Reevaluation of Anaerobic Nitrite Production as an Index for the Measurement of Metabolic Pool of Nitrate

Received for publication October 27, 1980 and in revised form February 23, 1981

MUHAMMAD ASLAM
Crop Science Section, Agriculture Canada Research Station, Harrow, Ontario NOR 1G0 Canada

ABSTRACT

The use of anaerobic nitrite production as an index for the measurement of metabolic pool of nitrate was reevaluated using primary leaves of 7-day-old barley and 10-day-old soybean seedlings. The seedlings were grown in nutrient solutions containing 5 to 15 millimolar nitrate. The nitrate-free in vivo assay system of nitrate reductase was used for measuring the production of nitrite. Both the duration and extent of nitrite production were dependent on the level of endogenous nitrate in the tissue. At cessation of nitrite production, 30 to 50% of the endogenous nitrate was reduced to nitrite. Nitrate from the tissue leaked continuously into the surrounding medium so that, at cessation of nitrite production, nitrate supply from the tissue was exhausted. The cessation of nitrite production, therefore, may have been caused by the depletion of endogenous nitrate from the tissue. It is concluded that anaerobic nitrite production is not a valid index for the measurement of the size of the metabolic pool of nitrate.

The total amount of nitrite produced in the in vivo assay during anaerobic reduction of endogenous nitrate in plant cells and tissues has been used to measure the size of the metabolic pool of nitrate (2, 3, 8). The method was first proposed by Ferrari et al. (8), who observed that nitrate reductase activity and energy supply for nitrate reduction were not factors limiting nitrite production in tobacco cells. Hageman et al. (9) observed that, in wheat leaves, nitrite production could be stopped by the lack of energy and loss of nitrate reductase activity. Nitrate availability within the tissue is not considered to be limiting to nitrite production (8, 9). However, my preliminary studies with soybean leaves show that, when nitrite production ceased, nitrate supply from the tissue became exhausted. Therefore, in the present investigation, the fate of tissue nitrate during anaerobic nitrite production was followed by analyzing the tissue and the assay medium for both nitrate and nitrite. The results suggest that cessation in nitrite production (nitrate reduction) was caused by the depletion of nitrate from the tissue.

MATERIALS AND METHODS

Plant Material. Seedlings of soybean (Glycine max [L.] Merr. cv. Amsoy) and barley (Hordeum vulgare L. cv. Numar) were grown in vermiculite and subirrigated with a modified Hoagland solution containing 5 to 15 mm nitrate (14). Seedlings were grown for 7 (barley) or 10 (soybean) days in a controlled environment growth chamber under a 16-h photoperiod at 50% RH and 25 C. Quantum flux at the tops of the plants was 500 µE m⁻² s⁻¹ and was supplied by incandescent and cool white fluorescent lamps. Primary leaves were sampled for assays during the middle of the photoperiod.

Measurement of Anaerobic Nitrite Production. Anaerobic nitrite production (accumulation) was determined by the following methods.

Method A. Leaf-discs from soybean (having a diameter of 9 mm each) or leaf sections from the top 8 cm of barley leaves (measuring approximately 5 × 5 mm or less) were prepared and thoroughly mixed. A weighed leaf sample, approximately 0.3 g (soybean) to 0.4 g (barley), was placed in a 25-ml Erlenmeyer flask containing 10 ml of either 0.1 M K-phosphate (pH 7.5) or 2 mm CaSO₄ solution. Preliminary studies showed that increasing the tissue weight up to 0.4 g did not affect the rate or the extent of nitrite production. Chloramphenicol (300 µg/ml) was routinely added to the assay medium to stop bacterial contamination. In some studies, 1% (v/v) 1-propanol was added to the assay medium. The flasks containing the tissue (3 replicates per treatment) were placed under vacuum (at 0.2 atm) for 2 min, releasing the vacuum every 30 s. During infiltration, the tissue became waterlogged and sank to the bottom of the flasks. After vacuum infiltration, the flasks were stopped and incubated in the dark at 28 C in a shaking water bath. Following incubation for different time intervals, both the tissue and the incubation medium were analyzed for nitrate and nitrite.

Method A-I. The method was the same as Method A, except that nitrite accumulation was followed by sequential removal of aliquots (0.05 to 0.1 ml) from the medium of the same assay flasks at intervals. Volume corrections were made whenever sequential sampling was used. Although, with this method, sample to sample variability was reduced, the initial rates of nitrite accumulation in barley leaves were underestimated by as much as 20 to 30% because of retention of nitrite in the tissue. However, at the cessation of nitrite accumulation, the amount of nitrite retained in the tissue was negligible (<5%). Therefore, the values of the size of metabolic pool of nitrate determined by this method were comparable to those determined by Method A.

Method B. This method was also similar to Method A, except that anaerobiosis was achieved by N₂ instead of vacuum infiltration. Barley leaf slices (approximately 0.4 g) were added to flasks containing 10 ml of 2 mm CaSO₄ which had been purged with N₂ for 15 min. N₂ was bubbled through the contents of each flask for 3 min after the leaf sections were added. The flask was then sealed with serum caps, purged with N₂ for an additional 1 min, and incubated in the dark at 28 C in a shaking water bath. The rest of the procedure was the same as in Method A.

Nitrate and Nitrite Analysis. The tissue was homogenized with a cold mortar and pestle in eight volumes of cold deion sol hydrated water. The homogenate was centrifuged at 30,000g for 15 min, and the supernatant was used to measure nitrate and nitrite.

1 During these studies, the author was the recipient of a visiting Fellowship from Natural Sciences and Engineering Research Council of Canada.

2 Present address: Plant Growth Laboratory, University of California, Davis, CA 95616.
Nitrate was measured as nitrite after enzymic reduction with dissimilatory nitrate reductase obtained from *Klebsiella pneumoniae* (1). Nitrite was determined colorimetrically after color development with 1 ml 1% (w/v) sulfanilamide in 1.5 N HCl and 1 ml 0.02% (v/v) N-(1-naphthyl)-ethylenediamine dihydrochloride for 15 min. Absorbance was read at 540 nm. Nitrate and nitrite were calculated as µmol per g fresh weight.

**RESULTS**

**Effect of Mode of Anaerobiosis.** Figure 1 shows the time course of nitrite accumulation and nitrate loss from barley leaf sections as affected by the mode of anaerobiosis, achieved either by purging with N₂ or by vacuum infiltration. Initially, the rate of nitrite accumulation was somewhat lower with N₂ than with vacuum infiltration, but, after 1 h, similar rates of nitrite accumulation were observed in both treatments. Consequently, under N₂ the total amount of nitrite accumulated remained at a slightly lower level throughout the incubation period than it did with vacuum infiltration. The loss of nitrate from the tissue into the medium was not affected by the method of achieving anaerobiosis. In further studies, therefore, vacuum infiltration was used to achieve anaerobiosis.

**Influence of Incubation Medium.** Nitrite production (both rate and extent) in soybean leaves incubated in CaSO₄ was considerably decreased compared to that in K-phosphate (Fig. 2A). In contrast, in barley leaves nitrite accumulation was not affected, whether the leaves were incubated in CaSO₄ or in K-phosphate (Fig. 2B). Propanol also had a different effect on nitrite accumulation in barley and soybean leaves. Whereas no enhancement of nitrite accumulation was observed in barley leaves (Fig. 2B), propanol in soybean leaves stimulated nitrite accumulation considerably in both CaSO₄ and K-phosphate (Fig. 2A). The stimulation by propanol was more pronounced in K-phosphate than in CaSO₄. Ethanol and 2,4-dinitrophenol also stimulated nitrite accumulation in soybean leaves (data not shown).

**Nitrite Production and Leakage of Nitrate.** The data show that, during nitrite production (Fig. 3A), there was a continuous leakage of nitrate from the soybean leaf discs into the incubation medium (Fig. 3B). After 6 h of incubation, the supply of nitrate from the tissue was almost exhausted, and 38 µmol of nitrate (60% of that originally present in the leaves) were recovered in the incubation medium.

In barley leaves, nitrite production continued for at least 14 to 15 h (Fig. 4). During the same period, nitrate concentration of the tissue decreased continuously and reached a minimal level as
FIG. 4. Time course of nitrite accumulation and loss of nitrate from barley leaf sections in darkness under anaerobic conditions. Seedlings were grown in 15 mM nitrate solution. Leaf-sections were incubated in 2 mM CaSO₄ solution, according to Method A. Tissue was analyzed for both nitrate and nitrite, and the assay medium was analyzed for nitrite only. Bars on the points represent SE.

Table I. Effect of Endogenous Nitrate on the Duration and Extent of Nitrite Accumulation in Barley Leaves

<table>
<thead>
<tr>
<th>Growth Nitrate Level</th>
<th>Initial Rates of Nitrite Accumulation</th>
<th>Total Nitrite Accumulation</th>
<th>Cessation Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td></td>
</tr>
<tr>
<td>5.0 μmol/g·h</td>
<td>10.4 ± 0.8</td>
<td>1.2 ± 0.3</td>
<td>5.0 ± 0.9</td>
</tr>
<tr>
<td>7.5 μmol/g·h</td>
<td>22.5 ± 1.9</td>
<td>2.0 ± 0.4</td>
<td>11.5 ± 1.0</td>
</tr>
<tr>
<td>10.0 μmol/g·h</td>
<td>41.0 ± 1.7</td>
<td>3.2 ± 0.4</td>
<td>21.2 ± 1.1</td>
</tr>
<tr>
<td>15.0 μmol/g·h</td>
<td>70.5 ± 1.8</td>
<td>4.8 ± 0.6</td>
<td>32.5 ± 1.7</td>
</tr>
</tbody>
</table>

* Means ± SE.

** Cessation time is the time when nitrite production ceased.

FIG. 5. Effect of cold incubation on anaerobic nitrite accumulation and nitrate loss from barley leaves grown in 15 mM nitrate. Leaf sections were incubated in 2 mM CaSO₄ solution, according to Method A. One set of flask was incubated at 28°C (○), the other set at 0°C (●). At the 10th hour, one-half of the flasks from 0°C were transferred to 28°C (△). The values indicated by arrows are percentages of endogenous nitrate (70 μmol/g fresh weight) remaining in the tissue at that particular time of incubation. Bars on the points represent SE.

** DISCUSSION**

The measurement of the metabolic pool of nitrate is based on the assumptions that (a), the cessation of nitrite production is caused by the complete reduction of nitrate from the metabolic pool and (b), leakage of nitrate from the tissue into the surrounding medium does not affect the measurement of the size of metabolic pool of nitrate (8). Inasmuch as metabolic nitrate is considered to be a fraction of the total nitrate in the tissue (8), large amounts of nitrate should still be present in the tissue when nitrite production ceases. The evidence presented here indicates that, as nitrite production ceased, nitrate supply from the tissue was essentially exhausted (Figs. 3 and 4). This implies that cessation of nitrite production may have been caused by the depletion of nitrate from the tissue rather than by the complete reduction of the metabolic nitrate. In contrast, Ferrari et al. (8) observed that most of the nitrate was still present in tobacco cells as cessation in nitrite production was approached. However, Hageman et al. (9) have shown recently that the system used by Ferrari et al. (8) probably was not completely anaerobic, and cessation of nitrite production could have been caused by the lack of complete anaerobiosis in the assay system. The presence of O₂ in the system of Ferrari et al. (8) may have not only prevented the leakage of nitrate from tobacco cells (6) but also inhibited nitrate reduction.
(4, 15), thus causing apparent cessation in nitrite production. Hageman et al. (9) also concluded that, in wheat leaves, availability of nitrate is not a limiting factor in causing cessation of nitrite production. They observed that similar amounts of nitrate were produced whether the tissue was incubated in medium containing or in medium lacking nitrate. However, they did not determine the nitrate concentration of the tissue. In the study reported herein, although at the time of cessation in nitrite production endogenous nitrate supply from the tissue was exhausted (Fig. 4), exogenous nitrate had no marked effect on nitrite production (data not shown). These data are in contrast to those previously reported by Aslam et al. (3) and Ferrari et al. (8), who observed higher rates of nitrite production when nitrate was added in the infiltration medium. This stimulation in nitrite production on the addition of massive doses of nitrate to a partially anaerobic system could have been due to the diversion of the reducing equivalents from NADH towards nitrate rather than towards O₂ (16).

Since nitrate first accumulates in the metabolic pool and only excess nitrate is accumulated in the storage pool (3, 12), at low nitrate supply to the tissue most of the nitrate absorbed is expected to accumulate in the metabolic pool, whereas, at higher nitrate supply (saturation conditions), the proportion of the absorbed nitrate accumulating in the metabolic pool will be low. If under anaerobic conditions, nitrate in the metabolic pool is the only source of nitrate available for reduction, then in tissue with low levels of endogenous nitrate, a higher proportion of the endogenous nitrate should be recovered as nitrite and vice versa. However, the evidence indicates that the total amount of nitrite produced was directly proportional to the level of endogenous nitrate in the tissue (Table I), suggesting that, under anaerobic conditions, nitrate from the storage pool also becomes readily available for reduction. A similar conclusion was also reached by Subbalakshmi et al. (16), who observed that, in intact leaves of wheat and rice, about two-thirds of the endogenous nitrate was reduced under anaerobic conditions.

During cold incubation, solute loss from the tissue is accelerated (13), whereas in vivo reduction of nitrate to nitrite is prevented (10). The incubation of the tissue at cold temperature, therefore, should allow nitrate to leak into the surrounding medium without concomitant nitrite production (8). Similar amounts of nitrite production by the tissue, whether preincubated at cold temperature or incubated continuously at 28 °C (Fig. 5), would then suggest that, during cold preincubation, leakage of nitrate occurred from the storage pool only. However, during cold incubation, the loss of the endogenous nitrate from the tissue was negligible (Fig. 5). During cold incubation, the leakage of nitrate from the tissue was prevented, and, on warming the tissue, nitrite production resumed and ceased only when nitrate supply from the tissue became exhausted (Fig. 5).

It is well documented that Ca²⁺ or Mg²⁺ is necessary for the maintenance of membrane selectivity of ion uptake and ion retention by plant cells (7). These divalent cations also prevent the loss of solutes induced by anaerobiosis (6). Incubation of leaf sections in medium containing Ca²⁺ should, therefore, prevent the leakage of nitrate from the tissue, and, consequently, more nitrate should be available for anaerobic reduction yielding more nitrite. However, Ca²⁺ decreased nitrite production in soybean leaves, whereas, it had no effect in barley leaves (Fig. 2). In contrast, a monovalent cation (K⁺) stimulated nitrite production in soybean leaves (data not shown). Heuer and Plaut (11) also observed that, in alfalfa leaves, K⁺ stimulated and Ca²⁺ inhibited nitrite accumulation. They argued that Ca²⁺ altered the membrane permeability so that more nitrate was retained by the tissue. However, they did not determine the nitrite content of the tissue. Evidence indicates that Ca²⁺ does not help prevent the leakage of nitrate (data not shown) and nitrite (Fig. 2) from the soybean leaf discs, suggesting that the effect of Ca²⁺ on anaerobic reduction of nitrate may be more complex than simply maintaining membrane integrity.

How propanol, ethanol, and 2,4-dinitrophenol stimulate anaerobic nitrite production is speculative. It is presumed that these chemicals either increase the entry and accessibility of nitrate within the cell (5, 8), or increase the leakage of nitrite from the cell (5). However, the differential effect of propanol in soybean and barley leaves and the fact that the leaves incubated in propanol accumulated more nitrite (Fig. 2) indicate the complexity of the influence of alcohols on anaerobic nitrite production.

In summary, the results presented here indicate that (a), nitrite production ceased when nitrate supply from the tissue was exhausted; (b), the duration and extent of nitrite production were directly proportional to the level of endogenous nitrate in the tissue; and (c), nitrite production (both the rate and quantity) was differently affected in soybean and barley leaves by Ca²⁺ and propanol. I concluded that anaerobic production of nitrite is not a valid index for the measurement of the size of the metabolic pool of nitrate in higher plants.

Acknowledgments—The author is grateful to Dr. J. M. Fulton, Director, Agriculture Canada Research Station, Harrow, Ontario, Canada, for providing the laboratory facilities. The author is also grateful to Dr. R. C. Huffer, and Dr. J. R. Thayer for barley seeds and cultures of Klebsiella pneumoniae, respectively. Critical reading of the manuscript by Dr. Ann Oaks is also greatly appreciated.

LITERATURE CITED

10. HALLMARK WB, RC HUFFERK 1978 The influence of ambient nitrate, temperature, and light on nitrate assimilation in sugarcane seedlings. Physiol Plant 44: 147-152
15. SAWINENSKY SK, MS NAIAK, DJD NICHOLAS 1978 Regulation of nitrate reduction by light, ATP and mitochondrial respiration in wheat leaves. Nature (Lond) 272: 647-648
16. SUBBALAKSHMI B, SP SINGH, SF PRAKASH, MS NAIAK 1979 Regulation of nitrate reduction in wheat and rice leaves by oxygen and NADH supply. Plant Sci Lett 14: 133-137

Copyright © 1981 American Society of Plant Biologists. All rights reserved.