Influence of Phenolic Acids on Ion Uptake

I. INHIBITION OF PHOSPHATE UPTAKE

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ABSTRACT

The influence of naturally occurring phenolic acids on phosphate uptake by barley (*Hordeum vulgare* L. cv. Karlsberg) roots was examined using ^{32}P-labeled phosphate. Without exception, all compounds tested, namely, benzoic, 2-hydroxybenzoic, 4-hydroxybenzoic, 3,4-dihydroxybenzoic, 3,4,5-trihydroxybenzoic, 4-hydroxy-3-methoxybenzoic, 4-hydroxy-3,5-dimethoxybenzoic, cinnamic, 2-hydroxycinnamic, 4-hydroxycinnamic, 3,4-dihydroxycinnamic, 4-hydroxy-3-methoxycinnamic, and 4-hydroxy-3,5-dimethoxycinnamic acids, inhibited uptake.

The degree of inhibition correlated well with the lipid solubility of these phenolic compounds. This as well as kinetic data support the hypothesis of inhibition through altered membrane properties.

Phenolic compounds are ubiquitous in nature, occurring in high concentrations in plant tissues (13), microorganisms (19), soils (21, 23), and, as has been demonstrated recently, in industrial effluent waters (18). Many of the physiological effects of phenolic compounds have been recognized for some time (16), but more recently they have been implicated as important factors in plant-environment interactions. The influence of such compounds in suppressing the growth and distribution of certain species (the phenomenon of allelopathy) is considered to be extremely common (24, 25).

It has been demonstrated that in both higher plants (8) and animals (1) simple phenols such as hydroquinone, phlorizin, and phloretin are capable of inhibiting the process of active transport. While these latter compounds have been considered in some detail in terms of the mechanism of their inhibition (1, 8), they are in fact limited in their natural distribution, and consequently their biological activities do not represent realistic environmental interactions. By contrast, phenolic acids, particularly benzoic and cinnamic acid derivatives, are ubiquitous in angiosperms (3), gymnosperms (12), and ferns (7). Through decomposition of plant material, rain leaching from leaves and bark, and exudation from roots a wide variety of phenolic acids may be introduced into the soil solution. Whitehead (23) sampled a variety of different soil types and reported the presence of 4-hydroxybenzoic, 4-hydroxy-3-methoxybenzoic, 4-hydroxycinnamic, and 4-hydroxy-3-methoxycinnamic acids as the free acids at soil solution concentrations equivalent to 5×10^{-5} M.

The present study examines the influence of these naturally occurring phenolic acids upon inorganic phosphate uptake by barley roots. Concentrations employed were in the range reported by Whitehead (23). In the main, experiments were of short duration ranging from several minutes to 3 hr.

MATERIALS AND METHODS

Barley seeds (*Hordeum vulgare* L. cv. Karlsberg) were germinated and prepared for treatment according to the methods described by Epstein and Hagen (5). All experiments were performed with 4-day-old excised roots, approximately 3 cm long. After excision the roots were gently mixed in a large volume of distilled water, filtered through two layers of cheesecloth, and spun by hand in a cheesecloth bag to remove excess water.

Root samples weighing 2 g were placed in 150-ml beakers containing 50 ml of solution, buffered at pH 7, containing 5×10^{-5} M Pi, 5×10^{-4} M CaCl$_2$, and 0.05 μc of ^{32}P-labeled phosphate. All experiments were performed at room temperature (23 C), and solutions were aerated continuously during the uptake period. The various phenolic acids which were added to the treatment solutions were dissolved in 1 ml of 95% ethanol. In most experiments, the final concentrations of phenolic acids experienced by the root tissue were 5×10^{-4} M, slightly in excess of the values reported by Whitehead (23) for soil solution. In the preliminary kinetic experiments involving vanillic acid, concentrations ranged from 5×10^{-5} M to 1×10^{-3} M.

The uptake of phosphate by root tissue was determined by the removal of 1-ml aliquots from the incubation medium at prescribed intervals. The radioactivity of each sample was determined in a Packard scintillation spectrometer, and uptake was determined by loss of activity from the solution.

Preliminary experiments indicated that the uptake of phosphate, in both control treatments (in the absence of inhibitors) and vanillic acid treatments, had become linear within 5 to 10 min of immersion of the tissue in the phosphate solutions. In the comparative experiments which followed, therefore, the first samples were removed 20 min after immersion. This was taken as the zero time. Further samples were removed 60 and 180 min after zero time.

Uptake values reported in the graphs are expressed as μmoles of Pi/g fresh weight of root and individual points are the means of three replicates. Inhibition values reported in the table are the means of three separate experiments.

RESULTS

Preliminary experiments established that the rate of uptake of phosphate under control conditions was 0.54 ± 0.005 μmoles/g fresh weight·hr. This rate of uptake was sustained for periods in excess of 4 hr without any indication of reduced rates even though up to 50% of the available phosphate was absorbed during this period. Figure 1 shows the pattern of
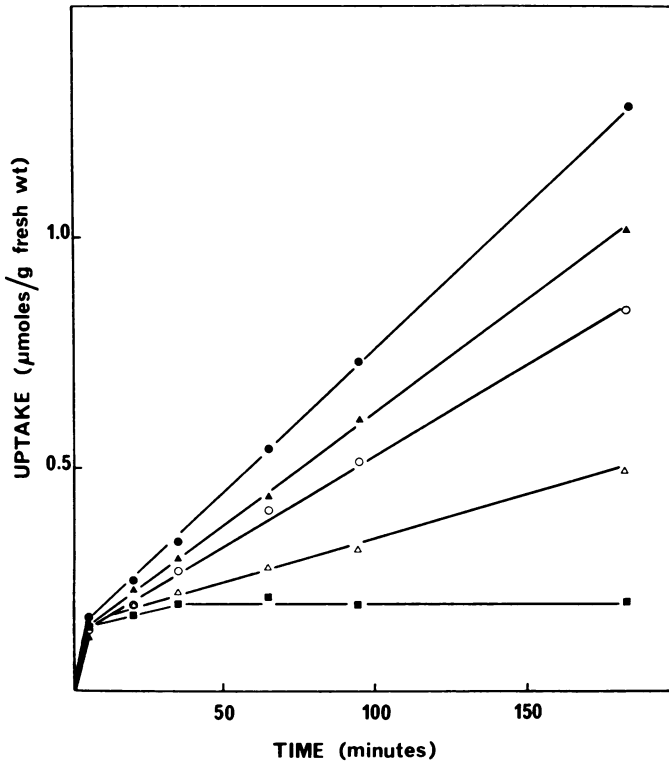
FIG. 1. The uptake of phosphate by excised barley roots, as influenced by various concentrations of vanillic acid. Control (●) (no vanillic acid); 5×10^{-5} M vanillic acid (▲); 1×10^{-4} M vanillic acid (○); 5×10^{-4} M vanillic acid (△); 1×10^{-3} M vanillic acid (■).

uptake for a typical experiment. The initial rapid uptake within the first 15 min corresponds to the penetration of the free space volume. Figure 1 also indicates the influence of 4-hydroxy-3-methoxybenzoic acid (vanillic acid) upon the uptake of phosphate. At concentrations of 5×10^{-5} M, 1×10^{-4} M, 5×10^{-4} M, and 1×10^{-3} M, this compound reduced the uptake by 24, 39, 71, and 90%, respectively. The K_i for this compound was obtained by drawing the double reciprocal plot for inhibition versus concentration as shown in Figure 2. The value for this constant was 1.66×10^{-4} M. Figure 3 shows the effect of various concentrations of vanillic acid upon the uptake of phosphate from solutions varying in phosphate concentration from 1.25×10^{-5} M to 1×10^{-4} M. Uptake of phosphate in this experiment was determined by sampling at zero time (as defined in "Materials and Methods") and 180 min after zero time. The data are presented in the form of Lineweaver-Burk plots. The form of the inhibition is certainly not competitive but, at low concentrations of the inhibitor, resembles uncompetitive inhibition. At higher concentrations of phosphate, the effect of the inhibitor is less pronounced.

Reversibility of Inhibition. The rapidity of the inhibition due to vanillic acid is quite apparent from Figure 1 in which differences in rate of uptake can be recognized within the first 30 min. However, the picture is somewhat complicated by the times involved in passive penetration of the free space by both phosphate and inhibitor. Figure 4 shows the results of experiments in which roots were allowed to absorb phosphate for 50 min prior to the addition of the inhibitor, o-hydroxybenzoic acid (salicylic acid) at a final concentration of 5×10^{-4} M. Within the first 10 min. there was no observable effect on rate of uptake. Clearly, penetration of the inhibitor to its site of
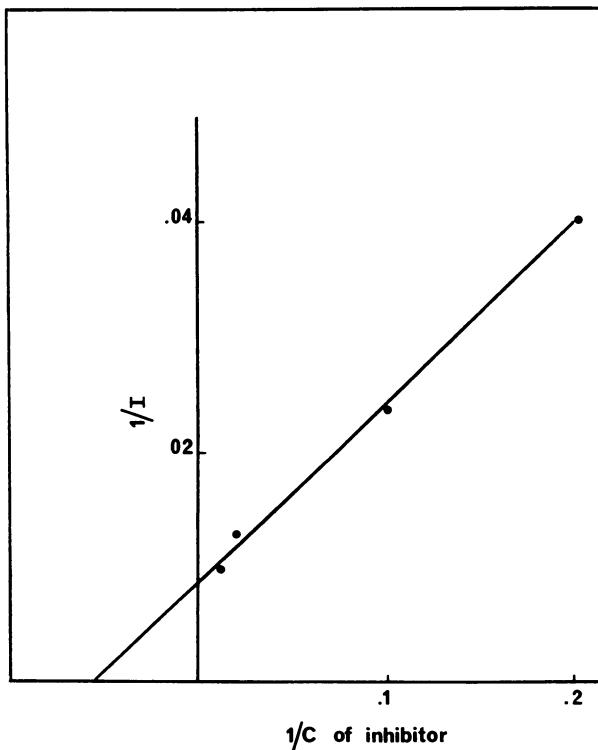

FIG. 2. Lineweaver-Burk plot for the inhibition of phosphate uptake by vanillic acid. The initial concentrations of phosphate were 5×10^{-5} M. I: % inhibition; C: concentration of vanillic acid $\times$ 10^5 M; K_i for inhibition: 1.6×10^{-4} M.
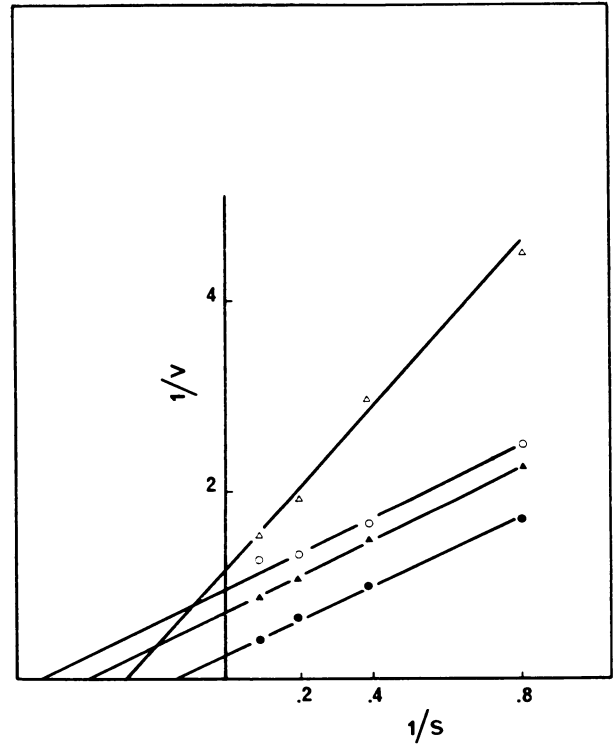

FIG. 3. Lineweaver-Burk plots for the uptake of phosphate in the presence of various concentrations of vanillic acid. V: rate of uptake measured in μmoles/g fresh weight; S: concentration of phosphate $\times$ 10^5 M. The control (●) was vanillic acid-free, other treatments contained 1.25×10^{-4} M vanillic acid (▲); 2.5×10^{-4} M vanillic acid (○); 5×10^{-4} M vanillic acid (△).
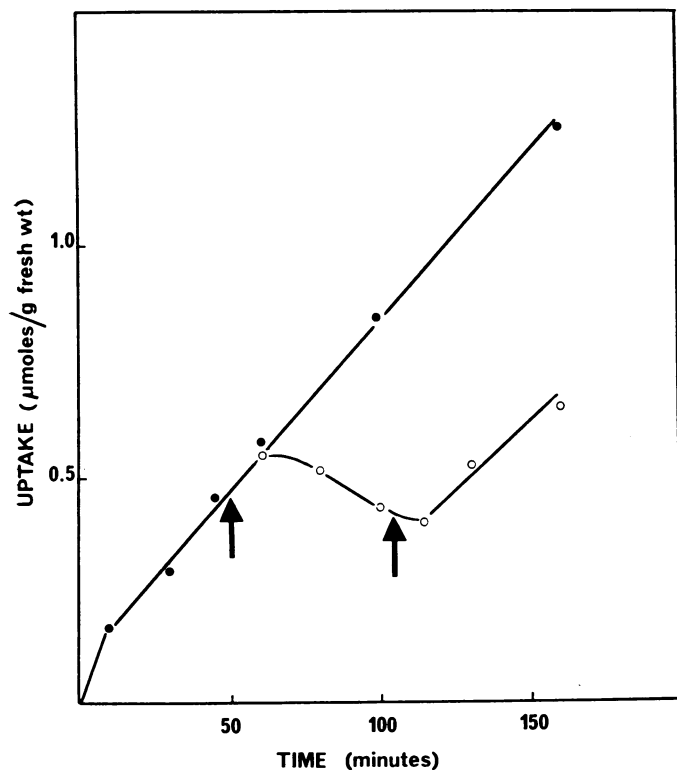
FIG. 4. The uptake of phosphate by excised barley roots as influenced by the addition of salicylic acid. Control (●) (no salicylic acid) and 5×10^{-4} M salicylic acid (○). The first arrow indicates the time when salicylic acid was added to the root samples. At the time indicated by the second arrow roots were rinsed briefly and returned to salicylic acid-free uptake solutions.

action required at least 10 min. Within 30 min there was a strong inhibition of uptake. In fact, there was a clear loss of phosphate from the tissue. Sixty minutes after the addition of salicylate the roots were removed from the solution, rinsed briefly in salicylate-free labeled phosphate solution, and then returned to fresh inhibitor-free uptake solution. Within the first 10 min after transfer, phosphate continued to be lost from solution, but after 25 min the rate of uptake was restored to almost that of the controls.

Comparative Experiments. The influence of several naturally occurring benzoic and cinnamic acids upon uptake of phosphate is shown in Table I. All these compounds were tested at a final concentration of 5×10^{-4} M. Inhibition by these compounds varied considerably, from 18% for 3,4,5-trihydroxybenzoic acid (gallic acid) to 93% for cinnamic acid.

DISCUSSION

The presence in soils of a wide variety of potentially phytotoxic chemicals, released by plants, is well documented (20, 21, 24, 25). Furthermore, the allelopathic influences of compounds such as the phenolic acids have been appreciated for some time (4, 17, 22, 25). To define the precise nature of the interaction between soil phenolics and the growth of higher plants, it seemed appropriate to examine the primary site of interaction between the plant and its environment, namely the cell membrane and its activities, particularly transport phenomena.

The present study clearly establishes that benzoic and cinnamic acid derivatives at concentrations ranging from 5×10^{-5} M to 1×10^{-3} M may significantly reduce the uptake of Pi. The

potency of the compounds examined varies considerably according to the pattern of substitution. In general, polyhydroxylated compounds are less effective than nonhydroxylated or methoxylated derivatives. It is quite apparent that the position of the phenolic hydroxyl substituent is extremely important; for example, p-hydroxybenzoic acid reduced uptake by 41% at 5×10^{-4} M, whereas, o-hydroxybenzoic acid at the same concentration reduced uptake by 85%. In general, the cinnamic acid derivatives were more potent than the corresponding benzoic acid derivatives.

By examining the influence of benzoic and cinnamic acid derivatives upon the growth of sugarcane, Wang (22) observed a considerable reduction of growth in both roots and shoots grown in Hoagland's solutions supplemented with varying concentrations of these acids. In their studies, up to 81% reduction in top weight was induced by p-hydroxycinnamic acid at 6×10^{-4} M.

In attempting to establish an hypothesis to account for the inhibitory effect of these phenolic compounds upon the uptake of phosphate, several possible mechanisms may be considered: (a) denaturation of specific membrane carriers; (b) uncoupling of mitochondrial electron transfer analogous to the effect of DNP; (c) utilization of ATP equivalence (phenolics are glycosylated within the tissue by glycosyl transferases utilizing UDPG or analogous nucleotide sugars); (d) alteration of membrane properties by the solution of the phenolic compound in the lipid component of the membrane.

The capacity of phenolic compounds for denaturing enzymes is well known to enzymologists working with plant tissues (14). However, in the present study it would appear that the inhibitory effect of phenolics such as salicylate is readily reversible even after application for periods of up to 60 min. Furthermore, the generalized inhibition of uptake of sugars (1), phenolic glucosides (1, 8), phenolic aglycones (8), and inorganic ions (8) is more suggestive of a nonspecific permeability effect. Figure 3 illustrates the effect of varying concentrations of vanillic acid upon phosphate uptake in the form of Lineweaver-Burk plots. Almost identical plots have been described for phenolic inhibition of glucose transport in animal small intestine (1), phenolic glucoside transport in barley roots (8), and phenolic aglycone uptake in barley roots (8). Since phenolic

Table I. *The Inhibition of Phosphate Uptake by Various Phenolic Acids*

The incubation medium was buffered at pH 7 and contained 5×10^{-5} M phosphate, 5×10^{-4} M CaCl₂, and 0.05 μc of ³²P-labeled phosphate. The inhibition values are based upon the uptake over a 3-hr period. Compounds were tested at a final concentration of 5×10^{-4} M.

Acid	Inhibition
	%
Benzoic acid	54
o-Hydroxybenzoic acid	85
p-Hydroxybenzoic acid	41
3,4-Dihydroxybenzoic acid	28
3,4,5-Trihydroxybenzoic acid	18
4-Hydroxy-3-methoxybenzoic acid	71
4-Hydroxy-3,5-dimethoxybenzoic acid	38
Cinnamic acid	93
o-Hydroxycinnamic acid	91
p-Hydroxycinnamic acid	80
3,4-Dihydroxycinnamic acid	39
4-Hydroxy-3-methoxycinnamic acid	90
4-Hydroxy-3,5-dimethoxycinnamic acid	67

Table II. *Octanol-Water Partition Coefficient of the Benzoic Acids*

The acids are arranged in order of their increasing inhibitory capacity.

Acid	Inhibition	Partition Coefficient
	%	
3,4,5-Trihydroxybenzoic acid	18	0.83
3,4-Dihydroxybenzoic acid	28	1.2
4-Hydroxy-3,5-dimethoxybenzoic acid	38	1.28
p-Hydroxybenzoic acid	41	1.58
Benzoic acid	54	1.87
4-Hydroxy-3-methoxybenzoic acid	71	1.72
o-Hydroxybenzoic acid	85	2.21

aglycones are considered to penetrate tissues by passive processes (8, 11), the similarity of inhibition characteristics argues against an effect upon membrane carriers.

Studies of the influence of phenols such as DNP and its analogues upon respiration and transport phenomena suggest an independence of action upon the two phenomena (6). Thus concentrations of nitrophenols which caused appreciable reduction of uptake of glucose (6) or phosphate (10) had little or no effect upon respiration. Nevertheless, according to Mitchell's (15) chemiosmotic hypothesis of phosphorylation, uncoupling agents such as DNP are considered to act through alterations in permeability which result in the discharge of electrochemical gradients. It may be that the same generalized permeability effects can account for both transport and respiration reduction. The greater sensitivity of the transport systems would depend upon their exposure to the higher concentrations of the external medium. Within the tissues, phenolics are rapidly conjugated with glucose or other molecules, and in consequence their toxicity is reduced. In studies of the uptake of hydroquinone in barley roots (8), it was found impossible to recover the free phenol despite uptake rates of 0.16 mg hydroquinone/g fresh weight·hr. One hundred per cent of the radioactivity due to absorbed hydroquinone was accountable for by the corresponding glucoside, arbutin. The present experiments involved much lower levels of phenolics and thus it is unlikely that any significant quantities of free phenols accumulated within the tissues.

Harborne and Corner (9) have established that when cinnamic acids are administered to the leaves of higher plants the major products are the corresponding glucose esters. When, in fact, salicylic acid was administered to excised barley roots, the major product extracted from the tissues was the glucose ester.

The third hypothesis, namely that glucosylation of penetrating phenolics poses a drain upon ATP supplies is highly unlikely. Root tissues have been found to be capable of glucosylating phenolics at external concentrations 10 times those involved in the current experiments (8).

The benzoic acids employed in this study, when arranged in order of their increasing inhibitory capacity, form a series which corresponds to the order of their increasing lipid solubility as based upon their octanol-water partition coefficients (2). This is shown in Table II.

This strong correlation between lipid solubility and inhibition of phosphate uptake leads to the last hypothesis, that inhibition is due to the penetration of and alteration of membrane properties. The internal concentration of the applied phenolic compounds is maintained close to zero by the high capacity for

glucosylation demonstrated by root tissue (8). Therefore it seems likely that it is during their passage through the membrane that the effects of these compounds are exerted. Recent studies of the influence of phenolics such as salicylate and other benzoic acids upon membrane potential in animal neurons have shown that such compounds are capable of rapid and reversible alterations in membrane potentials attributed to altered permeability to specific ions (2). The compounds studied in the present experiments probably distribute themselves between the aqueous incubation medium and the lipid phases of the membrane depending upon their partition coefficients. In the case of highly lipid-soluble compounds such as salicylate, the effects upon membrane permeability are sufficiently great to result in rapid loss of previously absorbed phosphate (Fig. 4).

It is remarkable that almost without exception both plants and animals respond to the administering of phenolic aglycones by conjugation of these compounds with highly polar molecules such as glucose (8), glucuronic acid (26), sulfate (26). By contrast, methylation is rare (26). The reduced lipid solubility achieved by these detoxication mechanisms clearly reduces their capacity for disruption of cellular activities through alteration of membrane properties, and provides an explanation for the nature of the detoxifying mechanism.

Considering the ubiquity of phenolic compounds, and, in particular, the high concentrations of phenolic acids which occur in soils (21, 23), it is somewhat difficult to understand why instances of severe metabolic inhibition are not more common. It may be that in nature soil phenolics are adsorbed onto soil particles and not free to penetrate plant tissues. Or perhaps exposure to phenolic compounds over a period of time results in the development of greater tolerance by plants. Wang's studies (22) indicated considerable species differences in sensitivity to the applied phenolics and underlines the potential of phenolic compounds for allelochemical effects. The inhibition of ion uptake provides a possible mechanism for such phenomena.

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