Ion Binding by Surfaces of Isolated Cuticular Membranes

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One approach to the solution of problems relating to the intake and loss of nutrients by plant foliage is to separate the leaf into its various components. Ion penetration, binding, and exchange phenomena for each can then be studied. A logical beginning is with the cuticular layer which may, with some species, be separated in relatively large segments as an intact membrane. Two such enzymically isolated membranes, the stomatous green onion leaf cuticle and the stomatous tomato fruit cuticle, have been subjected to rigorous study (10). Permeability of these cuticular membranes for both inorganic cations and anions was greater from the outer to the inner surface than from the inner to the outer surfaces. Furthermore, the rate of penetration was positively related to the extent of ion binding on the surface opposite the site of initial entry (11).

The degree of ion binding on the inner and outer cuticular surfaces and its relationship to surface morphology is herein described.

Materials and Methods

Cuticular Membranes. Intact cuticles were enzymically separated as previously reported from segments of stomatous surfaces of mature green onion leaves and the stomatous surfaces of ripe tomato fruits (10, 11).

Morphological Observations. Silicone rubber impressions (negatives) of the isolated membranes were prepared, after they were washed and blotted. Positive impressions were made from these negatives by applying a thin film of cellulose acetate. This was stripped off after drying and examined under a microscope, and appropriate samples photographed. The method was essentially that described in detail by Zelitch (12) for the observation and measurement of stomatal apertures on leaf surfaces. Transverse sections (12 μ) were prepared from cuticular membranes stained with crystal violet and safranin O. They were then embedded in paraffin, cut with a microtome, and stained with hematoxylin. These sections were observed microscopically and photomicrographs prepared.

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Measurement of Ion Binding. Discs of 1.3 cm in diameter were cut with a sharp punch from isolated cuticular membranes of ripe tomato fruits and green onion leaves. Ten properly oriented discs were then floated with either the inner or outer surface only, in contact with 1.0 mM solutions of Ca45 labeled CaCl2 (26 μCi/μmole) or S35 labeled K2SO4 (30 μCi/μmole). The contact duration was always 5 minutes except where time was a variable. Following 5 minutes of contact with the labeled solutions the cuticle discs were removed and the adhering solution blotted from the surfaces with soft tissue paper. They were then washed by shaking in deionized water for 5 minutes. Those ions retained by the cuticular surfaces were considered as the bound fraction. The same discs were then washed for 5 minutes in a nonlabeled salt solution otherwise identical and isotonic to that on which the discs were originally floated. The fraction retained by this procedure was considered as the nonexchangeable, and the fraction removed as the exchangeable.

The variables of time and concentration of the applied solution used to influence ion binding on tomato fruit cuticular surfaces are detailed in figure 2. After soaking in the isotopic solutions for the indicated time intervals, the adhering solutions were blotted off with tissue paper and the discs were washed in deionized water for 5 minutes. Concentration effects of Ca45Cl2 and K2S35O4 on ion binding were determined after blotting and washing except where indicated otherwise.

The adherence of ions to cuticular surfaces after blotting, washing, and exchange was determined by sequential direct radioassay of the same cuticular discs. Discs were oriented so that the treated side (outer or inner) was always toward the window of the G-M tube attached to a standard scaler circuit. This minimized errors of self-absorption.

Effects of Chemicals on Ion Binding. Discs (1.3 cm diameter), both nonboiled and boiled (5 minutes at 100°), were immersed for 1 hour in 10-4 M solutions of DNP, Na2S2O4, kinetin (Nutritional Biochemicals Corporation), or a 10-3 M solution of KCN. The discs were then blotted between layers of soft tissue paper, and immersed for 5 minutes in a 1.0 mM solution of Ca45 labeled CaCl2. After blotting again and shaking for 5 minutes in deionized water the intact discs were radioassayed.

All experiments were repeated with at least 10 discs. The values reported in table I and II and figure 2 are typical of those obtained for many discs.
Results

Morphological Observations. Outer surfaces of the stomatous tomato fruit cuticles appeared smooth (fig 1A). The inner surface, however, consisted of an irregular network of protrusions corresponding with the anticlinal walls of the epidermal cells (fig 1C). The transverse view (fig 1E) depicts the tomato fruit cuticle as not only a covering over the surface of epidermal cells, but completely surrounding them. Occasionally the residue of some epidermal cells appeared to be present.

The outer surface of the stomatous green onion cuticle was also smooth and waxy but contained numerous stomatal pores. No guard cells were associated with the stomatal apertures when viewed (fig 1B) from the outer surface. However, clear impressions of guard cells were observed on the inner surface (fig 1D). Transverse sections (fig 1F) of the much thinner onion leaf cuticles were inadequate for distinguishing cuticular or cell components.

Binding of Cations and Anions on Cuticular Surfaces. The binding of Ca\(^{++}\) and SO\(_{4}\)^{--} on the outer and inner surfaces of the 2 types of enzymically isolated cuticular membranes under several experimental conditions is recorded in table I. Ion binding per unit area was greater with green onion leaf cuticles than with those from the tomato fruit. This difference was more pronounced with Ca\(^{++}\) than SO\(_{4}\)^{--}. Equally significant was the markedly greater retention of both ions by the inner cuticular surfaces. These differences were most striking with Ca\(^{++}\) on tomato fruit cuticles and SO\(_{4}\)^{--} anion. This was true for all 3 removal forces: blotting, washing, or exchange. Complete removal of SO\(_{4}\)^{--} was effected by exchange on both outer and inner cuticular surfaces, but some Ca\(^{++}\) ions were retained especially on the inner surfaces (table I).

Saturation of the cation and anion binding sites on surfaces of tomato fruit cuticle, as indexed by ion retention after 5 minutes of washing in deionized water, was a function of both time of exposure to, and concentrations of the applied solutions (fig 2). Within 3 minutes saturation occurred for Ca\(^{++}\) supplied as Ca\(^{45}\)Cl\(_{2}\). For SO\(_{4}\)^{--}, supplied as K\(_{2}\)S\(^{35}\)O\(_{4}\), an equilibrium was not approached however, until after 200 minutes, and then only with a 0.2 mM solution. The total binding capacity for Ca\(^{++}\) exceeded by 500-fold that for SO\(_{4}\)^{--} when sufficient time was allowed and suitable concentrations were used for surface saturation to occur. Calcium binding was proportional to the concentrations of the applied solutions until saturation occurred on both cuticular surfaces at approximately the 5 mM level. Sulfate binding as determined by that remaining after washing was linear through the range of concentrations used, but of a very low magnitude as compared with that for calcium.

Effects of Chemicals on Ion Binding. Chemical pretreatments of cuticular discs derived from ripe tomato fruit had a marked effect on surface binding of calcium ions (table II). KCN and kinetin treated cuticles retained more and DNP treated cuticles much less calcium than those receiving no chemical treatment. These differences were largely eliminated if boiled rather than nonboiled cuticles were used. The calcium ion binding capacity of a dialyzing membrane similarly exposed to the various chemical pretreatments was not altered.

| Table 1. Retention of Ions by Outer and Inner Surfaces of Ripe Tomato Fruit and Green Onion Leaf Cuticles |
|-----------------|--------|---------|---------|---------|
| Ion             | Surface | Blotting | Washing | Exchange |
|                 |        | (m\textmu mole per cm\(^2\)) |
| Ripe tomato fruit cuticle |        |         |         |         |
| Ca\(^{++}\)     | Outer  | 1.6     | 0.9     | 0.4     |
|                 | Inner  | 18.0    | 13.0    | 4.0     |
| SO\(_{4}\)^{--} | Outer  | 0.4     | 0.01    | 0       |
|                 | Inner  | 1.1     | 0.02    | 0       |
| Green onion leaf cuticle |        |         |         |         |
| Ca\(^{++}\)     | Outer  | 58      | 48      | 15      |
|                 | Inner  | 149     | 126     | 33      |
| SO\(_{4}\)^{--} | Outer  | 0.11    | 0.05    | 0       |
|                 | Inner  | 3.40    | 0.32    | 0       |
Table II. Effects of DNP, KCN, NaN₄, and Kinetin on Retention of Calcium Ions by Ripe Tomato Fruit Cuticles

<table>
<thead>
<tr>
<th>Membrane</th>
<th>No chemical treatment</th>
<th>10⁻⁴ M DNP</th>
<th>10⁻³ M KCN</th>
<th>10⁻⁴ M NaN₄</th>
<th>10⁻⁴ M Kinetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cuticle, nonboiled</td>
<td>13.3</td>
<td>8.8</td>
<td>18.3</td>
<td>12.4</td>
<td>21.3</td>
</tr>
<tr>
<td>Cuticle, boiled</td>
<td>14.3</td>
<td>12.1</td>
<td>15.0</td>
<td>...</td>
<td>13.2</td>
</tr>
<tr>
<td>Dialyzing membrane</td>
<td>4.0</td>
<td>3.8</td>
<td>3.7</td>
<td>3.9</td>
<td>...</td>
</tr>
</tbody>
</table>

(No. 70160-1, Central Scientific Co.)

Discussion

Cuticular penetration is a prerequisite for foliar intake of nutrient ions. Increasingly greater attention has been directed toward the importance of this common barrier to foliar entry of solutes. The morphology, physiology, and biochemistry of plant cuticles have been the subject of several recent reports (1, 4, 5, 6, 7).

A significant feature of permeability of the 2 cuticular membranes that have in this report, and in others (10,11), been subjected to detailed study is that penetration by inorganic cations and anions is much greater from the outer surface to the inner surface as compared with movement from the inner to the outer surface (11). The directional differences in permeability appear associated with the extent of ion binding on the surface opposite initial entry. The much greater binding for the divalent Ca⁺⁺ cation than for the SO₄⁻⁻ anion, the markedly greater retention of ions, both cation and anion, by the inner compared with the outer surfaces, and the considerably higher exchange capacities for green onion leaf versus tomato fruit cuticles (table I) must be related to differences in the physical and chemical properties of the 2 membranes and their surfaces.

There are marked differences in surface morphology of cuticular membranes enzymically separated from ripe tomato fruit and green onion leaves. Skoss (8) has pointed out that the cuticular layer on the foliage of different plants varies in thickness and the degree to which the vertical walls of the epidermal cells are cutinized. For example, on the upper leaf surfaces of Hoya carnosa the cuticle extends vertically along the cell walls such as to give an outline of the epidermal cells. This was what was also observed by Baker, et al. (2) for leaf cuticles of Euonymus japonicus, and in the transverse sections of tomato fruit cuticle herein illustrated (fig 1E).

Differential ion binding capacities of the cuticular membrane surfaces may be related to surface morphology. Outer surfaces are smooth (fig 1A and B), while inner surfaces are irregular with many protrusions, cavities, attached fragments, with perhaps even cellular remnants. We have been unable, thus far, to prepare comparable transverse sections of green onion leaf cuticles. Such preparations might provide a morphological explanation for the many-fold greater ion binding capacities of both inner and outer surfaces as compared with the tomato fruit cuticle (table I).

A further possible explanation for differential ion binding by the 2 surfaces may be related to differences in physical and chemical properties. The outer cuticular surface is believed to be composed of layers of highly polymerized and oxidized fatty acids. The inner surface, in contrast, presents a more heterogeneous chemical composition. In addition to the fatty acids, which are less saturated than at the surface, cellulose, and perhaps pectic substances would be present. There is evidence of a gradient from low polarity on the exterior to a relatively high polarity in the layers bordering the epidermal cell wall (3). In any case the negative charges characteristics of cuticular membranes offer reasonable explanations for permeability differences between cations and anions as well as the greater binding of cations on the inner surfaces as compared with anions; and why cationic nutrients, in general, are absorbed more readily by plant foliage than the anionic (9).

The effects of biologically active chemicals on the binding of calcium ions by tomato fruit cuticle is an interesting area for further exploration. Similarity in binding by boiled and nonboiled, and the absence of respiratory activity (O₂ uptake) in nonboiled cuticular membranes would suggest that the binding described, herein, is of a chemical-physical nature. Cuticular isolation and storage procedures employed in these studies were not conducive for preservation of enzymic activity and hence these data do not preclude that active binding may not exist in intact or freshly prepared cuticle.

The Ca retentate (nonexchangeable) was 44 and 31% of that remaining after washing on the outer and inner surfaces, respectively, of the tomato fruit cuticle (table I). The remainder must have been bound on exchangeable sites. Similar amounts, 31 and 26%, respectively, were retained after exchange on green onion leaf cuticular surfaces. Bound ions include those which are exchangeable. Yamada (10) has reported that green onion leaf and tomato fruit cuticles contain approximately 3% ash. Thus, Ca⁺⁺ or SO₄⁻⁻ might exchange for Ca or SO₄ already in the cuticle. A more likely possibility, however, is that cation exchange occurs with hydrogen on the carboxyl...
radicals present in the polyfatty acids of the cuticle.

A final consideration lies in the possible value of the data for determining total exchange capacities of cuticular membrane surfaces. For calcium ions the concentration (1 mM) and time exposure (5 minutes) was sufficient for equilibrium (fig 2). Conversely, the time intervals required for saturation with sulfate were not practical for laboratory experiments, and even after prolonged contact values were only a small fraction of those for calcium. Comparative values for exchangeable calcium (derived from the differences between washing and exchange in table 1) on the outer and inner surfaces of tomato fruit cuticles were 0.5 and 9.0 μmole/cm², respectively. Corresponding values for green onion leaf cuticular surfaces were 33 and 93. These values are somewhat higher than those reported previously, but where equilibrium was not established (11).

**Summary**

A markedly greater binding of Ca++ from Ca⁴⁺Cl₂ and SO₄⁻⁻ from K₂S³⁵O₄ occurred on the inner compared to the outer surfaces of enzymatically isolated cuticular membranes of stomatous ripe tomato fruit cuticles and stomatous green onion leaf cuticles. This was related to morphological differences in the cuticular surfaces. The outer surfaces were smooth, while the inner surfaces showed protrusions and cuticular fragments outlining the epidermal cell walls, including the guard cells in the onion.

Retention of ions, on comparable cuticular surfaces, against blotting, washing, and exchange was greater for Ca++ than SO₄⁻⁻, and for onion leaf than tomato fruit. Total removal for SO₄⁻⁻ but not for Ca++ was accomplished by exchange.

Maximum fixation of Ca++ on surfaces of tomato fruit cuticle occurred within 3 minutes from a concentration of 1 mM CaCl₂. Saturation with SO₄⁻⁻ was not achieved even after 200 minutes.

Ion binding on fresh but not on boiled cuticular surfaces of tomato fruit, or a dialyzing membrane, was enhanced by KCN and kinetin, but greatly reduced by DNP.

**Acknowledgments**

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**Literature Cited**

Auxin Transport in the Physiology of Fruit Development 1, 2, 3

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One of the more interesting problems in the auxin control of plant growth and morphogenesis is the development of the ovary of a flower into a fruit. If pollination and fertilization do not take place, the ovary usually ceases growth and the entire flower abscises. A study by Muir (3) has shown that diffusible auxin is not present in the ovary of tobacco at anthesis but, following pollination, diffusible auxin appears in the style and later in the ovary. This diffusible auxin is correlated with the growth of the ovary into a fruit. Gustafson (2) found a higher auxin content in ovaries of certain varieties of oranges, lemons, and grapes which develop into fruits without pollination and fertilization than was present in the ovaries of the varieties requiring pollination and fertilization for growth to occur. This finding suggests that perhaps in some instances the auxin in the ovary is not produced there but is derived from the parent plant.

The possibility of transport of auxin from the parent plant to the ovary and subsequent parthenocarpy has been examined in the present study. Radioactive and nonradioactive indoleacetic acid (IAA) was supplied to various regions of the pedicel of a variety of tomato which does not produce naturally seedless fruits and a variety of cucumber which produces naturally both seeded and seedless fruits. The effects of gibberellic acid (GA) and 2,4-dichlorophenoxyacetic acid (2,4-D) on parthenocarpy of tomato were compared with those of IAA.

Materials and Methods

Tomato plants (Lycopersicon esculentum Mill. var. Pan American) and cucumber plants (Cucumis sativus L. var. Long Green) were grown in pots in the greenhouse. All treatments were started at the time the plants began flowering. Stamens and styles were removed from flower buds ready to open. One ovary on each plant was used. Untreated ovaries showing any sign of development were removed. Parthenocarpic ovaries were cut open in order to confirm the absence of seeds.

Lanolin Treatment. Lanolin paste containing IAA was applied to the proximal region (toward stem) or distal region (near ovary) of tomato pedicels or to the cut surface of the pistil.

Solution Treatment. Aqueous solutions of labeled and unlabeled IAA were supplied to tomato and cucumber pedicels and solutions of GA and 2,4-D were supplied to pedicels of tomato through glass tubes that tapered to not more than 0.5 mm in diameter. The tubes were inserted directly into the pedicel or attached to the pedicel by 2 strands of thread (tied to cotton packed inside the neck of the glass tube) which were drawn through the pedicel with a very thin needle. The tip of the tube was held in position by tying the thread at the undersurface of the pedicel and the larger end of the tube was suspended from a support. This method of treatment is shown in figure 1. In some instances, the thread was tied around the pedicel. The tomato and cucumber ovaries were observed every 3 days for periods of 20 to 35 days.

Methylene-C14-labeled IAA having a specific activity of 1.10 μc/μmole, and carboxyl-C14-labeled

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2 This paper is based on part of a thesis of the author submitted in partial fulfillment of the requirements of the Ph.D. in the Department of Botany, University of Iowa, Iowa City.
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