Influence of Decenylsuccinic Acid on Water Permeability of Plant Cells

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ABSTRACT

Decenylsuccinic acid altered permeability to water of epidermal cells of bulb scales of Allium cepa and of the leaf midrib of Rhoeo discolor. Water permeability, as determined by deplasmolysis time measurements, was related to the dose of undissociated decenylsuccinic acid (mM undissociated decenylsuccinic acid \( \times \) minute). No relationship was found between permeability and total dose of decenylsuccinic acid, or dose of dissociated decenylsuccinic acid, suggesting that the undissociated molecule was the active factor in permeability changes and injury.

At doses which did not damage cells (0.0008 to 0.6 [mM of the undissociated molecule \( \times \) minute]) decenylsuccinic acid decreased water permeability. At higher doses (e.g., 4 to 8 [mM \( \times \) minute]) injury to cells was common and decenylsuccinic acid increased permeability. Doses above the 10 to 20 (mM \( \times \) minute) range were generally lethal. The plasmolysis form of uninjured cells was altered and protoplasmic swelling occasionally was observed. The dose-dependent reversal of water permeability changes (decreased to increased permeability) may reflect decenylsuccinic acid-induced changes in membrane structure. Reported effects of decenylsuccinic acid on temperature dependence of permeability and frost resistance were not verified.

Various external or internal factors are assumed to alter permeability of cell membranes to water or solutes (cf. 20). Studies of entire plants or parts of plants may lead to erroneous conclusions about cell permeability because of other factors involved and because cell viability usually is not tested. With the methods now available, changes in permeability can be investigated directly at the cellular level (19). The difficulties in evaluating cell permeability changes with multicellular structures is exemplified by the conflicting conclusions reached by workers who have used detopped plants to study the influence of decenylsuccinic acid in water permeability. DSA\(^{1}\) is a waxy substance which has a molecular weight of 256 and the formula

\[
\text{CH}_4 - (\text{CH}_2)_k - \text{CH} = \text{CH} - \text{CH}_2 - \text{CH}(	ext{COOH}) - \text{CH}_2 - \text{COOH}\]

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Abbreviations: DSA: decenylsuccinic acid; RCD: relative change in deplasmolysis time.

MATERIALS AND METHODS

DSA, supplied by Humphrey-Wilkinson, Inc., North Haven, Connecticut was dissolved in water at 40 C. At concentrations above 5.0 mM, a precipitate formed at room temperature. At 1.0 mM the DSA solution was colloidial and translucent and had a low surface tension. At concentrations of 0.5 mM or lower DSA gave a clear solution with a surface tension close to that of pure water (no foaming).

When pH values of the 1.0 mM DSA solutions were adjusted to neutrality with a few drops of NaOH or KOH, the solutions cleared and lost their colloidial appearance. The pK values of DSA were estimated to be 4.5 and 5.7 by titration with a recording Corning Model 12 potentiometric pH meter. With these values the concentration, C, of undissociated DSA is
calculated:

\[
C = A \cdot \frac{1 - \alpha_2}{1 + \alpha_1 \cdot \frac{\alpha_2}{1 - \alpha_2}}
\]

(1)

where \( A \) = total DSA concentration of the solution; \( \alpha_1 \) and \( \alpha_2 \) are calculated from the relation \( pH = pK_a + \log \left[ \alpha_2/(1 - \alpha_2) \right] = pK_s + \log \left[ \alpha_1/(1 - \alpha_1) \right] \alpha_3 \) and \( \alpha_4 \) are the degrees of dissociation of the undissociated molecule and monobasic ion, respectively.

The influence of DSA on water permeability of cells of the adaxial epidermis of onion (\textit{Allium cepa}) bulb scales from three sources (variety Granex from Texas, Trapps Downing from Minnesota, and market onions of unknown origin) was determined at room temperature by measuring the deplasmolysis time. The time required for complete deplasmolysis depends mainly upon the permeability of the protoplasmic layer to water (the greater the deplasmolysis time, the lower the water permeability). Differences in the deplasmolysis time between treated and control tissues where all other external factors are the same indicate changes in water permeability. Deplasmolysis time is a convenient means to obtain relative measures of water permeability.

The difference in deplasmolysis time (treated--control) was expressed as percentage of the deplasmolysis time of the control:

\[
\frac{\text{RCD}}{\text{deplasmolysis time (treated) - deplasmolysis time (control)}} \times 100
\]

Negative values of RCD indicate higher water permeability of the treated sample with regard to the control and vice versa.

Similar studies were conducted on cells of the abaxial leaf midrib of a single clone of \textit{Rhoeo discolor}. In a few \textit{Rhoeo} experiments, the DSA effect on permeability was evaluated at lower than room temperatures (e.g., 10--15°C).

In a single study, six intact \textit{Rhoeo} plants in a controlled environment chamber each growing in a 15-cm pot containing mixtures of 2 parts soil, 1 part sand, and 1 part peat moss (v/v) were treated daily with 1 mm DSA, pH 7, (200 ml of soil application per pot and foliar spray to run off) for 21 days. Six control plants randomly distributed in the same chamber were treated similarly with distilled water. On the 16th through 21st days, all plants were subjected to progressively lower freezing temperatures for 3 hr during the dark period. Air temperature was measured at plant height each night during test freezing and frost injury was rated visually on the following day.

Figure 1 presents a flow diagram which outlines the deplasmolysis technique used in these studies. The adaxial epidermis from the third bulb scale of \textit{Allium} and the lower epidermis from the leaf midrib of \textit{Rhoeo} were collected as previously described (3, 21). Paired adjacent sections were included in each run; one a control and the other treated with DSA. Inasmuch as the control and the treated section of epidermis could not be handled simultaneously with the same equipment, one of the epidermal sections, alternating between treatments in successive experiments, was held in spring water\(^\ast\) (from Glenwood, Minneapolis; total hardness as CaCO\(_3\) 325 mg/l, Ca\(^{+}\) 78 mg/l, HCO\(_3\) 330 mg/l) until evaluation of the first section was terminated.

Pretreatment consisted of transferring epidermal sections to

\(^\ast\) Complete analysis available upon request.

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* IN SOME EXPERIMENTS

FIG. 1. Flow diagram showing the individual steps of a typical plasmolysis study.

Spring water or a KCl-CaCl\(_2\) solution, 20 mm each. These pretreatments may counteract possible Ca deficiencies in the plasmalemma and tonoplast and hence yield more uniform values for the permeability constant and improve protoplast rounding during plasmolysis (cf. 13, 17 [p. 93], 23, 24, 26 [p. 56]).

The initial gradual plasmolysis (initial plasmolysis) in near isotonic concentrations of mannitol (0.5--0.6 mm for \textit{Allium}; 0.30--0.35 mm for \textit{Rhoeo}) is a tempering treatment which reduces osmotic shock. DSA treatments were administered during initial plasmolysis. Strength of treatment is calculated as dose (DSA concentration applied \(\times\) time; the actual effective strength, however, may be modified by such factors as concentration effects of diffusion leakage out of the membranes). After 15 min to 2 hr of initial plasmolysis, the tissue was transferred in the same solution to a perfusion chamber (27) on a microscope stage. The chamber was sealed with a cover slip and vaseline.

Final plasmolysis was accomplished by passing mannitol (0.7 mm for \textit{Allium} and 0.5 mm for \textit{Rhoeo}) through the perfusion chamber. After 0.5 to 1 hr, protoplasts had reached the final

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degree of contraction (to about two-thirds or one-half of the cell volume) and osmotic equilibrium was established between the vacuole and the external solution. In each experiment the control and the treated sample were plasmolyzed for the same length of time.

Deplasmolysis was induced by perfusing the sample with a hypotonic concentration of mannitol (usually 0.35 M for Allium and 0.1 M for Rhoeo). Deplasmolysis time was measured as the time elapsed between the beginning of perfusion with the hypotonic solution and deplasmolysis of 90% of the cells. The RCD was calculated from these values. Each determination of deplasmolysis time was based on observation of approximately 200 Allium cells or 30 to 40 Rhoeo cells.

Analyses were made of the time course of deplasmolysis frequency in several experiments by taking time-lapse photomicrographs (Fig. 2) during deplasmolysis (cf. 18). In this way the times for 50% and 90% deplasmolysis were determined exactly.

To test for cell viability, the cells were replasmolyzed following deplasmolysis by perfusion of plasmolyticum through the chamber. Injured cells which did not replasmolyze were excluded from the evaluation of deplasmolysis time.

In the temperature dependence studies the perfusion solutions were cooled in a salt-ice water bath and solution temperatures were recorded by thermocouples in the outlet and inlet tubes of the perfusion chamber. The given experimental temperatures were the means of these inlet and outlet temperatures.

RESULTS

Toxicity of DSA Solutions. The relationships between DSA concentration, pH, duration of treatment and toxicity were examined in a series of 33 preliminary tests which are summarized in Table I. Controls which were run in each study are not shown. The controls were exposed to the same concentra-

Fig. 2. Frame of a time lapse microphotograph series of Allium cepa epidermis about 30 sec after transfer from the plasmolyzing solution (0.8 M mannitol) into the deplasmolyzing solution (0.35 M mannitol).
Table 1. Viability of Allium and Rhoeo Epidermal Cells as Related to DSA Concentration, pH, and Duration of Treatment

<table>
<thead>
<tr>
<th>DSA Concn (mM)</th>
<th>pH</th>
<th>Treatment Time (min)</th>
<th>Conc of Undissociated DSA (μM)</th>
<th>Dose of Undissociated DSA (μM × min)</th>
<th>Cell Viability</th>
<th>RCD</th>
<th>No. of Experiments</th>
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</table>

1 DSA was applied in a 0.5 m mannitol solution.
2 (a) = pH of DSA solution adjusted with NaOH or KOH; (u) = pH of DSA solution unadjusted.
3 Calculated with equation 1.
4 Increase in concentration of undissociated DSA (mm) and the treatment time (min).
5 Increase in deplasmolysis time in the cells of treated tissue versus control (positive RCD values) indicates decrease in water permeability by treatment and vice versa.
6 Not measured.
7 Decrease in deplasmolysis time (increase in water permeability) is related to cell damage (note lower cell viability).

The data of Table I show that a threshold concentration or dose (concentration × time) of the undissociated DSA exists for survival of the cells. This is also illustrated by the smooth graph obtained, when the dose of undissociated DSA is plotted versus survival (Fig. 6, part 1). Partial or complete injury always occurred in the concentration range 0.06 to 7.3 mm (4–200 [mm × min]), while concentrations of 0.00006 to 0.06 mm undissociated DSA (0.0037–4.0 [mm × min]) did not cause injury. Figures 3 and 4 indicate that the duration of treatment affects significantly the RCD values. It is therefore assumed that neither concentration nor treatment time alone but rather the dose determines the effect on the cell.

Total DSA doses, disregarding dissociation, were not related to viability; e.g., in the various studies, total DSA doses over a range of 30 to 300 (mm × min) killed all cells and total doses over a range from 0.06 to 125 (mm × min) caused no apparent injury.

At the concentrations used, highly dissociated DSA was not injurious to cells. This is illustrated by examining the survival of Rhoeo cells treated with 1.0 mM DSA at pH values of 3.8, 4.2, 5.2, 5.5, 6.0, and 6.2. At a pH adjusted to 6.2, there was no injury even when treatment time was extended to 125 min, whereas 30 min of treatment at pH 4.2 (adjusted) or 3.8 (unadjusted) killed all cells. Freshly detached Rhoeo leaves with cut bases immersed in 1.0 mM DSA at pH 3.8 sustained general injury. Control cells in distilled water at pH values above 3.5 were never injured. Allium cells generally were more sensitive than those of Rhoeo to variation in DSA concentration, treatment time, temperature, and pH.

These results are especially noteworthy since previous work was conducted with 1 mM DSA at pH 3.65 (14) or at unspecified pH values (9, 10). Although species tolerance to DSA probably differs, these findings support Newman and Kramer's (14) contention that injury to roots may account for the DSA effects observed by Kuiper (9).

Effects of DSA on the Water Permeability of Cells. Rhoeo epidermal cells were partially injured and deplasmolized faster when the treatment pH was below 5.2 to 5.5. An increase in impermeability without detectable cell damage was very rarely observed.

In order to evaluate the influence of DSA on uninjured cells most permeability studies (37 studies on Allium and 35 on Rhoeo) were conducted at neutral pH over a wide range of DSA doses. The results are summarized in Figures 3 and 4. The experimental material consisted primarily of dormant Granex onions which had been stored at 7 C, and fresh, detached leaves of Rhoeo, respectively. Deplasmolysis times for control cells ranged from about 1 to 6 min for Allium and 15 to 25 min for Rhoeo.

Relative changes in deplasmolysis time shown in Figures 3 and 4 indicate that DSA treatments almost always decreased permeability to water of both Allium and Rhoeo cells. Rare exceptions (negative RCD values in Figs. 3 and 4) were invariably associated with partially damaged tissues in which only some of the cells were plasmolyzable. A trend toward decreasing permeability with increasing DSA dose was ap-
parent in both *Rhoeo* and *Allium*, but was more pronounced in the latter. The greatest decrease in permeability (RCD about +100 for *Allium* and +130 for *Rhoeo*, see Figs. 3 and 4) was observed at doses of 0.5 mM undissociated DSA for 70 to 100 min. With *Rhoeo*, where higher doses were also tested, the RCD decreased in two experiments where the dose was greater than 60 (µM × min), probably indicating that permeability increased with greater doses.

In order to obtain a quantitative evaluation of the time course of deplasmolysis, eight experiments were conducted in which time-lapse photomicrographs were taken of tissues during deplasmolysis at 5- to 10-sec intervals (see Fig. 2). Deplasmolysis frequencies of cells in four of these studies are given in Figure 5. Each curve is based on observations of about 200 cells. Only cells which were replasmolyzable were counted. Similar cell counts have been previously described for plasmolysis frequency only (15).

A and B of Figure 5 show the typical decrease in water permeability in healthy *Allium* and *Rhoeo* cells induced by DSA at room temperature. The curve for control tissue usually was much steeper than for DSA-treated epidermis, indicating higher uniformity of the control cells. Figure 5C shows the similar results which were obtained when the DSA treatment was administered at 0.5 C with subsequent perfusion at 12 to 15 C. Figure 5D illustrates the kind of anomalous results which were obtained when the treated epidermis was injured by a high dose of undissociated DSA. In this experiment only 60% of the cells were replasmolyzable.

In contrast to Kuiper's findings (9), the data of the present study indicate that DSA decreases rather than increases water permeability of cells and that the DSA response does not appear to alter the temperature dependence of permeability phenomena. These observations agree with Newman and Kramer's view (14) that the DSA-induced increase in water permeability described by Kuiper probably was an artifact.

We did not observe any temperature-dependent effects of DSA (see ref. 9) in additional studies in which the treatments were administered at 0 ± 1.5 C, and the plasmolyzing and deplasmolyzing solutions were cooled to between 10 C and 15 C. As expected, the low temperatures reduced permeability (increased deplasmolysis time), but the reduction was essentially the same in both treated and control cells.

**Cytomorphological Effects of DSA Treatments at Neutral pH.** Doses of undissociated DSA (0.004 [mM × min]) caused extremely concave plasmolysis in *Allium* and *Rhoeo* and the appearance of numerous thick protoplastic strands. These features were observed much more frequently in treated cells than in control cells and less frequently at low doses than at high doses. Low doses of DSA which did not affect deplasmolysis time generally did not alter plasmolysis forms. At doses of 0.004 (mM × min) undissociated DSA, deplasmolysis was often incomplete, probably as a result of an increase in protoplastic viscosity. This indicates that DSA may have led to irreversible changes in the membranes and/or protoplasm. Low temperatures caused concave plasmolysis in both treated and untreated cells, indicating increased protoplastic viscosity. Swollen protoplasts were occasionally observed in *Allium* cells at doses greater than about 2 (µM × min) undissociated DSA. The swollen protoplasm usually persisted until the final stages of deplasmolysis, disappearing when turgor pressure developed.
Hernial protuberances of the protoplasm occurred frequently during deplasmolysis, indicating hardening of external protoplasm. The protuberances rarely led to a bursting of the protoplasm, and disappeared as deplasmolysis progressed (cf. 18, p. 54 and ff.). The osmotic ground values (19) of cells were normally not affected by DSA treatment except at low pH as exosmosis occurred.

Effects of DSA on Frost Resistance. In a controlled freezing experiment on intact Rhoeo plants the temperature in a controlled environment chamber was lowered on successive nights to progressively lower freezing temperatures for 3 hr during the middle of the dark period. Control plants and plants that had been treated with DSA for 21 days prior to freezing stress were uninjured by exposure to −0.9 C and −1.4 C, but all were severely injured at −4 C. DSA did not appear to induce a striking increase in frost resistance as reported by Kuiper (10).

DISCUSSION

The analysis of DSA influence at the cellular level indicated that its effects are more complex than previously suggested (9). Many DSA treatments caused death of cells if the pH was not adjusted to neutrality. If the pH was adjusted to neutrality, or the dose was low enough to avoid killing the cells, DSA reduced permeability of cells to water. At marginally injurious concentrations, DSA seemed to increase water permeability of cells which survived the treatment. Dead cells, of course, are highly permeable to water.

Toxicity of DSA was closely related to the pH of the DSA solution. This relationship, and the DSA effects on permeability, can be explained quite simply if it is assumed that undissociated DSA molecules are the “effective” form that can penetrate the plasmalemma and enter the mesoplasm, while DSA ions either cannot penetrate or can enter only to such a limited extent that no alteration occurs in the cell. This idea is supported by the data when survival, permeability, and cytomorphological changes are plotted against the dose of undissociated DSA (Fig. 6). Plots of these data against the total or dissociated DSA dose do not reveal such clear-cut relationship. For example, there was little or no DSA toxicity at the highest total DSA ion doses tested in these experiments. Preferential penetration of the undissociated molecule of a dissociable organic compound through the plasmalemma and
FIG. 6. Effect of DSA treatment on survival, permeability, and plasmolysis. 1: Survival of epidermal cells tested by plasmolysis. •: *Rhoeo discolor*, leaf, abaxial surface. @: *Allium cepa*, bulb scale, adaxial epidermis. ★: *Phaseolus vulgaris*, root (tested by growth; calculated from Newman and Kramer's data; 14 p. 608). 2: Assumed relation between doses and permeability changes derived from the results of these experiments (--.-- no experiments available in this dose range). 3: Dose limits for observation of concave and convex plasmolysis forms in *Allium*. 4: Dose range for occasionally observed swollen protoplasts in *Allium*.

Low doses of undissociated DSA generally caused a decrease in permeability of cells to water, whereas high doses increased permeability. This indicates that more than one process may be involved in water permeability changes induced by DSA. Similar reversal of effects is known to occur in the permeability of cells treated with ionizing radiation (25, p. 490), fusaric acid (5, p. 352–353), metabolic inhibitors (14), and other substances. The reason for these reversals may lie in alterations of the membrane structure; e.g., decrease in water permeability could be caused by changes in the ratio of the micellar to leaflet configuration in the lipid component of the membrane (cf. 20). Narrowing or decrease in the number of assumed water channels around the globular membrane proteins (cf. 22; G. Vanderkooi, 1971, personal communication) could also cause the lower water permeability. Increased permeability to water at high doses may be the manifestation of changes associated with partial injury of the membranes.

The observation of cytomorphological changes induced by DSA provides a basis for speculating about DSA effects at the subcellular level. For example, the concave plasmolysis forms observed in treated *Allium* cells may indicate that DSA strengthens the binding of membrane proteins to the cell wall, thus impeding separation during plasmolysis. Hernial protuberances of the protoplasts of treated cells indicated that there may have been an alteration (hardening) of the external protoplasm. Concave plasmolysis indicates that DSA may have also increased protoplasmic viscosity. The incomplete de-plasmolysis which was sometimes observed in DSA-treated cells may also be attributable to increased protoplasmic viscosity. The swelling of protoplasm which was associated with DSA treatments further suggests that DSA may affect intermolecular bonding in the ground plasm, perhaps by increasing the water binding of macromolecules. This, however, is largely conjectural since the molecular factors which are responsible for determining protoplasmic qualities such as wall attachment or viscosity have not been resolved.

In the present study *Allium* and *Rhoeo* cells reacted similarly to DSA treatment although *Rhoeo* cells were generally more resistant than *Allium*, and cells from dormant *Allium* bulbs were more resistant to injury by DSA than cells from growing bulbs. Species differences have been previously observed by Newman and Kramer (14). The toxicity of DSA and our inability to demonstrate any DSA effect on temperature dependence of permeability or on frost resistance raises questions about conclusions reached in studies in which the pH of DSA treatments was not controlled and where no observations were made on cell viability following treatment.

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LITERATURE CITED