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Acetaldehyde Oxime, A Product Formed during the *In Vivo* Nitrate Reductase Assay of Soybean Leaves[1]

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ABSTRACT

Evolution of nitrogen oxides ($NO_{(x)}$, primarily as nitric oxide) from soybean (*Glycine max* [L.] Merr.) leaves during purged *in vivo* nitrate reductase assays had been reported; however, these reports were based on a method that had been used for determination of $NO_{(x)}$ in air. This method also detects other N compounds. Preliminary work led us to doubt that the evolved N was nitric oxide. Studies were undertaken to identify the N compound evolved from the *in vivo* assay that had been reported as $NO_{(x)}$. Material for identification was obtained by cryogenic trapping and fractional distillation, and by chemical trapping procedures. Mass spectrometry, ultraviolet spectroscopy, and ^{15}N-labeled nitrate were used to identify the compounds evolved and to determine whether these compounds were derived from nitrate. Acetaldehyde oxime was identified as the predominant N compound evolved and this compound is readily detected by the method for $NO_{(x)}$ determination. Substantial quantities of acetaldehyde oxime (16.2 micromoles per gram fresh weight per hour) were evolved during the *in vivo* assay. Small amounts of nitrous oxide (0.63 micrograms N per gram fresh weight per hour) were evolved, but this compound is not detected as $NO_{(x)}$. Acetaldehyde oxime and nitrous oxide were both produced as a result of nitrate ($^{15}NO_3^-$) reduction during the assay.

Various adaptations of the *in vivo* NR[2] assay have been used by plant scientists to estimate NR activity in plant parts, to study the factors affecting NO_3^- reduction in plant tissue, and to study the interaction of NO_3^- reduction with other physiological processes (*e.g.*, 6, 10). The reliability of the *in vivo* procedure for estimating *in situ* NR activity depends in part on a stoichiometric relationship between NO_3^- reduction and NO_2^- accumulation during the assay. Harper and co-workers (12, 22, 26) have reported production of nitrogen oxides ($NO_{(x)}$) during gas purging of the *in vivo* NR assay of soybean (*Glycine max* [L.] cv Wells and cv Williams) leaf sections. It was also reported that the $NO_{(x)}$ evolved was derived from NO_2^- (12, 26), although a stoichiometric relationship was not found. Evolution of $NO_{(x)}$ was detected whether the assay was purged with N_2, Ar, air, or O_2, with the highest rates occurring under anaerobic conditions (12). In addition, it was shown that $NO_{(x)}$ evolution was associated with a constitutive NR activity in young soybean leaf sections (22, 26). The procedure used by Harper and co-workers to measure $NO_{(x)}$ evolution from the *in vivo* NR assay involved sequentially passing the purging gas through (a) the NR assay medium, (b) a preoxidizer (glass beads coated with $Na_2Cr_2O_7$-H_2SO_4), and (c) into a trapping solution containing Griess-Saltzman reagents. The preoxidizer converts nitric oxide (NO) to nitrogen dioxide (NO_2), and NO_2 reacts readily with water in the trapping solution to form NO_2^- ($2\ NO_2 + H_2O \rightleftarrows NO_2^- + NO_3^- + 2\ H^+$ [29]). This NO_2^- then reacts with the Griess-Saltzman reagents to form an azo chromophore, which is determined spectrophotometrically. Because only trace amounts of NO_2^- were detected in the trapping solution when the gas stream from the *in vivo* NR assay was not passed through the preoxidizer, it was inferred that NO was the predominant product (12).

Our preliminary studies of the volatile nitrogenous products of the *in vivo* NR assay confirmed Harper's observation (12) that substantial amounts of a compound detectable by the preoxidizer/Griess-Saltzman method were evolved from the $+NO_3^-$ *in vivo* NR assay of young soybean leaves (cv Williams). However, our results did not support the inference that NO was the compound evolved. The objectives of this work were to (a) identify the nitrogen compound(s) evolved during the *in vivo* NR assay of young soybean leaves and detected by the preoxidizer/Griess-Saltzman procedure, (b) determine whether this compound(s) was derived from NO_3^- using ^{15}N, and (c) estimate the amount of this compound(s) evolved during the purged *in vivo* NR assay (anaerobic).

MATERIALS AND METHODS

Plant Material. Soybean seeds (*Glycine max* [L.] Merr. cv Williams) were germinated at 26°C in the dark on sterile paper towels moistened with deionized H_2O. At 5 DAP, the etiolated seedlings were transferred to 2-L pots (containing 2 mM $CaSO_4$) in growth chambers, and were provided with 14 h/28°C light periods and 10 h/19°C dark periods. The light intensity was 600 $\mu E \cdot m^{-2} \cdot s^{-1}$ supplied by incandescent and cool-white fluorescent lamps. At 6 DAP, the $CaSO_4$ solution was replaced with a modified Hoagland nutrient solution (macronutrients: 5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 1 mM K_2HPO_4-KH_2PO_4 [pH 6.5], 2 mM $MgSO_4$; micronutrients: 50 μM KCl, 35 μM Fe as sodium ferric diethylenetriamine pentaacetate, 25 μM H_3BO_3, 5 μM $MnSO_4$, 2 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 15 nM $(NH_4)_6Mo_7O_{24}$). The rooting media were aerated continuously and the nutrient solutions were changed at 9 and 14 DAP. At 10 DAP, the unifoliolate leaves had just reached full expansion and were harvested 4 h after the onset of illumination, when NR activity was maximal. Harvested leaves were stored in plastic bags and held on ice (dark, no longer than 30 min) prior to the *in vivo* NR assay. The first trifoliolate leaves were similarly harvested 15 DAP.

For ^{15}N studies, the plants were grown as described above, except that at 13 DAP the plants were transferred to low nitrate nutrient solution to reduce tissue NO_3^- levels, yet permit induction and maintenance of NR (macronutrients: 7 mM urea, 1 mM

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[2] Abbreviations and symbols: NR, nitrate reductase; $NO_{(x)}$, nitrogen oxides (refers to nitric oxide [NO] and nitrogen dioxide [NO_2], collectively); DAP, days after planting; N_2O, nitrous oxide; m/z, mass to charge ratio; M^+, molecular ion; bp, boiling point.

KNO_3, 5 mM $CaCl_2$, 2.5 mM K_2SO_4, 1 mM K_2HPO_4-KH_2PO_4 [pH 6.5], 2 mM $MgSO_4$; micronutrients as above). The nutrient solution pH was maintained at approximately 6.5. The first trifoliolate leaves were harvested 15 DAP and stored as described above prior to the *in vivo* NR assay.

+$^{15}NO_3^-$ *In Vivo* NR Assay. Leaf discs (2 g fresh weight, 1-cm diameter) were placed in a 500-ml flask containing 130 ml of buffer (0.1 M K_2HPO_4-KH_2PO_4, pH 7.5; 50 mM KNO_3 enriched with 65.6 atom % ^{15}N; flask covered with aluminum foil and held at 0°C) and were vacuum-infiltrated twice (10 mm Hg, 2 min each time). The flask was placed in a closed gas exchange system (see Fig. 1) and the atmosphere in the system was replaced by N_2 (at atmospheric pressure) via 10 evacuation-N_2 flushing cycles (12 min total time). The gas exchange system was then isolated from the N_2-vacuum manifold (by closing stopcock 1), the flask was placed in a 30°C bath, and the system atmosphere was circulated at 400 ml·min^{-1}. After 1 h, the gas sample tube was isolated and removed for determination of ^{15}N-labeled N_2O, N_2, and NO (N_2 was chosen for the background anaerobic atmosphere because the mass spectrometer ion pumps do not satisfactorily pump any of the noble gases). The leaf discs and assay medium were immediately frozen at −80°C and stored at −10°C for further analyses.

Determination of ^{15}N-Labeled N_2O, N_2, and NO. Mass spectra and isotope-ratios were obtained using a Nuclide model-3-60-RMS dual-inlet isotope-ratio magnetic sector mass spectrometer equipped with dual collectors, an electron impact ion source, and an integrating ratiometer. Determinations of ^{15}N-labeled N_2O and N_2 were performed by the methods of Siegel *et al.* (28) and Mulvaney and Kurtz (20). Nitric oxide was separated from N_2O, CO_2, and H_2O by freezing the gas sample tube at −160°C (to condense N_2O, CO_2, and H_2O), reduced to N_2 with a reduced Cu column (described by Mulvaney and Kurtz [20]) at 250°C, and determined as $^{15}N_2$.

Procedures for Collecting N Compounds. For each collection of compounds evolved from the +NO_3^- *in vivo* NR assay, leaf discs (3 g fresh weight, 1-cm diameter) were placed in 150 ml of buffer (0.1 M K_2HPO_4-KH_2PO_4, pH 7.5; 50 mM KNO_3; held in the dark at 0°C) and vacuum-infiltrated as described previously. The leaf discs plus buffer were then transferred to a 500-ml gas washing bottle covered with aluminum foil in a 30°C bath, and He was bubbled through the assay medium (250 ml·min^{-1}). The exiting gas stream was passed sequentially through (a) vacuum trap A (Pyrex 7729, held at −80°C to remove the bulk of the water), (b) vacuum trap B (Pyrex 7728 fitted with 1- to 3-mm-high vacuum stopcocks [Ace Glass, Inc.] at the top and side tubulations, held at −196°C to concentrate the volatile N compounds for identification), and (c) a rotameter flowmeter (Union Carbide model 201-4334). Preliminary tests showed that for these conditions, approximately half of the N evolved (as determined by the preoxidizer/Griess-Saltzman method [12]) was retained in each trap. Compounds evolved from the assay were collected for 1 h before removing vacuum trap B (at −196°C) from the system. High vacuum techniques were used to (a) remove residual He, (b) combine trap B material from five separate collections to obtain sufficient material for identification, (c) separate the bulked material by fractional distillation into four fractions (fraction I, components volatile from −120 to −196°C; fraction II, from −98 to −120°C; fraction III, from −78 to −98°C; fraction IV, above −78°C), and (d) dissolve fraction IV in water for characterization. Samples were protected from light during trapping and manipulation. To determine which fraction(s) contained N compounds detectable by the preoxidizer/Griess-Saltzman system, each fraction was thawed and swept into this system via a He carrier stream.

The N compound evolved during the *in vivo* NR assay of soybean leaves and detectable by the preoxidizer/Griess-Saltzman method (12) isomerized during sample manipulation (see "Results and Discussion"). All attempts to prevent this isomerization during cryogenic trapping and fractional distillation of the contents of vacuum trap B failed. To trap the N compound yet minimize isomerization, vacuum trap B was removed from the system and the gas stream was bubbled through a gas dispersion tube into trap C (0.1 mM K_2HPO_4-KH_2PO_4, pH 7.5; 15 ml in a 25- × 150-mm test tube), held at −10°C. After 1 h of collection, the trap C tube and contents were immediately frozen at −80°C and stored at −10°C prior to subsequent analyses.

Procedures for Identification. Mass spectra of fractions I through IV were obtained using the mass spectrometer described above. Because fractions I and IV were simple mixtures (two and three components, respectively), qualitative analyses were performed by spectrum stripping (11) and by plotting the mass

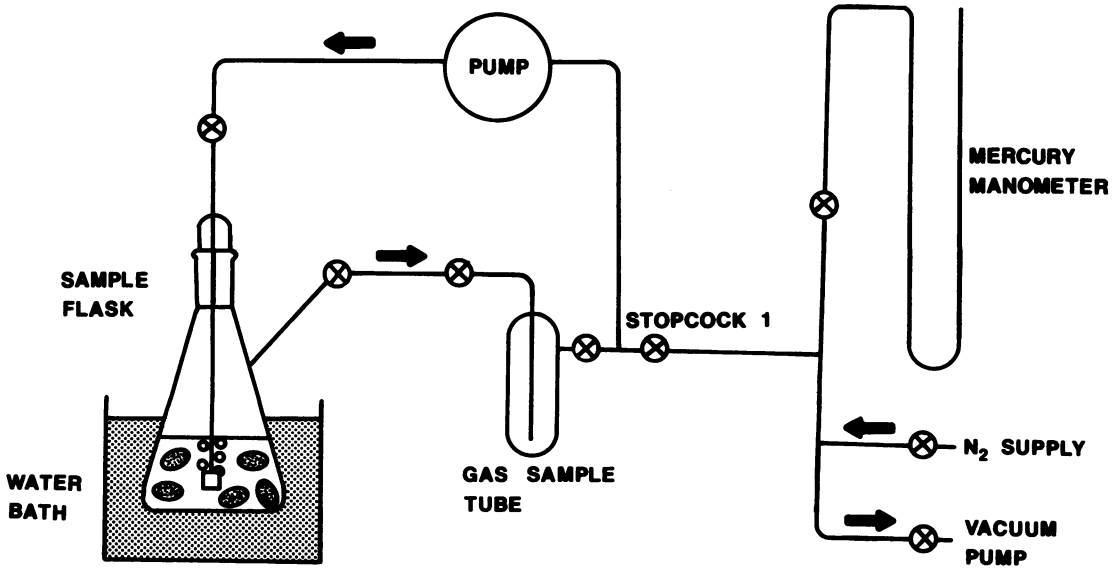

FIG. 1. Diagrammatic sketch of closed circulating gas exchange system used for determination of ^{15}N-labeled NO, N_2O, and N_2 produced during the +$^{15}NO_3^-$ *in vivo* NR assay of young soybean leaf discs. The system atmosphere was circulated at 400 ml·min^{-1} using a Cole-Parmer model K-7530-25 diaphragm pump (stopcock 1 closed). ⊗ = 1- to 3-mm-high vacuum stopcocks (Ace Glass, Inc.).

spectra obtained using varying ionization potentials (12–70 ev) and sample pressures (10^{-8} to 5×10^{-6} Torr) (3). UV-visible absorption spectra of aqueous solutions of fraction IV and of the N compound(s) in phosphate buffer (from trap C) were obtained using a Beckman model UV 5230 UV-visible spectrophotometer. The wavelengths of the absorbance maxima were somewhat concentration dependent, particularly for low concentrations of analyte. For comparison of UV-visible spectra of preparations obtained from the *in vivo* NR assay with solutions of standard compounds, the solutions were adjusted (with appropriate solvent) to similar absorbances at the wavelength of interest.

Estimation of Acetaldehyde Oxime Production. Acetaldehyde oxime (CH_3CH:NOH) evolved during the He-purged $+NO_3^-$ *in vivo* NR assay was cryogenically trapped in vacuum traps A and B by the procedure described previously for collection of evolved compounds (3 g fresh weight of leaf discs; 150 ml of 0.1 M K_2HPO_4–KH_2PO_4, pH 7.5; 50 mM KNO_3; dark at 30°C; He flow rate 250 ml · min^{-1}). After 1 h of collection, traps A and B were isolated, cooled at −196°C, the He headspace was pumped away, and the traps were incubated at 22°C for 1 week to ensure complete rearrangement of the oxime to acetamide [$CH_3(CO)NH_2$] (see "Results and Discussion"). The contents of both traps were transferred quantitatively with three 15-ml aliquots of water to a 100-ml volumetric flask, the resulting solution was diluted to volume, and acetamide assays were performed. Because acetamide (bp 221.2°C at 760 mm Hg) is less volatile than acetaldehyde oxime (bp 115°C at 760 mm Hg) (9), the amide is preferred for quantitative analyses in the liquid phase. After cryogenic collection of the evolved compounds, the *in vivo* assay medium and leaf discs were frozen (−80°C) and thawed three times (to increase permeability of the leaf material), and acetaldehyde oxime content was determined.

Assays. Acetaldehyde oxime was determined (a) as free NH_2OH by the 8-hydroxyquinoline method (18) after hydrolysis under mildly acidic conditions (35), and/or (b) it was determined as free NH_2OH by the method of Hucklesby and Hageman (14) after mildly alkaline hydrolysis (13). Acetamide-N was hydrolyzed to NH_4^+ using the following procedure. A 1-ml aliquot (containing up to 0.4 μmol $CH_3(CO)NH_2$) in a 25- × 150-mm tube was acidified with 1 ml of 5 N H_2SO_4. The tube was capped with a small glass funnel and glass marble, and was heated in a water bath at 95°C for 1 h. The tube contents were cooled and neutralized by the addition of 2 ml of 2.5 N NaOH, and the NH_3 produced was determined by the indophenol blue method (15).

^{15}N Enrichment of [^{15}N]Acetaldehyde Oxime. The medium from the $+^{15}NO_3^-$ *in vivo* NR assay was thawed, placed in a 500-ml gas washing bottle, purged with He for 30 min (250 ml · min^{-1}), and the oxime was trapped cryogenically (vacuum trap B) as described previously. The contents of vacuum trap B were separated by fractional distillation as described previously. Approximately 1 mm Hg of CO_2 was added to fraction IV (containing the oxime) and the mixture was incubated at 22°C for 1 week to ensure complete rearrangement of the oxime to the amide. The ^{15}N abundance of the amide was determined from the intensities of the peaks at m/z 59, 60, and 61 in mass spectra obtained from this preparation, after removal of CO_2 (7). The amide is preferred over the oxime for direct isotopic abundance determination because the amide produces a more intense molecular ion peak.

RESULTS AND DISCUSSION

Production of ^{15}N-Labeled NO, N_2O, and N_2. Nitric oxide was not detected by MS in gas samples obtained from the $+^{15}NO_3^-$ *in vivo* NR assay. Nitric oxide produces molecular ion peaks at m/z 30, 31, and 32 (corresponding to ions $^{14}N^{16}O^+$, $^{15}N^{16}O^+$, and $^{14}N^{18}O^+$) in the mass spectrometer. Harper and co-workers had reported that $NO_{(x)}$ evolved from the *in vivo* NR assay was derived from NO_2^- (12, 26), therefore $^{15}NO_3^-$ was used for this work because m/z 31 is the best peak for identification of NO by low resolution MS when N_2 is the background atmosphere (N_2 will produce a significant signal at m/z 30 due to natural abundance $^{15}N^{15}N^+$). No signal at m/z 31 was detected in mass spectra obtained, and isotope-ratio analyses confirmed that no detectable ^{15}NO was produced during the *in vivo* assay (Table I). The closed circulating gas exchange system employed, the assay conditions selected, and the plant material used were chosen to maximize NO production and enhance its accumulation in the gas phase during the *in vivo* assay. The detection limit for this system of 1.93 μg NO-N compares favorably with $NO_{(x)}$ evolution rates reported of about 100 μg N · g^{-1} fresh weight · h^{-1} for similar tissue and assay conditions (12). These results do not exclude the possibility that a trace amount of NO derived from unlabeled N compounds was produced during the assay; however, attempts to separate the components of the sample contributing to m/z 30 (and volatile at −160°C) via fractional distillation (using high efficiency traps cooled to −196°C) indicated that NO was not present in detectable quantities.

Additional evidence supporting the conclusion that the compound evolved from the *in vivo* NR assay and detected by the preoxidizer/Griess-Saltzman method (12) is not NO was obtained from preliminary work involving chemical trapping studies (1 N KOH:0.1 M $KMnO_4$ solutions), which showed that the compound(s) evolved was not detected by the method of Tedesco and Keeney (34) for determination of NO + NO_2. Significant evolution of $NO_{(x)}$ (primarily as NO) was reported when the assay medium was purged with air or O_2 (12). However, NO is rapidly oxidized by O_2 to NO_2 (29), and the finding that the compound(s) evolved from the *in vivo* NR assay of soybean leaves is not NO is in agreement with several studies in the literature which show that biological and chemical systems known to evolve NO under anaerobic conditions are not found to evolve this gas under aerobic conditions (*e.g.* 1, 21).

Small amounts of $^{15}N_2O$ derived from $^{15}NO_3^-$ were evolved from soybean leaves during the $+^{15}NO_3^-$ *in vivo* NR assay (Table I), however the amount produced was less than 1% of the amount of NO_2^--N accumulated in the assay medium, thus N_2O production resulting from NO_3^- reduction in the *in vivo* assay does not significantly influence the estimates of NO_3^- reduction from NO_2^- accumulation in the assay. Tests using high purity N_2O ($NO_{(x)} < 1$ μl · l^{-1}; 200 ml · min^{-1} for 30 min) showed that this compound is not detected by the preoxidizer/Griess-Saltzman method, therefore N_2O cannot be responsible for the results reported by Harper and co-workers (12, 22, 26). However, it can be inferred that the compound(s) detected by the preoxidizer/Griess-Saltzman method is an N compound because one of the N atoms in the azo chromophore is derived from the evolved compound (see 15). The preoxidizer ($Na_2Cr_2O_7$-H_2SO_4 coated on glass beads) is known to oxidize a variety of organic com-

Table I. *Amounts of ^{15}N-Labeled NO, N_2O, and N_2 Evolved from the $+^{15}NO_3^-$ In Vivo NR Assay of Soybean Leaf Discs*

The closed circulating gas exchange system (Fig. 1) was used. N_2O and N_2 were determined from isotope-ratio analyses (20, 28). NO was reduced to N_2 (in a reduced Cu column, 250°C) and determined as N_2.

Compound	Amount Evolved	Detection Limit	CV[a]	$^{15}X_N$[b]
	μg N · g^{-1} fresh wt · h^{-1}	μg N	%	
NO	0	1.93	1.3	
N_2O	0.63	0.33	1.5	51.8
N_2	0	1.93	1.2	

[a] CV, coefficient of variation.

[b] $^{15}X_N$, average mol fraction of ^{15}N, expressed as atom %.

pounds (17), and is not a specific oxidizing agent for NO. Production of $^{15}N_2$ was not detected (Table I).

Identification of the N Compounds. Of the four fractions produced by fractional distillation of the bulked material (cryogenically trapped in vacuum trap B) from the $+NO_3^-$ *in vivo* NR assay, only fractions I and IV contained detectable compounds. The contents of fractions II and III exerted pressures less than 1 mTorr (22°C, 60 ml volume), and the components of these fractions were not detected by MS or by the preoxidizer/Griess-Saltzman method. Therefore, these fractions were discarded. Figure 2 shows the mass spectrum obtained from fraction I. CO_2 was the primary component of this fraction, as evidenced by the intense peaks at m/z 44, 28, 16, and 12 (corresponding to ions CO_2^+, CO^+, O^+, and C^+). Nitrous oxide was a minor component of this fraction as indicated by the additional peaks at m/z 30 and 14 (corresponding to ions NO^+ and N^+), and by the C, N, and O isotopic abundances calculated for the molecular ions at m/z 44, 45, and 46. Subtraction of the CO_2 contribution from the mass spectrum of fraction I resulted in a spectrum consistent with that obtained for authentic N_2O using this instrument and operating conditions (Table II). These results are in agreement with the finding that a small amount of N_2O was evolved from the $+^{15}NO_3^-$ *in vivo* NR assay (Table I). Fraction I did not contain compounds detectable by the preoxidizer/Griess-Saltzman method.

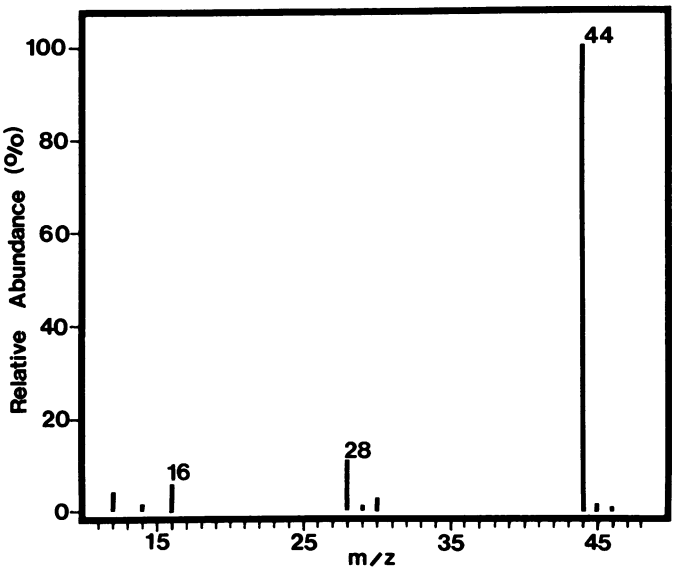

FIG. 2. Mass spectrum of fraction I. Fraction I was obtained by fractional distillation of the material retained in vacuum trap B and contained those compounds volatile from −120 to −196°C. (Ionization potential, 70 ev; acceleration potential, 3.0 kv; ion source and inlet at ambient temperature.)

Table II. *Calculated Mass Spectra for CO_2 and N_2O from the Mass Spectrum of Fraction I*

Experimental conditions are the same as for Figure 2.

m/z[a]	CO_2[b]		N_2O[c]	
	RI[d]	Ions	RI[d]	Ions
12	2.7	$^{12}C^+$		
14			3.8	$(^{14}N^{14}N)^{2+}$, $^{14}N^+$
16	6.1	$^{16}O^+$	5.2	$^{16}O^+$
22	0.9	$(^{12}C^{16}O^{16}O)^{2+}$		
28	11.0	$^{12}C^{16}O^+$	10.8	$^{14}N^{14}N^+$
29	0.1	$^{13}C^{16}O^+$		
30			31.1	$^{14}N^{16}O^+$
44	100.0	$^{12}C^{16}O^{16}O^+$	100.0	$^{14}N^{14}N^{16}O^+$
45	1.1	$^{13}C^{16}O^{16}O^+$, $^{12}C^{16}O^{17}O^+$	0.9	$^{14}N^{15}N^{16}O^+$, $^{14}N^{14}N^{17}O^+$
46	0.3	$^{12}C^{16}O^{18}O^+$	0.2	$^{14}N^{14}N^{18}O^+$

[a] m/z, mass to charge ratio.

[b] Calculated contribution of CO_2 to the mass spectrum of the mixture based upon spectra obtained using authentic CO_2.

[c] Contribution of N_2O obtained by subtraction of the CO_2 contribution from the spectrum of the mixture.

[d] RI, relative intensity (expressed as a percentage of the m/z 44 peak).

Production of N_2O from NO_3^- by a wide variety of microorganisms, including many dissimilatory nitrate-reducing bacteria (25) and several assimilatory nitrate-reducing fungi and bacteria (4), has been demonstrated. A review of the literature revealed only one report of N_2O production by higher plants (4). Bleakley and Tiedje (4) observed N_2O production by excised shoots of a number of plant species; however, these workers suggested that N_2O production was from microbial sources in the plant material because N_2O production was observed only after long incubations (6–10 h), and because production was inhibited by chloramphenicol. The possibility that microorganisms in the soybean leaves were responsible for the observed N_2O production reported here cannot be completely discounted; however, these studies were performed using short incubation times (1 h) and fresh soybean leaves with no visible indications of microbial growth. Bleakley and Tiedje (4) did not detect N_2O production from spinach leaves vacuum-infiltrated with unbuffered 5 mM KNO_3; however, considerably less plant material was used than in the work reported here. It would be expected that NO_3^- reduction by the material used (4) was minimal (the spinach used was obtained from local markets and stored, whereas NR activity declines rapidly during storage [10]; K-phosphate buffer [pH 7.5] and 50–200 mM NO_3^- are generally employed for optimal NR activity in higher plants [10]).

Fraction IV (compounds volatile above −78°C) contained the N compound(s) detected by the preoxidizer/Griess-Saltzman method. The finding that the compound(s) evolved is markedly less volatile at cryogenic temperatures than NO (see 5) is further evidence that the compound detected by Harper and co-workers (12, 22, 26) is not NO. Examination of fraction IV preparations by MS showed that this fraction contained three compounds: H_2O and two organic compounds, each having a mol wt of 59 amu. A series of analyses over time by the preoxidizer/Griess-Saltzman method and by MS of aliquots of fraction IV preparations (incubated at 22°C for up to 1 week prior to fractional distillation) showed that one of the organic compounds was produced in the preparation at the expense of the N compound initially detected by the preoxidizer/Griess-Saltzman method. These findings indicate that the N compound evolved from the *in vivo* NR assay isomerized during sample manipulation. The isomerization of the compound initially evolved was complete in less than 1 week of incubation at 22°C. The mass spectrum of the isomerization product (Fig. 3) is characteristic of that obtained for authentic acetamide using this instrument and operating conditions (peaks 16 through 20 are omitted from Fig. 3 because the fraction contained a substantial amount of H_2O). The peaks at m/z 15, 28, 44, and 59 indicate the principal ions produced are CH_3^+, CO^+, $CONH_2^+$, and $CH_3CONH_2^+$. Further support for identification of the isomerization product as acetamide is given in Figure 4, which shows the UV absorption characteristics of the isomerization product and authentic acetamide (aqueous solutions) are identical. Ammonium was produced when the isomerization product was subjected to acidic hydrolysis. Tests using 1 M acetamide solution (30°C) purged with He showed that this compound yields only trace responses in the preoxidizer/Griess-Saltzman system.

The finding that acetamide is the isomerization product of the

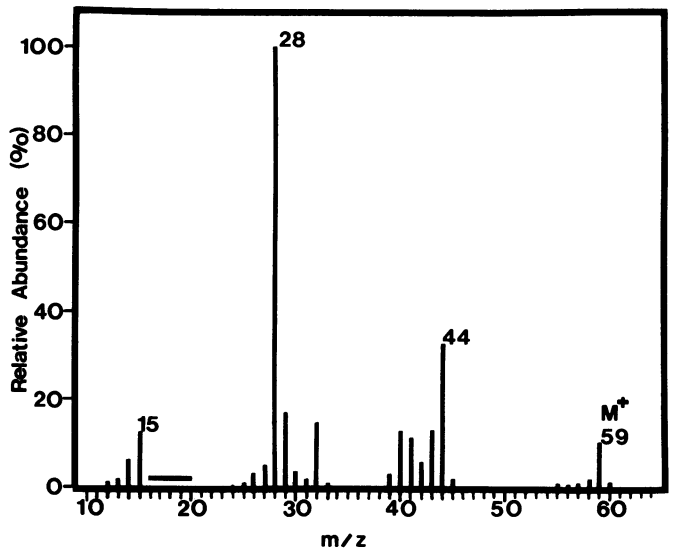

FIG. 3. Mass spectrum of fraction IV. Fraction IV was obtained by fractional distillation of the material retained in vacuum trap B and contained compounds volatile above −78°C. The volatile compounds trapped were incubated at 22°C for 1 week prior to distillation. Peaks at m/z 16 through 20 are omitted because water was present in fraction IV. (Ionization potential, 70 ev; acceleration potential, 2.4 kv; ion source, 65°C; inlet, 45°C.)

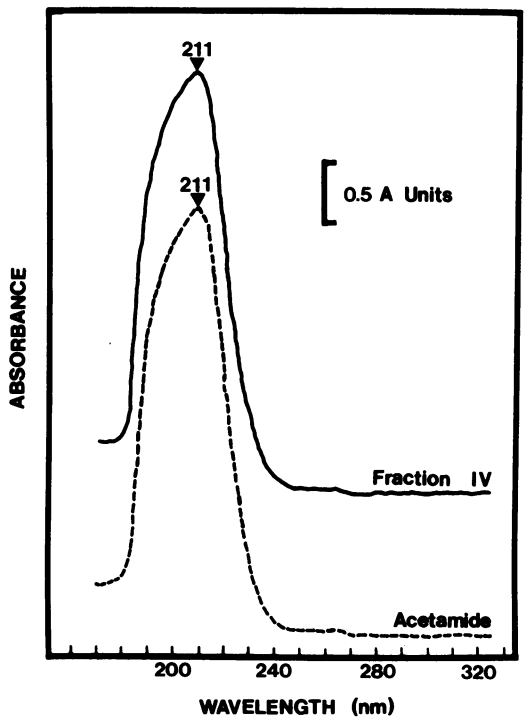

FIG. 4. UV absorption spectra of authentic acetamide (- - -) in H_2O and of fraction IV (——) in H_2O. Other details as in Figure 3 and "Materials and Methods."

N compound evolved from the *in vivo* NR assay and detected by the preoxidizer/Griess-Saltzman method indicates the molecular formula of the compound evolved is C_2H_5NO. A number of compound classes, most notably oximes, nitrones, and oxaziridines, undergo rearrangement reactions to produce amides (2, 27, 30–32); however, of the possible structures with the formula C_2H_5NO, only CH_3CH:NOH (acetaldehyde oxime) undergoes rearrangement to $CH_3(CO)NH_2$ (see 8, 30). Further evidence that the compound evolved from the *in vivo* NR assay is acetaldehyde oxime is provided in Figure 5, which shows the N compound evolved and retained in trap C (under conditions expected to minimize further reactions of the compound) had UV absorption characteristics identical to those of authentic acetaldehyde oxime (in phosphate buffer). Moreover, NH_2OH was produced when the compound (from trap C) was subjected to hydrolytic conditions, and this is a characteristic of oximes (32). Hydroxylamine was detected by two procedures (14, 18). A mean molar absorptivity at 196 nm (ϵ_{196}) of 5010 $M^{-1} \cdot cm^{-1}$ was calculated from the acetaldehyde oxime content and A_{196} of the solution from trap C, and is similar to molar absorptivity values reported in the literature for acetaldehyde oxime (9). Tests using 0.5 mM to 0.1 M acetaldehyde oxime solutions (30°C; in 0.1 M K_2HPO_4-KH_2PO_4, pH 7.5) purged with He showed that this compound is readily detected by the preoxidizer/Griess-Saltzman method, and yields only trace responses when the preoxidizer is omitted.

The Beckmann rearrangement of oximes to amides is generally catalyzed by acidic agents (8, 30). Mixtures of CO_2 and small amounts of H_2O were found to greatly promote rearrangement of authentic acetaldehyde oxime to acetamide in the apparatus used for cryogenic trapping and fractional distillation. Significant quantities of CO_2 were trapped along with the oxime in vacuum trap B during the *in vivo* NR assay, and it seems very likely that this CO_2 (presumably via H_2CO_3) was responsible for the rapid isomerization of the N compound during sample manipulation.

Estimation of Acetaldehyde Oxime Production. Substantial quantities of acetaldehyde oxime were produced during the He-purged *in vivo* NR assay (Table III). The rate of (CH_3CH:NOH)-

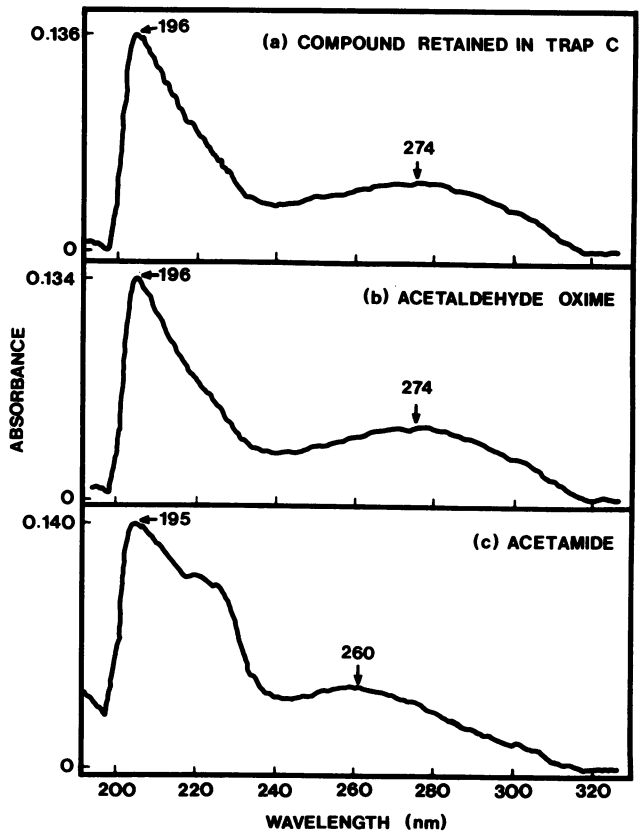

FIG. 5. UV absorption spectra of: (a) the volatile compound produced during the *in vivo* NR assay and retained in trap C (0.1 M K_2HPO_4-KH_2PO_4, pH 7.5); (b) authentic acetaldehyde oxime in 0.1 M K_2HPO_4-KH_2PO_4 (pH 7.5); and (c) authentic acetamide in 0.1 M K_2HPO_4-KH_2PO_4 (pH 7.5).

Table III. *Production of Acetaldehyde Oxime by Soybean Leaf Discs during the He-Purged + NO_3^- In Vivo NR Assay*

The *in vivo* assay medium and leaf discs from fully expanded unifoliolate leaves were purged with He (250 ml·min^{-1}). The acetaldehyde oxime evolved was trapped in vacuum traps A and B (held at −80°C and −196°C, respectively) for 1 h. Acetaldehyde oxime remaining in the leaf material and assay medium was also estimated.

Component	CH_3CH:NOH Produced[a]
	μmol·g^{-1} fresh wt·h^{-1}
Vacuum traps A + B	16.2 ± 1.0[b]
Remainder in leaf discs + medium	6.7 ± 0.3[c]

[a] Values reported as the mean (of three replicates) ± SD.

[b] The contents of vacuum traps A and B were incubated at 22°C for 1 week to ensure complete isomerization of the oxime to acetamide, which was determined as described in "Materials and Methods."

[c] Leaf discs plus medium were frozen (−80°C) and thawed three times to increase permeability of the leaf material. Acetaldehyde oxime was determined as free NH_2OH (18) after mildly acidic hydrolysis (35).

N evolution observed is higher (by almost 1.4 times) than the $NO_{(x)}$-N evolution rates reported by Harper (12) and Nelson *et al.* (22) for unifoliolate leaf sections of two soybean cultivars (Wells and Williams) under similar assay conditions. Because these workers did not employ a correction factor for conversion of $NO_{(x)}$ to NO_2^- in their preoxidizer/Griess-Saltzman system, the reported rates of $NO_{(x)}$ evolution would be underestimates. It is very likely that variations in plant material, bubbler design, temperature, flow rate of the purging gas, and other operating conditions will have substantial effects on the evolution rates obtained. It is noteworthy that the amount of of the oxime produced is roughly comparable to, or greater than, the amount of NO_2^- accumulated in the medium during the *in vivo* assay (see 12). Product removal (of oxime) by purging the assay medium would be expected to increase acetaldehyde oxime production.

Production of Acetaldehyde Oxime during Nitrate Reduction. [^{15}N]Acetaldehyde oxime purged from the +$^{15}NO_3^-$ *in vivo* NR assay medium, trapped cryogenically, and rearranged to [^{15}N] acetamide using CO_2 was found to be enriched with 43.4 atom % ^{15}N (SD = 2.2), which demonstrates that the oximino N was derived from NO_3^-. Several early studies reported oxime production from NO_2^- during nitrate assimilation and oximino N incorporation into protein by several bacteria, fungi, and higher plants (16, 33). An organic pathway of nitrate assimilation via NH_2OH and oximes was hypothesized (33). During the early 1960s, support for this organic assimilation pathway diminished because enzyme preparations active in oximino acid reduction to amino acids were not obtained, and because observed incorporation rates for oximino N into amino acids and proteins were significantly lower than those obtained from the inorganic nitrate assimilation pathway (via NH_3). Because NH_2OH condenses readily with carbonyl groups to produce oximes, Virtanen (36) proposed that oximes function as trapping agents that prevent NH_2OH accumulation when the assimilation pathway is not functioning optimally. During the 1950s and 1960s, enzyme preparations from higher plants and a *Scenedesmus* sp. which transferred oximino groups from donor oximes to acceptor carbonyl compounds (transoximases) were described (24, 37); however, no recent work regarding these enzymes appears in the literature. It has been reported (23) that when acetaldehyde was added as the acceptor carbonyl, acetaldehyde oxime was synthesized by nonspecific animal transoximase preparations. We are aware of no reports of acetaldehyde oxime production by plant materials. More recently, aldoximes have been shown to be intermediates in the conversion of amino acids to nitriles, cyanogenic glucosides, nitro compounds, and glucosinolates (19). It is reasonable that the accumulation of glycolytic products and NO_2^- associated with anaerobic conditions would promote acetaldehyde oxime production during the He-purged *in vivo* NR assay. This is supported by Harper's finding (12) that $NO_{(x)}$ production was 2- to 3-fold lower when the assay was purged with air than with N_2. However, we did not measure acetaldehyde oxime production under aerobic conditions.

Preliminary Work Using Maize. Studies of the volatile products of the +NO_3^- *in vivo* NR assay of maize [*Zea mays* L. hybrids B73 × Mol7, Pioneer 3382, and FS854] leaves indicate that evolution of N compounds during the assay, as determined by the preoxidizer/Griess-Saltzman method (12), is low (mean rates ranging from 0.10 to 0.16 μg N·g^{-1} fresh weight·30 min^{-1} as a function of genotype) compared with soybean. Attempts to identify the N compound(s) evolved by MS using collection and fractional distillation procedures developed using soybean leaves were unsuccessful because fraction IV preparations obtained from maize leaves were more complex than those obtained from soybean. Work is in progress to further separate these mixtures by GC for subsequent characterization by MS.

Conclusion. Acetaldehyde oxime and N_2O were identified as two new products of nitrate reduction and assimilation during the dark anaerobic +NO_3^- *in vivo* NR assay of young soybean leaves. Attempts to confirm production of NO during the assay by MS failed; however, rates of acetaldehyde oxime production observed (Table III) and the reactions of the oxime in the preoxidizer/Griess-Saltzman system would account for the $NO_{(x)}$ evolution rates reported by Harper and co-workers (12, 22). The possibility cannot be completely discounted that other N compounds detectable by the preoxidizer/Griess-Saltzman method are evolved during the *in vivo* NR assay of soybean leaves. However, comparison of the acetaldehyde oxime evolution rates obtained (Table III) with (a) rates obtained using the preoxidizer/Griess-Saltzman method (12, 22) and (b) rates obtained in preliminary chemical trapping studies (total N content of the trapping solutions was determined) indicate that acetaldehyde oxime is the predominant N product, and that evolution rates of other N compounds, if produced, would be low. Because acetaldehyde oxime-N is derived from nitrate, the magnitude of production of the oxime during the *in vivo* assay clearly demonstrates that NR activities determined from NO_2^- accumulation in the *in vivo* assay of young soybean leaves will be low, particularly if the assay medium is purged (as also noted by Harper [12] from $NO_{(x)}$ assays). Nitrous oxide production does not apparently significantly influence the accuracy of the *in vivo* NR assay for soybean. Conversely, evolution of N compounds detected by the preoxidizer/Griess-Saltzman method (12) during the +NO_3^- *in vivo* NR assay of maize leaves is low and does not significantly affect the reliability of the assay. The magnitude of acetaldehyde oxime production during the *in vivo* NR assay of soybean leaves and the finding by Harper and co-workers (22, 26) that production of this compound is associated with a constitutive NR activity suggests that the role of acetaldehyde oxime in nitrate assimilation by soybean warrants clarification.

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