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Klepper L, Flesher D & Hageman R H. Generation of reduced nicotinamide adenine dinucleotide for nitrate reduction in green leaves.

Plant Physiol. 48:580-90, 1971.

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This paper describes an *in vivo* assay to measure nitrate reductase activity within intact leaf discs. By experimentation with various substrates using this assay, it was concluded that the glycolytic oxidation of glyceraldehyde-3-phosphate and concomitant reduction of nicotinamide adenine dinucleotide (NAD) were the prime source of nicotinamide adenine dinucleotide hydrogen (NADH) for nitrate reduction in green leaves. [The SCJ[®] indicates that this paper has been cited in over 215 publications since 1971.]

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The research for this paper began as a spontaneous experiment to determine whether nitrate reduction could be measured in intact leaf discs. For specific reasons that I can no longer remember, I made leaf discs from jimsonweed. The leaf discs were placed in two test tubes containing nitrate and vacuum-filtered until they sank. One tube was covered with foil to exclude light (energy for nitrite reduction), and the other tube was placed in light. My theory was that if nitrate was reduced in these leaf discs, it would remain as nitrite in darkness and could be measured. However, if it was reduced further by nitrite reduction in light, no nitrite would be detected. Both tubes worked! I was certain that it could not

be that easy. I immediately accused two nearby graduate colleagues of tampering with my experiment. Within that next hour, I duplicated this simple experiment and apologized. For the past 17 years, I have apologized repeatedly, but these two, now well-established professors, have never let me forget the incident.

After these initial results, I refined the technique into an *in vivo* nitrate reductase assay and used it as the primary method to determine the metabolic pathway responsible for generating NADH for nitrate reduction and to identify the specific enzyme and substrate for the NADH-generating system. The basic assay has led to many other publications.^{1,2}

Donna Flesher provided excellent technical support. Dick Hageman, adviser and friend, guided me in discussions of future experiments and testing of hypotheses and provided the momentum needed by an inexperienced graduate student. This research was the result of cooperative efforts.

The paper has been cited frequently for several reasons. It offered a reasonable explanation for the link between carbon and nitrogen metabolism. Other researchers had often noted a relation between carbohydrate oxidation and nitrate reduction, but the exact mechanism was not known. It provided clarification concerning the effect of light (photosynthate) on nitrate reductase activity. Finally, the paper presented an *in vivo* assay for nitrate reductase activity that eliminated most of the problems of enzyme extraction present in the *in vitro* techniques. Looking back, since 1971, only minor changes made in the basic *in vivo* technique have accounted for a large number of analyses over a wide range of plant tissues.

1. Hageman R H & Reed A J. Nitrate reductase from higher plants. *Meth. Enzymology* 69:270-80, 1980.

2. Klepper L. Nitric oxide (NO) and nitrogen dioxide (NO₂) emissions from herbicide-treated soybean plants. *Atmos. Environ.* 13:537-42, 1979.