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Gas Chromatography-Mass Spectrometry of N-Heptafluorobutyryl Isobutyl Esters of Amino Acids in the Analysis of the Kinetics of $[^{15}N]H_4^+$ Assimilation in *Lemna minor* L.

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ABSTRACT

Rapid, sensitive, and selective methods for the determination of the ^{15}N abundance of amino acids in isotopic tracer experiments with plant tissues are described and discussed. Methodology has been directly tested in an analysis of the kinetics of $[^{15}N]H_4^+$ assimilation in *Lemna minor* L. The techniques utilize gas chromatography-mass spectrometry selected ion monitoring of major fragments containing the N moiety of *N*-heptafluorobutyryl isobutyl esters of amino acids. The ratio of selected ion pairs at the characteristic retention time of each amino acid derivative can be used to calculate ^{15}N abundance with an accuracy of ±1 atom % excess ^{15}N using samples containing as little as 30 picomoles of individual amino acids. Up to 11 individual amino acid derivatives can be selectively monitored in a single chromatogram of 30 minutes. It is suggested that these techniques will be useful in situations where the small quantities of N available for analysis have hitherto hindered the use of ^{15}N-labeled precursors.

There is a need for more detailed studies of the flow of N in the plant. Questions remain concerning the relative importance of primary ammonia-assimilation pathways (24), the pathways of utilization of the various N sources arriving in the growing leaf (2), and the mechanisms and significance of amino acid accumulation in response to environmental stress (31). One approach to these questions is through examination of the use of ^{15}N-labeled precursors (1, 2, 4, 8, 12, 24, 28, 29, 32, 33). However, ^{15}N labeling studies with plant tissues are hindered by the lack of sensitivity inherent in traditional MS and optical emission spectrometry methods (6, 9, 13, 14, 34) and by the complexities of the endogenous pools of amino acids which lead to problems in the interpretation of isotopic labeling kinetics in the absence of mathematical models (24, 29). A consequence of these combined constraints is that relatively few ^{15}N experiments have been performed with the precision necessary to yield definitive conclusions regarding precursor-product relationships, rates of synthesis and utilization, and metabolic compartmentation of the components of the pathways of amino acid biosynthesis in plant cells (18). In this paper, we specifically address the problem of limitations of sensitivity in methods for the determination of the ^{15}N abundance of amino acids in isotopic tracer experiments with plant tissues.

Since the pioneering work of Rittenberg (25), the mass spectrometric determination of the ^{15}N abundance of amino acids has routinely involved the following: (a) the isolation of individual amino acids by ion-exchange chromatography (29) or preparative high-voltage electrophoresis and paper chromatography (2); (b) quantitative conversion of the amino and amide groups to N_2 in evacuated vessels (23, 25); and (c) introduction of N_2 to the mass spectrometer for ^{15}N analysis by monitoring the ratios of ions 28, 29, and 30 (25, 29). Careful corrections for traces of residual N_2 in the mass spectrometer and for atmospheric N_2 as a contaminant of the sample introduced for ^{15}N analysis are required. Consequently, samples of less than 10 μg of N cannot be subjected to ^{15}N analysis with any degree of accuracy. Quantities of N in excess of 0.3 mg are typically required. These traditional methods are, thus, time-consuming when applied to a large number of amino acids in several independent extracts, and they become technically demanding when dealing with amino acid constituents present at relatively low levels in plant tissues.

Optical emission spectrometry has been more recently developed as an alternative method for ^{15}N analysis of plant tissues (6, 7, 9, 13, 14, 21, 22, 34). Although this method is generally more sensitive than MS, when dealing with samples of less than 0.2 μg N, helium and xenon are required to 'plug' the inner surface of the quartz vessel to prevent N absorption and to sustain N discharge (6, 34). The technique requires combustion of chromatographically isolated samples to N_2 at 850°C in vacuum with copper oxide and a highly activated calcium oxide briquet (6). Again, contamination of the sample by atmospheric N_2 and N contamination from reagents are of critical importance (9, 13, 21). Neither MS nor emission spectrometry methods appear to be suitable for the determination of the ^{15}N abundance of nmol quantities of amino acids in complex mixtures extracted from plant tissues.

N-HFBI[1] esters of amino acids can be formed quantitatively in a relatively simple two-stage derivatization procedure, and complex mixtures of these amino acid derivatives can be reproducibly separated with high resolution by GLC (15, 16). The question arises as to whether GC-MS of these volatile derivatives could be adapted as a rapid, automated, sensitive, and selective method for the determination of the ^{15}N abundance of small quantities of amino acids with the minimum of prepurification. GC-MS of volatile derivatives of ^{15}N-labeled amino acids has recently been applied to studies of the release and refixation of ammonia during photorespiration in spinach (36), to the flux of alanine and glycine in man (10), to $[^{15}N]O_3^-$ assimilation in seedlings (26), and to aspartate and glutamate analysis in urine (27). In this paper, we evaluate the feasibility of this type of approach using automated selected ion monitoring of *N*-HFBI derivatives. The methodology has been directly tested in an analysis of $[^{15}N]H_4^+$ assimilation in *Lemna minor*.

MATERIALS AND METHODS

Organism and Growth Conditions. Plants of *L. minor* L. were grown on the basal medium described by Stewart (30), with 2 mM

[1] Abbreviations: *N*-HFBI, *N*-heptafluorobutyryl isobutyl.

KNO_3 as nitrogen source in 250-ml Pyrex conical flasks containing 50 ml growth medium. Flasks were innoculated with about 50 mg fresh weight of plant tissue and were grown for 4 to 5 days at 21°C at a light intensity of 1,200 ft-c provided by a bank of fluorescent lights. At midexponential growth phase (0.25–0.3 g fresh weight of tissue/flask), 20 flasks were harvested and washed with 1 L N-free medium at 21°C. Fronds were resuspended in 2 L N-free growth medium at 21°C at the same light intensity. The growth vessel consisted of a 46- × 37-cm polypropylene tub (white) with medium at a depth of 1.5 cm stirred by the action of two magnetic stir bars. After 3 h of N starvation, $[^{15}N]H_4Cl$ (99 atom %) (Merck) was added to the growth medium to give a final concentration of 0.17 mM, and the plants were allowed to assimilate the new N supply for a further 4 h. Samples of 10 ml medium were removed for ammonia determination, and samples of plant tissue were removed at intervals for analysis of soluble N pools and total N content.

Extraction of Soluble Nitrogen Pools. Samples of 0.3 to 0.8 g fresh weight of plant tissue were harvested using a wire mesh strainer, washed with H_2O, blotted dry, weighed, and extracted in 10 ml methanol. After storage for 48 h at 4°C, the residual plant material was removed by filtration, and the filtrate was phase-separated by addition of 2 ml methanol, 6 ml H_2O, and 7 ml chloroform. The upper aqueous layer was rotary evaporated to dryness under vacuum at 30°C and dissolved in 1 ml H_2O.

Determination and Isolation of Ammonia. Ammonia was determined in the aqueous extracts and in the growth medium by the phenol-hypochlorite reaction (35). Ammonia was isolated from the aqueous extracts by Dowex-HCRW-2 Na^+ (J. T. Baker (Chemical Co., Phillipsburg, NJ) ion-exchange chromatography (24). The 1-ml samples of the aqueous extracts were applied to 4.5- × 1.0-cm columns of resin (20–50 mesh) equilibrated with H_2O. Neutral and acidic amino acids were eluted with 6 ml H_2O, and ammonia was recovered by eluting with 40 ml 0.2 M Na_2HPO_4/NaH_2PO_4 buffer (pH 7.5). The ammonia was subsequently distilled in a Labconco steam distillation apparatus (Labconco Corp., Kansas City, MO) and was collected as 25 ml distillate in 2 ml 0.2 M HCl following addition of 8 ml 2 M NaOH. The NH_4Cl recovered was rotary evaporated to dryness at 30°C and redissolved in 1 ml H_2O. Ammonia in the growth medium was directly steam distilled by addition of 8 ml 2 M NaOH to 10 ml medium and was similarly recovered as 1-ml samples of NH_4Cl in H_2O.

Determination of Total Nitrogen of Plant Material. Samples of 0.1 to 0.2 g fresh weight of plant tissue were washed with H_2O, blotted dry, weighed, and dissolved in 2 ml concentrated H_2SO_4 containing 1 g salicylic acid per 40 ml H_2SO_4 (13) in 30 ml Kjeldahl flasks. After 1 h at room temperature, 0.25 g sodium thiosulfate was added to each sample, and heat was applied for 10 to 15 min until frothing ceased. Digestion was completed following the procedures of Nichols *et al.* (19) by addition of 4 ml 5 M H_2SO_4 containing 20 g/L $CuSO_4 \cdot 5H_2O$ and 20 g/L sodium selenate and application of heat until decolorization of the samples occurred. After cooling, the digested material was diluted to 50 ml with H_2O, and 5-ml aliquots were subjected to steam distillation by addition of 20 ml 2 M NaOH. Ammonia was collected as 25 ml distillate in 2 ml 0.2 M HCl and was concentrated to 1 ml by rotary evaporation. Total N as NH_4^+ was determined by the phenol-hypochlorite reaction (35).

Isolation of Glutamate and Aspartate. Glutamate and aspartate were isolated from the neutral and acidic amino acid fraction (following removal of NH_4^+ from the aqueous extracts) by Dowex-1-acetate ion-exchange chromatography (28). The neutral and acidic amino acid fraction was applied to a 2- × 1-cm column of the resin (200–400 mesh) equilibrated with H_2O. Neutral amino acids and amides were collected by washing with 5 ml H_2O, and glutamate and aspartate were jointly eluted with 20 ml 2 M acetic acid. The latter fraction was rotary evaporated to dryness at 30°C and dissolved in 1 ml 60% methanol.

Purification of Neutral Amino Acids and Amides. The neutral amino acid/amide fraction from Dowex-1-acetate chromatography was rotary evaporated to dryness at 30°C and redissolved in 1 ml H_2O. Samples of 0.4 ml were applied to 1- × 1-cm columns of Dowex-50W H^+ (200–400 mesh) equilibrated with H_2O. Columns were washed with 10 ml H_2O, and the neutral amino acids and amides were jointly eluted with 6 ml 6 M NH_4OH. Each sample was rotary evaporated to dryness and dissolved in 0.4 ml 60% methanol.

Conversion of Ammonia to Glutamate. Purified samples of ammonia derived from steam distillation of the ammonia pool of the cell, the growth medium, or total N digests were neutralized to pH 7.0 by addition of small volumes (20–50 μl) of 200 mM Tris. To 1-ml (approximate) samples of neutralized ammonium were added 0.2 ml α-ketoglutarate (0.292 g of the free acid per 21 ml of 200 mM Tris, final pH 7.3), 0.1 ml NADH (20 mg of NADH per 1.2 ml of 100 mM Tris-acetate, pH 7.3), and 0.1 ml glutamate dehydrogenase (EC 1.4.1.3 type III from bovine liver [Sigma]; 400 units per 1.2 ml 100 mM Tris-acetate, pH 7.3). Samples were incubated at 30°C for 3 h and then applied to 1- × 1-cm columns of Dowex-50W H^+, as described above, for neutral amino acids. Glutamate was recovered by elution with 6 ml 6 M NH_4OH and was concentrated to 0.4 ml in 60% methanol. These same procedures were used for the synthesis and purification of glutamate from $[^{14}N]H_4Cl$ (0.37 atom % ^{15}N natural abundance) and $[^{15}N]$-H_4Cl (99 atom % ^{15}N) standards.

Derivatization of Amino Acids. Procedures similar to those described by MacKenzie and Tenaschuk (15, 16) were used. Aliquots of 0.1 to 0.4 ml of the amino acids in 60% methanol were placed in 1-ml microreaction vessels and were evaporated to dryness under a stream of compressed air dried over a column of Drierite (W. A. Hammond Drierite Co., Xenia, OH) at room temperature. The samples were further dried with 2- × 100-μl aliquots of methylene chloride. Aliquots of 0.1 ml of a freshly prepared solution of isobutanol-HCl (0.4 ml of acetyl chloride was mixed with 2 ml ice-cold isobutanol in a sealed vial at 4°C with continuous stirring) were added to each vial, and vials were sealed with cap and Teflon coated septum, vigorously mixed, sonicated for 30 s at 22°C, and then heated under reflux in a heating block at 120°C for 20 min. After cooling, excess isobutanol-HCl was evaporated under a stream of compressed, dried air, and 50 μl of heptafluorobutyric anhydride was added. The vials were again sealed with fresh septa and heated at 110°C for 10 min. After cooling, the samples were evaporated to incipient dryness under a stream of dry air and were finally dissolved in 20 μl of ethyl acetate:acetic anhydride (1:1, v/v). Routinely, 1-μl injections were utilized for the determination of the levels of individual amino acids by GLC, and the 20-μl samples were routinely diluted to 420 μl with ethyl acetate:acetic anhydride (1:1, v/v) for GC-MS analysis of 2-μl aliquots (see below).

Quantitation of Amino Acids by GLC. The levels of the individual amino acids in the acidic and neutral amino acid/amide fractions of the plant extracts were quantitated by coderivatization of 0.37 μmol pipecolic acid as internal standard with the aliquots of the amino acids. Samples of 1 μl of the final amino acid derivatives in ethyl acetate:acetic anhydride (1:1, v/v) were subjected to GLC using a Varian model 3700 gas chromatograph equipped with a fused silica capillary column of SE30 (12 m × 0.2 mm) (J and W Scientific, Rancho Cordova, CA). The split ratio at the injector port was 99:1 with a column pressure of 10 p.s.i. He carrier gas, 30 ml/min He sweep gas at the detector (flame ionization), 300 ml/min air, and 40 ml/min H_2 at the detector. Column temperature program conditions were 90°C for 4 min to 250°C at 4°C/min. Injector temperature was 280°C, detector temperature was 280°C, and attenuation was routinely maintained

in the range 16 to 64 × 10^{-12} amp/millivolt. Peak areas were determined by an interface of the gas chromatograph with a HP3354 data system via a HP18652A A/D converter (Hewlett-Packard) and a Teletype model 43 (Continental Resources, Santa Clara, CA). Peak areas were related to the area of the internal standard (pipecolic acid), and the response factors of each amino acid derivative were determined from GLC of *N*-HFBI esters of an amino acid standard mixture (Sigma) (see Refs. 15 and 16 for details of typical response factors and retention times of *N*-HFBI derivatives of amino acids).

GC-MS of *N*-HFBI Esters of Amino Acids. GC-MS of the amino acid derivatives was performed on a model HP5985A GC-MS system (Hewlett-Packard) in electron impact mode. The GC-MS system was interfaced with a HP21MX E computer equipped with a HP7900 disc drive, a Tektronix terminal (Tektronix, Irvine, CA), and Tektronix hard copy unit. The source temperature was routinely maintained at 200°C and ion energy at 70 electron volt. All analyses were performed on a 2-m × 2-mm glass column of 3% OV-101 with He carrier gas at 40 ml/min. Temperature programs varied with the type of sample. For samples containing only the glutamate derivative of interest, the temperature program used was 150°C to 300°C at 5°C/min. For samples containing only glutamate and aspartate derivatives of interest, the temperature program was 130°C to 300°C at 10°C/min. For neutral amino acid/amide fractions, the chromatographic parameters used were 100°C for 13 min to 300°C at 5°C/min. The injector temperature was maintained at 250°C for all applications. Interface temperature was maintained at 275°C. In extracted ion current profile mode, complete scans over the mass range 50 to 500 were performed throughout the chromatogram every 2 to 3 s. Relevant data concerning the major fragments containing the N moiety of individual amino acid derivatives were obtained from this type of analysis by subsequent retrieval of the intensity of individual ions from full scan spectra stored on magnetic disc. In selected ion monitoring mode, a group of four ions could be monitored at any one time in the chromatogram with scans of these masses every 200 milliseconds. A maximum of five mass groups each containing four selected ions could be programmed for each chromatogram. This mode proved particularly useful for automation of the monitoring of ion pairs associated with fragmentation of individual amino acids at their characteristic retention times. Injection was controlled by an HP7671A autosampler (Hewlett-Packard). Automatic data acquisition and output of the areas of each of the ions in the five mass groups monitored during the chromatogram were achieved by selected ion monitoring software (see Fig. 6 for details of selected ion monitoring parameters used).

RESULTS

GC-MS of *N*-HFBI Glutamate. To evaluate the potential of GC-MS of volatile derivatives of amino acids as a method for the determination of ^{15}N abundance, a comparative analysis was performed with *N*-HFBI esters of glutamate synthesized from either $[^{14}N]H_4^+$ (0.37 atom % ^{15}N natural abundance) or $[^{15}N]H_4^+$ (99 atom % ^{15}N) by the glutamate dehydrogenase reaction. Samples of 1 nmol of the glutamate derivatives were subjected to GC-MS, and mass spectra of the two glutamate derivatives differing in ^{15}N abundance are illustrated in Figure 1. Major ion fragments containing the N moiety were observed at m/e 252.1, 280.1, and 298.1 for $[^{14}N]$HFBI glutamate (Fig. 1A). These fragments were shifted by precisely + 1 mass unit in the $[^{15}N]$HFBI glutamate sample (Fig. 1B). Extracted ion current profiles of the *N*-HFBI glutamate samples are shown in Figure 2 for ions 280.1 and 281.1. A marked shift in the ratio of these ions is clearly associated with ^{15}N substitution. The relative area counts of these closely associated ion pairs can, thus, be used to calculate the ^{15}N abundance of glutamate derivatives from the following equation, provided that

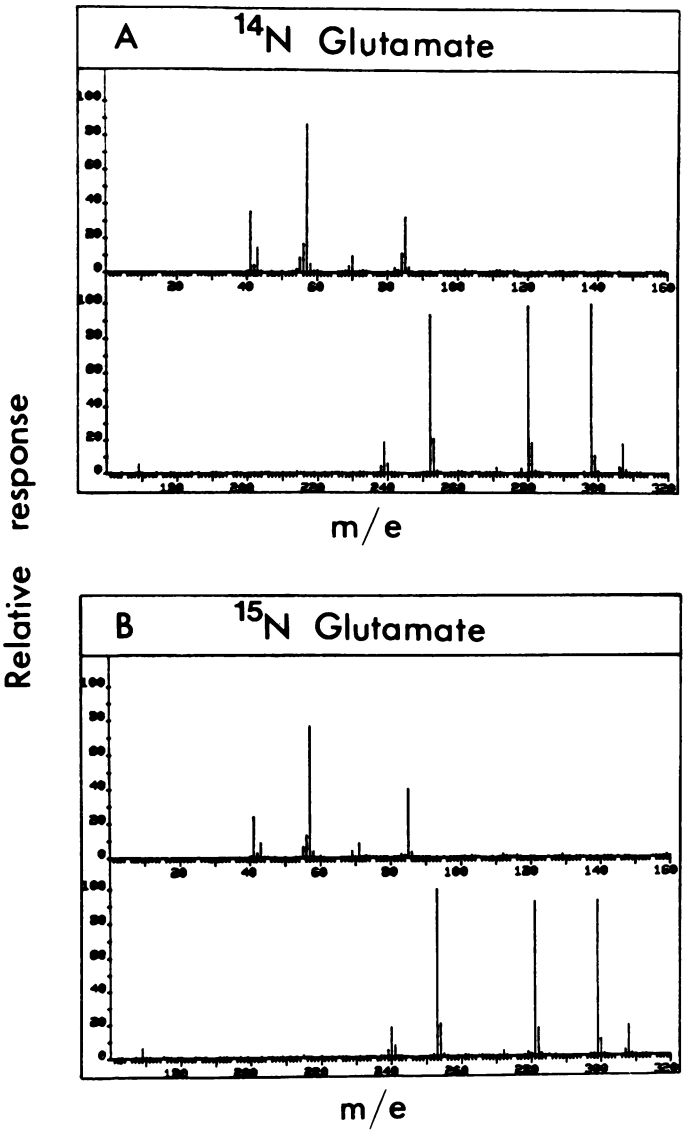

FIG. 1. Mass spectra of the *N*-HFBI derivatives of $[^{14}N]$- and $[^{15}N]$glutamate. Samples of 1.0 nmol of the *N*-HFBI esters of $[^{14}N]$glutamate (A) synthesized from 0.37 atom % $[^{15}N]H_4Cl$ and $[^{15}N]$glutamate (B) synthesized from 99 atom % $[^{15}N]H_4Cl$ were subjected to electron impact GC-MS on a column of 3% OV-101 temperature-programmed from 150°C to 300°C at 5°C/min. Mass spectra of $[^{14}N]$glutamate (A) and $[^{15}N]$-glutamate (B) derivatives were obtained by retrieval of data from magnetic disc following total ion chromatography. The retention time of the derivative of glutamate was 5.7 min.

the ratio of these closely associated ion pairs is known for $[^{14}N]$HFBI glutamate (0.37 atom % ^{15}N natural abundance):

$$^{15}\text{N abundance (atom percent)} = \frac{(Z \times 100)}{(Z + Y)} + 0.37$$

where

$$Z = \left(100 - \frac{[X \times Y]}{100}\right)$$

and

$$X = \frac{\text{Area of ion 281.1}}{\text{Area of ion 280.1}} \times 100 \text{ for } [^{14}N]\text{HFBI glutamate standard}$$

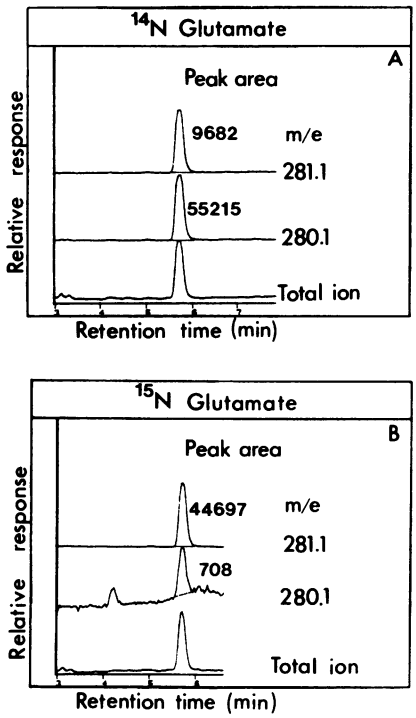

FIG. 2. Extracted ion current profiles of the *N*-HFBI derivatives of [^{14}N]- and [^{15}N]glutamate. The intensity of ions 280.1 and 281.1 was extracted from magnetic disc following total ion chromatography of [^{14}N]- and [^{15}N]HFBI glutamate. A, ions 280.1 and 281.1 arising from fragmentation of [^{14}N]glutamate (0.37 atom % ^{15}N). B, ions 280.1 and 281.1 arising from fragmentation of [^{15}N]glutamate (99 atom % ^{15}N). Peak areas of ions 280.1 and 281.1 are shown.

and

$$Y = \frac{\text{Area of ion 280.1}}{\text{Area of ion 281.1}} \times 100 \text{ for [}^{15}\text{N]HFBI glutamate unknown}$$

Similar calculations can be performed from the ion ratios, 298.1:299.1 or 252.1:253.1, for the derivative of glutamate, and, hence, three independent measurements of ^{15}N abundance can be derived from a single total ion chromatogram. Note that the ions directly preceding these mass pairs are of very low intensity (Fig. 1A). Table I shows a statistical analysis of the ratios of ions 280.1, 281.1, 298.1, and 299.1 for [^{14}N]HFBI glutamate (0.37 atom % ^{15}N) and [^{15}N]HFBI glutamate (99 atom % ^{15}N) in several independent chromatograms of samples containing 0.1 to 1.0 nmol. Computations of the ^{15}N abundance of the [^{15}N]HFBI glutamate sample synthesized from 99 atom % [^{15}N]H_4^+ from the data of Table I using the equation above yielded an observed ^{15}N abundance of 98.72 ± 0.2% from the ion ratio 280.1:281.1 and a ^{15}N abundance of 98.9 ± 0.1% from the ion ratio 298.1:299.1.

These results indicated sufficient precision and sensitivity in the techniques to warrant an extension of these basic principles to other amino acids and to merit practical applications in the analysis of isotopic labeling in plant tissues.

Assimilation of [^{15}N]H_4^+ by *L. minor*.

Incorporation of [^{15}N] into the Free Ammonia Pool and Total Nitrogen. Plants of *L. minor* in exponential growth on 2 mM KNO_3 as N source were transferred to 2,000 ml of fresh, N-free growth medium for 3 h and then supplied for a further 4 h with [^{15}N]H_4Cl (99 atom %) at an initial concentration of 0.17 mM. Samples were removed at intervals for analysis of the ammonia content of the medium, the size of the soluble N pool of the plant material, and the total N content of the plant tissue. A balance sheet of the fresh weight of tissue in the growth vessel, the ammonia content of the

Table I. *Statistical Analysis of the Ratios of Specific Ion Pairs Associated with the Fragmentation of the N-HFBI Esters of [^{14}N]- and [^{15}N]-Glutamate*

GC-MS conditions were as described in Figures 1 and 2. Four independent analyses of 0.1- to 1.0-nmol samples of the glutamate derivatives monitored the ratios of ions 280.1 and 281.1 alone. Five more samples were analyzed simultaneously for the ion ratios 280.1:281.1 and 298.1:299.1 in successive chromatograms.

Sample	Ratio (m/e) Monitored	Mean	SD	No. of Samples
		%		
[^{14}N]glutamate				
0.37 atom % ^{15}N	$\frac{281.1}{280.1} \times 100$	16.56	0.794	9
0.37 atom % ^{15}N	$\frac{299.1}{298.1} \times 100$	10.28	0.294	5
[^{15}N]glutamate				
99 atom % ^{15}N	$\frac{280.1}{281.1} \times 100$	1.666	0.259	9
99 atom % ^{15}N	$\frac{298.1}{299.1} \times 100$	1.386	0.109	5

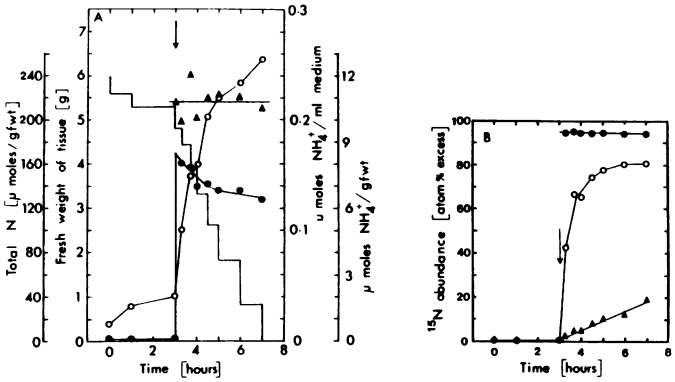

FIG. 3. Incorporation of ^{15}N into the free ammonia pool and total N of *L. minor*. A, The ammonia content of the growth medium (●), free ammonia pool of the cell (○), total nitrogen content of the plant material (▲), and total fresh weight of tissue in the growth vessel (stepped line, declining due to sample removal) are shown for the time course of the labeling experiment. [^{15}N]H_4Cl (99 atom %) was supplied at a concentration of 0.17 mM at 3 h (arrow). B, The ^{15}N abundance of the ammonia of the growth medium (●), the free ammonia pool of the cell (○), and total N content of the plant material (▲) was determined as described in the text.

growth medium, the intracellular ammonia pool, and total N content of the plant throughout the time-course of the experiment is shown in Figure 3A.

Ammonia derived from the growth medium, intracellular ammonia pool, and total N digests was isolated by ion-exchange chromatography and/or steam distillation and was ultimately converted to glutamate by the glutamate dehydrogenase reaction *in vitro*. The *N*-HFBI esters of glutamate were then subjected to GC-MS, as described above, for standard glutamate samples. The ^{15}N abundance of the growth medium ammonia, intracellular ammonia pool, and total N was, thus, derived from measurements of the ion ratios 280.1:281.1 and 298.1:299.1 (Fig. 3B). A comparison of the rate of depletion of the NH_4^+ from the medium per unit fresh weight of tissue in the growth vessel (Fig. 3A), with the rate of incorporation of ^{15}N into the total N of the plant material (Fig. 3B), provided a quantitative test of the method for ^{15}N analysis. Good agreement between the rate of NH_4^+ depletion from the medium (10–12 μmol/h·g fresh weight) and the rate of

incorporation of ^{15}N into total N (11 μmol/h·g fresh weight) was obtained.

Determination of the ^{15}N Abundance of Glutamate and Aspartate. Glutamate and aspartate pools were isolated from the aqueous extracts by Dowex-1-acetate ion-exchange chromatography, and the mixture of these acidic amino acids was derivatized and subjected to GC-MS. Figure 4 illustrates typical extracted ion current profiles of aspartate and glutamate pools at the time of addition of $[^{15}N]H_4^+$ (t = 3 h) (Fig. 4A) and after 4-h assimilation (t = 7 h) (Fig. 4B). A substantial shift in the ratio of closely associated ion pairs (284.1 and 285.1 for aspartate and 298.1 and 299.1 for glutamate) was observed to be associated with $[^{15}N]H_4^+$ assimilation. Figure 5A illustrates the changes in the mean ion ratios of the glutamate and aspartate derivatives during the time course of the experiment relative to the mean ratios of $[^{14}N]$HFBI aspartate and $[^{14}N]$HFBI glutamate standards (0.37 atom % ^{15}N). The ^{15}N abundance of glutamate and aspartate derived from these ratios is shown, together with measurements of the pool sizes of these amino acids during the time course of the experiment (Fig. 5B). The ^{15}N abundance of glutamate reached 75 atom % excess after 4-h assimilation (t = 7 h), and that of aspartate reached 73 atom % excess after 4-h assimilation. A transient accumulation of aspartate and, particularly, glutamate was observed immediately following addition of $[^{15}N]H_4^+$ to the growth medium (Fig. 5B).

Determination of the $[^{15}N]$ Abundance of Neutral Amino Acids and the Amino-Nitrogen Groups of Asparagine and Glutamine. After isolation of free ammonia, glutamate, and aspartate from the aqueous extract, the neutral amino acid fraction was purified by Dowex-50H$^+$ ion-exchange chromatography and derivatized as the *N*-HFBI esters. Taking advantage of the unique retention times of individual amino acid derivatives (15, 16) and making use of the selected ion-monitoring capabilities of the GC-MS, we were able to derive the ^{15}N abundance of alanine, glycine, valine, threonine, serine, leucine, isoleucine, proline, asparagine-amino N, phenylalanine, and glutamine-amino N sequentially in a single chromatogram of each extract using automatic injection and automatic data acquisition capabilities of the GC-MS.

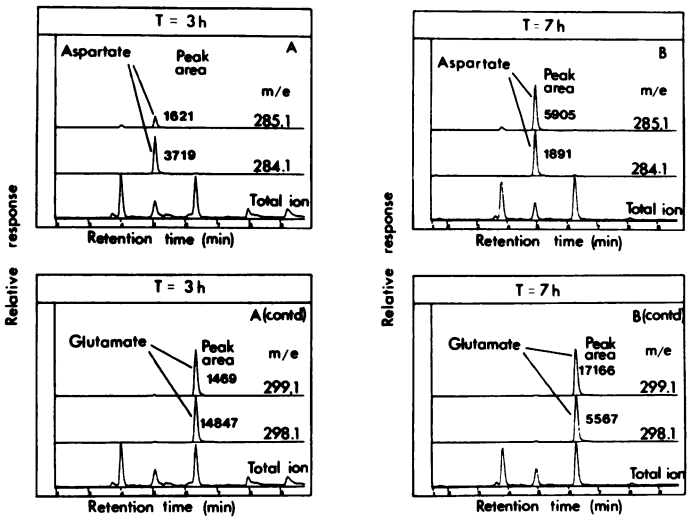

FIG. 4. Extracted ion current profiles of the derivatives of the acidic amino acid fraction of *L. minor*. Extracted ion chromatograms of *N*-HFBI derivatives of the acidic amino acid fraction of *L. minor* before addition of $[^{15}N]H_4^+$ to the growth medium (t = 3 h) (A) and after 4-h $[^{15}N]H_4^+$ assimilation (t = 7 h) (B) are shown. The aspartate derivative (retention time, 5.1 min) exhibited major ion fragments containing the N moiety at m/e 284.1 and 285.1. The glutamate derivative (retention time, 6.3 min) exhibited major ion fragments containing the N moiety at m/e 298.1 and 299.1. The compound chromatographing at retention time = 3.9 – 4.1 min was identified as the heptafluorobutyryl isobutyl derivative of malic acid. Peak areas for the relevant ions are shown. Temperature program conditions were 130°C to 300°C at 5°C/min.

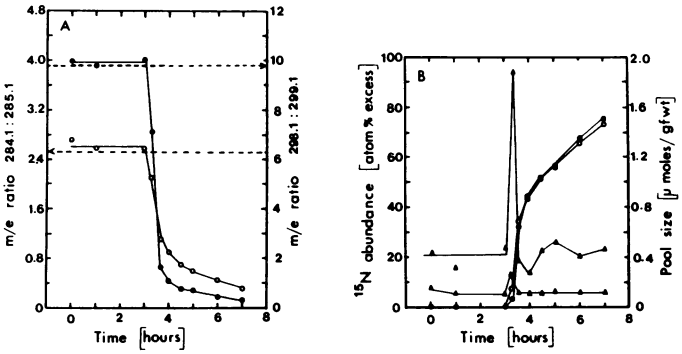

FIG. 5. Ratios of specific ion pairs associated with fragmentation of the *N*-HFBI esters of aspartate and glutamate and calculations of pool sizes and ^{15}N abundance of these amino acids in *L. minor*. A, Extracted ion current profiles of the derivatives of aspartate (ions 284.1 and 285.1) and glutamate (ions 298.1 and 299.1) were used to determine the ratios of these ion pairs, as described in Figure 4 legend, (○, 284.1:285.1; ●, 298.1:299.1). These ratios for the time course of the experiment are related to the mean ratios of standard $[^{14}N]$aspartate and $[^{14}N]$glutamate derivatives (0.37 atom % 15N) (- - -). B, Computations of the ^{15}N abundance of aspartate (○) and glutamate (●) were derived from the ion ratios shown in Figure 5A, using the equation described in the text. Pool sizes of aspartate (△) and glutamate (▲) were determined by quantitative GLC using pipecolic acid as internal standard, as described in "Materials and Methods."

Chromatography was initially standardized with injections of the derivatives of an amino acid standard mixture containing 125 pmol of each amino acid. In selected ion-monitoring mode, a series of five mass groups, each containing four ions, was programmed to monitor specifically key fragment pairs containing the N moiety at the characteristic retention times of the amino acids. Examples of two selected ion chromatograms from a single injection of the amino acid standard mixture are illustrated in Figure 6. Mass group 2 (Fig. 6A) shows ions 268 and 269 arising from fragmentation of *N*-HFBI valine and ions 253 and 254 arising from fragmentation of *N*-HFBI threonine. Mass group 3 (Fig. 6B) shows ion 239 arising from fragmentation of *N*-HFBI serine and ions 282 and 283 arising from leucine and isoleucine. The relative response from ion 240 of *N*-HFBI serine appeared diminished due to normalization on the higher level of the 240 ion present in *N*-HFBI leucine. Theoretically, it also was possible to derive relevant isotopic data for hydroxyproline, γ-aminobutyrate, cysteine, methionine, cystine, lysine, tyrosine, arginine, and histidine in complex amino acid mixtures using this type of analysis. However, the levels of the latter amino acids in the free amino acid pool of *L. minor* were extremely low (less than 10 nmol/g fresh weight) and did not merit special considerations for these components in the present analysis.

The ratios of selected ion pairs associated with each of the amino acids monitored in the neutral/amide fraction of the amino acid pool of *L. minor* are summarized with the mean ratios and standard deviations observed in five independent chromatograms of $[^{14}N]$amino acid standards (Table II). Calculations of the ^{15}N abundance of the amino acids based on these ion ratios are summarized with determinations of the pool sizes of each component (Table III).

Although both $[^{14}N]$HFBI glutamate and $[^{14}N]$HFBI aspartate do not give rise to major ion fragments at the m/e value directly preceding the doublet monitored (*i.e.* at m/e 297 and 283, respectively), this is not the case for certain other amino acid derivatives. Table IV shows the relative intensities of the ions preceding the mass pair monitored for each $[^{14}N]$amino acid standard. Notably,

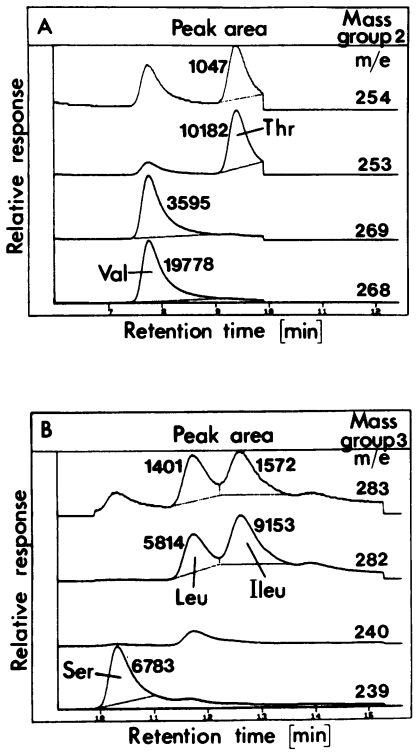

FIG. 6. Selected ion monitoring of complex mixtures of amino acid derivatives. Two typical selected ion chromatograms of the *N*-HFBI derivatives of an amino acid standard mixture containing 125 pmol of each of the following amino acids are shown: alanine, RT (retention time) = 4.0 min; glycine, RT = 4.5 min; valine, RT = 7.7 min; threonine, RT = 9.4 min; serine, RT = 10.3 min; leucine, RT = 11.7 min; isoleucine, RT = 12.6 min; proline, RT = 17.3 min; aspartate, RT = 23.9 min; phenylalanine, RT = 24.8 min; and glutamate, RT = 26.9 min. Temperature program conditions were 100°C for 13 min to 300°C at 5°C/min. Selected ion monitoring parameters were as follows: mass group 1 (3.0–5.8 min), ion pair 240, 241 = alanine and ion pair 226, 227 = glycine; mass group 2 (5.8–9.9 min), ion pair 268, 269 = valine and ion pair 253, 254 = threonine; mass group 3 (9.9–15.4 min), ion pair 239, 240 = serine and ion pair 282, 283 = leucine and isoleucine; mass group 4 (15.4–24.4 min), ion pair 266, 267 = proline and ion pair 284, 285 = aspartate or asparagine-amino N; mass group 5 (24.4–35.0 min), ion pair 316, 317 = phenylalanine and ion pair 298, 299 = glutamate or glutamine-amino N. A, Mass group 2; B, mass group 3. Peak areas are shown. All amino acids were assumed to be at natural ^{15}N abundance (0.37 atom % ^{15}N).

the derivatives of threonine and serine gave rise to major fragments at m/e 252 and 238, respectively. With ^{15}N substitution, the contribution of these preceding ions to the ratios monitored would lead to an underestimation of ^{15}N abundance if these ratios were applied directly to the equation described earlier. A computer program was used to generate theoretical relationships between ^{15}N abundance and the ratio of the ion pair monitored, taking into account the contribution from these preceding ions (Table IV). Table III shows ^{15}N abundance values computed with these corrections.

Glutamine-amino N and alanine were rapidly labeled, achieving 75 atom % excess ^{15}N after 4-h assimilation (t = 7 h). The pool of glutamine increased markedly in response to NH_4^+ supply. Other neutral amino acids labeled less rapidly: glycine achieved 48.1 atom % excess ^{15}N; serine, 59.2%; phenylalanine, 46.6%; proline, 36.7%; valine, 31%; leucine and isoleucine, 22%; asparagine-amino N, 20.5%; and threonine, 22%; after 4-h assimilation. Several of the amino acids monitored (proline, leucine, and isoleucine) were present at levels of less than 30 nmol/g fresh weight, and, hence, at levels of less than 30 pmol in the samples used for ^{15}N analysis.

Determination of the ^{15}N Abundance of Amino Acid Standards. As a final test of the method, a series of amino acid standards were prepared by mixing known quantities of 0.37 atom % [^{15}N]glycine, aspartate, and glutamate with known quantities of 99 atom % [^{15}N]glycine and aspartate and 95 atom % [^{15}N]glutamate to give a series of samples with ^{15}N abundance ranging from 0 to 98.6 atom % excess at approximately 10% intervals. Ratios of ion pairs (226:227 for glycine, 284:285 for aspartate, and 298:299 for glutamate) were measured using selected ion monitoring, as described above, with injections of 125 pmol of each amino acid derivative. The observed *versus* predicted ^{15}N abundances of the samples calculated from these specific ion ratios are shown in Figure 7. Linear regression analysis of observed *versus* expected ^{15}N abundance revealed a slope of 0.998, an intercept of −0.12%, and a correlation coefficient of 0.999. The combined error due to dilution of standards and measurement of ^{15}N abundance never exceeded 2.1 atom % excess. These results support the view that the ^{15}N analyses reported here for *L. minor* are accurate to within ±1 atom % ^{15}N excess. This agrees favorably with the results of Samukawa *et al.* (27), who have recently shown that low concentrations of ^{15}N in *N*-trifluoroacetyl butyl esters can be measured with a relative standard deviation of <0.6%.

DISCUSSION

The objective of the present study has been to evaluate the potential of an alternative mass spectrometric technique for the determination of the ^{15}N abundance of amino acids during isotopic tracer experiments with plant tissues. We have attempted to overcome the need for extensive purification of amino acids by ion-exchange chromatography (29), high voltage electrophoresis, and paper chromatography (2), or TLC (34), and to overcome the need for conversion of each nitrogen moiety to N_2 gas for ^{15}N analysis (7, 9, 23, 25). The latter techniques, using the more sensitive method of optical emission spectrometry, are, at best, limited to samples containing at least 0.1 μg N (34). The present investigations have illustrated the potential to derive the ^{15}N abundance of up to 11 individual amino acids simultaneously in a single chromatogram of 30 to 35 min, detecting small changes in the ratios of key ion fragments of *N*-HFBI esters present at levels of less than 30 pmol (0.0005 μg of N). The ability to automate sample injection, selected ion monitoring, and data acquisition, with a sensitivity 100- to 200-fold greater than with optical emission spectrometry and with a precision of ±1 atom % ^{15}N excess, clearly offers powerful analytical capabilities. Therefore, we suggest that the techniques reported here (see Refs. 10, 26, 27, 36) provide considerable potential in rapid analysis of the ^{15}N composition of amino acid mixtures and that these techniques should be particularly useful in situations where the small quantities of nitrogen available for analysis have hitherto hindered the use of ^{15}N-labeled precursors. The method seems to be particularly suited to rapid analysis of the ^{15}N composition of trace levels of N in the xylem and phloem stream in studies of the transport of nitrogenous solutes around the plant (20).

A major precaution to be taken in the use of these techniques is the concomitant appearance of interfering masses. Problems of this nature can be identified readily by the discrepency between the selected ion pair ratios of [^{14}N]amino acid standards and those of the [^{14}N]amino acids extracted from the plant material during standardization of purification methods. The ion-exchange purification methods and GC-MS conditions utilized here have eliminated problems of cochromatography, coderivatization, and cofragmentation of other metabolites with the amino acids of *L. minor*. Potential users of these techniques should also be aware of systematic errors in GC-MS isotope ratio measurements discussed by Matthews and Hayes (17).

Table II. *Summary of Ratios of Peak Areas of Selected Ion Fragments of N-HFBI Esters of Amino Acid Standards and the Amino Acids of the Neutral/Amide Fraction of L. minor*

GC-MS conditions were as described in Figure 6. Mean ratios and SDs of ^{14}N amino acid standards were derived from five independent analyses of an amino acid standard mixture containing 125 pmol of each amino acid derivative per injection.

Amino Acid as Derivatives	Retention Time	Ratio (m/e) Monitored	Mean m/e Ratio for ^{14}N Standard	SD	Ratio (m/e) of Samples Isolated from *L. minor*						
					t = 3.33 h	t = 3.66 h	t = 4.0 h	t = 4.5 h	t = 5.0 h	t = 6.0 h	t = 7.0 h
	min										
Alanine	4.0	240:241	2.702	0.145	1.610	1.067	0.761	0.569	0.483	0.414	0.295
Glycine	4.5	226:227	1.679	0.054	1.539	1.359	1.046	0.925	0.722	0.665	0.657
Valine	7.7	268:269	5.233	0.270	5.024	4.259	3.356	2.800	2.406	1.889	1.573
Threonine	9.4	253:254	9.614	0.265	8.309	7.379	6.363	4.944	4.165	2.654	2.987
Serine	10.3	239:240	10.995	0.394	6.612	2.653	1.764	1.539	1.449	1.188	1.186
Leucine	11.7	282:283	4.498	0.251	3.822	3.735	3.296	2.818	2.536	2.408	1.985
Isoleucine	12.6	282:283	6.155	0.326	5.414	5.372	4.803	3.639	3.021	3.482	2.263
Proline	17.3	266:267	9.575	0.358	7.893	6.445	4.411	3.238	2.607	1.832	1.472
Asparagine-amino N	23.9	284:285	2.360	0.134	2.300	2.239	2.234	2.024	1.962	1.706	1.482
Phenylalanine	24.8	316:317	6.866	0.473	4.578	4.614	3.711	2.863	1.347	1.773	1.093
Glutamine-amino N	26.9	298:299	9.639	0.378	2.373	1.448	0.998	0.681	0.494	0.404	0.314

Table III. *Summary of Calculated ^{15}N Abundances and Pool Sizes of Amino Acids in the Neutral/Amide Amino Acid Fraction of L. minor*

Data was derived from Table II and quantitative GLC of the amino acid derivatives using pipecolic acid as internal standard (see "Materials and Methods"). See text for details of method of calculation of isotopic abundance.

Amino Acid	^{15}N Abundance						
	t = 3.33 h	t = 3.66 h	t = 4.0 h	t = 4.5 h	t = 5.0 h	t = 6.0 h	t = 7.0 h
	atom % excess						
Alanine	20.20 (75)[a]	36.30 (105)	48.80 (152)	58.40 (163)	63.25 (277)	67.50 (221)	75.55 (166)
Glycine	5.10 (86)	12.29 (102)	26.49 (94)	32.65 (44)	44.10 (75)	47.59 (80)	48.10 (68)
Valine	0.79 (145)	4.20 (152)	9.70 (137)	14.35 (140)	18.43 (154)	25.47 (74)	31.00 (58)
Threonine	1.73 (146)	3.31 (137)	5.51 (115)	9.96 (96)	13.59 (93)	25.71 (97)	22.19 (95)
Serine	6.19 (580)	26.70 (305)	41.30 (251)	47.05 (213)	49.70 (315)	59.11 (325)	59.20 (386)
Leucine	3.81 (26)	4.36 (25)	7.54 (17)	11.79 (9)	14.79 (14)	16.30 (15)	22.15 (14)
Isoleucine	2.16 (16)	2.33 (12)	4.39 (14)	10.15 (12)	14.50 (12)	11.15 (13)	21.95 (10)
Proline	2.18 (35)	4.83 (32)	10.98 (35)	17.01 (26)	21.93 (34)	30.82 (28)	36.77 (38)
Asparagine-amino N	1.12 (1,492)	2.29 (1,450)	2.38 (955)	6.70 (1,045)	8.05 (1,076)	14.25 (963)	20.50 (1,171)
Phenylalanine	6.95 (55)	6.81 (83)	11.35 (74)	17.57 (62)	39.75 (94)	31.10 (84)	46.57 (89)
Glutamine-amino N	24.13 (184)	37.00 (391)	47.40 (755)	57.80 (1,564)	65.89 (1,536)	70.49 (1,792)	75.65 (2,646)

[a] Numbers in parentheses, pool sizes (nmol/g fresh weight).

In the present studies, we have not determined the ^{15}N abundance of the amide groups of glutamine and asparagine. Theoretically, each of these amide groups can be liberated as NH_4^+ by incubation with glutaminase and asparaginase, respectively (24), the NH_4^+ isolated by Dowex-HCRW-2-Na^+ chromatography and steam distillation, and the NH_4^+ then converted to glutamate by the glutamate dehydrogenase reaction for ^{15}N analysis by GC-MS. In practice, however, this approach demanded substantially more nitrogen than was available in the plant extracts. At least 500 nmol were required in order to achieve good recovery of NH_4^+ in the steam distillation apparatus without substantial isotope dilution from traces of ammonia in the buffers involved at various stages in the enzyme incubations and purifications. This represents a limitation of the purification methods rather than a limitation in the sensitivity of the mass spectrometer. The use of micro-steam distillation apparatus and micro-ion-exchange columns for purification of the ammonia derived from the amide groups might overcome some of these constraints. Where the amide groups are to be monitored, we recommend scaling up the quantity of plant tissue to be extracted.

Table IV. *Computations of Relationship between ^{15}N Abundance and Ion Pair ratio of N-HFBI Amino Acids Correcting for Contribution from the Ion Directly Preceding the Ion Pair Selectively Monitored*

Full scan spectra of ^{14}N amino acid standard mixtures as *N*-HFBI derivatives were used to derive the relative intensities of the ions directly preceding the ion pairs selectively monitored in Table II. An iterative computer program was used to generate the theoretical relationships between ^{15}N abundance and ion pair ratio for each amino acid derivative, taking into account the contribution from the preceding ion. Values of ion ratios are shown at 20% intervals of ^{15}N abundance.

^{14}N Amino Acid Derivative	Relative Intensity of Preceding Ion	Relative Intensities of Selected Ion Pairs		Computed Ratio of ^{15}N Abundance (Atom % Excess)					
				0	20	40	60	80	100
	m − *1*	*m*	*m* + *1*	*m*/(*m* + *1*)					
Alanine (m = 240)	0.663	100	37.009	2.70	1.61	0.97	0.54	0.23	0.01
Glycine (m = 226)	0	100	59.568	1.68	1.18	0.79	0.48	0.22	0
Valine (m = 268)	1.71	100	19.111	5.23	2.28	1.18	0.61	0.25	0.02
Threonine (m = 253)	56.30	100	10.402	9.61	3.22	1.78	1.15	0.79	0.56
Serine (m = 239)	57.10	100	9.095	10.99	3.35	1.82	1.17	0.80	0.57
Leucine (m = 282)	2.33	100	22.23	4.50	2.13	1.14	0.60	0.26	0.02
Isoleucine (m = 282)	1.79	100	16.248	6.15	2.43	1.22	0.62	0.26	0.02
Proline (m = 266)	1.66	100	10.444	9.57	2.83	1.31	0.64	0.26	0.02
Aspartate (m = 284)	4.05	100	42.366	2.36	1.50	0.94	0.55	0.26	0.04
Phenylalanine (m = 316)	12.90	100	14.566	6.87	2.61	1.34	0.73	0.37	0.13
Glutamate (m = 298)	0.28	100	10.375	9.64	2.83	1.30	0.63	0.25	0.003

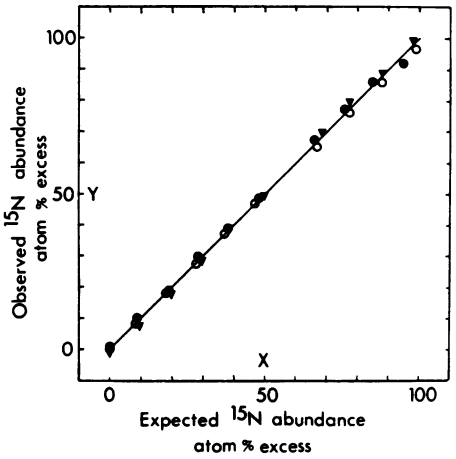

FIG. 7. Comparison of observed *versus* expected ^{15}N abundance of a series of ^{15}N amino acid standards. Known quantities (determined by the ninhydrin reaction [28]) of 0.37 atom % [^{15}N]glycine, aspartate, and glutamate were mixed with known quantities of 99 atom % [^{15}N]glycine, 99 atom % [^{15}N]aspartate, and 95 atom % [^{15}N]glutamate (Merck) to give 10 samples containing glycine, aspartate, and glutamate with expected ^{15}N abundance ranging from 0 to 98.6 atom % excess ^{15}N at approximately 10% intervals. The mixtures were derivatized as the *N*-HFBI esters and subjected to GC-MS using automated selected ion monitoring, as described in Figure 6 legend, with injections of 125 pmol of each amino acid derivative. The ion ratios 226:227 for glycine, 284:285 for aspartate, and 298:299 for glutamate were used to calculate the ^{15}N abundance of these amino acid mixtures (▼, glycine; ○, aspartate; ●, glutamate). Observed ^{15}N abundance (Y) was related to expected ^{15}N abundance (X), using least square linear regression analysis of Y on X. The equation of best fit is represented by the line drawn ($Y = 0.998X - 0.119$; correlation coefficient [r] = 0.999).

Hydrolysis of the amide groups of glutamine and asparagine occurs during derivatization, and, thus, glutamine cochromatographs with glutamate and asparagine with aspartate, yielding isotopic data only for the amino groups. Separation of glutamine from glutamate and asparagine from aspartate is essential in order to discriminate among the amino groups of these components.

In the present studies of [^{15}N]H_4^+ assimilation in *L. minor*, we have not attempted a detailed analysis of the isotopic labeling kinetics. In view of the complexities of the amino acid pools of *L. minor* under steady-state growth conditions (24), an adequate consideration of the implications of the present isotopic labeling kinetics during non-steady-state ammonia assimilation would demand the development of complex computer models. Such an approach is beyond the scope of the objectives of the present paper. However, certain features of the results are worthy of brief notes.

(a) The ^{15}N abundance of the intracellular ammonia pool did not achieve the level of the external ammonia supply but, rather, saturated at an abundance of 80% ^{15}N. This indicates either the occurrence of a storage pool of NH_4^+ in the cell or the occurrence of pathways for the release of relatively unlabeled NH_4^+ within the tissue. It is possible that protein turnover was activated by the period of N starvation prior to ^{15}N supply and that this protein N was specifically mobilized by the photorespiratory N cycle yielding [^{14}N]H_4^+ (5, 18, 36). Appreciable input of relatively unlabeled amino nitrogen into glycine is indicated by the observation that glycine achieved apparent saturation of ^{15}N abundance at a level of 44 to 48 atom % excess.

(b) The isotopic labeling of glutamine-amino N and glutamate was virtually identical throughout the time course of incorporation, suggesting that, under conditions of prior N starvation, the subsequent assimilation of NH_4^+ occurs via a pathway which involves a rapid equilibrium between the amino groups of glutamine and glutamate, namely the glutamate synthase cycle (18, 24). Surprisingly, after 0.33 h of ^{15}N assimilation (t = 3.33 h), the label in glutamate was far less than that in glutamine-amino N, aspartate, or alanine. This appears to be inconsistent with the role of glutamate as precursor to the latter amino acids, but this observation can be reconciled if it is assumed that there is compartmentation of glutamate pools within the cell (24).

(c) The labeling of aspartate suggests that there is rapid transamination between glutamate and aspartate at the primary site of ammonia assimilation, presumably the chloroplast (18, 24). The labeling of aspartate was more than sufficient to accommodate the labeling of asparagine-amino N and threonine, assuming that aspartate is the primary N donor for these N groups (3, 11).

Future application of this method, in conjunction with computer simulation (24), should be useful in elucidating the precise contri-

bution of the photorespiratory N cycle to N turnover in plants (5, 18, 36) and in developing models to account for the complex labeling kinetics observed in tracer experiments with higher plant tissues.

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