Inhibitory Effects of 2,3,5-Triiodobenzoic Acid on Ion Absorption, Respiration, and Carbon Metabolism in Excised Barley Roots

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ABSTRACT

The ability of 2,3,5-triiodobenzoic acid (TIBA) to alter ion absorption, respiration, carbon metabolism, and the permeability of the cell membranes of excised barley roots has been examined. Roots pretreated in either H2O, KCl, or TIBA followed by treatment in KCl, TIBA, or KCl and TIBA demonstrated that inhibition of ion uptake due to TIBA was reversible. These studies also suggest that ions already accumulated within the vacuole remain sequestered after the addition of TIBA, whereas cytoplasmic ions leak out into the external medium. A 20-minute lag period was present prior to the onset of inhibition of O2 consumption by TIBA. A b-type cytochrome from corn that is apparently associated with the plasmalemma and possibly involved in respiration or ion uptake, or both, was unaffected by TIBA. The addition of TIBA to treatment solutions resulted in the synthesis and accumulation of ethanol. Analysis of organic acids showed that only the malate concentration was affected by treatment with TIBA. A reduction of 26% was noted for malate in the presence of 2 micromolar TIBA. These combined results suggest that the inhibitory action of TIBA in barley roots involves an alteration of mitochondrial respiration and not a direct depolarization of the plasmalemma.

MATERIALS AND METHODS
Barley seeds, Hordeum vulgare L. cv. Briggs, were grown as described previously by Jacobson and Jacobson (17). When seedlings were 6 days old, the roots were excised, cut into sections approximately 2 cm long, washed, and centrifuged at 65g for 5 min.

The experimental procedure for the treatment of barley roots was described previously (17). Briefly, 1-g sections of root were incubated in 3.5 liters aerated treatment solutions consisting of CaSO4 (1 mM) alone or CaSO4 with KCl (5 mM), TIBA, or KCl and TIBA combined. For reasons previously discussed (17), the pH of the treatment solution was 4 and the temperature was 25°C. At the conclusion of the incubation period, roots were separated from solutions by a fine nylon mesh filter and washed.

Inorganic ions were extracted from the roots by treatment with HNO3. The acid extracts were filtered, made up to volume, and analyzed for K by flame photometry and for Cl by a coulometric-amperometric procedure.

In determining ethanol content, 20 g roots were treated as described above for 4 h. Following this treatment, washed roots were placed in Erlenmeyer flasks, frozen with liquid N2, and stored in a freezer for later analysis. Aliquots of the treatment solutions also were obtained and stored in a refrigerator. After removing the roots from the freezer, 20 ml distilled H2O warmed to 80°C was added to the flask and gently heated on a steam bath. Ethanol concentration was determined by measuring the reduction of NAD at 340 nm (2) when ethanol is oxidized to acetaldehyde in the presence of alcohol dehydrogenase (EC 1.1.1.1, Sigma). To ensure that ethanol was not volatilized, aliquots were analyzed by this method and recoveries in excess of 99% were obtained. Ethanol prepared by the method of Horwitz (14) was used as a standard.

The determination of organic acids was accomplished by extraction and subsequent fractionation. After 2 h treatment, 15 g treated roots were frozen in liquid N2, lyophilized, and ground in a Wiley mill (40 mesh). The extraction procedure was similar to that described by Pucher et al. (29). The ground root material (0.7 g) was mixed with 4 ml 4 N H2SO4 and 5 g asbestos and quantitatively transferred to an extraction thimble. The thimble was placed in a Soxhlet extraction apparatus containing anhydrous ethyl ether. After an 18- to 24-h extraction period, 5 ml H2O was added to the flask and the ether evaporated. The organic acid-containing aqueous phase was passed through Whatman No. 1 filter paper, made basic by titration with 0.1 N NaOH using phenol red indicator (1% w/v), and reduced to near dryness on a steam bath. Final drying was accomplished in vacuo over P2O5. When dry, the organic fraction was redissolved in 0.5 ml of the appropriate H2SO4 concentration to reacidify the organic acids.

The extracted organic acids were partitioned by silica gel column chromatography (6) and the resulting fractions were quantified by titration with 0.0100 N NaOH. Elution was accomplished by step-wise increases of 1-butanol in chloroform. The combined

A large number of compounds that inhibit aerobic respiration or oxidative phosphorylation also inhibit ion uptake in barley roots (17, 24, 28). The addition of respiratory substrates, such as succinate, overcomes the inhibition caused by these compounds. In corn roots, Lauchli and Epstein (21) demonstrated that the uncoupler carbonyl cyanide m-chlorophenylhydrazone and the inhibitor of ATP synthesis, oligomycin, reduced the absorption and translocation of CI. Similar results were obtained for K (23). These papers support the hypothesis that a causal relationship exists between aerobic respiration and ion uptake.

The effects of the auxin antagonist TIBA on aerobic respiration appear somewhat variable. In sunflower stem sections, TIBA had no effect on O2 consumption (27), whereas, in cucumber mitochondria, it reduced respiration (32). Previously, we reported (17) that TIBA inhibited the absorption of K and CI and that this inhibition appeared to be mediated through a reduction in aerobic respiration. Here, we present further studies of the effects of TIBA on respiration, ion absorption, carbon metabolism, and membrane permeability.

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2 Abbreviation: TIBA, 2,3,5-triiodobenzoic acid.
fractions eluted with the highest 1-butanol concentration (35%) were dried, reacidified, and repartitioned in 35% 1-butanol-chloroform solvent on a freshly prepared column. This improved recovery and peak resolution.

Further quantification of malate was performed by the method of Gutmann and Wahlfield (11) with the following modifications in the buffer solution: (a) 2 m glycine, (b) 0.8 m hydrazine sulfate, (c) Na2EDTA was added at 11 m, and (d) equal portions of this mixture and 2.0 m NaOH were combined. The assay involves measuring the reduction of NAD in the presence of malate dehydrogenase (EC 1.1.1.37, Calbiochem).

Measurement of O2 consumption by excised roots was performed as described previously (17).

The corn membrane Cyt studies were performed by the method of Goldsmith et al. (8).

RESULTS

Respiration Time Course. The consumption of O2 by excised barley roots in the presence or absence of TIBA for time intervals of up to 120 min was determined (Fig. 1). These solutions contained KCl and CaSO4 and were adjusted to pH 4. O2 consumption in the presence or absence of TIBA was essentially the same for the first 20 min. After 20 min, roots incubated without TIBA continued to respire at a constant rate (slope = 0.25), whereas those treated with TIBA show a decreased rate of respiration (slope = 0.18). These results correlate with those previously published (17) concerning the effects of TIBA on K and Cl absorption.

Pretreatment Studies. Excised barley roots were pretreated in solutions adjusted to pH 4 and containing either CaSO4 (1 mm) alone or CaSO4, with KCl (5 mm) or TIBA (1 μm). After a 2-h pretreatment, roots were washed and transferred to various solutions (pH 4) for a 2-h treatment period. All treatment solutions contained CaSO4 (1 mm). Examination of Table I shows that there was no significant difference in ion absorption between roots aged for 2 h in H2O followed by a KCl treatment (IV) and tissues not aged (III).

Absorption of K and Cl by roots pretreated in TIBA and subsequently transferred to KCl treatment solutions (Table I, V) demonstrated about 78% recovery of the inhibition caused by TIBA when compared to the KCl control (Table I, IV). Pretreatments in TIBA followed by a treatment in KCl and TIBA (Table I, VI) yielded an uptake value not as great as that obtained by treatment in KCl alone (Table I, V). The inhibition due to TIBA appeared to be reversible. When compared to the KCl control (Table I, IV), pretreatment in KCl followed by a 2-h treatment period in TIBA (Table I, VII) resulted in a loss of only 11% of the accumulated K or Cl. This suggests the presence of an effective mechanism of sequestering ions. The data also suggest that TIBA may not affect the permeability of the tonoplast in a manner similar to that of the plasmalemma.

Membrane Cytochrome Studies. A membrane fraction from corn coleoptiles that appears to be plasmalemma in nature has been isolated, and a b-type Cyt from it has been partially characterized (19). This Cyt is known to undergo reduction in the presence of riboflavin and blue light (8) or methylene blue and red light (5). Since this Cyt might be involved in the phenomenon of either ion uptake or respiration, or both, its response to TIBA was determined. TIBA was supplied to a similar corn membrane fraction to determine whether it could interact in any way with the light-sensitive electron transport pathway of the above Cyt. TIBA has no discernible effect on the system (Fig. 2). In darkness, following irradiation, the Cyt loses its electron and becomes oxidized, whereas subsequent actinic irradiation causes the Cyt to become reduced again. On its return to darkness, the Cyt reoxidizes slowly to the dark level. If TIBA interacted with the Cyt to alter the flow of electrons in the plasmalemma, a change either in the extent of photoreduction or the kinetics of dark decay, or both, should be observed. The data demonstrated that TIBA did not influence the oxidation-reduction of this Cyt under the conditions used.

Ethanol Studies. Since TIBA was effective in decreasing aerobic respiration (Fig. 1), a study was initiated to determine the site of TIBA inhibition. As a first approximation, the production of ethanol by excised barley roots, as a result of TIBA inhibition was measured. An increase in ethanol suggests that TIBA somehow affects Krebs cycle metabolism and requires the subsequent oxidation of the terminal products of glycolysis by an anaerobic mechanism. No increase in ethanol would imply that TIBA affected some site in glycolysis. Preliminary experiments were performed to determine if the root segments used here would produce and accumulate ethanol. Roots exposed to a nitrogen environment produced 140 μg ethanol/g root tissue but essentially none under an aerobic environment (Table II). The negative values in the initial and air-treated samples were due to a slight decrease in absorption at 340 nm caused by the sample.

![Fig. 1. The time course of O2 consumption (μmol g⁻¹) by excised barley roots in the presence (○) and absence (□) of TIBA. Concentrations were: KCl, 5 mm; CaSO4, 1 mm; TIBA, 1 μm; the pH value was 4. Values are expressed as means ± SD.](image)

Table I. K and Cl Absorption in Response to Various Pretreatment and Treatment Regimes

<table>
<thead>
<tr>
<th>Pretreatment Solution</th>
<th>Treatment Solution</th>
<th>Absorption</th>
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<tr>
<td></td>
<td></td>
<td>K</td>
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<td></td>
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<td>Cl</td>
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<tr>
<td>I H2O</td>
<td></td>
<td>-2.10 ± 0.53</td>
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<tr>
<td>II TIBA</td>
<td></td>
<td>-2.43 ± 0.22</td>
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<tr>
<td>III KCl</td>
<td></td>
<td>17.41 ± 0.94</td>
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<tr>
<td>IV H2O® KCl</td>
<td>KCl</td>
<td>16.98 ± 0.88</td>
</tr>
<tr>
<td>V TIBA KCl</td>
<td></td>
<td>13.26 ± 0.50</td>
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<tr>
<td>VI TIBA KCl + TIBA</td>
<td></td>
<td>9.43 ± 0.27</td>
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<tr>
<td>VII KCl TIBA</td>
<td></td>
<td>15.17 ± 1.11</td>
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<tr>
<td>VIII KCl</td>
<td></td>
<td>32.47 ± 1.67</td>
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<tr>
<td>IX KCl + KCl + TIBA</td>
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<td>24.23 ± 1.21</td>
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*a* Control containing only 1 m CaSO4.

*b* KCl control.
malate was the only acid to show a significant response. As a result, it was further quantified by measuring the reduction of NAD via malate dehydrogenase. The values presented in Table III for malate were determined by this method. Because of the large concentrations involved, the change in malate was probably vacuolar in nature and reflective of changes in ionic content. Treatments with TIBA resulted in malate values less than the initial concentration and suggested that TIBA promoted its decarboxylation. The changes observed in fumaric acid concentration probably resulted from its conversion to malic acid catalyzed by the reversible enzyme, fumarase.

Examination of the K and Cl content of these roots (Table III) showed that Cl absorption was greater than K. Additionally, the total measured anion content, inorganic and organic, was greater than the cation content. Because electrostatic neutrality must be maintained, ionic changes other than those observed here must have occurred within the root.

**DISCUSSION**

The data presented support the earlier assumption (17) that, in excised barley roots, the benzoate derivative TIBA inhibits cellular processes by altering respiratory metabolism and not by directly antagonizing the plasma membrane. The accumulation of ethanol in roots treated with TIBA suggested that entry into the Krebs cycle or some biochemical reaction within it was being blocked. The only Krebs cycle acid to show any significant decrease in response to TIBA was malic acid. The small decrease observed in fumarate was probably a result of the equilibrium that exists between these two acids ($K_{eq} = 4.4$). Because of its cyclic nature, if entry into the Krebs cycle were inhibited, probably no decrease in constituent concentration would occur, but a reduction in the turnover rate of the acids might occur. It seemed that TIBA may have inhibited metabolite entry into the Krebs cycle possibly by altering the physical or chemical nature of the mitochondrial membrane(s). Pyruvate, the precursor to the cycle, showed no change in concentration when treated with TIBA, but this would be expected since it can undergo further reduction to lactate and/or decarboxylation to ethanol. The study presented here showed that TIBA-treated roots accumulated ethanol (Table II), which allowed for the reoxidation of NADH and continuation of glycolysis. Bourke et al. (3) have shown that TIBA altered glucose

When air was bubbled through solutions containing CaSO$_4$ (1 mN) and KCl (5 mN), the addition of 5 µM TIBA caused a marked production of ethanol (Table II). When concentrations as low as 1 µM TIBA were used (data not shown), similar, although smaller, amounts of ethanol were produced. TIBA concentrations greater than 5 µM resulted in further, although not proportionately greater, increases in ethanol production. Further evidence that TIBA affects Krebs cycle metabolism and not glycolysis was secured from solutions containing both N$_2$ and TIBA. The ethanol concentration in this case was practically equal to solutions containing both air and TIBA.

**Organic Acid Analysis.** Having demonstrated that glycolysis was not affected by TIBA, the investigation for the metabolic site of TIBA inhibition centered on measuring the concentration of organic acids in barley roots. These results, presented in Table III, had deviations from the mean of several experiments of less than 5% and, in most cases, 1 to 2%. With the addition of 2 µM TIBA,
metabolism by increasing its catabolism while having no effect on anaabolism. Thus, TIBA seems to induce conditions which biochemically resemble anaerobiosis.

A number of earlier studies have shown malate to be the predominant acid in barley roots (18), snap-bean roots (15), and onion and soybean roots (9). Similar results were obtained here. During KCl treatment and in the absence of TIBA, the malate concentration increased by 32% over the initial concentration, whereas, in the presence of TIBA, it decreased by 26%. This change in malate concentration was probably not the result of the normal function of the Krebs cycle. Because this is a cycle, malate must serve as the product of one reaction and the substrate for the following reaction. Hart (13) and Jacobson (16) have reviewed a number of possible enzymic reactions leading to the synthesis of malate. These enzymic reactions have been characterized in a number of higher plants (33), including excised barley roots (10, 13). Lips and Beevers (22) demonstrated that, in corn roots, compartmentalization exists in the form of mitochondrial and extramitochondrial pools of malate and that these pools have different biosynthetic origins.

It has been suggested that, in barley roots, organic acids aid in maintaining electrostatic neutrality during ion uptake (18). Jacobson (18) has shown that the acetate found in barley roots appears to be in a bound and unusable form for electrostatic balancing. In corn roots, labeled acetate was shown to be a precursor for mitochondrial and extramitochondrial malate (22). Our results (Table III) appear to support the idea that acetate was not involved since treatment with TIBA did not affect its concentration. The role of acetate in this material remains an enigma.

The O₂ time curve in Figure 1 closely resembles the time curve for K and Cl absorption previously published (17). Both processes have similar kinetics and lag times of 20 to 30 min. The close parallelism observed for these two physiological processes suggests a cause and effect relationship between the inhibition of aerobic respiration and ion uptake. The results of this experiment as well as the ethanol and organic acid data agree with the hypotheses previously suggested (17) that TIBA can penetrate into and be transported across the plasmalemma entering the cytoplasm where it may interact with cellular metabolism. In contrast, Glass and Dunlop (7) suggest that the inhibition of ion uptake by a number of hydroxyl and methyl benzoates may be due to the depolarization of the plasmalemma by these compounds.

Had the inhibition of ion uptake caused by the addition of TIBA been the result of depolarization of the plasmalemma, then an explanation must exist for the respiration data described here and previously (17). A b-type Cyt associated with a plasmalemma fraction has been reported in Neurospora mycelium (4), Dicyostelium (26), and corn seedlings (19). If this Cyt were capable of interacting and participating in the reduction of O₂, then a blocking of the electron flow by TIBA would account for the depolarization and inhibition of aerobic respiration. TIBA did not interact with the plasmalemma cytochrome (Fig. 2). Thus, it seems that the reduction in aerobic respiration was probably cytoplasmic in origin. The O₂ consumption attributable to such a Cyt system probably involves a minute and possibly undetectable amount of O₂ consumption in barley roots when compared to that associated with the mitochondria and to the degree of respiratory inhibition by TIBA.

The data from Table I suggest that TIBA did not affect the permeability of the tonoplast in a similar manner as the plasmalemma. Previously, it was shown that exposing the cell to TIBA without KCl resulted in an efflux of absorbed ions (16). Although lacking a compartmental analysis, the data presented here similarly suggest that an efflux of cytoplasmic ions occurred and that those ions accumulated within the vacuole remained sequestered. The sequestering of inorganic ions and organic acid anions within the vacuole has been previously suggested (18). The mechanism by which vacuoles of higher plants accumulate inorganic and organic compounds is unclear, but possibly it involves a tonoplast-bound permease that is not linked directly to metabolism (25).

The reversibility of the TIBA-induced inhibition of ion uptake is shown in the experiments where roots are pretreated in TIBA and subsequently transferred to KCl (Table I). This reversibility may be due to the metabolism of TIBA to less toxic forms. In soybean (31) and rats (1) TIBA is metabolized to the diiodobenzoic acids and to unidentified polar metabolites. Haborne and Corner (12) found that, when cinnamic acids were administered to leaves of several higher plants, the major products recovered were the corresponding glucose esters. Halide-containing compounds, such as the herbicides atrazine (2-chloro-4-ethylamino-6-isopropylamino-S-triazine) and propachlor (2-chloro-N-isopropylacetanilide), when applied to corn, barley, sorghum, and sugarcane leaves, appeared to undergo a nucleophilic chloride displacement with reduced glutathione to form the detoxified glutathione-herbicide conjugate (20, 30). A similar reaction may occur between the iodine component of TIBA and glutathione. Another possibility for detoxification would be compartmentalization of TIBA within the plant, with the vacuole as a possible compartment.

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