Effect of Aerobic and Anaerobic Conditions on the in Vivo Nitrate Reductase Assay in Spinach Leaves

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Abstract. ¹⁵N-labelled nitrate was used to show that nitrate reduction by leaf discs in darkness was suppressed by oxygen, whereas nitrite present within the cell could be reduced under aerobic dark conditions. In other experiments, unlabelled nitrite, allowed to accumulate in the tissue during the dark anaerobic reduction of nitrate was shown by chemical analysis to be metabolised during a subsequent dark aerobic period. Leaves of intact plants resembled incubated leaf discs in accumulating nitrite under anaerobic conditions. Nitrate, n-propanol and several respiratory inhibitors or uncouplers partly reversed the inhibitory effect of oxygen on nitrate reduction in leaf discs in the dark. Of these nitrate and propanol acted synergistically. Reversal was usually associated with inhibition of respiration but some concentrations of 2,4-dinitrophenol (DNP) and ioxynil reversed inhibition without affecting respiratory rates. Respiratory inhibitors and uncouplers stimulated nitrate reduction in the anaerobic in vivo assay i.e. in conditions where the respiratory process is non-functional. Freezing and thawing leaf discs diminished but did not eliminate the sensitivity of nitrate reduction to oxygen inhibition.

Key words: Nitrate reduction – Oxygen – Regulation – Spinacia.

Introduction

The in vivo assay of nitrate reductase is commonly used as a guide to the nitrate reduction capability of plant tissue. This assay, in which nitrite formation by leaf discs is measured, is dependent on the endogenous generation of NADH and is performed in the dark to prevent further reduction of nitrite by the tissue (Ferrari and Varner, 1970; Klepper et al., 1971).

Anaerobic conditions favour nitrite accumulation in this assay (Ferrari and Varner, 1970; Radin, 1973; Klepper et al., 1971; Canvin and Atkins, 1974) and it has been supposed (Ferrari and Varner, 1971; Klepper et al., 1971; Klepper, 1974) that the removal of oxygen is necessary for the complete elimination of nitrite reduction. The evidence of Canvin and Atkins (1974) using direct measurements with ¹⁵N labelled substrates indicated however that nitrite was not reduced in darkness under nitrogen or in air. Nitrate assimilation was negligible in experiments of Canvin and Atkins (1974) under dark aerobic conditions. From this they argued (Atkins and Canvin, 1975) that nitrate reduction ceased under dark aerobic conditions, attributing this to competition between nitrate and oxygen for reductant. Radin (1973) while also favouring this explanation found that some nitrite was reduced under dark aerobic conditions.

The addition of the respiratory inhibitors antimycin A and 8-hydroxyquinoline-N-oxide (HOQNO) to the dark aerobic in vivo assay resulted in nitrite accumulation (Ferrari and Varner, 1970) perhaps indicating competition between oxygen and nitrate for reductant.

We report further experiments to clarify the extent
of nitrate and nitrite reduction under dark aerobic or anaerobic conditions and in relation to concentrations of nicotinamide nucleotides together with more detailed studies of the effects of respiratory inhibitors on the apparent inhibition by oxygen of nitrite accumulation.

Materials and Methods

Spinach (Spinacea oleracea L.) cv. Yates True Hybrid 102 (Samuel Yates and Co., Macclesfield, Cheshire) was grown in sand and water culture in a greenhouse according to the general methods of Hewitt (1966) or by nutrient film technique, using a nutrient-based nutrient solution.

The routine aerobic/anaerobic in vivo nitrate reductase assay was as follows: 50–100 mg of 4 mm diameter leaf discs were sliced in half to increase uptake area and placed in 5 x 1.5 cm glass specimen tubes with 2 ml buffer (0.1 M KH2PO4-K2HPO4, 1 mM EDTA, pH 7.5). The tube was sealed with a Suba-seal stopper No. 25 in which inlet and outlet hypodermic syringe needles (No. 19G) were fixed. The inlet needle opened beneath the liquid enabling gas to be bubbled through the sample. The gas, supplied as nitrogen (or nitrogen-oxygen mixtures experiment of Fig. 3, only) or as air from a diaphragm pump, was first bubbled through 1.5 l of deionized water in order to reduce evaporation. The gas flow was adjusted to the minimum required for free bubbling in all tubes. The samples were incubated at 27.5°C in a water bath and tubes were covered with aluminium foil to exclude light. Corresponding light treatments were provided by illuminating tubes from underneath in a glass tank with 2 x 150 W Osram spotlights (50 K lux incident on tubes). After incubation the tubes were placed in a boiling water bath for 4 min in order to release the nitrite from the tissue. A sample was then assayed for nitrate (as Mann et al., 1978).

Respiratory measurements on similar leaf disc samples were made at 27°C using a standard Warburg apparatus (Umbreit et al., 1964).

For nitrate assimilation studies with 15NO3, leaf material was sampled from plants which had been starved of nitrate for several days and then watered with N/3 nitrate for four days before use to ensure the presence of minimal endogenous nitrate. The plants were not deficient in nitrogen, and in vivo nitrate reductase activity was similar to that of normally grown plants. Leaf discs were incubated as for the aerobic-anaerobic assay with the buffer containing 0.1 M KH2PO4-K2HPO4, pH 7.5, at neutrality.

Results and Discussion

In illuminated leaf tissues, nitrite is reduced faster than it is generated from nitrate and therefore does not accumulate. The assimilation of exogenous nitrite by illuminated leaf discs from an aerated solution, however, is dependent upon the pH of the solution. In our experiments, no loss of nitrite occurred at pH 7.5 or above from the external solution or from the total system (solution + discs), whereas rates of 1–3 μmol h–1 g–1 fresh weight were obtained at pH 5.5. We suspect that nitrite is unable to enter the cells at neutrality.

Spinach leaf discs incubated aerobically in darkness also showed no change in external or total (i.e. tissue + external) nitrite concentration (Table 1). Some nitrite production occurred when discs were incubated anaerobically in darkness with nitrite solutions (Table 1) which can be explained by reduction of endogenous nitrate. Inability of nitrite to penetrate the tissue at pH 7.5 would explain the failure of Canvin and Atkins (1974) to demonstrate nitrite reduction in dark aerobic conditions in experiments where they supplied 15N-labelled nitrite. Their conclusion — that nitrite is not reduced under these conditions — is therefore not necessarily valid. We conclude from our data that endogenous nitrite is reduced in dark aerobic conditions, although at a slower rate than in light. Thus in experiments in which 15NO3 was supplied of ethanol/min at pH 8.8 and 25°C alcohol dehydrogenase, while the NADPH assay solution contained 2.5 μmol glucose-6-phosphate and 10 units (1 unit oxidises 1 μmol of glucose-6-phosphate/min at pH 7.4 and 25°C) of glucose-6-phosphate dehydrogenase. The reaction was started by the addition of either alcohol dehydrogenase or glucose-6-phosphate dehydrogenase. Calibration curves were linear from 0–0.7 μmol NAD(H) and from 0–0.7 μmol NADPH and were unaffected by the extract. Recovery of NAD(H) was greater than 95%.

Nitrate assays were carried out by the method of Canvin and Atkins (1974) but using a cadmium reductant prepared by the method of Sloan and Sublett (1966). The resulting nitrite was measured using the alkaline iodine method as described by Mann et al., 1978, but omitting the Tris buffer.

Chemicals

Amytal, antimony A, ATP, CCCP (carboxyl cyanide m-chlorophenylhydrzone), DCPIP (2,6-dichlorophenolindophenol), HOQNO (8-hydroxyquinoline-N-oxide), NAD+, NADP+, NADPH, gramicidin, oligomycin, alcohol dehydrogenase, glucose-6-phosphate dehydrogenase, Tes (N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid) and Hepes (N-2-hydroxyethylpiperazin-2-ethanesulfonic acid) buffers were obtained from Sigma Chemical Co. Tris and 2,4-dinitrophenol were supplied by British Drug Houses, Poole, U.K. NADH was purchased from Boehringer, Mannheim. We thank A.H. Marks and Co. Ltd., Bradford for a gift of isoxynil (4-hydroxy-3,5 diiodobenzonitrile).