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Review

# The in vivo nitrogen isotope discrimination among organic plant compounds

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**Abstract**

The bulk $\delta^{15}$N-value of plant (leaf) biomass is determined by that of the inorganic primary nitrogen sources $NO_3^-$, $NH_4^+$ and $N_2$, and by isotope discriminations on their uptake or assimilation. $NH_4^+$ from these is transferred into "organic N" mainly by the glutamine synthetase reaction. The involved kinetic nitrogen isotope effect does not become manifest, because the turnover is quantitative. From the product glutamine any further conversion proceeds in a "closed system", where kinetic isotope effects become only efficient in connection with metabolic branching. The central and most important corresponding process is the GOGAT-reaction, involved in the de novo nitrogen binding and in recycling processes like the phenylpropanoid biosynthesis and photorespiration. The reaction yields relatively $^{15}$N-depleted glutamate and remaining glutamine, source of $^{15}$N-enriched amide-N in heteroaromatic compounds. Glutamate provides nitrogen for all amino acids and some other compounds with different $^{15}$N-abundances. An isotope equilibration is not connected to transamination; the relative $\delta^{15}$N-value of individual amino acids is determined by their metabolic tasks. Relative to the bulk $\delta^{15}$N-value of the plant cell, proteins are generally $^{15}$N-enriched, secondary products like chlorophyll, lipids, amino sugars and alkaloids are depleted in $^{15}$N. Global $\delta^{15}$N-values and $^{15}$N-patterns of compounds with several N-atoms can be calculated from those of their precursors and isotope discriminations in their biosyntheses.


*Keywords:* $\delta^{15}$N-Value; $^{15}$N-Kinetic isotope effect; In vivo nitrogen isotope discrimination; Biosynthesis; Nitrogen recycling; Nitrogen transport; Nitrogen pools; Natural compounds; Amino acids; GOGAT; $NH_4^+$; $NO_3^-$

**Contents**



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## 1. Introduction

Nitrogen is one of the essential main bioelements, indispensable for the structure and the function of proteins, glycoproteines, nucleic acids, and many other plant molecules. The macromolecules are nitrogen sinks containing nitrogen in the main binding types in organic natural compounds, namely amino/amide groups, and nitrogen in heteroaromatic rings, respectively. The relative proportion of nitrogen in the terrestrial live organic biosphere is only 1.5% of the total organic N on earth ($0.9 \times 10^{18}$g), which by itself is not more than 0.023% of the N-amount in atmospheric $N_2$ ($3.9 \times 10^{21}$g), the latter representing itself 4% of the total earth nitrogen ($10^{23}$g) (Stankiewicz and van Bergen, 1998). In contrast to its functional importance, in plants the relative amount of nitrogen is less than 1% of dry biomass. Nitrogen forms in contact with the biosphere are in the upper earth crust $NO_3^-$, (bound) $NH_4^+$ and degradation products of soil organic sediments, e.g. amino acids, polyamines and aminoglucans (Muzzarelli and Muzzarelli, 1998) and in the atmosphere $N_2$, $NH_3$ and $NO_x$ (Wirén et al., 1977). To be available for plants, the latter nitrogen sources must be converted (by microorganisms) into $NO_3^-$, $NH_4^+$ or special transport metabolites. On the other hand, nitrogen from excretion or decay products of plants and animals is converted, again by microorganisms, into the above mentioned organic and inorganic compounds. In some cases atmospheric $NO_x$ and $NH_3$ can directly and significantly contribute to the nitrogen economy of plants (Siegwolf et al., 2001; Schjoerring et al., 2000). The exploitation of primary sources of nitrogen by higher plants is therefore considerably more complicated than that of oxygen, which they can directly assimilate from the three primary sources $H_2O$, $CO_2$ and $O_2$ (Schmidt et al., 2001).

As nitrogen is often an essential growth limiting factor for plants, isotopic shifts in the nitrogen cycle have found large interest in context with questions of plant nutrition, plant physiology and ecology. Therefore, so far most studies on nitrogen uptake, transport and metabolism, on metabolic fluxes, and on the correlations of the main compartments in the global nitrogen cycle and on isotope discriminations implied in these processes, using measurements of natural $^{15}$N-abundances, have predominantly focused on the turnover and balance of main global nitrogen fractions (inorganic primary material, total plant material, inorganic/organic nitrogen in plants). Handley and Raven (1992) and Fogel and Cifuentes (1993) have compiled corresponding data, e.g. on $^{15}$N-discriminations connected to bacterial nitrification and denitrification, to in vivo nitrogen fixation, assimilation and transport of primary compounds. They also compiled data on kinetic isotope effects on some individual enzyme catalysed reactions involved, and discussed them in relation to metabolic correlations and metabolite transfers between the main nitrogen pools in the biosphere. Correspondingly, Kohl and Shearer (1995) gave an extensive report on the use of natural $^{15}$N-abundance variations for the investigation of N-cycle processes. Waser et al. (1998a, b) reported on nitrogen isotope fractionations accompanying the uptake of different nitrogen compounds by marine diatoms, Handley et al. (1999) discussed the importance of mycorrhiza for the nitrogen isotope discrimination in plant systems, and Uhle et al. (1997) used natural abundance nitrogen isotope ratio measurements for proof of nitrogen transfers in marine symbiotic systems. Several global metabolic flux system models have been conceived (Handley and Scrimgeour, 1997; Robinson et al., 1998; Hobbie et al., 1999b), based on data on natural nitrogen isotope abundances and their variations.

A recent short review on "Physiological mechanisms influencing plant nitrogen isotope composition" (Evans, 2001) mainly concentrates on isotope fractionations in context with nitrogen uptake and transport, but states that "future work must address acquisition of organic nitrogen from the soil, the role of mycorrhiza, and internal transformations within the plant". It is exactly the latter aspect, which is the centre of interest of the present contribution. First investigations on the nitrogen isotope signatures and correlations of individual nitrogen containing organic compounds from plants, and on the importance of defined kinetic isotope effects on reactions involved in their metabolism, were performed and discussed by

Yoneyama (1995) and Yoneyama et al. (1998a). In the same year Schmidt and Kexel (1998) correlated data on $\delta^{15}$N-values[1] of nitrogen containing natural compounds to nitrogen kinetic isotope effects on enzyme reactions implied in their biosynthesis, and thereby attempted to deduce from here the importance of organic nitrogen pools and their correlations in plants. Since then our understanding of the importance of different factors such as kinetic and thermodynamic isotope effects, reversibility and irreversibility of involved reactions, and of branching in metabolic systems for the in vivo isotope discriminations and on their interferences has greatly increased (Schmidt et al., 2001). We have therefore re-examined intermolecular and intramolecular nitrogen isotope distributions in natural organic compounds from plants in the light of this knowledge.

As the global $\delta^{15}$N-value of any plant biomass is primarily determined by that of the actual nitrogen source (see for example Denton et al., 2001), the comparability of compounds from different origins is quite difficult. Also only quite few examples exist with $\delta^{15}$N-data on different nitrogen containing substances from the same source, indispensable for intrinsic standardisation. This could partially be overcome by the "normalization" of the $^{15}$N-abundance of individual compounds relative to that of the $\delta^{15}$N-value of the bulk biomass or of proteins from the same origin, also by the implication of results from $^{15}$N-tracer experiments. Finally, as only a limited amount of monomer compounds with more than one nitrogen atom per molecule exists, and as from practically none of them $^{15}$N-patterns have been available, these were indirectly derived from their precursors and verified via the corresponding global $\delta^{15}$N-values. Only $\delta^{15}$N-data from products originating from plants and autotrophic microorganisms were used, because the origin of nitrogen in N-containing compounds from heterotrophs is depending on the individual nitrogen sources and nutrition conditions (Macko and Estep, 1984).

[1] [Note: $\delta^{15}N[‰]_{AIR\text{-}N_2} = \left[\frac{(^{15}N/^{14}N)_{Sample}}{(^{15}N/^{14}N)_{Air\text{-}N_2}} - 1\right] * 1000$ stable isotope abundances of bioelements are generally expressed as the relative difference of the isotope ratio of a compound to that of an international standard in ‰. The international standard for nitrogen isotope ratio measurements is: AIR-$N_2$ = Nitrogen gas originating from atmospheric air (Mariotti, 1983, 1984; Coplen et al., 1992) with an $^{15}N/^{14}N$ ratio of $(3678.2 \pm 1.5) \times 10^{-6}$ (De Bièvre et al., 1996). For more information about referencing strategies and techniques in stable isotope ratio measurements refer to e.g. Werner and Brand (2001). Measurement equipment is described in e.g. Brand et al. (1994) or Werner et al. (1999) and literature cited therein].

## 2. Results and discussion

### 2.1. Primary sources of nitrogen for higher plants and reactions involved in their conversion to organic nitrogen

The main assimilation forms of inorganic nitrogen by plant roots are nitrate and ammonium (Marschner, 1995); their uptake, transport, accumulation and excretion depends on individual conditions and mechanisms, and is submitted to regulation processes (Wirén et al., 1997; Evans, 2001). Nitrate is transported to the chloroplasts, where it is reduced by nitrate reductase and nitrite reductase to ammonia; in $C_4$-plants this is probably occurring only in the mesophyll cells (Richter, 1996). External ammonium is accumulated and transported by special carrier systems. Ammonia from the nitrogen assimilation by symbiotic bacteria is transported as $NH_4^+$ across the peribacteroid membrane (Tyerman et al., 1995); in some plant species the fixed nitrogen is transported preferably in form of alanine (Waters et al., 1998; Allaway et al., 2000), into the host interstitial cells, where it is bound either in form of glutamine or asparagine, or via the purine biosynthesis and degradation in allantoic acid and allantoine (ureides) as transport metabolites; sometimes also citrulline has this function (Wheeler et al., 1992). These compounds, either directly or after hydrolysis, provide nitrogen for biosyntheses to other plant cells. In legumes with symbiotic nitrogen assimilation the pathway of nitrogen to secondary plant products is therefore especially complex. Correspondingly, mycorrhiza provide nitrogen to higher plants already bound in organic form. In the chloroplasts, ammonia from different origins is bound by the glutamine synthetase reaction to the amide N-form, and from here by the reductive glutamine: 2-oxoglutarate aminotransferase-(GOGAT)-reaction into the α-amino group of glutamate, from where it is available by transamination for any other amino acid. Isoenzymes of glutamine synthetase and of GOGAT are present in chloroplasts and cytosol of higher plants and in the cytosol of nodules (Richter, 1996). Several N-transport and/or N-storage metabolites are in use by plants, often specific for special plant taxa, e.g. glutamine and asparagine (*Asparagus*), serine and arginine (Rosaceae, Saxifragaceae), citrulline (Betulaceae), canavanine (Leguminosae), allantoine and allantoic acid (*Acer*), urea (special fungi), ammonia salts of organic acids (*Rheum*) and diverse indol derivates like tyramine, serotonin (e.g. *Musa*, Sitte et al., 1998).

In any case, the main primary form of organic nitrogen in plants is amide-N in glutamine, the source of α-amino-N in amino acids and of nitrogen in heteroaromatic compounds. From here, any further nitrogen conversion and nitrogen isotope discrimination must occur as in a closed system [except for losses of $NH_3$ into the atmosphere (Harper and Sharpe, 1998) or in

Table 1
$^{15}$N-Kinetic isotope effects on enzyme catalysed reactions of importance in plant organic nitrogen metabolism. Enzymes from plants, where possible

| Reactions | Enzyme and source | $k_{14}/k_{15}$ | Reference |
|---|---|---|---|
| *Oxidoreductions* | | | |
| $NO_3^- + NADH/H^+ \rightarrow NO_2^- + NAD^+ + H_2O$ | Nitrate reductase from *Spinacera oleracea*<br>*Zea mays*/*Chlorella vulgaris* | 1.015<br>1.030 | Medina et al., 1982<br>Olleros-Izard, 1983 |
| $NH_4^+ + 2\text{-oxoglutarate} + NADPH/H^+ \rightarrow \text{glutamate} + NADP^+ + H_2O + H^+$ | Glutamate dehydrogenase from bovine liver | 1.018 (pH 5.8) | Weiss et al., 1988 |
| $NH_4^+ + \text{pyruvate} + NADH/H^+ \rightarrow \text{alanine} + NAD^+ + H_2O + H^+$ | Alanine dehydrogenase from *Bacillus subtilis* | 1.0106 (pH 7.1) | Weiss et al., 1988 |
| *Amidations and transaminations* | | | |
| $\text{Glutamate} + NH_3 + ATP \rightarrow \text{glutamine} + AMP + PP + H_2O$ | Glutamine synthetase from *Spinacera oleracea* | 1.0165[a] | Yoneyama et al., 1993 |
| Aspartate + glutamine + ATP → asparagine + glutamate + AMP + PP | Asparagine synthetase from *E. coli* | 1.0222 | Stoker et al., 1996 |
| $\text{Glutamine} + HCO_3^- + 2\ ATP \rightarrow \text{carbamyl}\sim P + 2\ ADP + P_i + \text{glutamate}$ | Carbamylphosphate synthetase from *E. coli* | 1.0217[b] | Rishavy et al., 2000 |
| *Transaminations* | | | |
| Glutamate + oxaloacetate → α-oxoglutarate + aspartate | Oxaloacetate:glutamate aminotransferase from porcine heart | 1.0083 | Macko et al., 1986 |
| Aspartate + α-oxoglutarate → glutamate + oxaloacetate | Oxaloacetate:glutamate aminotransferase from porcine heart | 1.0017 | Macko et al., 1986 |
| Aspartate + PLP-E → oxaloacetate + PNP-E[c] | Aspartate aminotransferase from *E. coli* | 1.0056 | Rishavy and Cleland, 2000 |
| *Lyase reactions* | | | |
| Phenylalanine → cinnamic acid + $NH_3$ | Phenylalanine ammonia lyase from *Rodotorula glutinus* | 1.0021 | Hermes et al., 1985 |
| 3-Methylaspartate → 3-methylfumarate + $NH_3$ | 3-Methylaspartate ammonia lyase from *Clostridium tetanomorphum* | 1.0246±0.0013 | Gani et al., 1999 |
| Aspartate → fumarate + $NH_3$ | 3-Methylaspartate ammonia lyase from *Clostridium tetanomorphum* | 1.0390±0.0031 | Gani et al., 1999 |
| Argininosuccinate → arginine + fumarate | Argininosuccinate lyase from bovine liver | 0.9964±0.0003 | Kim and Raushel, 1986 |
| Glutamate → γ-aminobutyrate + $CO_2$ | Glutamate decarboxylase from *E. coli* | 0.9855±0.0006[d] | Abell and O'Leary, 1988 |

(*continued*)

Table 1 (*continued*)

| Reactions | Enzyme and source | $k_{14}/k_{15}$ | Reference |
|---|---|---|---|
| *Hydrolyses* | | | |
| Benzoyl-L-argininamide + $H_2O$ → benzoylarginine + $NH_3$ | Papain from *Carica papaya* | 1.024 | O'Leary et al., 1974 |
| Adenosinemonophosphate + $H_2O$ → adenine + ribose-5-phosphate | AMP-nucleosidase from *Azotobacter vinelandii* | 1.034 … 1.021 | Parkin et al., 1991 |
| Arginine + $H_2O$ → ornithine + urea | Arginase from calf liver | 1.01 ± 0.001 | Medina et al., 1982 |
| Urea + $H_2O$ → 2 $NH_3$ + $CO_2$ | Urease from *Canavalia ensiformis* | 1.008 ± 0.0005 | Medina et al., 1982 |
| Adenosinemonophosphate + $H_2O$ → inosinemonophosphate + $NH_3$ | AMP-desaminase from *S. cerevisiae* | 1.010 ± 0.002 | Merkler et al., 1993 |

[a] Calculated from in vivo isotope fractionation.
[b] KIE value on hydrolysis of glutamine.
[c] First half-reaction of transamination; PLP-E = pyridoxalphosphate enzyme, PNP-E = pyridoxaminephosphate enzyme.
[d] Interpreted as isotope effect on the Schiff-base formation.

some cases loss of organic N in form of root exudates (Jones et al., 1994)], implying a strict isotope balance, where $\delta^{15}$N-values of minor products must be preferably affected. The main reactions involved in the subsequent primary and secondary plant nitrogen metabolism are reductions, amidations, transaminations, hydrolyses and lyase reactions, all of them implying the potential for nitrogen kinetic isotope effects. In vivo, these can only become manifest as nitrogen isotope discriminations and lead to intermolecular or intramolecular isotopic patterns under specific prerequisites in the cells, mainly concerning pools, metabolic fluxes and branching in the course of biosyntheses. The following investigations have for aim the study of these conditions and their influence on isotopic discriminations between organic compounds in the system plant.

### 2.2. In vitro $^{15}$N-kinetic isotope effects and in vivo nitrogen isotope discriminations

Kinetic isotope effects (KIEs) on the reactions in question have preferably been studied in context with the investigation of reaction mechanisms and related problems; therefore many detailed corresponding data are available. However, in the present context only representative examples have been compiled (Table 1) with possible connections to corresponding in vivo isotope discriminations (Handley and Raven, 1992) or with potential importance for observed intrinsic isotopic shifts between organic nitrogen compounds within the system plant.

Before studying these correlations, we have to remember some principal differences between in vivo and in vitro investigations and measurements of isotopic shifts. In vivo aspects concern mostly whole processes occurring between extrinsic and intrinsic metabolic pools and compartments. Typical for them is to study bulk shifts between the $\delta^{15}$N-values of a primary precursor from a large or infinite (extrinsic) pool and the global $^{15}$N-abundance of the biological system in question after an infinite time. The observed relative $^{15}$N-depletion of the final product (organic N) is described by an isotope discrimination factor, e.g. $\alpha = 1 + (\delta^{15}N_{source} - \delta^{15}N_{sink})/1000$, but the shift cannot always be assigned to a defined individual step of the process, for example it can be due to an isotope effect on the uptake of the primary compound, its conversion by an enzyme catalysed key reaction, or the overlap of both. The assignment to one of these alternatives may become possible via the (indirect) exclusion of one of these alternatives, as will be demonstrated later on for nitrate assimilation.

Kinetic isotope effects on enzyme catalysed reactions measured in vitro are preferably determined in respect to the elucidation of reaction mechanisms, the identification of (functional) groups involved in the catalysis

and for the understanding of enzyme activity regulations. They are defined as the ratio of the turnover rates or velocity constants of the isotopomer molecules in the reaction in question, and measured as the change of the isotope abundance of substrate or product after a defined partial turnover of a limited substrate pool. A kinetic isotope effect on an enzyme catalysed reaction is theoretically correlated to $V_{max}$ and $K_M$ values of a defined enzyme catalysed step, however, practically measured and expressed as the ratio of the rate constants of the whole reaction, for nitrogen as $k_{14}/k_{15}$. In regard to the elucidation of the reaction mechanism it has to be taken into account that the experimental isotope effect may be the product of isotope effects on several steps, and therefore the "first irreversible isotope sensitive partial step" has to be identified and discussed in context with the reaction mechanism. This requires also the knowledge of possible intermediate steps and equilibria. As for example the proper substrate of the glutamate dehydrogenase or the glutamine synthetase reaction, respectively, is $NH_3$ (and this is the only substrate to be taken into account in mechanistical considerations), the measured experimental kinetic isotope effect must be corrected for the equilibrium isotope effect on the deprotonation of $NH_4^+$ ($NH_4^+ \leftrightarrow NH_3 + H^+$; $^{15}K_{eq} = 1.0192$; Hermes et al., 1985; Rishavy and Cleland, 1999). Yet in the present context, only the global experimental isotope effect is of importance for discussion with in vivo observed isotopic discriminations; as far as possible, data on plant enzymes have been compiled in Table 1.

### 2.3. Isotope discriminations on uptake and transport of nitrogen

At a first level, the global $\delta^{15}N$-value of the biomass of a higher plant is determined by that of the primary nitrogen source (soil nitrogen, fertiliser, $N_2$). Some of the corresponding inorganic compounds are immediately assimilated by the plants, others by microorganisms, which then provide "organic" N to the plant. The involved assimilation reactions may be similar in both systems and hence imply corresponding isotope discriminations. As one can assume that in a system microorganism/plant a kind of a steady state of the nitrogen flux and its $^{15}N$-abundance will be attained, the activity of symbiotic microorganisms will thus probably contribute a constant shift of the plant $\delta^{15}N$-value relative to that of the source. Therefore, the isotope discriminations connected to the direct and the indirect availability of nitrogen for higher plants are of prominent interest.

#### 2.3.1. Global N-assimilation processes and implied nitrogen isotope discriminations

As already pointed out, the uptake of nitrogen by plants or symbiotic microorganisms or the transport between these is mostly catalysed by special carrier systems. Corresponding in vitro measurements on implied (equilibrium) isotope effects are not available. On the other hand, observed in vivo isotope discrimination measurements are often overlapped by kinetic isotope effects on correlated enzyme catalysed reactions. So the discussion of isotope discriminations on the in vivo nitrate assimilation often comprises the isotopic shift between an external nitrate pool (via $NH_4^+$) and the total bound nitrogen in the organic biomass; $\alpha$-factors between 1.003 and 1.03 have been found (Handley and Raven, 1992; Fogel and Cifuentes, 1993). Independent of the mechanism and the individual steps, the proper uptake of nitrate by higher plants does obviously proceed without notable nitrogen isotope discrimination (Yoneyama et al., 1998a; 2001); the in vivo measured isotope discrimination is hence probably mainly caused by the nitrogen isotope effect on the assimilatory nitrate reductase reaction. Although for the $^{15}N$-isotope effect on this reaction quite large differences have been found (Table 1), these cannot explain the whole scale of in vivo isotope discriminations. This must therefore be due to the overlap of the kinetic isotope effect on the nitrate reduction with other effects. Mariotti et al. (1982) observed influences of external nitrate concentration, Yoneyama (1991) contributions of nitrate transfers between different compartments. The main reason must, however, be the different excretion of the non-reduced, $^{15}N$-enriched nitrate (Medina and Schmidt, 1982; Yoneyama, 1995), attaining between 5 and 80% of the corresponding uptake, depending on organism and external conditions (Handley and Raven, 1992 and cited literature therein). In an investigation with bacteria Shearer et al. (1991) have even found a direct correlation between the $^{15}N$-discrimination on the nitrate assimilation and the ratio of influx and efflux of the ion. Similar results have been obtained with $^{15}N$- and $^{13}N$-labelled nitrate on soybeans (Clarkson et al., 1996). Nitrate reductase activity can be found in roots and/or shoots depending on plant taxa. Nitrate transport to shoots via xylem is assumed to be irreversible (Comstock, 2001 and cited literature therein). Therefore the kinetic isotope effect on the nitrate reductase reaction can only become efficient as in vivo nitrogen isotope discrimination in roots with nitrate excretion or in leaves with remarkable nitrate accumulation. In summary, it can thus be assumed that the nitrogen isotope discrimination connected to the assimilatory nitrate reduction is caused by the overlap of the kinetic isotope effect on the nitrate reductase reaction and the extent of the re-diffusion or accumulation of non-converted nitrate, but not by an isotope effect on the transportation of this ion itself (no isotope effect on diffusion of charged ionic species like $NO_3^-$ or $NH_4^+$ in aqueous solution is expected (Shearer and Kohl, 1986; 1989a) because of the large sphere of hydration of these ions

(Comstock, 2001)). This parallels the biological carbon fixation with an overlap of the $^{13}$C-KIE on enzymatic $CO_2$-binding reactions with diffusive $CO_2$-fluxes in and out of plant's stomata ($k_{12}/k_{13}$ on the diffusion of $CO_2$ in air is $\sim$1.0044) as a cause for the variation of $\delta^{13}$C-values in organic plant material (O'Leary and Osmond, 1980; O'Leary et al., 1992).

Ammonium is accumulated by means of specific transporters; it is rapidly distributed to various organelles, and converted into organic binding preferably by the glutamine synthetase reaction (Howitt and Udvardi, 2000). While this enzymatic reaction implies a kinetic nitrogen isotope effect but not an isotope discrimination (see later), here the uptake itself implies a remarkable $^{15}$N-depletion (depending on the external ammonium concentration up to $-29$‰), as described for rice plants (Yoneyama et al., 2001) and for microorganisms (Hoch et al., 1992). Additionally also efflux of $NH_4^+$ in rice and other plant species has been described (Britto et al., 2001).

Symbiotic mycorrhiza bind, like plants, nitrogen from $NO_3^-$ and $NH_4^+$ into organic forms, and provide it to the host plant preferably in the form of glutamine in exchange with glutamic acid (for details of mycorrhizal symbiosis, also giving results on $^{15}$N-tracer experiments with various N-sources, see Smith and Read, 1997). The whole process is obviously accompanied by a nitrogen isotope discrimination, because mycorrhiza can be, but are not necessarily $^{15}$N-enriched relative to soil-N and to plant-N (Högberg et al., 1999; Hobbie et al., 1999a; Kohzu et al., 2000; Tjepkema et al., 2000), and will often contribute to the isotope shift observed between plant- and soil-N (Handley et al., 1999). A large $^{15}$N-depletion ($\delta^{15}$N-values near $-10$‰) of chitin relative to the protein was found in ectomycorrhizal fungi (Taylor et al., 1997). The molecular basis of this nitrogen isotope discrimination is probably connected to the synthesis (Turnbull et al., 1996) and to the transport (Kohzu et al., 2000) of glutamate. Furthermore, plants can even directly assimilate organic nitrogen from soil (Kielland, 1994; Wirén et al., 1997; Kakkar et al., 1998; Näsholm et al., 2000). Nitrogen isotope fractionations on these processes are not indicated, but were outside the scope of these authors' interest.

For plants with symbiotic nitrogen fixating rhizobia, the starting material $N_2$ is occurring in an infinite pool with constant $\delta^{15}$N-value. Isotope fractionation factors for $N_2$-fixation are close to unity (Kohl and Shearer, 1980; Handley and Raven, 1992), and no or only a small kinetic isotope effect on the nitrogenase reaction in *Azotobacter* has been reported [$k_{14}/k_{15}$: 0.9963–1.0022 (Hoering and Ford, 1960), $k_{14}/k_{15}$: $\sim$1.0039 (Delwiche and Steyn, 1970), $k_{14}/k_{15}$: $\sim$1.0032 (Rowell et al., 1998)]. The $NH_3$ produced by the nitrogenase reaction diffuses across the bacteroid membrane into the peribacteroid space (Udvardi and Day, 1997) and is then actively transferred (across the peribacteroid membrane) into the host cell in the form of $NH_4^+$ ($NH_3$, see below, Tyerman et al., 1995) or via an alanine shuttle (Waters et al., 1998; Allaway et al., 2000), and is then immediately and completely transferred into organic binding by the glutamate synthetase reaction, hence the nitrogen isotope effect on this reaction does not become efficient (Evans et al., 1996; Yoneyama et al., 1998a). This is in line with the observation that most nitrogen isotope fractionation factors described for the nitrogen assimilation, measured on the $\delta^{15}$N-value of the global biological material, are below 1.003 (Handley and Raven, 1992; Shearer and Kohl, 1993), and that global $\delta^{15}$N-values of biomass (bulk material) from N-assimilating legumes are the lowest of all known $\delta^{15}$N-values at all. In this case the intrinsic nitrogen metabolism is thus not preceded by a remarkable isotope discrimination accompanying the nitrogen uptake. However, as with mycorrhiza, obviously a nitrogen isotope discrimination is implied in the transport of assimilation products across the peribacterial membrane (diffusive $NH_3$, transport? Day et al., 2001), because nodules can be, depending on the host/guest system, $^{15}$N-enriched relative to the plants (Turner and Bergerson, 1983). Finally, plants can absorb and excrete gaseous ammonia (Francis et al., 1997; Pearson et al., 1998; Harper and Sharpe, 1998), the latter probably in context with photorespiration, however, data on accompanying nitrogen isotope discriminations were not available. If the uptake/loss of $NH_3$ from plant stomata is rate-limited by the diffusion of $NH_3$ in air, the transported $NH_3$ will be depleted in $^{15}$N by $\sim$18‰ relative to the $\delta^{15}$N of the source (Farquhar et al., 1983). $NH_3$ in the atmosphere originating from biological processes can have $\delta^{15}$N-values $< -10$‰ (Heaton, 1986). In some cases also N-transport in form of $NH_3$ from one plant to another has been observed (Sharpe and Harper, 1997); such a transport is also possible in form of root exudates (Paynel et al., 2001).

### 2.3.2. $\delta^{15}$N-Values and correlations of amino acids and transport metabolites in nodules

Yoneyama and his group have performed extensive work on $\delta^{15}$N-values of primary N-binding and translocation compounds in symbiotic and nitrate assimilating plants (Yoneyama, 1995; Yoneyama et al., 1998a). Quite often, nodules of symbiotic plants are enriched in $^{15}$N towards the host plant (Turner and Bergersen, 1983; Shearer and Kohl, 1989b; Yoneyama, 1995). Free ammonia from roots or nodules of symbiotic plants can have relatively positive $\delta^{15}$N-values, probably due to the nitrogen kinetic isotope effect on the glutamine synthetase reaction; this ammonia is not the immediate precursor of the bacteroid protein. There is, however, a strong isotopic correlation of this bacteroid protein to the amide-N in the nodules (Shearer and Kohl, 1989b).

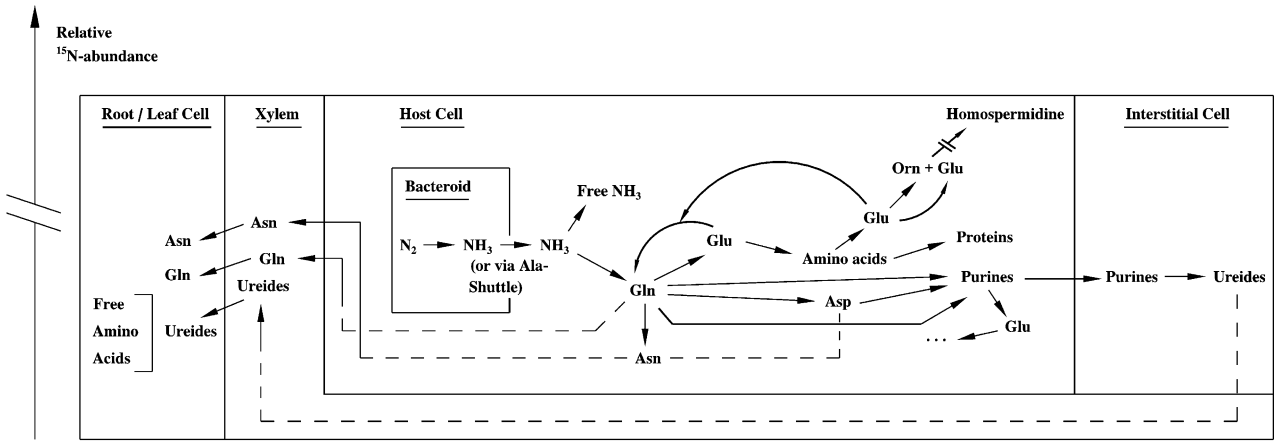


Fig. 1. Metabolic correlations and relative $^{15}N$-abundances of transport metabolites in and from nodules of $N_2$-assimilating plants. The scale is arbitrary and not linear. Asn = asparagine, Gln = glutamine, Glu = glutamic acid, Orn = ornithine.

Amide-N of glutamine and ureide-N were always, asparagine-N was in most cases depleted in $^{15}N$ against bacteroid protein and ammonia in the nodules (Bergersen et al., 1988; Yoneyama, 1995; Yoneyama et al., 1998a). The expected corresponding mass and isotope balance compensation was found in the $^{15}N$-enrichment of polyamines, especially of homospermidine, which attained a $\delta^{15}N$-value of +48‰ in the nodules of *Sesbania rostrata* (Yoneyama et al., 1998b). Polyamines are synthesised in a complicated (N-recycling ?) reaction sequence from ornithine and arginine via citrulline and carbamoyl-putrescine, and the condensation of putrescine with glutamic and aspartic acid semialdehydes, respectively (Kindl, 1991). Although most of the nitrogen atoms of the polyamines are thus originating from glutamic acid in the course of this biosynthesis, it is difficult to understand so far, why these products are $^{15}N$-enriched towards their precursor. Nevertheless the large $^{15}N$-enrichment of homospermidine is in line with the high $\delta^{15}N$-value of the δ-amino groups of arginine from bacteria (Medina and Schmidt, 1982).

Tracer experiments with $^{15}NH_3$ showed that the labelling of asparagine, occurring in relatively high concentration in nodules, proceeds only slowly; it is therefore supposed that the amide is part of a recycling system (Parsons and Baker, 1996). The transport of the nitrogen carrier molecules themselves seems to be accompanied by isotope discriminations, as for example in nodule-free roots and in leaves of soybeans, a slight $^{15}N$-enrichment of the ureides towards free and bound amino-N was found, and as amino acids and soluble nitrogen containing compounds extracted from leaves of several plant tissues followed the $^{15}N$-abundance sequence aspartic acid/asparagine > glutamic acid/glutamine > threonine, proline, valine > glycine + alanine + serine, γ-aminobutyric acid and phenylalanine (Yoneyama and Tanaka, 1999).

The formation and translocation of the transport metabolites imply a couple of metabolic branching, recycling and unidirectional processes, including the glutamine and asparagine synthetase reactions, the GOGAT reaction, the synthesis and degradation of purines as well as shuttle processes for their transport between different compartments, all of them being potentially accompanied by isotope effects; therefore, an assignment of their relative isotopic depletion or enrichment to a defined step is not yet possible. However, it seems that the synthesis of the transport metabolites in the nodules includes a general $^{15}N$-depletion, while their excretion into the xylem is accompanied by a secondary $^{15}N$-enrichment (Fig. 1). Although the relative $^{15}N$-enrichment of the transport metabolites aspartic acid/asparagine and glutamic acid/glutamine is also found in the acceptor organs, this can be due to the nitrogen isotope discriminations in these organs itself. In summary, transport from the nodules is a continuous unidirectional process, however, working under steady state conditions, with a more or less complete transfer of the bound nitrogen after a positive or negative $\delta^{15}N$-shift relative to that of the primary source.

### 2.4. $\delta^{15}N$-Values of and $^{15}N$-discriminations between organic nitrogen containing plant products

The central primary inorganic compound in plants is ammonium, the main first organic one is glutamine. From here $^{15}N$-discriminations in the intrinsic plant nitrogen metabolism demand the combination of kinetic isotope effects and branching events. So, for example in komatsuna plants (*Brassica campestris* L.) and in spinach (*Spinacera oleracea*) a large depletion in $^{15}N$ of ammonium relative to the remaining nitrate has been observed, while the amide-N could be depleted or enriched in $^{15}N$ relative to this ammonia (Yoneyama, 1995). This proves that the in vitro measured kinetic isotope effect on the nitrate reductase reaction is efficient in vivo, provided it is combined to a partial nitrate excretion. On the other hand the isotope effect on the

glutamine synthetase reaction (Yoneyama et al., 1993) does in most cases not become efficient in vivo because of the quantitative consumption of the ammonia (Evans et al., 1996). The observed variations of the $^{15}N$-abundance of ammonia relative to that of the amide-N could rather be due to actual relative activities of glutamine synthetase and glutamate dehydrogenase (Schlee et al., 1994), the latter being more involved in $NH_3$-liberation, or to local and actual conditions of nitrogen fluxes.

#### 2.4.1. Main organic N-compounds: proteins, heteroaromates, aminoglucans, porphyrins and lipids

In spite of the very complex correlations between nitrogen pools and nitrogen containing compounds in higher plants the verification of some regularities for in vivo nitrogen isotope discriminations is possible by comparing the $\delta^{15}N$-values of representatives of their main groups. The problem of a generalisation is that most $\delta^{15}N$-values of nitrogen containing plant products available have been measured under different aspects, are originating from different sources and cannot easily be "normalised" among each other. The most promising way is to correlate their $\delta^{15}N$-value to that of the proteins from the same source, because proteins represent normally the largest nitrogen pool in plants. In this context it has to be mentioned that as early as 1963, Gaebler et al. (1963) have stated that the amide-N of plant proteins is relatively enriched in $^{15}N$ as compared to the α-amino-N, and that the $^{15}N$-abundance of alkaloids is at the lower edge of that of proteins.

A more quantitative information on nitrogen isotope discrimination between the main fractions of nitrogen containing organic compounds from plants can be derived from investigations of Gonzáles-Prieto et al. (1995) with $^{15}N$-labelled compounds. These authors have grown alders on $K^{15}NO_3$ as the nitrogen source and fractionated the leaves by treatment with 1 and 3 N HCl. Eighty per cent of the total organic N was extractable from the *Alnus cordate* material, and the $^{15}N$-content in the non-extractable and extractable fractions was practically identical to that of the total biomass (0.5 at.% enrichment and 0.1 at.% depletion in $^{15}N$, respectively). Among the compounds in these HCl-extracts, the amino acid-N fraction (41%) showed a relative $^{15}N$-enrichment above the bulk value by 1.7 at.%, the fraction "hydrolysable unidentified N" (31%), which is regarded as "heterocyclic-N of nucleotides, ureides and probably chlorophyll", was enriched by 4.9 at.%, while the minor fractions (hexosamine-N, $NH_4^+$-N and amide-N) were distinctly $^{15}N$-depleted. In the corresponding fractions from *Alnus incana* the sequence of the $^{15}N$ content was in principle identical, although not so distinct. The authors discuss their results as typical and in line with those from other corresponding investigations. Keeping in mind that the $NH_4^+$- and amide-N fractions in their experiments are not identical with the corresponding soluble compounds in life plant material, but products of hydrolysis, and that ureides and chlorophyll are certainly only a minor part in the "hydrolysable unidentified N-fraction", it can be derived from these results that N in heteroaromatic binding is really enriched in $^{15}N$ as compared to α-amino-N from amino acids, while secondary products are $^{15}N$-depleted.

Hofmann et al. (1997) measured for the "buffer extractable protein" from wheat a $\delta^{15}N$-value +5.5‰, for the "extracted non-protein-N" (among others nucleic acids ?) +10‰ and for the non-soluble N-containing fraction ("structural protein", probably lipids, chlorophyll and aminoglucans) +2.5‰. This again confirms the relative $^{15}N$-enrichment of the heteroaromatically bound nitrogen and the $^{15}N$-depletion of the indicated secondary compounds relative to the protein. As the total N-amount in all three fractions was about the same, the mean bulk $\delta^{15}N$-value of the biomass can be calculated to be around +6‰. Proteins from microorganisms showed a 3.5‰ enrichment in $^{15}N$ relative to the total biomass (Macko et al., 1987). This result is probably due to a larger amount or to a larger $^{15}N$-depletion of N-containing cell wall compounds. The latter explanation is in line with the finding that chitin from various sources is always relatively $^{15}N$-depleted, e.g. up to 9‰ to protein from the same source (Macko et al., 1990; Taylor et al., 1997; Schimmelmann et al., 1998).

Fitting into the present context are also the results of investigations on the $\delta^{15}N$-values of chlorophylls and N-containing lipids relative to those of the total biomass ($\delta^{15}N$ between +4 and +20‰). In four out of six investigated higher plants, the lipids were slightly ($\Delta\delta = -1$ to $-2$‰) depleted or identical in $^{15}N$, in two others $^{15}N$-enriched ($\Delta\delta = +2.9$ and +6‰) relative to the bulk material; chlorophylls were all relatively $^{15}N$-depleted ($\Delta\delta = 0$ to $-6$‰), and no significant difference was observed between chlorophyll a and chlorophyll b (Kennicutt et al., 1992; see also Bidigare et al., 1991). A mean $^{15}N$-depletion of −5.1‰ relative to the $\delta^{15}N$-value of bulk biomass (−7 to +7‰) is also described for the chlorophyll of marine phytoplankton (Sachs et al., 1999). More differentiated are $\delta^{15}N$ data reported by Beaumont et al. (2000) for compounds of *Rhodobacter capsulatus*, a purple nonsulfur bacterium, and of the cyanobacterium *Anabaena cylindrica*. The authors found for the lipids of *R. capsulatus* relative to the $\delta^{15}N$-value of the bulk biomass a mean $^{15}N$-depletion by ∼−5.6‰, preferably for the "neutral lipids" (glycolipids ?, $\Delta\delta^{15}N = \sim -9$‰), less for the "polar lipids" (choline containing representatives ?, $\Delta\delta^{15}N = \sim -1$‰), indicating a nitrogen isotope discrimination at the branching of the biosyntheses of choline and sphingosine, respectively, from their common precursor serine. The bacteriochlorophyll a from *R. capsulatus* was $^{15}N$-depleted towards the bulk biomass

Table 2
$\Delta\delta^{15}$N-Values [‰]$_{AIR\text{-}N_2}$ of amino acids from protein hydrolysates and of free amino acids from defined origin, normalized by difference to Glu (for *Triticum aestivum* I to Ala) = 0‰. *Triticum aestivum* I = soluble protein extracted at the two-leaf stage, *Triticum aestivum* II at the anthesis stage

| Protein and origin | $\Delta\delta^{15}$N[‰]$_{AIR\text{-}N_2}$ in α-amino-N and total N of amino acid | | | | | | | | | | | | | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Asp | Gly | Ala | Val | Leu | Ile | Ser | Thr | Phe | Tyr | Lys | His | Arg | Trp | Pro | |
| *Anabaena* sp. strain IF on $N_2$ | +1.5 | −3.5 | −3.0 | −2.5 | −8.5 | −7.5 | −10.0 | −2.5 | −1.0 | −5.0 | −4.5 | −6.0 | −7.5 | n.d. | n.d. | Macko et al., 1987 |
| *Anabaena* sp. strain IF on $NO_3^-$ | +2.0 | −2.0 | −1.5 | −2.5 | −9.5 | −4.5 | −8.0 | −2.5 | +2.0 | −1.5 | −2.5 | −6.5 | −5.0 | n.d. | n.d. | Macko et al., 1987 |
| *Triticum aestivum*, I, greenhouse | +2.0 | −5.5 | 0.0 | 0.0 | −2.0 | 0.0 | −3.0 | [+12.0] | n.d. | +4.5 | −3.0 | n.d. | n.d. | n.d. | n.d. | Hofmann et al., 1997 |
| *Triticum aestivum*, II, greenhouse | +1.0 | −6.0 | +1.0 | −3.5 | −5.0 | −2.0 | −4.5 | +3.0 | n.d. | +3.0 | −4.5 | n.d. | −1.0 | n.d. | −3.5 | Hofmann et al., 1997 |
| *Glycine max*. (10%)/ *Hordeum vulg*. (78%) | +1.5 | −2.1 | +0.3 | n.d. | n.d. | n.d. | −4.1 | −3.8 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | +1.2 | Hare et al., 1991 |
| Mean $C_3$-plants, homotroph | +1.6 | −3.8 | −0.65 | −2.1 | −6.25 | −3.5 | −5.9 | −1.45 | +0.5 | +0.25 | −3.6 | −6.25 | −4.5 | n.d. | −1.15 | |
| *Zea mais* | +2.3 | +2.5 | +1.4 | +4.5 | n.d. | n.d. | +1.0 | −1.2 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | +3.5 | Hare et al., 1991 |
| *Vibrio harvey* strain B-352 on Glu | −9.0 | −5.0 | −3.5 | −5.5 | −6.0 | −3.5 | −6.5 | −3.5 | −2.5 | −3.5 | −4.5 | −5.5 | −2.0 | n.d. | n.d. | Macko et al., 1987 |
| Bovine collagen | −2.3 | −5.5 | −3.8 | −0.8 | −2.0 | −1.3 | −6.5 | −10.5 | −1.8 | −6.2 | −5.3 | −7.5 | n.d. | −5.5 | −0.5 | Hürzeler, 1997 |
| Achilles tendon collagen | n.d. | −3.8 | n.d. | +1.1 | −1.2 | n.d. | n.d. | n.d. | n.d. | −3.8 | −5.7 | n.d. | −4.8 | n.d. | 0.0 | Minagawa et al., 1992 |
| Free amino acids from pea (*Pisum sativum*) nodules | +7.1 | +0.4 | n.d. | +7.5 | n.d. | n.d. | n.d. | −0.1 | n.d. | n.d. | n.d. | n.d. | +8.1 | n.d. | n.d. | Yoneyama et al., 1998b |

The mean absolute $\delta^{15}$N-value of Glu from plant proteins is ~ +7.0‰, that of total proteins ~ +5‰. n.d. = not determined. $\delta^{15}$N-value in [ ] is not considered for mean $\delta^{15}$N-value calculation. Some of the $\delta^{15}$N-values have been taken from graphical displays.

($\Delta\delta^{15}N = -7.5$‰), while that of *A. cylindrica* was enriched in $^{15}$N by $\Delta\delta^{15}N = +8.5$‰. The authors explain this difference quite convincingly on the basis of the different biosyntheses of δ-amino-levulinic acid in the two microorganisms, proceeding in *R. capsulatus* from the relatively $^{15}$N-depleted precursor serine via glycine (see Table 2), however, in *A. cylindrica* directly from the relatively $^{15}$N-enriched glutamic acid via glutamate-1-semialdehyde; the latter "$C_5$-pathway" is typical for higher plants, archaebacteria and some eubacteria (Richter, 1996). Serine has thus been recognised as a central intermediate in the nitrogen metabolism, leading to different descendents and implying $^{15}$N-discriminations in its metabolism.

In summarising these results on the main nitrogen containing plant materials it can be stated that, relative to the dominant nitrogen pool of α-amino-N, represented by the proteins (mostly enriched in $^{15}$N relative to the global $^{15}$N-abundance of the corresponding total biomass), in general aromatic and heteroaromatic compounds, receiving defined N-atoms directly from the amide-N of glutamine (Purich, 1998; Zalkin and Smith, 1998), are $^{15}$N-enriched, while lipids are sometimes, chlorophyll and aminoglucans, generally $^{15}$N-depleted. The branching points for these isotope discriminations are between the glutamine and glutamate pools, probably with a kinetic nitrogen isotope effect on the GOGAT reaction, and on an unknown reaction in which serine is involved.

### 2.4.2. Transamination and potential isotopic equilibration in the α-amino-N pool

Normally the largest nitrogen pool in plants is the α-amino-N pool, and its most important sink products are the proteins. The nitrogen in all amino acids is originating from glutamic acid as primary N-donor, however, in principle all amino acids can exchange, directly or via glutamic acid, amino groups among each other by transamination. This reaction is reversible, and the comparison of the $\delta^{15}$N-values of the α-amino-N from individual amino acids should indicate, whether this pool is isotopically equilibrated or not. The individual biosyntheses and conversions of the different amino acids are controlled and adapted to the actual demands. A generally limiting factor is the availability of nitrogen itself. As it can be assumed that the ribosomal biosynthesis of proteins and their (quantitative) hydrolysis do not imply nitrogen isotope discriminations, the $\delta^{15}$N-values of the individual amino acids of a protein hydrolysate should reflect their relative $^{15}$N-abundances in the common α-amino-N pool before the protein biosynthesis.

Macko et al. (1987) have measured the $^{15}$N-kinetic isotope effect on the glutamate:oxaloacetate transaminase

reaction in both directions (Table 1); on the basis of their values a corresponding equilibrium nitrogen isotope effect $k_{14}/k_{15}$ of 1.0066 can be calculated. This value is distinctly different from unity, and it indicates a slight relative $^{15}$N-enrichment in favour of glutamate for the system in equilibrium.

$$\text{glutamate} + \text{oxaloacetate} \leftrightarrow \alpha\text{-oxoglutarate} + \text{aspartate}$$

On the first glance it is astonishing that this difference exists between these quite similar homologues. An independent criterion for a possible influence of the side chain on thermodynamic properties of the α-amino group would be the p$K$-values; these, for aspartic acid (p$K_1$ = 1.99; p$K_2$ = 9.90; p$K_3$ = 3.90, Voet and Voet, 1992), and for glutamic acid (p$K_1$ = 2.10; p$K_2$ = 9.47; p$K_3$ = 4.07), reveal a quite remarkable difference, especially for p$K_2$, characterising the basicity of the α-amino group. By a corresponding comparison of the p$K$-values of other amino acids as a potential criterion for the relative shifts of their $\delta^{15}$N-values, two groups, one with p$K_1$ = 2.29–2.35 and p$K_2$ = 9.74–9.87, represented by the amino acids Gly, Ala, Val, Leu and Ile, and another one including Ser, Thre, Met, Phe and Tyr with p$K_1$ = 2.09–2.20 and p$K_2$ = 9.10–9.28 can be discussed.

Table 2 shows that there is no correlation between the relative $\delta^{15}$N-values of these amino acids and these p$K$-values. Therefore, the relative $\delta^{15}$N-values are not determined by thermodynamic factors, and the α-amino N-pool in plants is certainly not in isotope equilibrium. Obviously, most amino acids rather "appear to have isotope fractionations associated with the metabolic pathways in their synthesis" (Macko et al., 1987).

This may be the case for some amino acids like valine, leucine and isoleucine, serving preferably only as precursors for proteins. It is also obvious that the relative $\delta^{15}$N-value of some amino acids can be assigned to that of their precursor, e.g. proline to glutamic acid. However, the $\delta^{15}$N-values of most other proteinogenic amino acids must additionally be influenced by their various other functions, like transport or recycling of nitrogen or by being precursors of secondary compounds. For example, glutamic acid is, in agreement with its general donor function, relatively enriched in $^{15}$N to nearly all other amino acids, and the $^{15}$N-enrichment of aspartic acid may also be due to its function as transport or storage metabolite. On the other hand, the $\delta^{15}$N-values of phenylalanine and tyrosine, although slightly more positive than those of other acceptor amino acids, do by far not reflect the large importance of these aromatic amino acids in context with the phenylpropanoid biosynthesis in plants. Correspondingly, the relative $^{15}$N-depletion of serine and glycine in proteins from $C_3$-plants and their relative $^{15}$N-enrichment in $C_4$-plants (Table 2), may be due to their implication in the photorespiratory nitrogen cycle, but are by far less affected than would be expected from the large turnover of this reaction sequence. Therefore, the above mentioned statement of Macko et al. (1987), that the nitrogen isotopic characteristics of amino acids are determined by their biosyntheses, needs some complementary explanations. We believe that also their various metabolic functions, grouping them to pools with metabolic branching points to different directions, catalysed by enzyme reactions implying kinetic isotope effects have additionally to be taken into account.

### *2.4.3. Amino acids involved in intrinsic recycling processes: phenylpropanoid biosynthesis and photorespiration*

As mentioned in Section 2.4.2, the $\delta^{15}$N-values of amino acids synthesized by intrinsic recycling processes as by-products are obviously not extremely different from those of other amino acids, although from their large turnover rate a remarkable isotopic peculiarity would be expected (Schmidt and Kexel, 1998). For example phenylalanine and, to a lesser extent, tyrosine are the key intermediates in the synthesis of phenylpropanoids, which, however, only preserve the carbon skeleton of this precursor, while the ammonia, liberated by the phenylalanine:ammonia lyase reaction, is recycled by the glutamine synthetase and GOGAT reactions, to become again available for the conversion of prephenate to arogenate by transamination (Razal et al., 1996; Singh et al., 1998). Raven et al. (1992) estimate that this process revolves approximately 66% of the primary assimilated nitrogen by all terrestrial plants. Provided that the relatively small amount of phenylalanine (and tyrosine) needed for protein and alkaloid syntheses is not independently produced, e.g. in another compartment, one would expect that, due to the isotope effect on the phenylalanine:ammonia lyase reaction (Table 1), this small aliquot should show a relative quite positive $\delta^{15}$N-value. As this is not the case (Table 2), it has to be assumed that the main irreversible pathway attains a kind of steady state with variable absolute turnover, but a metabolic pool of phenylalanine with a constant nitrogen isotopic abundance, which does not affect that of the small net production of the amino acid (Fig. 2).

This may globally be similar with the amino acids serine and glycine in context with the photorespiratory nitrogen cycle (Wingler et al., 2000). This process, extending over different plant compartments, is said to be responsible for the largest nitrogen flux in leaves of most $C_3$-plants in the light (Keys et al., 1978; Yu and Woo, 1991; Raven et al., 1993). It implies, in regard to nitrogen recycling, several reactions with potential $^{15}$N-kinetic isotope effects, including transaminations, the glycine decarboxylase, the glutamine synthetase and the GOGAT reactions (Betsche and Eising, 1986; Yu and Woo, 1991), also, e.g. in *Chlorella* species, the glutamate dehydrogenase reaction (Kalinkina and Naumova,

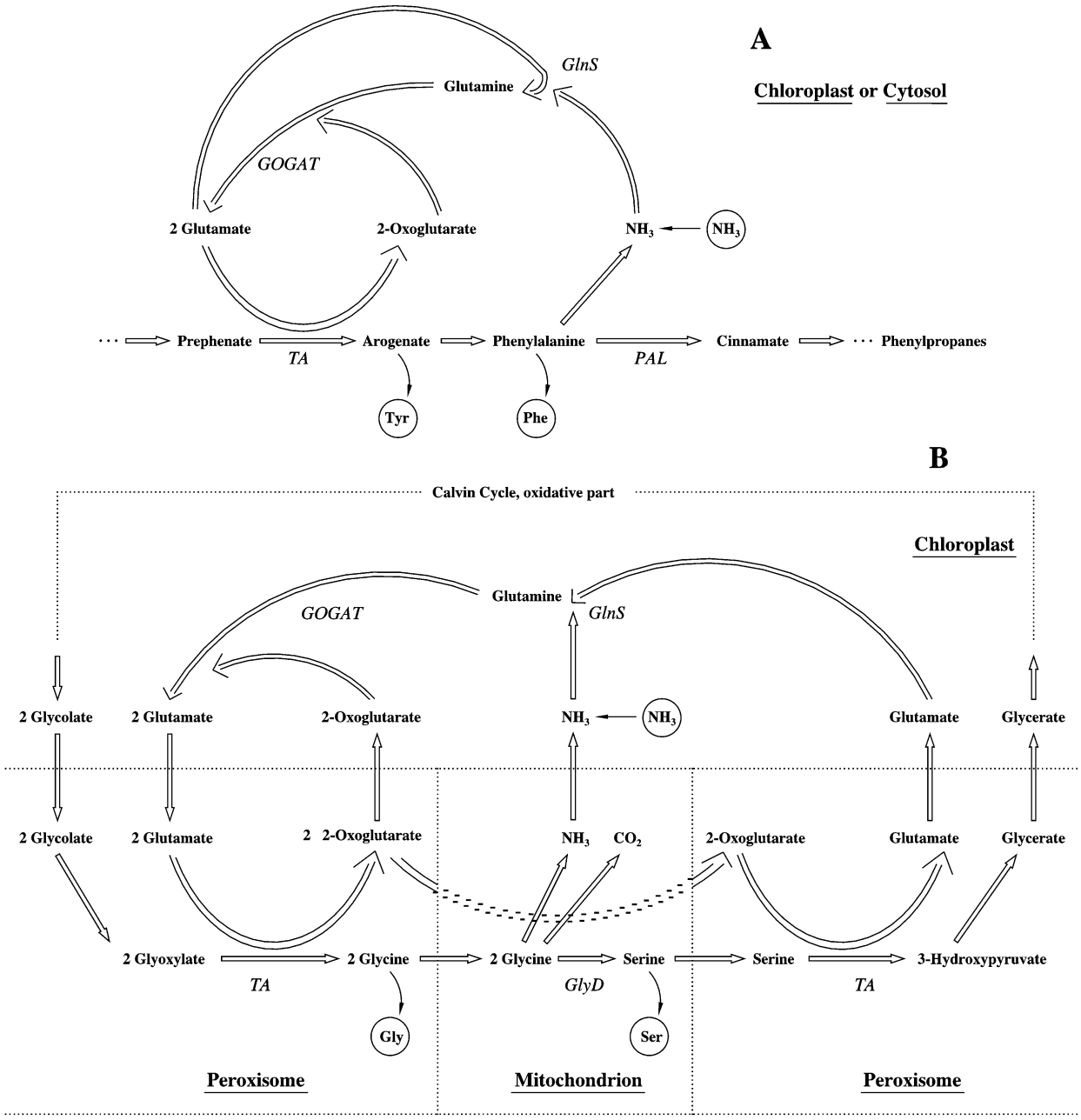


Fig. 2. Ammonia recycling in the phenylpropanoid biosynthesis with phenylalanine/tyrosine as key substances (A) and in the photorespiratory cycle of serine/glycine (B), respectively. Double arrows indicate main metabolite fluxes, small arrows net input of ammonia and output of amino acids, respectively, whose carbon skeletons are originating from the cycles. Their net biosynthesis ($NH_3$ and outcoming amino acids are encircled) is thus a small unidirectional side path of the main metabolite turnover near "steady state" with large (isotopically) constant pools of intermediates, conditions, under which kinetic isotope effects cannot become efficient. Names of enzymes are in italics, *TA* = transaminases, *PAL* = phenylalanine:ammonia lyase, *GlnS* = glutamine synthetase, *GlyD* = glycine decarboxylase, *GOGAT* = glutamine:2-oxoglutarate amino transferase.

1993). Normally, the kinetic isotope effects on these reactions should not become efficient in the case of a total recycling of the intermediates without input or net production of substrates and products. Yet a net production of glycine and serine must exist, because both amino acids are, independent from their function in proteins, involved in many metabolic processes, e.g. serine in the $C_1$-metabolism (Weilacher et al., 1996; Hanson and Roje, 2001) and as seen before in the lipid biosynthesis. However, again the net production is not or only slightly influenced by the recycling process in a quasi-steady state, and only its variable absolute turnover may have an effect on the $\delta^{15}$N-value of the "free" serine. As a matter of fact, the turnover of the respiratory cycle is influenced by external conditions like light intensity and availability of primary nitrogen sources (Zhu et al., 2000), and it is quite different in $C_3$- and $C_4$-plants (Lacuesta et al., 1997). Therefore the observed

small difference of the $\delta^{15}$N-values of glycine and serine between these two plant types (Table 2, Hare et al., 1991) has to be discussed on this background, but it has to be verified on the base of more samples.

A partial nitrogen recycling is also integrated in the tetrapyrrol (chlorophyll, porphyrine) biosynthesis. However, this reaction sequence comprises a net recycling of only one half of the input nitrogen, while the other half is irreversibly incorporated into the sink compound. The condensation of two moles δ-aminolevulinic acid to porphobilinogen will probably imply an (intermolecular ?) isotope effect, leading to the preferred incorporation of "light" nitrogen into the pyrrol rings. However, this possible $^{15}$N-depletion will probably be compensated by the $^{15}$N-enrichment of the precursor glutamic acid, in line with the observation that in higher plants chlorophyll is only slightly depleted in $^{15}$N relative to the bulk nitrogen isotope abundance (see Section 2.4.1, Kennicutt et al., 1992).

### *2.4.4. $\delta^{15}$N-Values of alkaloids and other secondary plant products*

Besides polymers [proteins, nucleic acids, cell wall glycoproteins ("extensines", counting for approximately 20% of the cell wall dry matter)] some small monomolecular compounds like alkaloids can be sink products for nitrogen in plants. The biosyntheses of alkaloids can occur in different plant compartments (Kindl, 1991); sometimes alkaloids are preferably produced in roots, and transported from here into other plant organs. Precursors and nitrogen sources for most alkaloids are defined amino acids, so for morphine/heroin tyrosine, for cocaine ornithine, obviously with the δ-amino-N atom as sole nitrogen source, indicating that the biosynthesis proceeds via an unsymmetrical intermediate (cf. biosynthesis of the piperidine ring of anabasine, Watson et al., 1990). On the other side, the pyrrolidine ring of nicotine is originating from the same source, however, synthesised via the symmetrical intermediate putrescine (Walton et al., 1990; Botte et al., 1997), which has for consequence that the N-atom in the product is equally originating from both amino groups of ornithine. Recent data of Saad et al. (2001) on the ornithine homologue lysine showed that the $\delta^{15}$N-value of the α-amino-N of this amino acid is in the normal range (+3.5‰), while that of the ϵ-amino-N group shows a distinct depletion in $^{15}$N (−3.8‰); this is in line with the unusually complicated biosynthetic introduction of the ϵ-amino-N group into this amino acid. Therefore in the present context a corresponding difference between the $^{15}$N-abundances of the α- and the δ-amino-N atoms of ornithine can be assumed. Tyrosine, precursor of morphine and heroin, is an amino acid with a relatively high $^{15}$N-content, and an approximate $\delta^{15}$N-value of +6‰ in proteins (see Table 2). Finally, the N-atom in methyl-*N*-methyl-anthranilate is not originating from the α-amino-N pool, but via anthranilate from glutamine amide-N, for which a $\delta^{15}$N-value of $+11\pm3$‰ has to be assumed.

From these precursors the expected (respectively found) mean $\delta^{15}$N-values are for morphine/heroin +6‰ (0‰), for cocaine −3.5‰ (−8‰), for the pyrrolidine ring of nicotine 0‰ (−5.2‰), and for methyl-*N*-methyl-anthranilate +11‰ (+6.25‰). The differences between the expected to the found $\delta^{15}$N-values are in all cases from −4.5 to −6‰, reasonable values for the depletion in $^{15}$N of a minor product from a larger pool. On the other hand, the $^{15}$N-depletions of these secondary products relative to the bulk $\delta^{15}$N-values of leaves and pulp (Table 3), respectively, from the same origin (the $\delta^{15}$N-value of the corresponding protein would be about 3.5‰ more positive), are extremely different, namely −14.7‰ for cocaine, −8.9‰ for nicotine and 0‰ for methyl-*N*-methyl-anthranilate, proving that their N-atoms are not originating from a global metabolic pool but from defined precursors.

The correlation of the N-atom in the pyridine ring of nicotine ($\delta^{15}$N-value −5.2‰) to the precursor aspartic acid ($\delta^{15}$N-value +8.5‰, as derived from Table 2, from where a $^{15}$N-depletion of +2‰ would be expected) is not that clear. It has therefore to be discussed, whether the N-atoms of secondary products from this amino acid are immediately (isotopically) connected to the large pools of aspartic acid as transport metabolite or for protein synthesis, or whether they can be treated as originating from independent "secondary" metabolic pools, eventually in individual cells and cell compartments. The complicated metabolism of aspartic acid and its amide (Richards and Schuster, 1998) will include remarkable isotope discriminations in any case. Aspartic acid provides N-atoms to other amino acids by transaminations, to some secondary products (e.g. purines) by lyase reactions, but it can also be completely incorporated into the carbon skeletons of secondary products (e.g. pyrimidines, pyridine ring of nicotine). Any of the involved reactions may imply individual nitrogen isotope effects, leading to the observed $\delta^{15}$N-value differences from protein originating aspartate and in between the secondary products themselves. In the case of the observed $^{15}$N-depletion of the δ-amino-N group of ornithine in the present context and its large $^{15}$N-enrichment in context with the biosynthesis of polyamines in nodules, certainly the influence of different reactions and metabolic pools are involved.

Assuming that the experimentally found $\delta^{15}$N-values for secondary products have a general importance, it must be possible to calculate global $\delta^{15}$N-values of other representatives with more than one N-atom from these $\delta^{15}$N-values by correlating them to the corresponding precursors. In this regard we have to assign, on the base of the above reported results, in such compounds for

Table 3
$\delta^{15}$N-Values [‰]$_{AIR\text{-}N2}$ of secondary natural compounds

| Compound (*n* in parentheses) | | Origin, plant (reference and its $\delta^{15}$N[‰]$_{AIR\text{-}N_2}$) | Biosynthetic N-precursor ($\delta^{15}$N[‰]$_{AIR\text{-}N_2}$ in proteins) | $\delta^{15}$N[‰]$_{AIR\text{-}N_2}$ | Reference |
|---|---|---|---|---|---|
| Heroin[a] | (5) | *Papaver somniferum* | Tyrosine (~ +6.0‰) | −3.6 to +1.7 | Ihle and Schmidt, 1996 |
| | (?) | | | −8.5 to −1.5 | Avak et al., 1996 |
| | (20) | | | −1.6 to +1.3 | Zimmer, 1999 |
| | (?) | | | −4.3 to +0.5 | Besacier and Chaudron-Thozet, 1999 |
| Heroin[a], Morphine | (20) | | | −2.5 to +2.5 | Ehleringer et al., 1999 |
| Cocaine | (4) | *Erythroxylon coca* | Ornithine (δ-amino N) | −13.7 to −5.4 | Ihle and Schmidt, 1996 |
| | (?) | (coca leaves +6.5‰)[b] | (~−4.0‰) | −13.0 to −5.5 | Avak et al., 1996 |
| | (20) | | | −12.0 to −3.5 | Zimmer, 1996 |
| | (20) | | | −12.0 to −5.0 | Ehleringer et al., 1999 |
| Nicotine | (21) | *Nicotiana tabacum* (tobacco leaves +2.9‰)[c] | Ornithine (α-+δ-amino N) (0‰), aspartic acid (+8.5‰) | −5.2±0.5 | Jamin et al., 1997 |
| Caffeine | (22) | *Coffea arabica*, *Theobroma sinensis* | Glycine (+2.5‰), glutamine (2 *+ 6‰), aspartic acid (−5‰) | +2.6±2.0 | Danho et al., 1992 |
| | (?) | | | +3.0 to +5.0 | Weilacher et al., 1996 |
| Methyl-*N*-methyl-anthranilate | (20) | Mandarin essential oil (fruit pulp +6‰)[d] | glutamine (+11‰) | +4.2 to +8.3 | Faulhaber et al., 1997 |

Mean $\delta^{15}$N-values used in text are calculated from the references.
[a] Semisynthetic from morphine.
[b] $\delta^{15}$N-Values of coca leaves: +0.1 to +13.0‰ (Ehleringer et al., 2000).
[c] $\delta^{15}$N-Values of leaves from the same tobacco plants: 0 to +10.3‰ (Jamin, pers. commun.).
[d] $\delta^{15}$N-Values of pulp from orange juices: +4 to +8‰ (Kornexl et al., 1996).

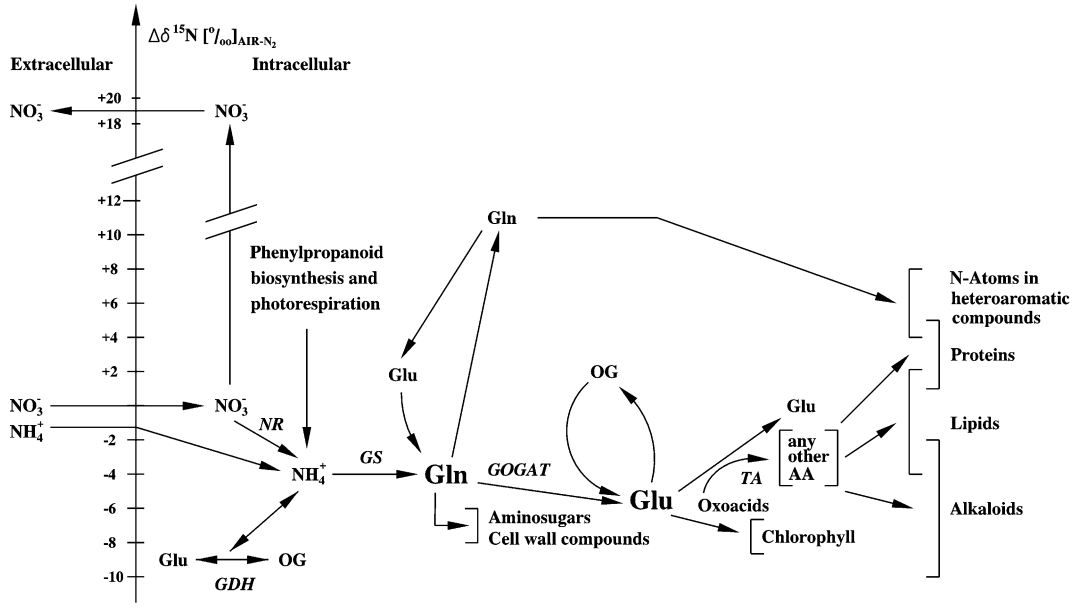


Fig. 3. Generalised mean $\delta^{15}$N-value shifts between organic compounds within the system plant. The first intrinsic precursor with a small metabolic pool, but large turnover is $NH_4^+$, originating from different sources. From here, the plant can be more or less regarded as a "closed system" (except for losses of $NH_3$). The display does not integrate the effects of compartmentation, pools and metabolite transports. Central reaction of nitrogen isotope discrimination is the GOGAT reaction, involved in the net primary production of α-amino-N, but also in the N-recycling processes phenylpropanoid biosynthesis and photorespiration. Enzymes: *NR* = nitrate (+ nitrite) reductase, *GS* = glutamine synthetase, *GOGAT* = glutamine:2-oxoglutarate amino transferase, *GDH* = glutamate dehydrogenase, *TA* = transaminases, *GF* = glutamine:fructose-6-P amino transferase. Substrates: OG = 2-oxoglutarate, OA = oxoacids, AA = amino acids.

glutamine originating amide-N-atoms a $\delta^{15}$N-value $+6\pm3$‰, to α-amino-N originating ones $+1\pm3$‰ taking into account the relative $^{15}$N-abundance order for individual amino acids according to Table 2), ω-amino-N originating ones $-4\pm3$‰, and to those from aspartic acid α-amino-N $-5\pm3$‰.

Applied to tryptophane, the side chain of this amino acid, originating from serine, should be characterised by a relative low $\delta^{15}$N-value for α-amino-N, namely −2‰, while the indole ring with an N-atom from glutamine should attain +6‰. The calculated global $\delta^{15}$N-value +2‰ is in good agreement with experimental $\delta^{15}$ N-values of indole alkaloids (brucine, strychnine), namely −0.3 to +1.5‰, obtained from data of Gaebler et al. (1963). Correspondingly, the global $\delta^{15}$N-value of histidine with N-atoms from aspartic acid (−5‰), α-amino-N (+1‰) and glutamine (+6‰) is calculated to be 0‰, while the experimentally determined $\delta^{15}$N-value, obtained from Table 2, is +0.75‰. Finally, the corresponding calculation for caffeine, alkaloid with four N-atoms, one of them from glycine (+2.5‰), one from aspartic acid (−5‰), and two from glutamine (+6‰) yields +2.4‰ in excellent agreement with the data in Table 3. It has, however, to be kept in mind that the two N-atoms from glutamine are introduced by two quite different enzymes and via different reaction mechanisms, which may also imply different kinetic nitrogen isotope effects. Martin (1995) tried to determine the $^{15}$N-pattern of natural caffeine by quantitative $^{15}$N-NMR. He found different signal heights for the individual positions, however, as these were partially due to differences in the nitrogen relaxation times, a quantitative interpretation was not possible.

## 3. Conclusions

A synopsis of the compiled isotope data and the correlations of N-containing compounds from higher plants permits to establish a global view of the metabolic flux of nitrogen between organic compounds in plants and the implied isotope discriminations and shifts. The display in Fig. 3 does not include carrier compounds from mycorrhiza or symbiotic nodules, and it does not take care of possible nitrogen isotope discriminations within the plant due to compartmentation and transport.

A definite nitrogen isotope discrimination is implied in the nitrate reduction, leading to relatively $^{15}$N-depleted $NH_4^+$ and highly $^{15}$N-enriched $NO_3^-$, which is accumulated and/or excreted; a depletion in $^{15}$N of $NH_4^+$ towards an external source is obviously implied in the transport itself. From $NH_4^+$ and glutamine as intrinsic starting materials the subsequent nitrogen metabolism must practically elapse like in a closed system, which means that an isotopic shift in one direction must be balanced by a reverse one in another direction. The glutamine synthetase reaction itself, dominant process for the transfer of ammonia into organic binding in higher plants (Gerendas et al., 1997), although implying a kinetic nitrogen isotope effect, does normally not contribute to a $^{15}$N-discrimination, due to its irreversibility and quantitative performance. A sometimes observed small positive or negative shift of the $\delta^{15}$N-value of ammonia relative to that of glutamine (Yoneyama, 1995) under special conditions of metabolism (Schlee et al., 1994) is rather due to an interference of the glutamate dehydrogenase reaction, preferably working in the direction of $NH_3$-liberation, or to variations of the $NH_3$-flux.

The central role of the amide nitrogen of glutamine and the various enzymes involved for the $NH_2$-transfer from here has recently been reviewed by Zalkin and Smith (1998). Correspondingly, we attribute the predominant nitrogen isotope discrimination in plants (and in mycorrhiza and nodules as well) to isotope effects on these transaminations, mainly to the GOGAT reaction (for which a kinetic isotope effect in vitro has so far not yet been measured), leading to a remarkable $^{15}$N-enrichment of non-converted substrate and to metabolic pools of relatively $^{15}$N-depleted products, mainly glutamic acid. In this context it has to be reminded that the GOGAT reaction is not only involved in the de novo nitrogen binding, but also constitutes an essential part of the N-recycling in the phenylpropanoid biosynthesis and the photorespiration, and is therefore not only located in chloroplasts but also in the cytosol. A probable nitrogen isotope effect on the glutamine:fructose-6-P transaminase reaction (He et al., 2000), which has also not yet been determined, will additionally contribute to the observed $^{15}$N-enrichment of the residual glutamine. The latter is the N-source of anthranilic acid (Haslam, 1993) and its descendents, as well as of certain positions in heteroaromates.

The glutamic acid formed by the GOGAT reaction is by itself the centre of a recycling system, feeding the α-amino-N pool of all other amino acids. A kinetic isotope effect on the transaminations involved must be the cause for the relative $^{15}$N-depletion of most amino acids and a $^{15}$N-enrichment of glutamic acid; the nitrogen isotope effect of the transamination to ω-positions must be extraordinarily large. A central importance has also to be attributed to aspartic acid/aparagine. The overlap of influences of transport, cell compartmentation, metabolic pools and kinetic isotope effects on different conversion reactions of this amino acid has been discussed in Section 2.4.4. Like glutamic acid and aspartic acid, all amino acids are sources for proteins, many of them also for secondary products (lipids, alkaloids), a condition, which probably leads to a slight $^{15}$N-enrichment of the former and remarkable depletions in $^{15}$N of

the latter compounds relative to the mean global $^{15}N$-abundance of the plant biomass.

Data for a possible contribution of compartmentation and transport phenomena within the plant to nitrogen isotope discriminations do not exist so far. The mechanisms of plant intrinsic transports of organic compounds between compartments or organs must be similar to those described for transports between symbiontic microorganisms and plants (Section 2.3.1.), hence also the implied isotope discriminations. These are depending on actual flux conditions. So, for example metabolic fluxes between different plant organs can change their direction in the course of the development of the plant (Masclaux et al., 2000), and the $^{15}N$-abundance of amino acids in these plant organs can vary with the development state (Yoneyama et al., 1997). Also in context with the discussed recycling processes (phenylpropane biosynthesis and photorespiration) metabolite transports are needed, and they must be adapted to individual conditions. Many processes, e.g. the phenylpropanoid biosynthesis, are occurring in different compartments and correspondingly, differently regulated isoenzymes of phenylalanine:ammonia lyase are existing. Similarly purines and pyrimidines are synthesised in the cytosol and in the chloroplasts as well, and the metabolic turnover varies with the actual cell status.

A final aspect to be discussed in context with the nitrogen isotopic abundance in plant products is, whether it is thermodynamically directed. A "thermodynamic order" of the relative $^{15}N$-abundances in dependence on individual binding forms would demand a complete equilibrium or steady state for all reactions involved in the corresponding biosyntheses (Schmidt, unpublished). As has been shown, this is not even true for the transamination within the α-amino-N pool. A global check of a possible partial thermodynamic order of a nitrogen isotope distribution between organic nitrogen in different binding forms can be performed on the base of isotope effects on binding enthalpies (Ivlev et al., 1974; Galimov, 1985), and on equilibrium nitrogen isotope effects on protonations (Rishavy and Cleland, 1999). The following relative $^{15}N$-abundance sequence turns out: Heteroaromatic N $\geqslant$ tertiary amine- and amide-N (?) > secondary amine- and amino acid-N > primary amine-N > ammonia-N.

Some of the experimental $\delta^{15}N$ data seem to be in line with this sequence, however, after our opinion only by chance. Many of the representatives of the nitrogen binding types in question are at the end of irreversible biosynthetic reaction sequences, and the central key reactions in the plant nitrogen metabolism, e.g. the glutamate synthetase reaction, are irreversible per se. Therefore the nitrogen isotope discriminations in plants must be dominated by kinetic isotope effects on enzyme catalysed reactions in connection with metabolic branching events. The reactions are probably mainly transaminations, among these as most important and efficient representative the GOGAT reaction.

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