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Transformation of plant biochemicals to geological macromolecules during early diagenesis

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Abstract Chemical and isotopic changes in plant biochemicals that were transformed into organic geochemicals have been measured in anaerobic, freshwater marsh environments. In two litter bag studies, plant biochemicals decayed extensively in the first year, as recorded by dry weight, C:N ratios, $\delta^{15}N$ of bulk tissue and amino acids, and $\delta^{13}C$ of individual amino acids. Molecular analyses of Rubisco revealed that the high-molecular-weight enzyme subunit could be recognized antigenically for at least 12 months, but concentrations and amounts declined. Geochemical compounds, advanced glycation endproducts, were not found in fresh plants, but formed gradually with first indications documented at 3 months. The organic remains of plants were reworked or replaced by microbial products from decomposition, as indicated by a shift in the isotopic composition of individual amino acids in total plant protein. In experiments with Rubisco, isotopic changes over time in the individual amino acids in the 50–60 kDa molecular weight range were substantial. These high-molecular-weight substances were no longer pristine molecules. Biochemical and isotopic tools for studying living processes have been demonstrated to be effective and novel approaches to identify and quantify altered geochemical remnants.

Key words Stable isotopes · Proteins · Decomposition · Diagenesis · Marshes

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Introduction

Tracing biochemicals through complex food webs can be fruitfully accomplished with stable isotopes at the natural abundance level. Since the pioneering work of Craig (1953), carbon isotope ratios of plants have proven invaluable for detecting primary photosynthetic pathways (Lajtha and Marshall 1994), for delineating food web relationships (Fry and Sherr 1984), and for monitoring environmental conditions (Marino and DeNiro 1987). Specific carbon molecules that form the backbone of the major biochemical moieties, namely carbohydrates, proteins, and lipids, transfer from plant to animal changing their molecular form and function, but often without significantly changing their isotopic compositions. The significant isotopic fractionations that go into the biosynthesis of high-molecular-weight molecules are integrated by measuring the isotopic compositions of the composite sample, or the bulk material.

Although isotope tracers in bulk material are exceedingly powerful delineating tools, new insights into physiological and biogeochemical processes can be addressed with stable isotopic analyses of separated compounds. The early work of Abelson and Hoering (1961) and Parker (1964) documented the molecular diversity in isotopic signatures within an organism or a specific biochemical class of compounds. The compound-specific isotope research in these early studies illustrated the potential that stable isotopes hold, but not until more user-friendly methods appeared did the application of compound-specific isotope analysis substantially impact the field (e.g., Freeman et al. 1990; Hayes et al. 1990). Almost every biochemical and geochemical compound that can be separated by gas chromatography (GC) can be potentially analyzed for its carbon isotopic composition.

In this paper, compound-specific isotope analysis of amino acids in intact, high-molecular-weight proteins is emphasized. Proteins have pronounced differences in primary, secondary, tertiary, and quaternary structures

that dictate their function in biological organisms. Proteins can be soluble or membrane bound, they can have long turnover times (e.g., collagen), or their concentrations can vary on daily time scales (e.g., serum albumin). Because these molecules are synthesized in distinct temporal and spatial regimes in organisms, there is great potential for measuring the differential incorporation of stable isotopes in proteins in living organisms.

When an organism is consumed, or dies and enters the geological environment, transformations in the physical, chemical and, potentially, isotopic compositions of the original biomolecules begin. Some original molecular-structure information, as well as the composite isotopic signature (e.g., Tuross et al. 1988; Fogel et al. 1997), may remain intact during diagenesis (Benner et al. 1991; Opsahl and Benner 1993). At the molecular level, however, proteins unfold, are hydrolyzed by microorganisms, or combine with free sugars to form chemical structures resistant to biological decay. With each of these processes, the potential for a change in the isotopic composition of the residual molecule increases. Moreover, original biochemicals can become contaminated with exogenous organic matter. All depositional environments contain microorganisms or fungi that degrade all but the most resistant biochemicals. Soluble and structural proteins from these organisms can potentially infiltrate original organic material, thereby altering not only molecular structure, but potentially the isotopic composition. Finally, the transformation of primary organic matter to secondary organic substances is a critical part of global carbon and nitrogen cycling. Understanding the formation of geochemical organic matter in soils and marshes, therefore, is important for interpreting some of the major carbon reservoirs in the terrestrial carbon cycle (Boutton and Yamasaki 1996; Hedges and Oades 1997).

Carbon cycling is intimately linked to nitrogen, often a limiting nutrient in both terrestrial and marine ecosystems. If nitrogen cycling controlled detrital food webs, then the survival and cycling of proteins, in addition to resistant biomolecules, is important. Litter bag studies from a variety of depositional environments, including freshwater marshes, soils, and salt marshes, determined that soluble, organic nitrogen was initially leached from plants by physical processes (Zieman et al. 1984; Benner et al. 1991; Lee 1992; Opsahl and Benner 1993). Nitrogen was then "immobilized" and entered the plant remains from unidentified, environmental sources.

The observation that protein material was being incorporated and retained in plant tissues did not fit with the paradigm of resistant biomolecules as the primary constituents of geochemical organic matter. What was the source of immobilized nitrogen? Does any of the original plant protein persist? From an ecological perspective these questions are of interest because carbon and nitrogen cycling in sediments and soils is linked to widespread changes in landscapes. From the geochemical perspective, confirmations of indigenous organic and isotopic signals in fossil materials should hold clues to forms of life and environmental signals throughout Earth's history.

As stable isotope mass spectrometry gained importance in the geological community, biologists and biochemists began developing the tools that today drive the burgeoning field of biotechnology. Protocols were developed to enable most biological laboratories to identify and quantify the presence of specific biomolecules. Specific proteins could be located in cells, their molecular weight distributions understood, and their amounts quantified (Harlowe and Lane 1988). In this paper, we couple analytical methods from biochemistry to compound-specific isotope techniques to demonstrate the potential power of the two, combined techniques. Two series of litter bag experiments were conducted to test the utility of these methods. The source and timing of nitrogen immobilization in organic matter in the geosphere was investigated. We were able to demonstrate that many of the original plant proteins were altered chemically and isotopically through the insertion of bacterial molecules during the fossilization or immobilization processes.

Materials and methods

Sample collection and deployment of litter bags

Plants for both experiments were collected in mid September 1990 and 1996, at the Jug Bay Wetlands Sanctuary in Anne Arundel County, Md. Senescence had started in late August to early September, and leaves were starting to turn from bright green to light green with small patches of brown. All samples were freeze-dried, and plant tissues were kept whole. The first series of litter bag experiments was conducted from 1991 to 1993. In this experiment, aerobic (surface) decomposition was compared with anaerobic (subsurface) decomposition. For surface deployments, leaf tissues were sewn into nylon mesh bags (100 μm pore size), whereas roots and/or rhizomes were incubated in polypropylene bags at least 30 cm belowground in anoxic sediments. Plants included wild rice (*Zizannia aquatica*) (leaves vs roots) and the marsh plant spatterdock (*Nuphar* sp.) (leaves vs. roots and rhizomes). In January 1991, litter bags were deployed in the freshwater tidal marsh where they had been originally collected. Bags were harvested periodically.

The second series of plants included holly (*Ilex opaca*), alder (*Alnus serrulata*), mountain laurel (*Kalmia latifolia*), and oak (*Quercus alba*) leaves, and pine (*Pinus virginiana*) and red cedar (*Juniperus virginiana*) needles. These samples were collected from trees adjacent to the freshwater tidal marsh. The rhizomes and roots of spatterdock, and locally grown corn stalk (*Zea mays*) were also sampled. All tissues were freeze-dried before initial weight determination, prior to sewing into litter bags. Bags with leaves contained between 3 and 10 g dry weight, but the wood and corn stalk bags held up to 75 g of material. At least ten samples of each plant type were prepared. All litter bags in this study were constructed with polypropylene mesh (100 μm). The litter bags were then buried in the marsh at a depth of at least 30 cm, so they would decay anaerobically. In this second study, weights are based on freeze-dried material.

Ash-free dry weights (550°C for 2 h) were determined on completely dried and ground plants. Results for dry weights and % initial nitrogen are calculated on an ash-free basis. Samples (0.5–4.5 mg) were analyzed for CHN on a Carlo Erba 1108 Elemental Analyzer with acetanilide used as the standard.

Amino acid analysis

Between 1–5 mg of dried plant material was hydrolyzed in 1 ml of constant boiling 6 N HCl (Pierce) for 20 h at 110°C. The acid was evaporated under a stream of N_2 at 110°C before derivatizing for GC analysis. For isotopic analysis, isopropyl-N-TFA derivatives of the amino acids were made as previously described (Silfer et al. 1991). Standard amino acid solutions were derivatized with each batch of samples that were processed. Amino acids were detected by fluorescence following reaction with orthothaldialdehyde (o-pa) post-column (Hare et al. 1985).

Stable isotope analyses

Dried, ground material (7–9 mg) was loaded into a pre-combusted quartz tube [6 mm outer diameter (OD) × 4 mm internal diameter (ID)] and sealed under vacuum. After combustion at 910°C for 1 h, samples were cooled under controlled conditions. Gases, N_2 and CO_2, were purified cryogenically on a vacuum line and analyzed by mass spectrometry. Nitrogen analyses were determined on a modified Nier-Johnson double-focusing mass spectrometer, and $\delta^{15}N$ measurements were reproducible and precise to within ±0.2‰. Carbon isotopes were measured on a Finnigan 252 mass spectrometer with precision and accuracy of ±0.1‰.

On-line GC analyses were determined on a Finnigan/Varian 3400 GC-combustion interface (Hayes et al. 1990). Briefly, mixtures of organic compounds were separated by GC, combusted at 940°C in a reactor furnace with CuO, Pt, and NiO wires. Standard amino acids of known isotopic composition were derivatized and measured to correct for carbon added during derivatization (Silfer et al. 1991). For C isotope analyses, about 2 μg of hydrolyzed protein is injected into a Varian 1750 split-splitless injector onto an HP-Ultra 1 column (0.53 mm OD × 0.32 mm ID × 25 m). After various splits in the injector (1:10) and the open split (1:2), about 10 ng of C per peak enters the ion source of the mass spectrometer. For nitrogen isotope analysis, about 3 μg of sample was injected in the splitless mode, so that roughly 100 ng of amino acid is converted to N_2 for analysis.

Each sample was analyzed in triplicate with corresponding standard amino acids. Reproducibility for C isotopes was typically ±0.4‰ with a range of ±0.2‰ to ±1.0‰. For nitrogen isotopes, because the derivative contains no added nitrogen, values were determined directly. Standard deviations on the major peaks, proline, aspartate, glutamate, lysine, and arginine, were about ±0.5‰. Well-resolved but very small peaks, i.e., valine and leucine, had higher errors ±1–2‰.

Polyclonal antibodies

An antibody to the large subunit of Rubisco (55 kDa) was raised in a goat (J.A. Berry, Plant Biology. Carnegie Institute of Washington). The Rubisco large subunit had been purified to homogeneity prior to injection into the animal. This antibody did not cross-react with any human serum proteins. For Western blots and enzyme-linked immunoassays (ELISAs), dilutions of 1:1000 were used. In addition, an antibody was raised in a rabbit to a synthetically produced melanoidin or advanced glycation endproduct (AGE) (Hoering 1973). This particular melanoidin was synthesized autocatalytically by reacting the amino acid, lysine, with glucose. The subsequent polymer had a high molecular weight and was essentially insoluble in water. This antibody was tested to common mammalian proteins and did not cross-react. A dilution of 1:200 was used for all experiments.

Gel electrophoresis

Plant material for protein and carbohydrate electrophoresis experiments was taken from frozen material (not heated) and subsequently ground under liquid N_2 to a fine powder. The grinding was critical, because proteins were not recovered from unground material, even after several days extraction. Proteins were extracted from plant samples with two different reagent solutions: (1) a reducing solution containing Tris-buffered saturated thiourea-urea with SDS and B-mercaptoethanol (Yates and Greaser 1983) or (2) a nonreducing solution containing 8.0 M urea at pH 7.0. With solution 1, about 30 mg of plant material was extracted with 1 ml of solution overnight at room temperature. With solution 2, zirconium beads (Mini Beadbeater) disrupted and extracted samples with three 90-s bursts. Remaining plant material was pelleted by centrifugation, and the supernatant recovered. The extract was then filtered through a 0.45-μm Acrodisc filter.

Protein extracts were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a Novex X-Cell Mini-Cell on 4–20% Tris-glycine gels (20 mA/gel). Standard protein mixtures (Rainbow Marker, Amersham) and protein extracts were mixed with sampling running buffer (Novex) at roughly 1:1 or 1:2 proportions. After separation, gels were stained with a solution of colloidal Coomassie blue (Novex), a general protein stain sensitive enough to detect nanogram quantities of protein. Glycoproteins were stained with Alcian blue according to the procedure of Fisher et al. (1983).

Transfers for Western blots and gold staining were performed with X-Cell Blot Modules (Novex) onto nitrocellulose or PDVF membranes. Transfer buffer for Western blots contained 20% methanol in Tris-glycine solution (4°C). PDVF transfers were performed in CAPS buffer rather than the glycine buffers (Johansen et al. 1992). Macromolecules were transferred from gels to blots for 20 min at 75 V, <650 mA. Blots were rinsed with three consecutive 20-min washes with distilled water to remove SDS, which is incompatible to many of the stains and antibodies. After biochemicals were blotted onto membranes, these membranes were used for a variety of biochemical tests, including gold staining, Western blots, or protein isolation. For total protein, nitrocellulose membranes were then stained with one capful of colloidal gold stain (Aurodye Forte, Amersham) until color developed (15–60 min). Reduced carbohydrates were detected by enzyme immunoassay using the DIG Glycan Detection Kit (Boehringer Mannheim).

High-molecular-weight fractions in the 55-kDa and 12-kDa regions of Western blots were excised from PDVF transfers. Excised pieces were incubated in glass boats, and bound proteins hydrolyzed by vapor-phase reaction with 6 N HCl at 165°C for 45 min. Samples were prepared for amino acid analysis by GC and HPLC analysis as described above.

Western blots

After washing out SDS, nitrocellulose blots containing bound protein molecules from the original gels were incubated with a 1% (w/v) solution of filtered nonfat dried milk (NFDM) in phosphate-buffered saline (PBS) overnight at 4°C. Between steps, the blots were washed three times for 15 min with Tris-buffered saline solution containing Tween (TBS-Tween, pH 7.4). The first antibody was diluted with 50 ml of filtered, phosphate-buffered NFDM. Incubation occurred during a 2-h period with shaking at room temperature.

The blot was carefully washed with TBS-Tween ensuring that each drop of solution was removed. Second antibody linked to horse radish peroxidase (HRP) was diluted with NFDM solution and incubated for 1 h. Electroblots were washed twice as above, followed by a third rinse in Tris-buffered saline with no Tween. Protein bands were visualized by color development (15 min to overnight) using 4-chloro-1-naphthol solution in the presence of H_2O_2.

Enzyme-linked immunoassays

ELISAs were performed on urea extracts of plant samples. Filtered extracts were diluted 1:10 with carbonate-bicarbonate buffer (Sig-

ma). An aliquot (200 µl) of this solution was diluted serially (1:2) along the wells of the ELISA plate. Antigen was allowed to bind to the wall of the plate for at least 1 h. Excess solution was vigorously tipped out of the plates and washed three times with TBS-Tween. Blocking with PBS-NFDM (200 µl/well) took place at 4°C overnight. First and second antibodies were prepared, incubated (100 µl/well), and washed as above. Color development of the antibody complexes occurred with HRP in citrate buffer with opd (Sigma) and H_2O_2. Quantification was determined by measuring the OD at 490 nm.

Results and discussion

Chemical and isotopic composition of bulk plants in early diagenesis

Decomposition rates were highly dependent on the type of plant: grasses, both roots and leaves, degraded more slowly than the leaves, roots and rhizomes of herbaceous tissues (e.g., wild rice vs spatterdock, Fig. 1A). Large storage rhizomes and carbohydrate-rich stalks, such as those from spatterdock or corn, lost a considerable portion of their weight in less than 3 months. For the spatterdock, no long-term distinction could be found between aerobic and anaerobic decomposition rates as measured by ash-free dry weight. In all of the 1- to 2-year decay experiments, a significant portion of the plant organic matter remained. The chemical nature of this resistant material was investigated with further measurements.

Considerable changes in the C:N ratios of the plants occurred with time. In the high C:N rhizome and stalk tissues, C:N decreased sharply, indicating removal of carbohydrates which contain only C, H, and O (Fig. 2A). Following these initial losses of C, the %C in degraded plants was relatively constant, while the %N values, typically between 0.5 and 1.5% of the total weight, showed the greatest relative changes (Fig. 2B). During aerobic degradation, nitrogen was leached from plant material without significant evidence for the immobilization of N. In anaerobically incubated tissues, nitrogen immobilization, as reflected by % initial N, varied considerably over a 2-year period. The changes were related to increases and decreases in temperature of the sediments, whereby increased temperature resulted in loss of N and decreased temperature resulted in immobilization of N.

Nitrogen isotopes in plant material changed after both aerobic and anaerobic incubation, but not in the same direction (Fig. 3A; Fogel and Tuross 1995). Some degraded plant tissues increased in $\delta^{15}N$, whereas in others $\delta^{15}N$ decreased with extent of degradation (Fig. 3B). Isotope shifts in $\delta^{15}N$ of anoxic incubations covaried with fluctuations in the %N initial. Thus, as N was mobilized and immobilized into plant tissue, its chemical and isotopic composition changed (Fig. 3C).

Carbon isotopic changes in all plants including leaves, roots or rhizomes, woods, or grasses were typi-

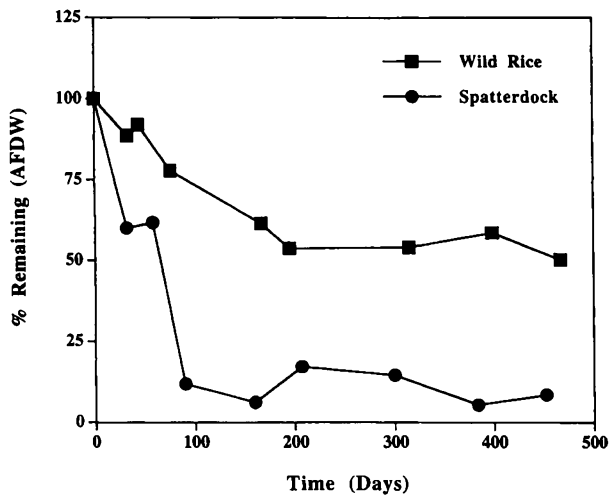


Fig. 1 Comparative decomposition of anaerobic incubations of wild rice (*Zizannia aquatica*) and spatterdock (*Nuphar*) from the 1991–1993 litter bag series (*AFDW* ash-free dry weight)

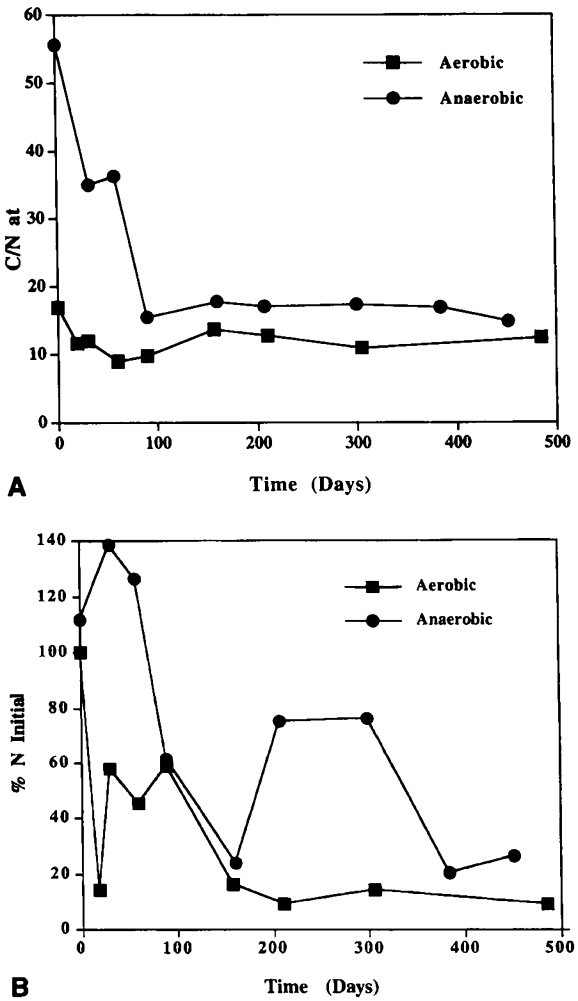
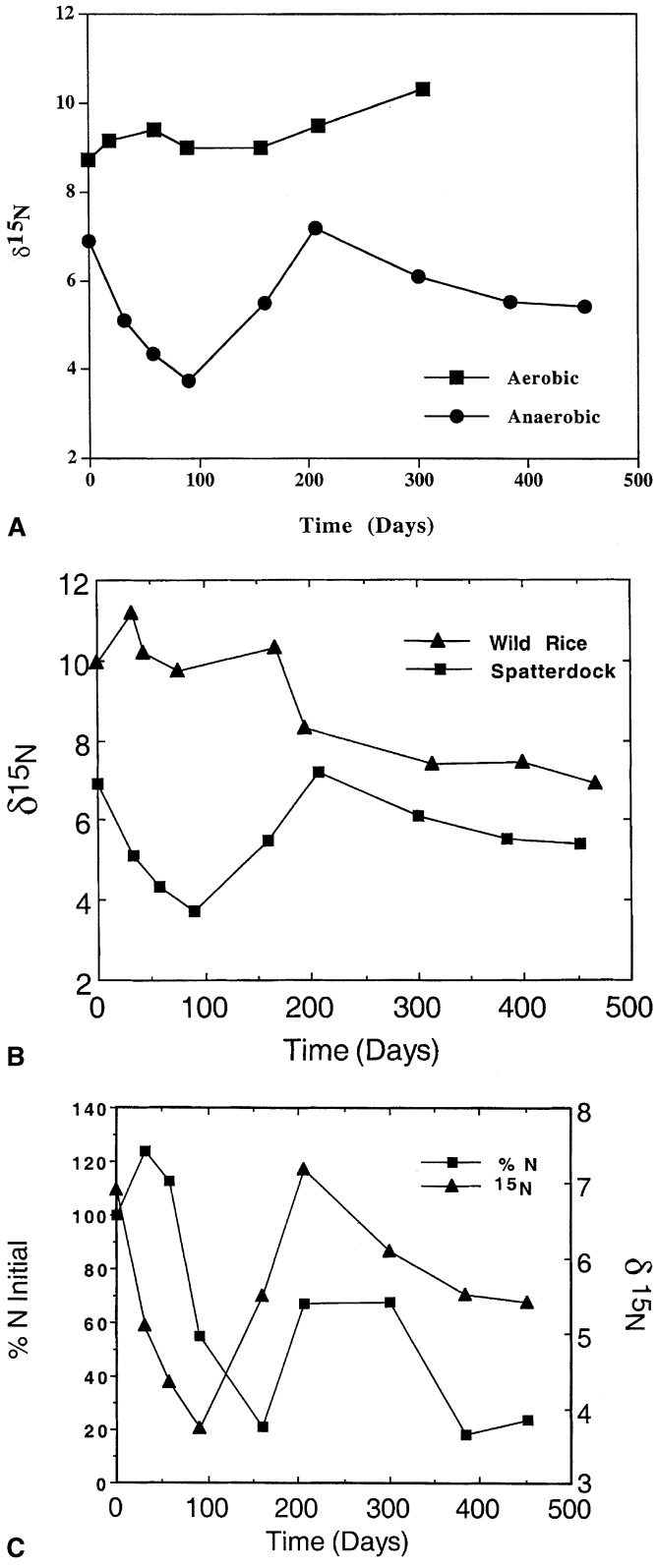


Fig. 2 **A** Changes in atomic C:N ratios during decomposition of spatterdock roots and rhizomes (anaerobic) as compared with leaves (aerobic). **B** Fluctuations in the %N initial vs time. Increases in this parameter indicate nitrogen immobilization, whereas decreases signify leaching and loss of nitrogen. Above- and belowground degradation processes have different patterns of N movement



cally within ± 1‰ of starting material. Although carbon isotopes in the bulk tissues exhibited virtually no change, the carbon isotopic compositions of the individual amino acids were significantly shifted by the decay

Fig. 3 **A** Nitrogen isotope ratios of spatterdock leaves and roots and rhizomes were slightly different in fresh plant material. As degradation proceeded, the isotopic compositions of both incubations changed by ± 3‰. **B** Although these two plants were incubated in the same region of the marsh, their isotopic compositions varied by as much as 7‰ in the early stages of diagenesis. With time, the $\delta^{15}N$ values began to parallel one another and were within 2‰ of each other. **C** Changes in the nitrogen isotopic composition of residual wild rice root material as they relate to fluctuations in the amounts of elemental nitrogen. As nitrogen was immobilized (% N increase) or mobilized (% N decrease), $\delta^{15}N$ values deviated

process (see Fogel and Tuross 1995). The typically wide range of $\delta^{13}C$ values of amino acids decreased from 23‰ in fresh tissue to 18‰ in degraded plants.

In addition, the amino acid concentrations of the plant tissues increased with increasing decomposition. On average, the total pool of hydrolyzable amino acids in litter bag samples increased over time. For example, the amino acid concentration in one of the wild rice litter bags was 60 μmol amino acids/g dry weight of plant material. After 14 months of decomposition, the decayed wild rice roots had 124 μmol amino acids/g dry weight of plant material. In terms of absolute amounts, there were 112 μmoles in the litter bag at the start of the experiment, but 141 μmoles at 14 months. Because there is a shift in the amount and the isotopic composition of the amino acids as immobilization progresses, carbon and nitrogen cycling have been extensive in the plant tissues during early diagenesis. Indeed, microbial processes strongly influenced the chemical and isotopic compositions of degraded plants.

Biochemical transformations in high-molecular-weight plant components

Protein degradation

Proteins extracted under reducing conditions were separated by SDS-PAGE. Distinct bands corresponding to major plant proteins were stained with Coomassie colloidal stain in both fresh and decayed plants (Fig. 4). Of the eight plant samples analyzed, all fresh plants had protein bands at 55 kDa, which represents the large subunit of the photosynthetic protein Rubisco (Miziorko and Lorimer 1983). By comparison, only five of the decayed plants showed distinct or faint bands at 55 kDa; the decayed corn sample contained a protein at 52 kDa. In the fresh plants, bands at 12–14 kDa corresponding to the small subunit of Rubisco were detected in seven plants, whereas six of the degraded plants had bands in this region. Seven of the fresh plants had higher-molecular-weight bands in the region from 70 to 100 kDa. The 100-kDa band has been linked to dimers of Rubisco. In contrast, only two of the decayed plants had higher-molecular-weight bands, and those were in the region of 200 kDa, a size considerably larger than found in fresh material. To summarize, the number of

distinct proteins and their corresponding molecular weights on average decreased, but did not disappear following 1 year of anaerobic decay.

Two different immunological techniques were used to confirm the presence of the large subunit of Rubisco, ELISA and Western blots. ELISA, the most sensitive immunological assay, is capable of detecting about 0.01–0.1 ng of protein (Harlowe and Lane 1988). It is a rapid and easy method yielding quantitative results. Western blotting combines the resolution of proteins by SDS-PAGE with immunological detection, so that protein molecular-weight can be estimated. A major drawback is the dependence of the method on the reaction of the antibodies with the protein that must be denatured for SDS-PAGE. Because antibodies often recognize the folded secondary or tertiary regions of a protein, the amount of protein that be detected by Western blot is considerably less: a minimum of 1 ng of protein is required.

On a weight-corrected basis, three different patterns of Rubisco degradation were detected as a function of time (Fig. 5). In corn, Rubisco levels dropped sharply in the first 3 months, but then remained constant for up to 1 year. In spatterdock, Rubisco decayed roughly parallel to the loss of weight in the total material, but in the mountain laurel leaves, Rubisco concentrations only changed slightly after 1 year. Analysis by Western blot revealed that the molecular weight of the Rubisco large subunit recognized by the antibody did not decrease with increased degradation, but the amount of protein recognized by the antibody was much less (Fig. 6). There

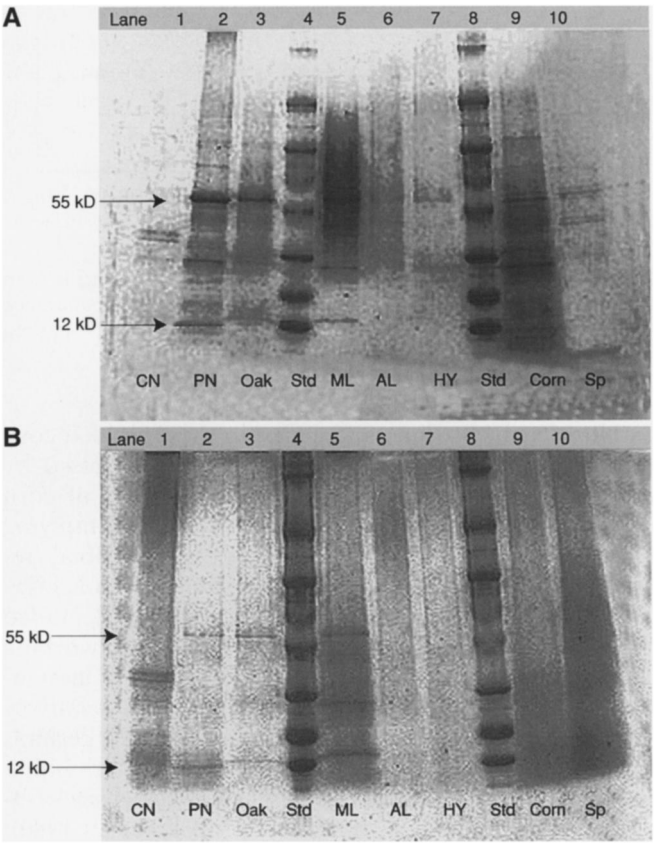


Fig. 4 SDS-PAGE of protein extracts from fresh (**A**) and decayed (**B**) plants. Tris-glycine gels (4–20%) were used with running buffer that included β-mercaptoethanol. Plant samples in lanes are as follows: *lane 1* cedar needles, *lane 2* pine needles, *lane 3* oak leaves, *lane 4* protein standard, *lane 5* mountain laurel, *lane 6* alder, *lane 7* holly leaves, *lane 8* protein standard, *lane 9* corn, *lane 10* spatterdock

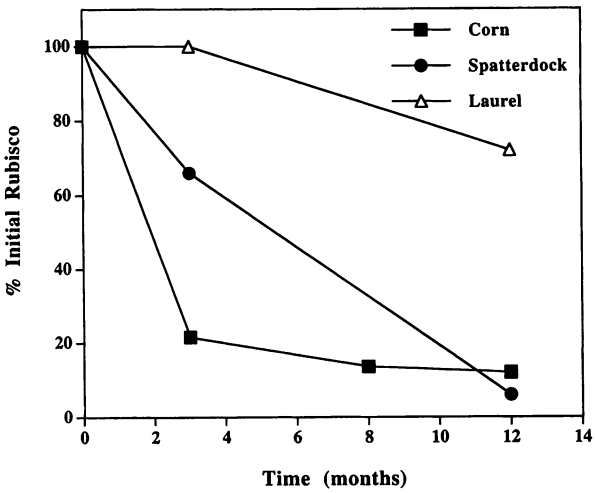


Fig. 5 Decrease in Rubisco in degraded plants as a function of extent of decomposition. *% Initial Rubisco* was calculated on a weight-corrected basis, similar to the %N initial. Rubisco concentrations were determined by ELISA. Purified Rubisco (Sigma) was used as a standard

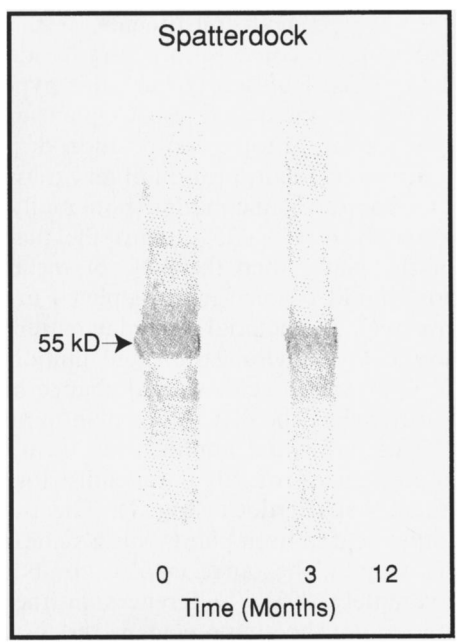


Fig. 6 Western blot of Spatterdock rhizome. After 3 months decomposition, a band corresponding to the large subunit of Rubisco can be clearly seen. In corn and mountain laurel samples, evidence for high-molecular-weight Rubisco at approximately 55 kDa was visualized up to 12 months (data not shown)

was no evidence for antigenically recognizable Rubisco at lower molecular weights. Either Rubisco was thoroughly degraded to very low molecular weight products, or if it remained at higher molecular weights, the large subunit was no longer recognized by the antibody.

To examine how degraded proteins are associated in the environment, PAGE was conducted under reducing (with sulfide reagents) and nonreducing (without sulfide reagents) conditions. Addition of β-mercaptoethanol is used in protein extraction buffers to cleave S-S bonds, such that proteins have a more uniform shape (i.e., linear) for migrating through the gel. Nonreducing conditions (i.e., S-S bond intact) are required for immunodetection, because reducing reagents can inhibit antibody binding.

After electrophoresis, proteins were transferred from gels to nitrocellulose and stained with colloidal gold. Under nonreducing conditions, the major protein bands, stained a reddish color, ranged from 20 to 80 kDa. By comparison with Western blot using Rubisco antibody, the 55-kDa band was part of the gold-stained protein in the 20- to 80-kDa molecular-weight range. Under reducing conditions, the molecular-weight distribution of the proteins decreased to 10–20 kDa with almost no stainable proteins in the 20- to 80-kDa range. Given this difference in staining, it appears that proteins in degraded samples may be held together by secondary disulfide bonds.

Isotopic changes in individual amino acids

If high-molecular-weight molecules of Rubisco remained, then amino acid constituents of this protein should have isotopic compositions almost identical to those in the original Rubisco. To test this hypothesis, a C_4 plant (corn) was incubated in a C_3-plant-dominated marsh. If changes in isotopic composition of plant material were driven by incorporation of an external source of soluble C_3-based organic matter (potentially Rubisco of approximately $\delta^{13}C = -25‰$) into the macroscopic remains of the plant, then the $\delta^{13}C$ of the individual amino acids should become more depleted in ^{13}C with time. Conversely, if bacterial organic matter replaced plant molecules by mineralization and immobilization, then the $\delta^{13}C$ of amino acids should change but would not necessarily reflect the $\delta^{13}C$ of C_3 plant amino acids.

The $\delta^{13}C$ of individual amino acids from the total proteins of corn had a roughly parallel distribution with those of the C_3 spatterdock (Fig. 7). The isotopically heaviest amino acid in both plants was glycine, while the lightest was valine. The range in $\delta^{13}C$ for both plants was approximately 20‰. Differences in the isotopic partitioning among the amino acids is the result of isotopic fractionation during biosynthesis (Abelson and Hoering 1961; Macko et al. 1987). Degraded plants had several amino acids with a $\delta^{13}C$ that shifted to more positive values: aspartate, alanine, glutamate, lysine, leucine, and valine in the spatterdock rhizome, and

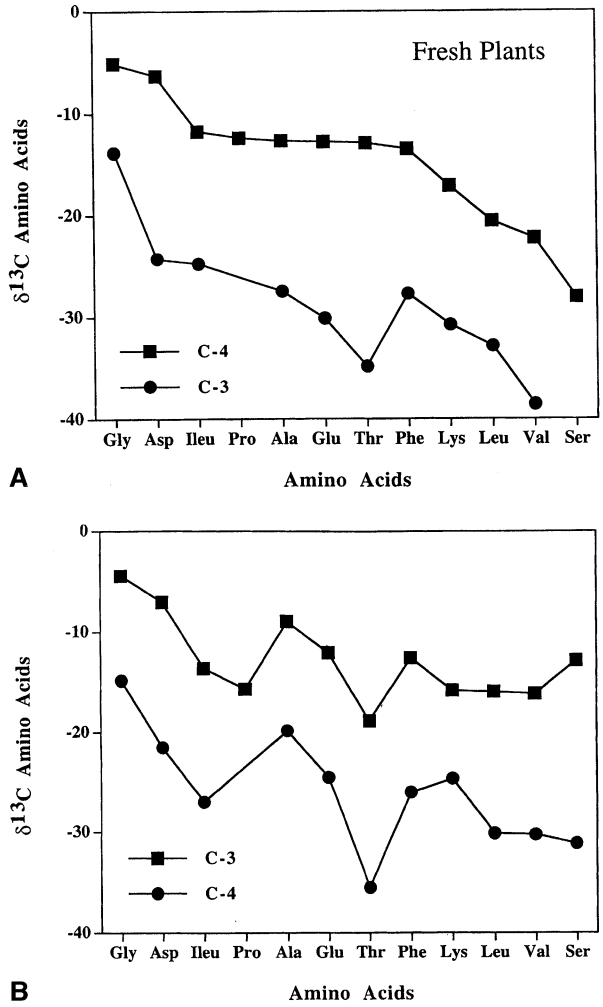


Fig. 7 Carbon isotopic compositions of individual amino acids from total proteins in fresh (**A**) and decayed (**B**) corn (C_4) and spatterdock (C_3). Note that traces are roughly parallel, but that the relative pattern in carbon isotopic labeling changes with degradation

alanine, leucine, valine, and serine in the degraded corn stalk. Ranges in $\delta^{13}C$ of the plants were depressed by only 15‰ (threonine excepted). The C_4 signal of corn did not collapse to that of the C_3 spatterdock, implying that the amino acids originated from microbial reworking of the corn rather than external amino acids.

Nitrogen isotopic shifts between fresh and degraded plants were even more diagnostic of bacterial reworking (Fig. 8). The range and distribution of $\delta^{15}N$ in individual amino acids of spatterdock (2–8‰) was relatively small in comparison to carbon isotopes (Fig. 7), perhaps because transamination enzymes that transfer amino groups from amino acids to the carbon skeletons of α-ketoacid precursors are limited (see Macko et al. 1986). With the exception of phenylalanine, all of the $\delta^{15}N$ of individual amino acids in degraded spatterdock were shifted, many of them to more negative $\delta^{15}N$. The porewaters of the sediments in which the decomposition occurred have very high concentrations of ammonium (millimolar levels were determined in the 1991 experi-

ments). Not only are bacteria resynthesizing carbon skeletons (Fig. 7), but they are also incorporating nitrogen directly into amino acids with large isotopic fractionations (Hoch et al. 1992, 1994). This uptake of nitrogen from porewater into proteins most likely represents the "immobilization" phase of decomposition.

To address whether the alterations in the $\delta^{13}C$ of amino acids affected the isotopic signature of Rubisco itself, distinct protein bands in two molecular-weight regions of the electroblot were analyzed for their amino acid isotopic compositions. An area of the blot that corresponded to 55 kDa (range 50–60 kDa), and presumed to represent the large subunit of Rubisco, was compared to a band at 12 kDa (range 10–14 kDa) (Fig. 9). The lower-molecular-weight material was the bulk of the protein in corn. Isotopic signatures of amino acids within individual proteins from fresh plant material were not identical (Table 1), especially with respect to aspartate, glycine, and glutamate. The $\delta^{13}C$ of amino acids from the separated protein bands were similar, but not identical to the $\delta^{13}C$ of the amino acids from the total corn. Therefore, if one specific protein is degraded relative to another, then overall isotopic composition of the bulk material can shift in response to preferential decay or preservation of particular molecules.

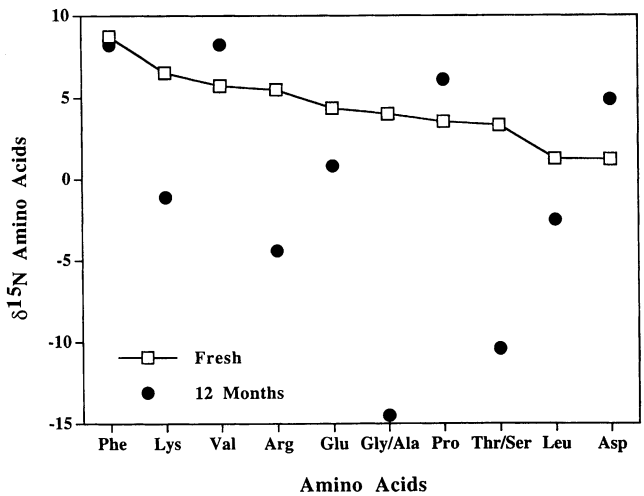


Fig. 8 Nitrogen isotopic compositions of individual amino acids from total spatterdock plant protein. Fresh material had very conservative values, whereas the degraded material was altered in almost every amino acid by up to −15‰

Alternatively, the isotopic composition of the original, higher-molecular-weight proteins could be modified over time. To test this, two additional samples, one at 3 months and another at 7 months, were subjected to the same procedure. On a dry-weight basis, the yield of protein amino acids in the degraded plants was about 10–50% of that in fresh material (Table 2). Numerous peaks corresponding to degradation products were detected on chromatograms. The $\delta^{13}C$ of the amino acids in the 55-kDa region lost their biosignature during decomposition (Table 1). In four amino acids, $\delta^{13}C$ values were more negative (e.g., serine, glycine, glutamate, and valine), in contrast to the more positive $\delta^{13}C$ in the total decayed corn. Based on the high percentage of degradation products in the extracts coupled with the direction of the isotopic shift, the composition of the 55-kDa region appeared to contain mangled proteins of plant origin.

Degradation of sugars

Carbohydrates comprise the bulk of higher plant material, and many bacteria have very specific enzymes for metabolizing cellulose and starch. In litter bag studies

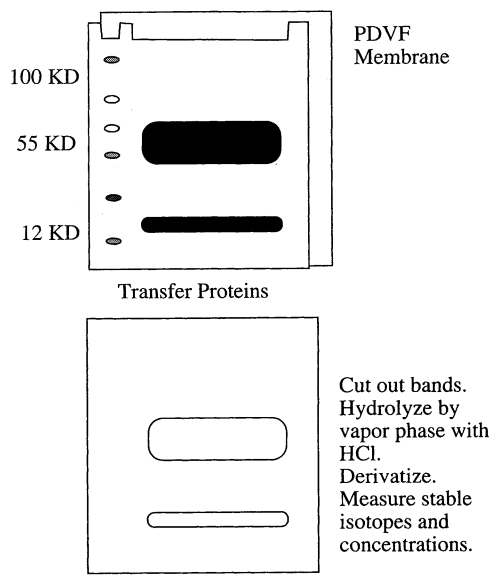


Fig. 9 Diagram of the procedure for separation of high-molecular-weight proteins and isolation from PDVF membrane

Table 1 Isotopic compositions ($\delta^{13}C$) of amino acids from total protein in fresh and decayed corn and from high-molecular-weight fractions of fresh and degraded corn isolated from blots

Amino acids	Total tissue		12-kDa band	55-kDa band		
	T_0	12 months	T_0	T_0	3 months	7 months
Ser	−28.1	−12.8	2.8	0.23	−9.4	−6.3
Pro	−12.4	−15.6	−5.6	−8.0		
Asp	−6.3	−7.0	−8.0	−18.5	−6.2	−24.9
Gly	−5.1	−4.4	−8.2	−16.5	−26.0	−24.7
Glu	−12.8	−12.0	−12.5	−19.4	−26.2	−20.3
Phe	−13.5	−12.5	−18.3	−14.7		
Val	−22.2	−16.1	−19.0	−21.2	−25.8	
Leu	−20.6	−15.9	−20.9	−20.0	−20.6	−14.7

Table 2 Amino concentrations and distributions in 55-kDa fractions of fresh and decayed corn taken from PDVF membranes. Similar weights of plant material were extracted for each sample. Degradation products were detected in extracts derivatized for GC-C-IRMS. Complete separation of glycine and alanine was not acheived

Amino acid	Fresh		3 months		7 mos.	
	pmol/μg dry weight	% total	pmol/μg dry weight	% total	pmol/μg dry weight	% total
Aspartate	0.43	10.8	0.07	3.8	0.24	4.2
Threonine	0.18	4.4	0.03	1.8	0.10	1.6
Serine	0.28	7.0	0.10	5.0	0.24	4.1
Glutamate	0.48	12.2	0.09	4.9	0.27	4.6
Glycine + alanine	1.60	40.4	0.80	42.1	2.45	41.5
Valine	0.35	8.7	0.06	2.9	0.17	2.8
Methione	0.10	2.4	0.00	0.0	0.00	0.0
Isoleucine	0.20	5.0	0.03	1.6	0.85	14.3
Leucine	0.36	9.0	0.72	37.9	1.50	25.3

(e.g., Opsahl and Benner 1999), decomposition of sugars, detected as monomers, proceeded at greater rates than that of other resistant molecules (e.g., lignin). The interrelationships between carbohydrate decomposition and protein diagenesis in high-molecular-weight molecules are considered in our study because of the geochemically significant reactions that can occur between sugars and proteins (Hoering 1973).

The degradation of high-molecular-weight sugars into glycoproteins and as reduced carbohydrates was examined by PAGE. The two pools of carbohydrate had distributions with distinct molecular-weight ranges that did not overlap. Glycoproteins stained with Alcian blue (Fisher et al. 1983) were detected in low-molecular-weight regions in the fresh and degraded corn plants. As decomposition proceeded, higher-molecular-weight smears appeared, possibly reflecting the influx of bacterial glycoproteins and/or the increase of aggregated, glycosylated degradation products. Reduced carbohydrates detected antigenically on electroblots were in the range of >50 kDa in fresh and degraded corn extracts. The molecular weights of the sugars decreased slightly after 1 year of decay. Nevertheless, carbohydrates and glycoproteins were relatively stable molecules that persisted in the plant material for at least 1 year.

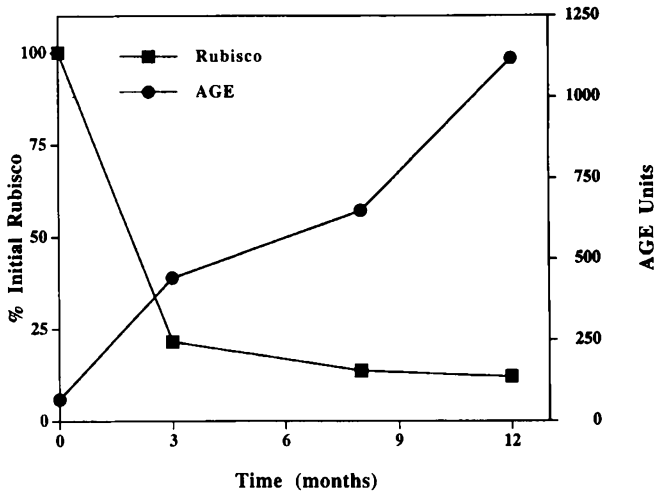


Fig. 10 Relative concentrations of melanoidins (*AGE*) with respect to Rubsico concentrations in corn. Control samples (buffer solution) were compared with plant extracts, and concentrations of melanoidins are relative units calculated from measuring the OD of the colored antibody reaction. As Rubisco decreased, melanoidins increased

Advanced glycation endproducts

Although carbohydrates were detected in all of the samples, the change in residual dry weight of the plants indicated that these compounds were mineralized by microorganisms. One hypothesis in the geochemical literature, popular in the 1970s, concerned the formation of insoluble complex organic compounds from sugars and proteins that are termed humic acids, fulvic acids, melanoidins, or today, advanced glycation endproducts (AGEs) (Hoering 1973; Sensi et al. 1991; Papoulis et al. 1995). AGEs are the presumed "sinks" for biochemical macromolecules in the geological environment. We hypothesized that AGEs would increase in concentration at the expense of a biochemical source, either proteins or sugars. The concentrations of AGEs increased in corn, spatterdock, and mountain laurel as a function of degradation time. The formation of AGEs occurred over a period of several months, and was inversely related to the degradation of Rubisco (Fig. 10). AGEs were high-molecular-weight substances in the range of 60–100 kDa, as confirmed by Western blot.

Summary

The timing of the disappearance of primary organic matter as it is transformed into geochemical organic compounds has been measured in anaerobic freshwater marsh environments. Under anaerobic conditions, higher plant biochemicals decayed rapidly in the first year. Extensive changes were recorded in dry weight, C:N ratios, $\delta^{15}N$ of bulk tissue and amino acids, and $\delta^{13}C$ of individual amino acids. The molecular analyses of Rubisco revealed that the high-molecular-weight

enzyme subunit could be recognized antigenically for at least 12 months, but concentrations and amounts declined. Geochemical compounds, AGEs, did not occur in fresh corn stalk, but were gradually formed over the period of a year with first indications documented at 3 months. From an ecosystem perspective, organic matter in soils and sediments should have similar, or greater, rates of transformation. After 1–2 years of aerobic or anaerobic decomposition, approximately 10–40% of the original material remained.

At this extent of decomposition, the organic remains of primary producers have been extensively reworked or replaced by microbial products from decomposition. The $\delta^{15}N$ and $\delta^{13}C$ of individual amino acids in the total amino acid pool shifted reproducibly. The $\delta^{13}C$ pattern was indicative of resynthesis of microbial protein from a pool of original plant carbon. As an example, the amino acids in degraded corn had C_4-based $\delta^{13}C$. Nitrogen immobilization was tracked with conventional parameters including C:N ratios, %N initial, and amino acid concentrations. Isotopic shifts at the bulk and compound-specific level showed that the changes in nitrogen chemistry were the result of bacterial synthesis including incorporation of sedimentary N into proteins. Future work in this laboratory will concentrate on determining the specific bacterial molecules that survive mineralization processes, e.g., the membrane porin proteins that have been found in dissolved organic matter in the ocean (Tanoue 1992).

Implications

Isotopic ratios of organic matter have been critical determinants of early life processes in the Precambrian (Schopf 1983) and for understanding carbon cycling through Earth's history (Hayes et al. 1990). Recently, nitrogen isotopes of organic matter in ocean sediments have been proposed as useful tracers of paleooceanographic nitrogen cycling (Altabet and Francois 1994). In our study, we documented extensive changes in the $\delta^{15}N$ of the residual organic material that were dependent on degradation pathways. These changes may preclude the use of $\delta^{15}N$ in plant fossil material or sedimentary organic matter for reconstructing paleoenvironments (Altabet and Francois 1994; Calvert et al. 1996). While our experiments were conducted with terrestrial plant material, parallel types of experiments have been conducted with degraded phytoplankton (Nguyen and Harvey 1997) demonstrating similar conversions of high-molecular-weight algal proteins in controlled experiments.

Conversely, $\delta^{13}C$ of the degraded tissue did not change extensively over a 2-year time frame. Bulk $\delta^{13}C$ is a robust parameter (Benner et al. 1991; Opsahl and Benner 1993; Wedin et al. 1995; Ficken et al. 1998) and only when there is extensive preservation of an isotopically distinct compound (e.g., lignin) is the bulk value seriously impacted. The isotopic compositions of specific amino acids in the total protein pool and isolated high-molecular-weight fractions, however, shifted substantially as a result of microbial incursion and diagenetic processes. It is possible that extensive alteration (e.g., deamination or decarboxylation) in the primary amino acid makeup of antigenically recognizable Rubisco was the total source of isotopic signals. However, it is also possible that a background of degradation products was included in the analyses. Resolution of the relative contribution from either of these possibilities will form the basis for future research.

Only 1 in 10,000 carbon atoms fixed in biological material as either carbohydrates, proteins, or lipids ever enters the geological record (Hedges and Keil 1995). Therefore, all occurrences of high-molecular-weight organic substances in geochemical environments deserve special attention. Future work should concentrate on the molecular nature of the immobilized protein and geochemically transformed molecules, which undoubtedly originate through bacterial (or if aerobic, fungal) activity. Organic fossils therefore could be thought of as reservoirs of bacterial information, not simply the of the primary, macroscopic organism. Morphological observations which are the strength of paleontology can then be combined with molecular information on diagenesis to understand not only living, but depositional, environments.

Because shreds of organic molecules do escape total mineralization by bacteria, identification of the fate of the mangled fragments is critical. In our study, plant proteins recombined with sugars to form AGEs, as early as 3 months. This transformation has the potential to remove molecules from our analytical windows, especially those techniques developed for use with biomolecules. Parent molecules could still exist, yet we may not be able to recognize them. These resistant geochemicals are the molecules that remain in detritus, soils, and kerogen. They are not the pristine biochemicals that entered the environment, and yet they form the backbone of total carbon, and nitrogen, in the Earth's crust. Biochemical tools for studying living processes need further refinement to identify and quantify these hybrid molecules – part biochemical signal and part alteration product. Stable isotopes as built-in tracers can be used to chronicle the history of these biological and chemical changes and hold the promise for studying large-scale ecosystem C and N cycling and the fossil record.

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