anions like uranine but also for small cations like the Fe$^{3+}$ ion, that are relevant to foliar fertilization.

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