Vein Loading in Seedlings of *Phaseolus vulgaris* Exposed to Excess Cobalt, Nickel, and Zinc

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**ABSTRACT**

Vein loading in unifoliate leaves of white bean seedlings exposed to excess Co²⁺, Ni²⁺, or Zn²⁺ for 1 to 4 days was studied by incubating leaf discs from the center of incubated 14-millimeter discs. Uptake of radiosucrose was greater particularly in discs from seedlings exposed to excess Ni²⁺ and Zn²⁺. The effect increased as exposure of the seedlings to metal increased up to 4 days. Autoradiographs showed vein loading of control leaf tissues with most of the radiosucrose accumulating in minor veins and little remaining in the mesophyll. In discs from metal-treated plants, most of the sucrose remained in the mesophyll without accumulating preferentially in the minor veins. This effect was evident within 24 hours of exposure to excess metal and intensified with longer exposures to metal. The inhibition of vein loading was also evident in *vitro*. Both the preferential accumulation of sucrose into the minor veins of control tissues and the accumulation into mesophyll of metal exposed tissues were sensitive to 2,4-dinitrophenol and the blockage of sulfhydryl groups. It is concluded that the inhibition of vein loading contributes markedly to the observed toxicological effects of reduced photosynthetic export and of accumulation of carbohydrates in fully expanded leaves of bean seedlings exposed to excess metal ions.

Reductions in growth and yield are commonly recorded symptoms of metal toxicity in plants. We have reported that exposure of white bean seedlings to excess Co²⁺, Ni²⁺, or Zn²⁺ inhibited the export of photosynthates from unifoliate leaves and that these leaves accumulated increased amounts of sucrose, reducing sugars, and starch (12, 13). This suggested that a primary effect of excess metal in causing growth reduction is to inhibit the export of assimilates from source regions to sink regions. Increased deposits of callose were evident on sieve plates of phloem in plants treated with metal, but no correlation was found between increased callose in petioles and the reduced translocation of photosynthates through them (11).

Sucrose is the major translocated sugar in *Phaseolus vulgaris* (5, 10). Accumulation of this carbohydrate in source leaves of metal-treated seedlings indicates either an entry of sucrose into a storage pool making it unavailable for translocation, or inhibition of loading of sucrose into the phloem of minor veins (6). Phloem loading, or vein loading, is the process by which the major translocated substances are selectively and actively delivered to the sieve tubes prior to translocation from the source region (6).

The aim of our work was to evaluate the capacity of leaf discs from bean seedlings exposed to excess Co²⁺, Ni²⁺, or Zn²⁺ to show vein loading of sucrose. We also studied the sensitivity of the uptake of radiosucrose to two known inhibitors of phloem loading.

**MATERIALS AND METHODS**

**Plant Material.** White bean seedlings (*Phaseolus vulgaris* L., var. Kentwood) were grown in hydroponic culture in a growth room (12, 13). Ten days after seeding the plants were exposed to 400 mM Co²⁺, 200 mM Ni²⁺, or 200 mM Zn²⁺ as the sulfate salts in fresh half-strength Hoagland solution (pH 5.0). These concentrations of metals cause the rapid development of visible toxicity symptoms (12). Considerable, albeit altered, metabolism must still be occurring under these circumstances since unifoliate leaves accumulate carbohydrates (13).

**Collection of Leaf Discs.** The upper surface of the basal half of unifoliate leaves was abraded gently with 320-grit carborundum which was then rinsed off with 5 mM K-phosphate (pH 6.5). One disc 14 mm in diameter was cut from each leaf half in the abraded areas between the midvein and the major lateral veins. The discs were floated with their abraded side down on phosphate buffer until used.

**Uptake of [¹⁴C]Sucrose.** The assay for uptake of radiosucrose was modeled on the work of others (8, 9, 14). Groups of eight 14-mm leaf discs were taken from the phosphate buffer, blotted, and placed abraded side down on 2 ml incubation medium in flat bottomed 50-mm i.d. glass dishes. The incubation medium contained 20 or 50 mM [U-¹⁴C]sucrose in 5 mM K-phosphate (pH 6.5) and at a specific radioactivity of 1 μCi/μmol. After a 30-min uptake period, the eight discs were washed twice in 40–45 ml of 1 mM CaCl₂ in Petri dishes. The 14-mm discs were then blotted and an inner 7-mm disc was cut from each. This was done because radiosucrose differentially infiltrated the edges of the leaf discs.

**Radioassay of Leaf Discs.** After washing, individual 7-mm discs were digested with 0.5 ml Protosol solubilizer and 0.25 ml of 30% (v/v) H₂O₂ at 55–60 °C in scintillation vials. Ten ml of Aquasol scintillation fluid were then added. Radioactivity was determined by a liquid scintillation spectrometer.

**Autoradiography of Leaf Discs.** Incubated and washed 7-mm discs or the outer rings from 14-mm discs were frozen and lyophilized. The dried tissues were compressed at 165 MPa (24,000 p.s.i.) pressure which reduced the leaf thickness from a typical 240 to 40 μm. Individual discs or rings were glued onto 2-cm squares of herbarium paper with rubber cement. The mounted tissues were then counted in a planchet counter. The discs and rings were exposed to x-ray film for enough days to accumulate 21–25 × 10⁶ counts (as per the glass flow counter). Films from any one experiment were developed at the same time.

**Analysis of Autoradiographs.** X-ray film images were projected onto color reversal paper with an enlarger with the filtration corrected to make the background white and the subject as black as possible. Some autoradiographs were scanned across one diameter with a microdensitometer. Mean peak height was deter-

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mined for each trace by measuring individual peak heights from the nearest trough. Total area under the trace was measured with a planimeter. An index was calculated by dividing the mean peak height in mm by the surface area under the trace in cm².

Uptake of [14C]Sucrose in Situ. Unifoliate leaves were abraded as for collection of leaf discs. After washing the leaf, a 3-mm-wide ring of 8-mm i.d. Tygon tubing was attached to the upper leaf surface in an area from which a leaf disc would be cut. The ring was sealed onto the leaf with silicone grease. One hundred µl of radioisocrose incubation medium were inserted in the well. After 30 min incubation, the medium was aspirated off and the well was washed with some 1 mM CaCl₂. A 7-mm disc was cut from the center of the well to be washed for 20 min in two changes of 1 mM CaCl₂ prior to freezing and autoradiography.

Inhibitor Studies. The effect of 4 mM DNP₂ on sucrose uptake was determined by incorporating the drug in the radioisoscrose incubation medium. Other discs were floated on 2 mM PCMBS dissolved in 20 mM K-phosphate (pH 7.0). After 15 min the discs were washed for a total of 15 min in three changes of the phosphate buffer. Some of the discs treated with PCMBS were then exposed for 10 min to 20 mM DTE dissolved in 20 mM K-phosphate (pH 7.0). Three washes in phosphate buffer over 15 min followed. The two types of pretreated discs were then processed through the standard assay for [14C]sucrose uptake.

Endogenous Contents of Sucrose in Leaf Discs. Two 7-mm leaf discs were cut from unabraded unifoliate leaves. One disc originated from the minor vein area normally used for radioisoscrose uptake, the other was centered over the midrib in the lower third of the lamina. Sucrose was determined on alcohol extracts of the weighed discs and the remaining leaf lamina by reducing sugar analysis before and after invertase digestion (13).

RESULTS

Uptake of Radioisoscrose. The margins of leaf discs became infiltrated with radioisoscrose which was not removed efficiently even with extensive washing of the discs in 1 mM CaCl₂. In the assays, tissue infiltration was eliminated by incubating 14-mm leaf discs, washing them, and then cutting an inner 7-mm disc for analysis (Fig. 1, A, B, C, and D). Analyses of different parts of leaf discs (Table I) indicated that leaves of metal-treated seedlings had more sucrose in the minor vein areas, precisely those tissues used for [14C]sucrose uptake estimations. Since specific radioactivities were not uniform among treatments, sucrose uptake is reported as dpm per disc for 30-min incubations.

Sucrose uptake by leaf discs from seedlings exposed to excess metal was greater than in control tissues, either in 20 mM sucrose (Table II) or in 50 mM sucrose (data not shown). The effect was evident on exposing seedlings to excess Ni²⁺ or Zn²⁺ for 1 day, to Co²⁺ for 2 days, and continued on to the 4th day.

AUTORADIOGRAPHS OF LEAF DISCS. Autoradiographs of discs from
opposite sides of the same leaves as reported in Table II are given in Figure 2. Vein loading of sucrose is demonstrated by a preferential accumulation of radioactivity in the minor veins with little radioactivity present in the mesophyll (6). On this basis leaf discs from control white bean seedlings showed definite vein loading in all cases (Fig. 2, A, E, I, and M). The discs from bean seedlings exposed for 1 day to excess Co²⁺, Ni²⁺ or Zn²⁺ showed reduced vein loading (Fig. 2, B, C, and D). In these discs, radioactivity accumulated in the minor veins but the mesophyll appeared darker than in control discs (Fig. 2A). Vein loading was inhibited markedly in all plants exposed to excess Co²⁺, Ni²⁺, or Zn²⁺ for 2–4 days (Fig. 2, F, G, H, J, K, L, N, O, and P).

Analysis of Autoradiographs by Microdensitometry. Microdensitometry was used for an objective analysis of the autoradiographs. Densitometer traces made from left to right across the middle of the discs shown in Figure 2, A, B, C, and D are depicted below each disc. One transect was made at random across a diameter of a disc. The peaks are equivalent to the veins encountered in the transect while the area under the trace is equivalent to the radioactivity accumulated in exposing the x-ray film. Definitive vein loading (Fig. 2A) was associated with a densitometer trace having tall peaks. For leaf discs which were judged to have reduced vein loading, the densitometer traces showed shorter peaks with troughs higher in the traces than in control discs.

From Table III it is evident that essentially the same number of veins were encountered in the discs from the various treatments. The areas under the curves were collectively significantly larger for the discs from metal exposed seedlings than from controls. This consistent difference in the apparent amount of radioactivity producing the image is attributed to the highly preferential localization of radiosucrose in the veins of control leaf discs. Although all x-ray films were exposed until accumulation of 21–25 × 10⁶ counts per disc, those regions with higher radioactivity could have saturated the x-ray film locally, thus preventing any further exposure of the film there.

The mean peak height for any densitometer trace divided by the area encompassed by the trace gave the index representing the amount of vein loading. Whereas Figure 2 illustrates only one disc from each treatment, the mean index of vein loading encompasses all eight replicate discs within any one treatment (Table IV). The indices of discs from seedlings exposed to excess Co²⁺, Ni²⁺, or Zn²⁺ were significantly lower than those of controls. Inhibition of vein loading was evident after the seedlings had been exposed to excess metal for 24 h and the effect was increased by 2 days exposure to metal ions.

Inhibition of vein loading similar to that found with excised leaf discs was evident when radiosucrose was supplied to abraded leaves for incubations in situ. Seedlings grown with excess metal ions for 2 days had mean indices (± SE) of 0.05 ± 0.01, 0.11 ± 0.02, and 0.10 ± 0.02 for Co²⁺, Ni²⁺, and Zn²⁺, respectively. Control plants had mean indices of 0.38 ± 0.02.

Effects of Inhibitors on Sucrose Uptake. The uptake of sucrose by control discs preferentially into minor veins was inhibited by both DNP and PCMBs (Table V). Leaf discs from seedlings exposed to excess metal for 3 days also showed inhibitions of sucrose uptake by DNP and PCMBs. These inhibitions were

Table III. Analysis of Autoradiographs by Microdensitometry

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Peaks</th>
<th>Relative Area of Leaf Discs cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22 ± 0.6</td>
<td>23.0 ± 0.1</td>
</tr>
<tr>
<td>Cobalt</td>
<td>24 ± 0.8</td>
<td>73.7 ± 2.7**</td>
</tr>
<tr>
<td>Nickel</td>
<td>25 ± 0.8</td>
<td>79.7 ± 1.7**</td>
</tr>
<tr>
<td>Zinc</td>
<td>22 ± 0.9</td>
<td>80.0 ± 3.1**</td>
</tr>
<tr>
<td>Day 2</td>
<td>Control</td>
<td>20 ± 0.9</td>
</tr>
<tr>
<td>Cobalt</td>
<td>22 ± 0.8</td>
<td>82.3 ± 1.9**</td>
</tr>
<tr>
<td>Nickel</td>
<td>19 ± 1.3</td>
<td>81.0 ± 2.3**</td>
</tr>
<tr>
<td>Zinc</td>
<td>21 ± 1.3</td>
<td>75.7 ± 2.5</td>
</tr>
</tbody>
</table>

** Indicates increased relative area over control of day at 0.01 level of probability.

Table IV. Indices of Vein Loading in Bean Seedlings Exposed to Excess Metal

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1</th>
<th>Day 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.39 ± 0.03</td>
<td>0.37 ± 0.03</td>
</tr>
<tr>
<td>Cobalt</td>
<td>0.23 ± 0.02**</td>
<td>0.08 ± 0.01**</td>
</tr>
<tr>
<td>Nickel</td>
<td>0.14 ± 0.01**</td>
<td>0.08 ± 0.01**</td>
</tr>
<tr>
<td>Zinc</td>
<td>0.17 ± 0.02**</td>
<td>0.11 ± 0.01**</td>
</tr>
</tbody>
</table>

** Indicates decreased indices of vein loading over control of day at 0.01 level of probability.
Fig. 2. Autoradiographs of leaf discs from seedlings exposed to excess metals. Leaf discs were collected from unifoliate leaves of control seedlings and seedlings exposed to 400 μM Co²⁺, 200 μM Ni²⁺, or 200 μM Zn²⁺ for 1–4 days. These autoradiographs are for discs from the opposite halves of the leaves used for radiosucrose uptake estimates (Table II). Fourteen-mm discs were incubated in 20 mM radiosucrose for 30 min. After washing in 1 mM CaCl₂, an inner 7-mm disc was cut from each incubated disc and used to prepare the autoradiographs. Microdensitometer traces of discs A, B, C, and D are depicted in the second row of panels. Number accompanying each densitometer trace is the index of vein loading for that disc.
Table V. Influence of Metabolic Inhibitors on Sucrose Uptake by Leaf Discs of Bean Seedlings Exposed to Excess Metal

Ten-day-old seedlings were exposed to 400 µM Co++, 200 µM Ni++, or 200 µM Zn++ for 3 days. Standard assay was used to incubate 14-mm discs in 20 mm radio-sucrose for 30 min. One plant provided one replicate of four discs required for inhibitor treatment. The 4 mm DNP was included in incubation medium. Leaf discs were floated on 2 mm PCMBS in buffer for 15 min and washed for 15 min in buffer. For PCMBS reversal, discs were floated on 20 mm DTE for 10 min followed by washing for 15 min with buffer. Sucrose uptake as a percentage of uptake in the absence of inhibitors is given in brackets. Mean ± SE of 8 replicates.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No Inhibitors</th>
<th>+DNP</th>
<th>+PCMBS</th>
<th>+PCMBS + DTE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm per disc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5,854 ± 914**</td>
<td>1,366 ± 88**</td>
<td>2,216 ± 259**</td>
<td>5,153 ± 761**</td>
</tr>
<tr>
<td>Cobalt</td>
<td>9,228 ± 814*</td>
<td>2,089 ± 101**</td>
<td>3,134 ± 112**</td>
<td>10,188 ± 543**</td>
</tr>
<tr>
<td>Nickel</td>
<td>6,515 ± 960</td>
<td>2,229 ± 280**</td>
<td>3,016 ± 186**</td>
<td>6,586 ± 756**</td>
</tr>
<tr>
<td>Zinc</td>
<td>13,993 ± 466**</td>
<td>1,544 ± 88**</td>
<td>3,154 ± 125**</td>
<td>12,912 ± 1,220**</td>
</tr>
</tbody>
</table>

* Indicates significant stimulation of sucrose uptake over controls in the absence of inhibitors at the 0.01 level of probability.
** Indicates inhibition of sucrose uptake by exposure to inhibitor at the 0.01 level of probability.

Table V shows that the uptake of sucrose by leaf discs from control white bean seedlings was comparable to that of inhibitors, with values similar to the control, indicating that the inhibitors did not significantly affect the sucrose uptake. However, in the presence of cobalt and nickel, the sucrose uptake was significantly reduced, indicating that these metals may inhibit the transport of sucrose across the leaf discs.

**DISCUSSION**

The rate of [$^{14}$C]sucrose uptake by leaf discs from control white bean seedlings varied between experiments. The rates were generally comparable to those reported for sugar beet (8, 9, 14), but lower than those of tobacco leaf discs (1). The leaf discs from seedlings exposed to excess Co++, Ni++, or Zn++ all had sucrose uptakes equal to or greater than those of controls. The increased uptake was evident in incubations with 20 mm sucrose (Tables II and V) and 50 mm sucrose. The variations in radiosucrose uptake necessitated estimating the radioactivity in individual discs prior to exposure of x-ray films. The enhanced uptake of sucrose by discs from metal treated seedlings was as sensitive to DNP and PCMBS as that of control discs (Table V).

Definitive vein loading was shown in all cases by leaf discs from control white bean seedlings (Fig. 2, A, E, I, and M). Exposure of white bean seedlings to excess Co++, Ni++, or Zn++ for 1 day reduced sucrose vein loading (Fig. 2, B, C, and D). Exposures of seedlings to excess metal for 2–4 days markedly inhibited vein loading in all cases (Fig. 2 and Table IV). This inhibition was also evident when radiosucrose was supplied to leaves in situ.

Analysis of autoradiographs by microdensitometry indicated that about the same number of veins and similar total silver grain densities were being considered in the transects across the discs (Table III). The autoradiographs, the sample microdensitometer traces, and the indices of vein loading (Fig. 2 and Table IV) indicate that the radioactivity present in leaf discs from metal-exposed seedlings was primarily located in mesophyll tissues to the detriment of preferential accumulation in minor veins. The partitioning of radiosucrose remains the same despite the total amount of radioactivity in the leaf discs. Consequently, reduced vein loading in leaf discs from metal-exposed seedlings is consistent with the demonstrated increased uptake of sucrose by these same disc as compared to controls (Table II).

Increasing concentrations of sucrose in the incubating medium reduce the intensity of radioactivity in the veins and cause greater accumulation in mesophyll cells (2, 6). This does not explain the inhibition of vein loading described here for discs from metal treated plants. First, the highest endogenous sucrose concentrations found in typical minor vein discs (about 50 mm, Table I) are lower than the 200 mm external sucrose solutions used by Geiger and co-workers (2, 6). Second, the autoradiographs for leaf discs incubated in 200 mm sucrose (2, 6) still exhibit much more vein loading than discs of bean seedlings treated with excess Co++, Ni++, or Zn++ for 1 or 2 days and incubated in 20 mm sucrose for 30 min (Fig. 2).

Exposing leaf discs to DNP or PCMBS inhibited the preferential sucrose uptake into minor veins in control tissues and also the uptake by mesophyll cells of unfoliate leaves from metal-exposed seedlings (Table V). The inhibition by PCMBS was totally eliminated by supplying the sulphydryl-protecting compound DTE. The effects of DNP, PCMBS, and DTE observed for white bean leaf discs are comparable to those reported for sugar beet leaf discs (8, 9, 14). Our results do not differentiate between the vein loading of sucrose from the apoplast or that from the symplast (6). In the case of leaf discs from metal-exposed seedlings vein loading is drastically inhibited, yet sucrose uptake by the discs into mesophyll is as sensitive to metabolic inhibitors as in control tissues where the uptake is preferentially into minor veins. Since both types of accumulations are equally sensitive to inhibition, we suggest that caution be taken in ascribing the effect of inhibitors in normal tissues exclusively on actions in the sieve element-companion cell complex or its membranes.

The nature of the inhibition of vein loading in unfoliate leaves of white bean seedlings exposed to excess Co++, Ni++, or Zn++ is unknown. Further experiments are required to establish the role of essential metal ions acting directly in situ or indirectly in eliciting compounds, such as phenolics (12), which might inhibit vein loading. Since phloem loading is an energy-dependent, carrier-mediated process (3, 9, 14), ATP may be limiting in the leaf discs from seedlings exposed to excess metal ions. For this alternative to apply the sieve element-companion cell complex must be affected specifically, unless the mechanism for sucrose phloem loading differs from that of sucrose uptake by mesophyll cells.

The reduction in yield of plants exposed to excess metals (4), particularly of roots (12), may in large part be accounted for by inhibition of vein loading, thus leading to reduced phloem translocation (13) which over time would cause decreased mass transfer of photosynthate to the sink regions. The agronomic significance of alterations in phloem loading was envisaged by Geiger and Sovonick (7) in their quest for more data on the effect of pollutants on carbohydrate translocation. We suggest that the inhibition of vein loading documented here is an example of an "essential to life" process (4) which has failed due to the toxicity of excess metal ions.

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**LITERATURE CITED**