Plant Physiol. (1974) 54, 855–858

Influence of Phenolic Acids on Ion Uptake

IV. DEPOLARIZATION OF MEMBRANE POTENTIALS

Received for publication March 22, 1974 and in revised form July 1, 1974

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ABSTRACT

The membrane potentials of aged, excised barley (*Hordeum vulgare* L.) root cells were rapidly depolarized by the addition of salicylic acid (*o*-hydroxybenzoic acid) to the buffered medium bathing root segments. Initial values for membrane potentials were restored very slowly (within 100 minutes) by replacing the phenolic solution by phenolic-free buffer. Several other naturally occurring benzoic and cinnamic acids depolarized cell membrane potentials. The cinnamic acids consistently caused a greater depolarization than the correspondingly substituted benzoic acids. A strong positive correlation was found between the depolarization values ($\triangle E$) for the benzoic acids and their lipid solubilities. This study supports the hypothesis that the inhibition of ion uptake brought about by naturally occurring phenolic acids is caused by a generalized increase in membrane permeability to inorganic ions.

The phenomenon of allelopathy, in which one species of plant may dramatically inhibit the development of others by producing and releasing specific compounds into the environment, is well documented (18). In a few cases, the allelopathic compounds and their mode of transmission have been elucidated (11, 12). In a large number of reported cases, however, the modes of action of the allelopathic compounds (allelochemicals) are not understood. This paper reports further investigations into the mode of action of an important group of allelochemicals, phenolic acids.

Earlier papers by Glass (3–5) have established that one ubiquitous group of phenolics, namely benzoic and cinnamic acids, are potent inhibitors of active K and Pi absorption by excised barley roots. The effect is readily reversible and the inhibitory capacity of the benzoic acids is strongly correlated with their lipid solubilities. From these results it was suggested that phenolics act directly on the cell membrane, modifying its permeability and thereby increasing the rate of efflux of ions. To test this hypothesis the effect of phenolics on cell membrane electrical potentials has been studied. According to the Goldman (7) equation:

$$E = \frac{RT}{F} \ln \frac{\sum(P_{j+}.C^{o}_{j+}) + \sum(P_{j-}.C^{i}_{j-})}{\sum(P_{j+}.C^{i}_{j+}) + \sum(P_{j-}.C^{o}_{j-})}$$

where E is the electropotential difference (PD) between o, the outer solution and i, the inner solution; R, the gas constant (joules degree·mole); T, the absolute temperature; F, the Faraday (coulombs equivalent^{-1}); P subscript is the permeability coefficient of a univalent cation, $j+$, or a univalent anion, $j-$; C_{j+}^{o}, the concentration of $j+$ outside, and C_{j+}^{i}, the concentration of $j+$ inside. Predictions of E based only upon K^+ and Cl^- characteristics, derived from the above equation, may under certain conditions be remarkably consistent with measured values for root cell membrane potentials (14). Clearly any alterations of the permeability properties of the cell membrane with regard to K^+ or Cl^- will be reflected by changes in E. If the cell membrane became extremely permeable, the imbalance of ion concentration across the cell membrane, which is necessary for E, would disappear and E would fall to near zero.

MATERIALS AND METHODS

Seedlings of *Hordeum vulgare* L. cv. Karlsberg were grown for 3 or 4 days in aerated 5×10^{-4} M $CaSO_4$ solution following a 1-day germination in aerated distilled water. At the end of this period, the last 1 to 1.5 cm of roots were excised from the root tip and allowed to age in aerated 5×10^{-4} M $CaSO_4$ solution. It was found that the aging process produced a very significant increase in the cell membrane potential of the order reported by Pitman *et al.* (14). Membrane potentials of cells in the root tips were measured with microcapillary electrodes using the apparatus described by Dunlop and Bowling (2). During determinations of membrane potentials the root tips were held in a 5 ml Plexiglas chamber which, under control conditions, contained a solution of KCl (5×10^{-5} M) and $CaCl_2$ (5×10^{-4} M) buffered at pH 7.2 (5 mM tris-HCl). Phenolic acids were added to the above buffered solution dissolved in 1 ml of 95% ethyl alcohol. The presence of this small quantity of ethyl alcohol (1%) was found to have no significant effect upon measured potentials.

Two methods were used to measure depolarizations caused by phenolic solutions. Initially the electrode was positioned in an epidermal cell, and when a steady voltage was recorded the solution in the chamber changed by operating the pumping system (solution flow rate was 5–10 ml/min). This method was employed to obtain the data shown in Figure 1, which are typical of several such experiments. This method suffered from the drawback that the movement of liquid in the chamber commonly caused the electrode to be displaced. Nevertheless, this method was essential for continuous observation. In those experiments where we required simply the net depolarization value for a particular solution, the electrode was positioned in several epidermal cells and in each case a steady potential recorded. The electrode was removed, the solution in the chamber was replaced, and then several new measurements of po-

Table I. *Influence of External K^+ Concentration upon E Values in the Presence of Salicylate*

Roots were allowed to stand for 15 min in the appropriate solutions before measurements of *E* were made.

	K^+Concn		ΔE
	5×10^{-5} M	5×10^{-4} M	
	mv		
Controls	-168.0 ± 2.0	-122.8 ± 1.7	45.2
$+ 2.5 \times 10^{-4}$ M Salicylate	-50.0 ± 1.4	-44.3 ± 1.8	5.7

Table II. *Influence of Various Benzoic and Cinnamic Acids upon Membrane Potential*

All compounds were tested at a final concentration of 2.5×10^{-4} M in solutions buffered at pH 7.2 containing $CaCl_2$ (5×10^{-4} M) and KCl (5×10^{-5} M).

Compound	Depolarization of Potential
	mv
Benzoic acids	
Salicylate	117.5 ± 7.0
4-Hydroxybenzoate	29.0 ± 6.2
3,4-Dihydroxybenzoate	40.5 ± 3.3
2,5-Dihydroxybenzoate	63.1 ± 1.6
4-Hydroxy-3-methoxybenzoate	62.4 ± 2.7
3,4,5-Trihydroxybenzoate	7.0 ± 4.2
4-Hydroxy-3,5-dimethoxybenzoate	55.2 ± 6.6
Cinnamic acids	
4-Hydroxycinnamate	92.4 ± 2.7
3,4-Dihydroxycinnamate	76.4 ± 2.8
4-Hydroxy-3-methoxycinnamate	107.5 ± 1.2

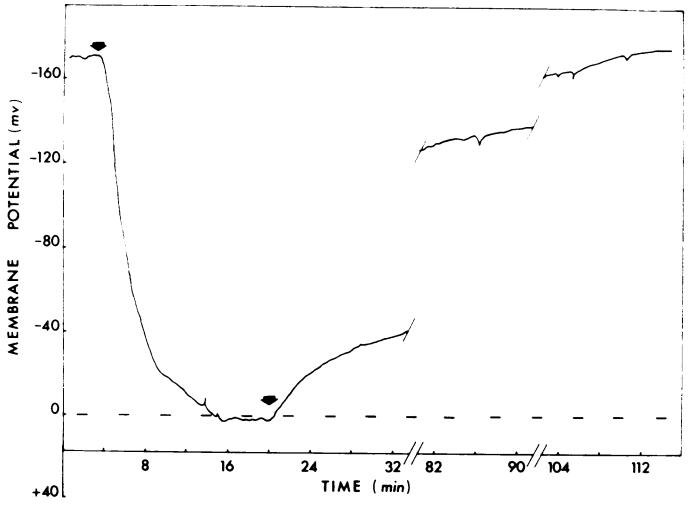

FIG. 1. Depolarization and recovery of root membrane potential following exposure to 5×10^{-4} M salicylic acid. The first arrow indicates the approximate time of arrival of the salicylate solution at the root chamber. The second arrow indicates the approximate time of removal of salicylate from the root chamber.

tential were obtained. This method was used to obtain the data shown in Tables I and II and Figures 2 and 3. In all cases, the experimental procedures were repeated several times. The data shown in Table I represent the means of at least three trials. Log *P* values shown in Figure 5 were obtained from the literature (9), and the regression line was calculated by the application of the method of least squares to the data.

Uptake of K^+ shown in Figure 4 was determined using K^+ solutions labeled with ^{86}Rb as described previously (5). Ten 2-g samples of roots were employed to measure uptake. After 50 min salicylic acid was added to 5 of the samples (dark circles, Fig. 4) while the remaining 5 samples were allowed to continue as before. One hour after the addition of salicylate the roots were removed, rinsed in cold phenolic-free solution and then returned to phenolic-free uptake solutions for the remainder of the experiment.

RESULTS

Figure 1 demonstrates the rapid and extensive depolarization of the membrane potential of a single cell in the cortical parenchyma resulting from exposure to 5×10^{-4} M salicylic acid. Although it was difficult to determine the exact instant when the cell came in contact with the salicylic acid, depolarization seemed to commence immediately, or at the most within 15 sec of contact. Depolarization was completed within 12 min and a stable potential of +2 mv was established. Following replacement of the phenolic solution by phenolic-free solution, the membrane potential began to recover immediately, but required 100 min to recover completely. This experiment was repeated several times with essentially no variation in the timing of responses. However, the initial and final potentials following treatment with salicylate varied from one sample to another. Based upon eight trials, the average initial potential was -148.6 ± 5.1 mv, the average depolarized potential was -6.9 ± 5.3 mv, and the average drop in potential was 141.7 ± 4.2 mv. Figure 2 shows a dose-response curve for salicylate. The times required for membrane potentials to respond to the presence of various concentrations of salicylate and to recover following their removal remained constant regardless of the concentration of salicylate. However, the extent of depolarization increased with increasing salicylate concentration.

Figure 3 shows a plot of the depolarization of root membrane potentials (ΔE) in the presence of the benzoic acids listed

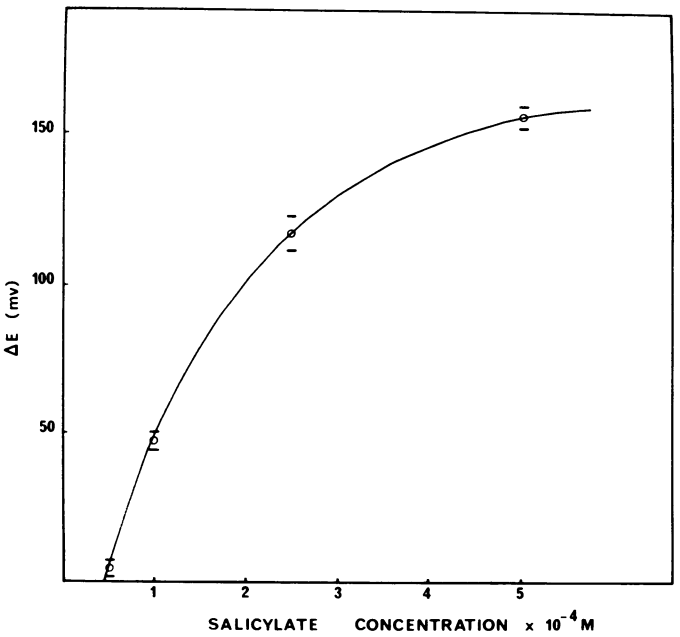

FIG. 2. Depolarization of root membrane potentials by different concentrations of salicylate.

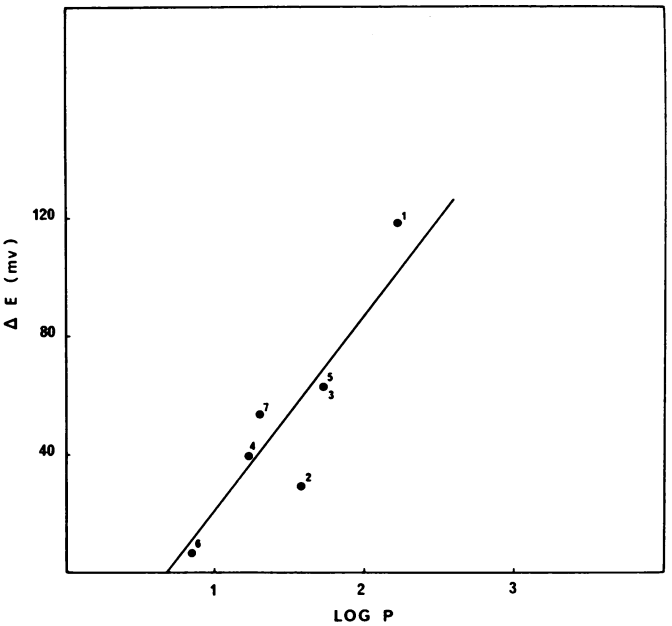

FIG. 3. Plot of depolarization of membrane potential ($\triangle E$) for benzoic acids at a final concentration of 2.5×10^{-4} M against the log of their partition coefficients (log P). The numbers beside the points identify the compounds in Table II.

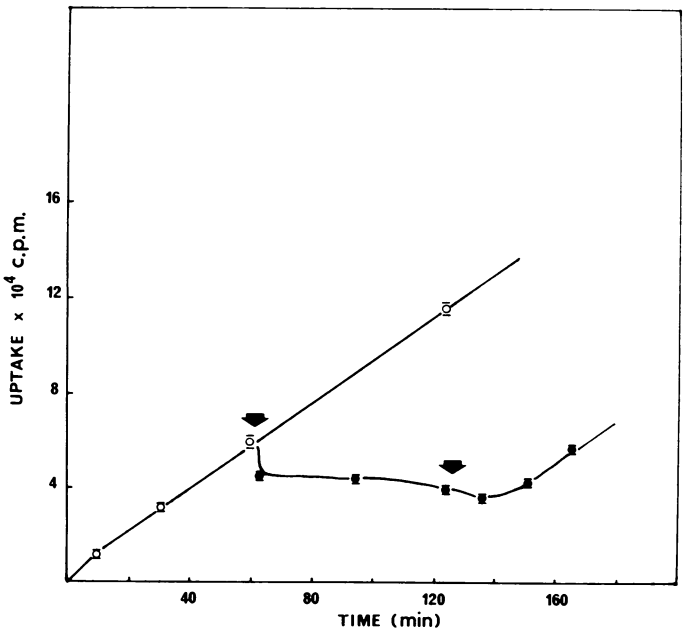

FIG. 4. Influence of salicylate (2.5×10^{-4} M) upon K^+ uptake by controls (○) and salicylate treated tissue (●). The first arrow indicates the time of addition of salicylate. The second arrow records the time of removal of salicylate.

in Table II, against the log of partition coefficients (log P), between octanol and water, of the compounds concerned. The plot demonstrates a strong positive correlation between these two variables.

DISCUSSION

It is difficult to validate the results of this study by comparing them with other published results because we have been unable to find any other data on the effect of naturally occurring phenolic compounds on plant membrane potentials. However, the effect of the respiratory uncoupler, 2,4-dinitrophenol has been tested and it seems to act in a similar way to the phenolics tested in this study, in so far as it causes a rapid depolarization of wheat root cells and *Neurospora* membrane potentials (16, 17). Furthermore, although recovery of the membrane potential started immediately upon removal of 2,4-dinitrophenol, it was relatively slow as found with the present compounds. In contrast, salicylic acid caused a hyperpolarization rather than a depolarization of the membrane potentials of mollusc neurons (1), but only at concentration 1,000 times greater than the concentration at which the maximum effect was exerted by phenolic compounds on barley roots. Furthermore, in mollusc neurons, increased permeability was limited to cations, whereas in barley roots there is apparently a generalized increase in permeability to both cations and anions. These differences are indicative of the basic differences in electrodiffusive properties between plant and animal cell membranes (15).

The immediate and dramatic effect of the phenolic compounds on E strongly implies that they act directly on the cell membrane. This is further supported by the immediate onset of recovery when phenolics are removed. If they were acting on some cytoplasmic process, it is reasonable to suppose that there would be some delay while the inhibitor was washed out or removed by detoxification. Little delay is in fact evident. The effect of salicylate in changing a net K^+ influx into an efflux is also very rapid, although recovery of initial rates of influx required about 20 min following removal of the inhibitor. This suggests that the depolarization of E and the loss of ability to take up ions are aspects of the same direct effect on the membrane. However, ion uptake ability completely recovers within 20 min whereas 100 min are required for recovery of E. This anomaly is discussed in the next paragraph, but it is interesting to note that both Macklon and Higinbotham (10) and Pitman (13, 14) found that E is not necessarily directly correlated with rate of K^+ uptake. The strong correlation between the lipid solubility of the benzoic acids and their effectiveness in depolarizing E, is further evidence that the membrane is the primary site of action. This parallels the earlier finding (3–5) of a close correlation between lipid solubility and inhibitory effect on ion uptake by benzoic acids. One further final result reported here that implies that phenolics directly affect the membrane is the effect of salicylate in eliminating the sensitivity of E to K^+_o (Table I). Considering the Goldman equation, it would be difficult to explain this as being anything other than a direct effect on membrane permeability.

In formulating a detailed explanation of the effects of these phenolic compounds, it is necessary to account for the following observations: (*a*) the efflux of K^+ (Fig. 4) appears to occur in two phases, an initial fairly massive loss of activity which is complete within minutes, followed by a much more gradual loss which is maintained until removal of the tissue from salicylate; (*b*) rate of K^+ uptake completely recovers long before E does.

The immediate efflux of ions on the application of these phenolic compounds indicates that one of the first effects is an increase in permeability of the cell membrane. In root tissue this involves a nonspecific increase in permeability to both cations and anions (3, 5). Reference to the Goldman equation indicates that this increase in permeability and the resultant efflux of ions will affect E in two ways. First, the increased permeability will be reflected by changes in the permeability coefficients, and second, the efflux of ions will tend to equalize

the internal and external ion concentration. These two processes can be expected to result in a rapid change in E.

The fact that a major proportion of the absorbed radioactivity is not lost even during a 60-min exposure to salicylate suggests that this represents a separate pool of ions, probably located in the vacuole. Root tissue possesses a highly developed system for detoxifying phenolic acids by conjugating them with glucose. This markedly reduces the lipid solubility of these compounds and, in consequence, their toxicity. Thus previous experiments have shown (6) that after exposure to phenolic compounds the internal concentration of unconjugated phenolics is extremely low. Studies of the effect of *p*-hydroxybenzoic acid on K^+ absorption by barley roots (5) gave no indication of a second phase of efflux observed in the case of salicylate. However, the *o*-hydroxybenzoic acids, which include salicylate, have been shown to possess structural impediments to the usual means of detoxication (4), which accounts for their greater toxicity. Thus we believe that the second phase of efflux is brought about as salicylate begins to accumulate within the cell cytoplasm. Benzoates which are not *o*-substituted would be detoxified more effectively, and therefore would not accumulate in sufficient concentrations to affect the tonoplast.

If the initial loss of ions is in fact from the cytoplasmic pool, this indicates a large loss of semipermeability of the plasmalemma, and it might be asked why E is not zero under these conditions. The residual potential difference could be attributable to either the continuing operation of an electrogenic ion pump (8), or to a potential difference across the tonoplast. In the latter case, the greatly changed ionic composition of cytoplasm makes it meaningless to compare the residual potential difference with the scant data on potential differences across the tonoplast of unpoisoned cells. On removal of the inhibitor the permeability coefficients would return to the normal values, and thus would contribute to the recovery of E. However it would take some time before ion uptake would build up the cytoplasmic composition to its original level, which would explain the slower rate of recovery by E than by rate of ion uptake.

The present report establishes that phenolic acids strongly influence cell membrane potentials and supports the hypothesis that their influence upon ion uptake is mediated largely through direct, nonspecific permeability effects upon the cell membrane. In the case of *o*-hydroxylated compounds, there may be a delayed effect upon internal membranous structures including the tonoplast and cell organelles. The latter effect may bring about a minor metabolic inhibition of active transport of ions. Clearly, these profound physiological changes which are brought about in root tissue by phenolic compounds have not been shown to be the basis for the allelopathic effects of phenolic compounds in nature. Nevertheless, it seems highly likely that such dramatic effects upon so important a structure as the root cell membrane must be of some consequence in allelopathy.

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