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Characterization of the Inhibition of K^+ Absorption in Oat Roots by Salicylic Acid[1]

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ABSTRACT

The phenolic compounds salicylic acid (*o*-hydroxybenzoic acid) and ferulic acid (4-hydroxy-3-methoxycinnamic acid) inhibited K^+ ($^{86}Rb^+$) absorption in excised oat (*Avena sativa* L. cv. Goodfield) root tissue. Salicylic acid was the most inhibitory. The degree of inhibition was both concentration- and pH-dependent. With decreasing pH, the inhibitory effect of the phenolic increased. During the early stages of incubation, the time required to inhibit K^+ absorption was also pH- and concentration-dependent. At pH 4.0, 5×10^{-4} molar salicylic acid inhibited K^+ absorption about 60% within 1 minute; whereas, at pH 6.5, this concentration affected absorption only after 10 to 15 minutes. However, at 5×10^{-3} molar and pH 6.5, salicylic acid was inhibitory within 1 minute. The capacity of the tissue to recover following a 1-hour treatment in 5×10^{-4} molar salicylic acid ranged from no recovery at pH 4.5 to complete recovery at pH 7.5. The absorption of salicylic acid was pH-dependent, also. As pH decreased, more of the phenolic compound was absorbed by the tissue. The increased absorption of the compound at low pH most likely contributed to apparent tissue damage at pH 4.5 and might have accounted for the lack of recovery of K^+ absorption as pH decreased.

Under the proper conditions of pH and concentration, phenolic acids such as salicylic acid could significantly affect mineral absorption by plants in the field.

For many years it has been postulated that some plants exert an influence on neighboring plants through the production and release of toxic compounds (13, 17). Some of these phytotoxins have recently been shown to inhibit ion absorption (4–6). The most commonly cited class of compounds involved in such toxic activity is that of phenolic compounds, especially derivatives of benzoic and cinnamic acid. Glass (5) reported that a number of these phenolic compounds, including salicylic acid (*o*-hydroxybenzoic acid) and ferulic acid (4-hydroxy-3-methoxycinnamic acid), inhibit K^+ absorption in barley roots. McClure *et al.* (11) found that FA[2] inhibited phosphate absorption in soybean roots but not nearly to the extent reported by Glass for barley roots. They suggested the different results might be due to differences between species or pH of the absorption medium. Scharff and Perry (14) reported that pH of the bathing medium affects K^+ efflux from yeast cells in the presence of SA. In a preliminary publication (7), we reported a similar effect of pH and SA on K^+ efflux from oat roots. As pH of the medium fell, K^+ efflux increased with 5×10^{-4} M SA present. Also, the herbicide 2,4-D inhibited K^+ absorption in wheat roots more extensively as pH decreased (18). More recently, Jacobson and Jacobson (9) reported the same effect of pH in barley roots using TIBA. Thus, pH appears to be an important factor to consider when studying phenolic acids and their derivatives.

The purpose of this research was to characterize extensively the action of the benzoic acid derivative, SA, on K^+ absorption in excised oat root tissue. Our intent was to identify conditions required for inhibition of K^+ absorption by SA and to provide the background for studying the physiological processes by which SA acts to inhibit absorption. We investigated the effects of pH, concentration of the inhibitor, and rapidity of action by the compound on K^+ absorption. In addition, the absorption of SA itself was measured. We found that pH was a major factor in determining the degree of inhibition of K^+ absorption by both SA and FA. We further observed that two parameters, pH and concentration of SA, interacted to produce complex effects on K^+ absorption at various times of exposure to SA.

MATERIALS AND METHODS

Plant Material. Oat seeds (*Avena sativa* L. cv. Goodfield) were germinated and grown aeroponically on moistened cheesecloth over 1 mM $CaSO_4$ for 5 days at room temperature (about 21°C) in darkness (8). The terminal 5 cm of the primary and two seminal roots were excised and cut into 1-cm segments for absorption studies. Segments were stored on moist filter paper over ice until used. Three plants were used for each sample (about 0.08 g). For comparison studies, maize (*Zea mays* L. cv. B73 × Mo17), barley (*Hordeum vulgare* L. cv. Beacon), and wheat (*Triticum aestivum* L. cv. Olaf) were germinated and grown employing the same methods.

Absorption Measurements. Absorption of K^+ and SA was measured using the radioisotopes $^{86}Rb^+$ (Amersham, Arlington Heights, IL), and [^{14}C]SA (New England Nuclear), respectively. Nonradioactive SA and FA were purchased from Sigma. Absorption solutions contained 0.5 mM KCl, 0.25 mM $CaSO_4$ with 25 mM Tris and 25 mM Mes buffers mixed to obtain the desired pH. Because the phenolic compounds were dissolved in absolute ethanol, all solutions including the controls contained a final concentration of 1% ethanol (v/v). Approximately 12 μCi/liter $^{86}Rb^+$ was added as a tracer for K^+. For SA absorption measurements, the solutions contained 5×10^{-4} M SA labeled with about 10 μCi/liter [^{14}C]SA.

Excised root segments were transferred to test tubes containing 10 ml continuously aerated absorption solution. Following the specified absorption period, segments were rapidly vacuum filtered in a Büchner funnel on No. 2 Whatman filter paper for 30 s. The tissue was washed for 2 min in ice-cold (pH 6.5) absorption

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[2] Abbreviations used: FA, ferulic acid; SA, salicylic acid; TIBA, 2,3,5-triiodobenzoic acid; SA°, undissociated salicylic acid; SA^-, dissociated salicylic acid.

solution lacking radioisotopes and phenolics. The segments were filtered again for 30 s, rapidly transferred to tared scintillation vials, and weighed. Each vial received 10 ml dioxane-methyl cellusolve scintillation cocktail (1). Radioactive content was determined by liquid scintillation spectroscopy with a Beckman model LS-200 scintillation counter.

Recovery of Absorption Capacity. The tissue was tested for its capacity to recover from the effects of 5×10^{-4} M SA on K^+ absorption. The root segments were treated for 1 h in absorption solution of the appropriate pH containing 5×10^{-4} M SA or 1% ethanol (control) but no radiotracer. Following this treatment, the tissue was vacuum filtered for 30 s as described above and washed for 2 min in ice-cold (pH 6.5) nonradioactive absorption solution. The tissue was filtered again for 30 s and transferred to $^{86}Rb^+$-labeled absorption solution containing no inhibitor for 1 h at the appropriate pH. The remainder of the experimental procedure was the same as described for "Absorption Measurements."

Absorbance and Conductivity Measurements. To determine if SA affected membrane permeability, changes in absorbance of the bathing medium were monitored at pH 4.5 and 6.5. Conductivity of the medium was monitored at pH 4.5, also. About 0.45 g excised root tissue was placed in 20 ml absorption solution containing 5×10^{-4} M SA or 1% ethanol (control). Absorbance or conductivity of the bathing medium was monitored every 20 min. Leakage of organic cell constituents was monitored as changes in absorbance at 260 nm with a Pye Unicam SP8-100 UV double beam spectrophotometer. This wavelength was chosen because of the interfering absorption spectrum of SA at other wavelengths in the UV region. Conductivity was measured at pH 4.5 using a Beckman Solu-Bridge conductivity meter equipped with a Beckman Model 425 temperature-compensated conductivity bridge having a 1 cm constant. The conductivity of the bathing medium at pH 6.5 was considerably larger than at pH 4.5. Sensitivity of the probe did not allow the detection of small changes in conductance at high conductivities. Thus, conductance changes at pH 6.5 could not be monitored.

Uniformity of Data. Each experiment was performed at least twice with three or more replicates per treatment. For each experimental treatment, the standard error of the mean did not exceed 10% of that mean.

RESULTS

Of the species tested, maize was the most sensitive to SA inhibition of K^+ absorption at pH 6.5, whereas barley was the least affected (Table I). The effect on absorption in wheat and oats was intermediate between that of maize and barley. Morphological differences rather than physiological differences between maize and cereal roots may have accounted for the relative sensitivities to SA. Although maize was the most sensitive species, the oat cultivar 'Goodfield' was chosen for further investigation because of our personal knowledge about the mechanism of ion absorption in this species.

Two phenolic compounds were compared for their effectiveness in inhibiting K^+ absorption across a pH gradient. One was a benzoic acid derivative (SA) and the other a cinnamic acid derivative (FA). Although pH considerably influenced K^+ absorption in both the controls and the treatments, the effectiveness of the inhibitor was greatly enhanced as pH decreased (Fig. 1, A and B). Both compounds produced the same trend across a pH gradient from pH 4.5 to 7.5. In the presence of 5×10^{-4} M SA, K^+ absorption (expressed as per cent control) ranged from only 8% at pH 4.5 to 82% at pH 7.5. On the other hand, K^+ absorption in the presence of 5×10^{-4} M FA was substantially higher, 42 and 92% at the respective pH values. Because SA was more inhibitory, further studies were limited to this compound.

To determine if K^+ absorption was a linear function of time, we measured absorption in the presence and absence of 5×10^{-4} M SA at pH 6.5 from 1 to 5 h. Both the treated and the control tissue appeared to absorb K^+ linearly over this time period (Fig. 2). The amount of absorption by the treated tissue averaged about 70% of the control in this experiment.

The concentration of SA required for a given degree of inhibition depended upon the pH of the absorption solution (Fig. 3). As the pH decreased, the amount of SA required to produce a given effect decreased. At concentrations of 10^{-5} M and higher, absorption was substantially inhibited at pH 4.5 and 5.5. No apparent inhibition occurred at pH 6.5 below concentrations of 10^{-4} M; and at pH 7.5, inhibition was observed only at concentrations of 5×10^{-4} M and higher. Being a weak acid with a pK_a of about 3, the undissociated species of SA (SA°) increases approximately 10-fold for each decrease of one pH unit from pH 7.5 to 4.5. Superficially

Table I. *Inhibition of K^+ Absorption in Four Crop Species by Salicylic Acid*

Species	K^+ Absorption		
	1% Ethanol	5×10^{-4} M Salicylic acid	Inhibition
	$\mu eq \cdot g^{-1} \cdot hr^{-1}$		% Control
Goodfield Oats	3.4	2.6	76
Olaf Wheat	3.3	2.3	70
Beacon Barley	4.2	3.4	81
B73 × Mo17 Maize	0.8	0.5	63

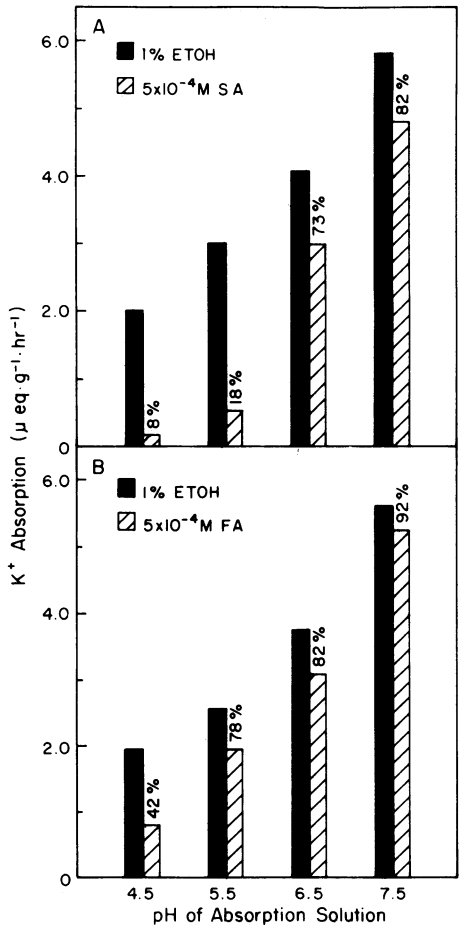

FIG. 1. K^+ absorption by excised oat root tissue in the presence of SA (A) or FA (B) compared to controls (1% ethanol) at four pH values. Percentages are as per cent of the corresponding control.

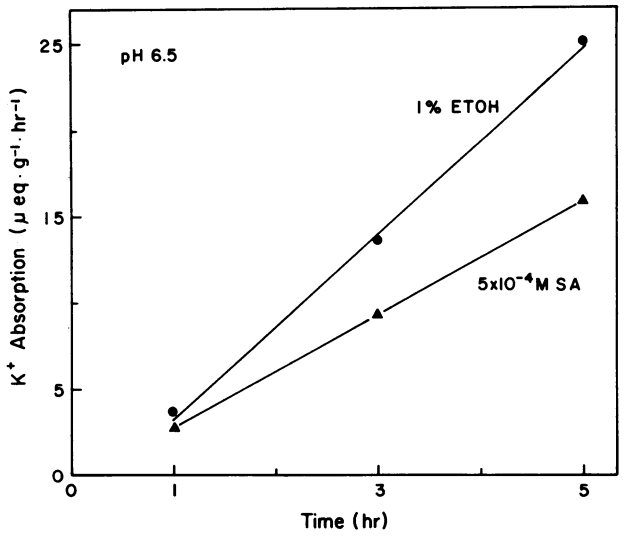

FIG. 2. Time course of K^+ absorption as influenced by 5×10^{-4} M SA at pH 6.5.

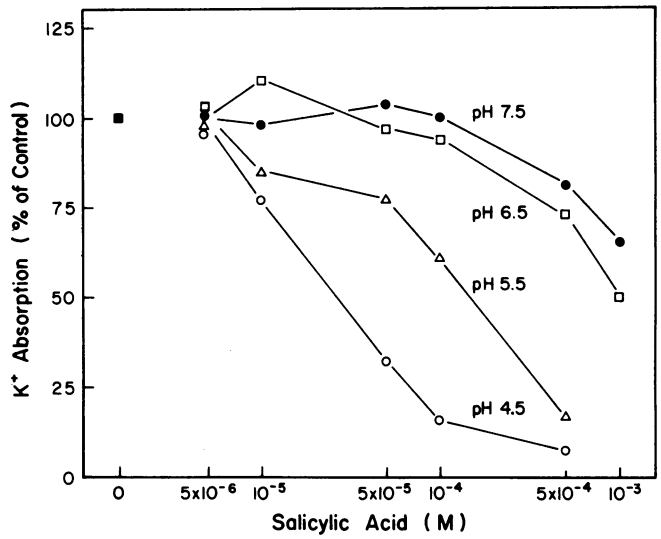

FIG. 3. Influence of concentration of SA on K^+ absorption at four different pH values.

this would seem to indicate that SA° is the species causing the increased inhibition as pH is decreased.

Time of exposure to the inhibitor was an additional factor affecting inhibition of K^+ absorption in the early stages of inhibition. When the pH was lowered to 4.0 with 5×10^{-4} M SA present, K^+ absorption was only 40% of the control by 1 min (Fig. 4A). At pH 5.5, absorption was about 50% of the control within 1 min (data not shown). At pH 6.5, inhibition of K^+ absorption by 5×10^{-4} M SA was not observed before about 10 min (Fig. 4B) and per cent inhibition was constant beyond 1 h (Fig. 2). However, increasing SA concentration to 5×10^{-3} M, at pH 6.5, resulted in absorption of only about 75% of the control within 1 min (Fig. 4C).

Because of the interactions of pH, concentration, and to some extent time, selection of appropriate combinations required to produce a desired level of inhibition was quite difficult. This problem also made it difficult to design experiments to determine the ability of root tissue to recover its capacity to absorb K^+ after treatment with SA. The majority of the absorption experiments were performed at 5×10^{-4} M SA for 1 h. Thus, we chose these conditions to investigate the recovery of K^+ absorption. After root

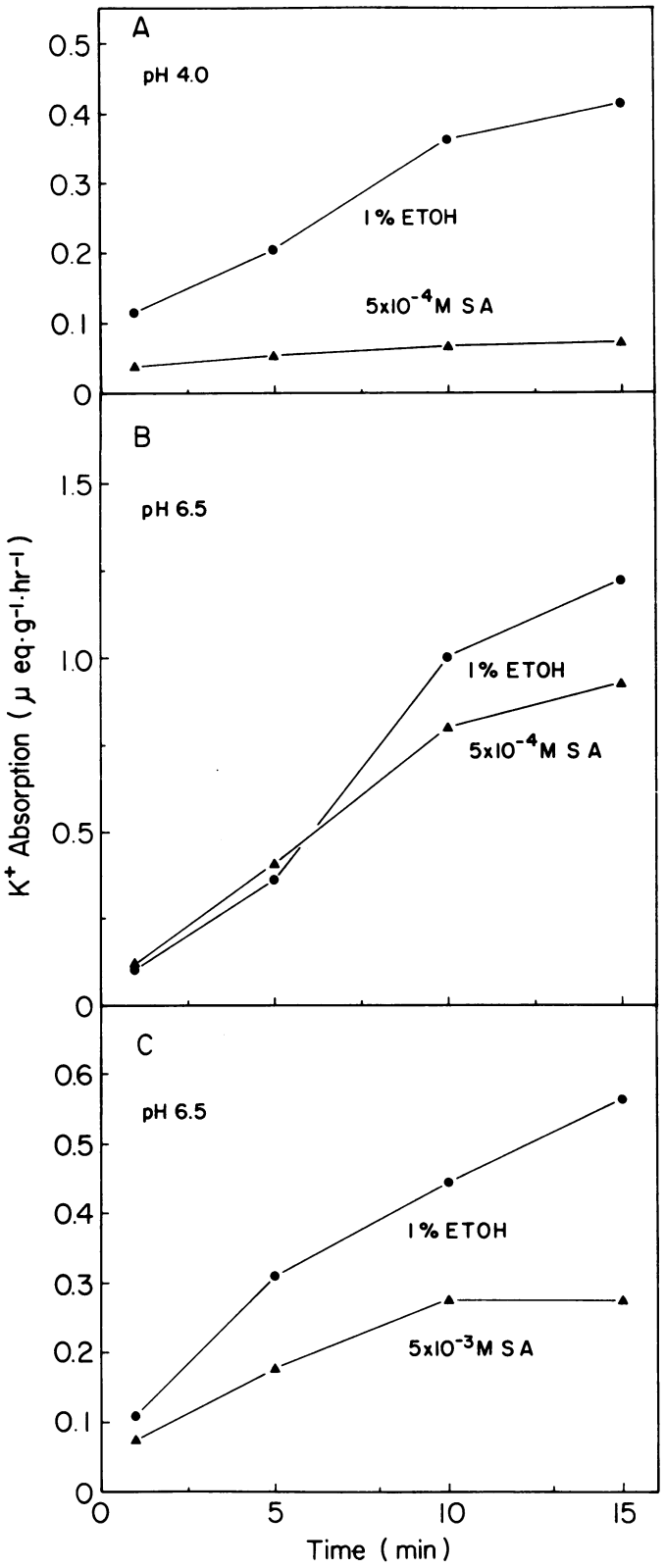

FIG. 4. Relationship between time, concentration, and pH on the inhibition of K^+ absorption by SA at short time intervals.

segments had incubated for 1 h in absorption solution buffered at pH 4.5, 5.5, 6.5, or 7.5 containing 5×10^{-4} M SA without $^{86}Rb^+$, the segments displayed differing capacities to absorb $K^+(^{86}Rb^+)$ from solutions not containing SA but buffered at the respective pH values (Fig. 5). At all pH values, except possibly pH 4.5, K^+

absorption was greater for the pretreated tissue (Fig. 5) than tissue treated with SA during the absorption period (Fig. 1). This indicated that the tissue was able to recover some or all of its absorption capacity subsequent to SA treatment. The extent of recovery increased with increasing pH, complete recovery being achieved at pH 7.5 (Fig. 5).

The possibility was considered that the increased inhibition of K^+ absorption observed at lower pH values might result from greater absorption of SA as pH decreased. Using [^{14}C]SA, the absorption of SA by root segments was measured across the pH gradient 4.5 to 7.5. There was an increase in SA absorption as pH decreased, with a drop in absorption at pH 4.5 (Fig. 6). The apparent drop at pH 4.5 may have resulted from tissue damage since it was noted that the tissue was flaccid after 1 h in 5×10^{-4} M SA at pH 4.5.

Root segments were tested at pH 4.5 and 6.5 for possible membrane damage resulting from exposure to 5×10^{-4} M SA. Membrane damage resulting in leakage of ions and organic solutes out of the root cells was assessed by measuring changes in conductivity and A_{260} of the tissue bathing medium with or without SA present (2, 12). At pH 4.5, differences in absorbance were observed between treated and untreated tissue only after 55 min, at which time leakage of cell contents which absorb at 260 nm became apparent (Fig. 7A). However, at pH 4.5, an increase in conductivity was observed within less than 40 min suggesting loss of electrolytes within this period. At pH 6.5, less material which absorbed at 260 nm was lost from the SA treated tissue than from the control (Fig. 7B). Replication of this experiment repeatedly confirmed the differences at pH 6.5. It appeared that 5×10^{-4} M SA treatment damaged oat root segments at pH 4.5, resulting in leakage of organic compounds and electrolytes from the cell (Fig. 7A). However, comparable absorption measurements at pH 6.5 suggested that SA did not increase membrane leakiness.

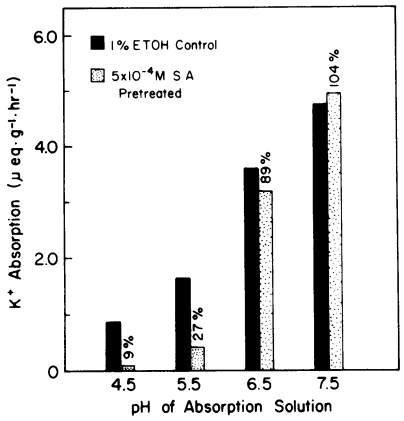

FIG. 5. Recovery of capacity of oat root segments to absorb K^+ after a 1-h pretreatment in 5×10^{-4} M SA at four pH values. The control tissue was treated for 1 h in absorption solution containing 1% ethanol but lacking $^{86}Rb^+$ and then transferred to absorption solution containing 1% ethanol and $^{86}Rb^+$ for 1 h. The 5×10^{-4} M SA pretreated tissue was treated for 1 h in absorption solution containing 5×10^{-4} M SA but lacking $^{86}Rb^+$ and then transferred to absorption solution containing 1% ethanol and $^{86}Rb^+$ for 1 h. Both the pretreatment solution and the labeled absorption solution were buffered at the same pH (4.5, 5.5, 6.5, or 7.5) for each individual treatment.

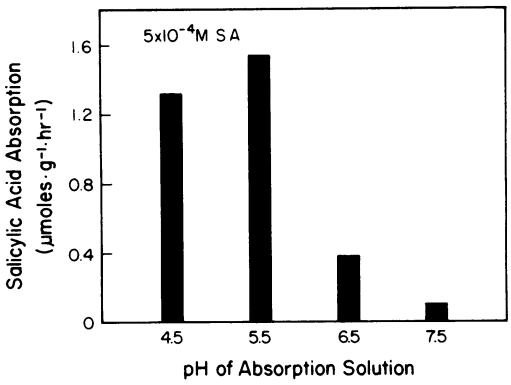

FIG. 6. Absorption of SA by oat root segments across a pH gradient from pH 4.5 to 7.5.

DISCUSSION

The capacity of either SA or the similar phenolic compound FA to inhibit K^+ absorption in excised oat root tissue is strongly pH-dependent (Figs. 1 and 3). Being a weak acid, with pK_a about 3, the undissociated form of SA (SA°) increases about 10-fold for each unit decrease in pH. This suggests that SA° is the active species. On the other hand, the species that is active in the cytoplasm may be the charged species (SA^-) with only SA° able to readily permeate the plasma membrane (15). If the cytoplasmic pH remains constant at about pH 7 regardless of the exterior pH (16), almost all molecules of SA° penetrating the plasmalemma would become dissociated in the cytoplasm. At pH 4.5 and 5.5, a substantial accumulation of SA in the cytoplasm could occur as suggested for IAA by Edwards and Goldsmith (3). Once dissociated inside the cell, SA^- would not freely diffuse across the membranes and out of the cytoplasm. Also, a concentration gradient for SA° would remain from the outside to the inside at low external pH as SA° moves into the cytoplasm and dissociates to SA^- and H^+. If SA° permeates the plasmalemma and most of it dissociates to SA^-, a given concentration of SA at a lower pH as is suggested by Figure 6. The decrease in SA content at pH 4.5 was

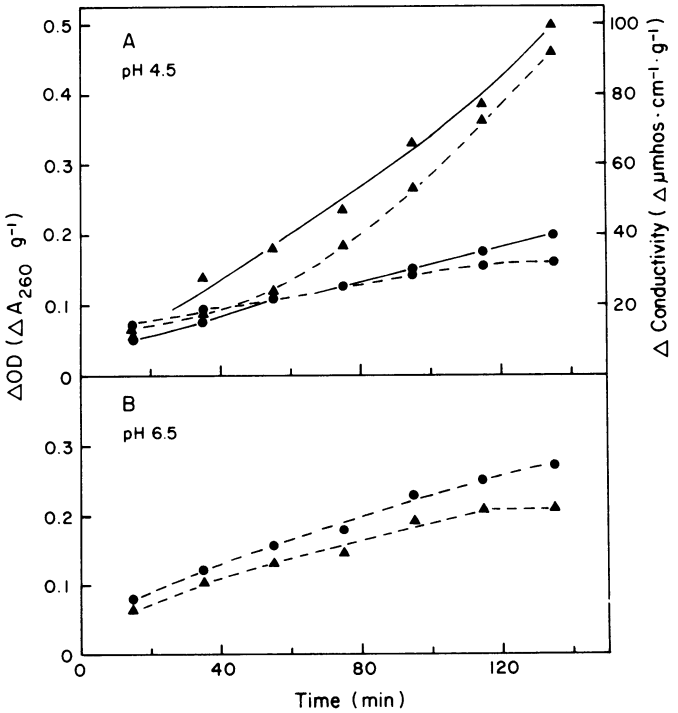

FIG. 7. Membrane damage by SA. Changes from time = 0 were determined for: (a) conductivity of the bathing medium of the control (●——●) and 5×10^{-4} M SA treated (▲——▲) root segments, and (b) A at 260 nm of the bathing medium of the control (●- - -●) and 5×10^{-4} M SA treated (▲- - -▲) root segments. Measurements were made at pH 4.5 (A) and pH 6.5 (B).

attributed to tissue damage (see later discussion). Thus, the observed pH effect may be a simple matter of higher internal concentrations of SA as pH decreases.

The rapid inhibition (within 1 min) suggests that there is an initial surface effect at the plasmalemma (Fig. 4). The fact that absorption appears linear with time from 1 to 5 h (Fig. 2) might be interpreted in two ways. One, the effect is at the surface of the membrane and achieves constant inhibition rapidly. Secondly, the effect is internal and the membrane is quite permeable to SA which is able to achieve rapid equilibration internally. If the membrane were less permeable to SA, then internal concentration would rise more slowly and an increase in inhibition would be expected with time rather than the observed linear response for periods longer than 1 h (Fig. 2).

At pH 4.5, the loss of turgidity, the apparent leakage of electrolytes and organic constituents (Fig. 7A), and the lack of recovery following treatment with 5×10^{-4} M SA (Fig. 5) indicate permanent damage by SA. This effect may be present at higher pH but expressed to a lesser degree because of lower internal concentrations of the inhibitor. However, such a permanent effect was not apparent from absorbance data at pH 6.5 (Fig. 7B), nor was a permanent effect indicated at pH 7.5 where full recovery was observed following SA treatment (Fig. 5).

Glass (4–6) has published extensively on the effects of phenolic acids on ion absorption. He reported that 2.5×10^{-4} M SA inhibited K^+ absorption in barley roots by 96% at pH 7.1 (5). In oat root segments, we found that twice the above concentration (5×10^{-4} M SA) inhibited K^+ absorption only about 27% at pH 6.5 (Fig. 1A). In a cultivar of barley, different from that used by Glass, the inhibition was even less (Table I). McClure *et al.* (11), working with FA, have reported similar discrepancies between their results for the inhibition of phosphate absorption by FA in soybean roots and that found by Glass (4) for barley roots. They suggest that the differences might be due to the plant material used and to the pH of the absorption solutions. However, McClure *et al.* used pH 6.0, whereas Glass used pH 7.1, and we have demonstrated that inhibition of K^+ absorption by FA should increase as pH decreases (Fig. 1B). Absorption of phosphate may differ under these conditions, but we think that is doubtful. We have no answers to these disparities.

Glass (6) reported that the capacity of most phenolic acids to inhibit ion absorption is highly correlated with their octanol-water partition coefficients. SA is quite lipid-soluble, having an octanol-water partition coefficient (log P) of about 2.2 (10). It may be that as pH is lowered and the concentration of SA° increases, more SA° becomes dissolved in cell membranes until a point is reached where membrane integrity is disrupted. Since SA° would tend to reach an equilibrium between membrane lipids and the aqueous surroundings, according to the partition coefficient of SA in the lipids, the concentration of SA° could be much higher in the membranes than in the bathing medium. The actual concentration in membranes could, therefore, be quite high. Zsoldos *et al.* (18) suggested that 2,4-D exerted its effect by disturbing lipid-protein interactions within the hydrophobic region of the membrane in a nonspecific manner. SA° may act in a similar fashion.

Although the exact mode of action of SA on K^+ absorption is unclear at this time, our research indicated certain characteristics of its action. The most obvious is the effect of pH on the action of SA. Also, based on our own experience and the work of others, it seems unlikely that SA acts in a specific manner or on one specific process, but rather has multiple effects in expressing activity. The inhibition of K^+ absorption in oat roots by SA is a function of pH and concentration of the inhibitor. These two parameters interact interdependently to produce varying effects on K^+ absorption. Lag time to the onset of inhibition was dependent upon the concentration of SA and pH. Table I suggests that the genetic potential for differential response to SA may exist among important crop species. This being the case, the above factors could be important in the inhibition of mineral absorption in nature and may be important in the expression of allelopathy by phenolic acids.

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