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Forms and nature of organic N in soil

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Abstract

Most of the N in surface soils occurs in organic forms. The organic N in soil plays a key role in plant nutrition and soil fertility through its effects on microbial activity and nutrient availability. Typically, about one-third of the fertilizer N applied to temperate-zone soils is immobilized and retained in organic forms at the end of the growing season. A significant portion of this newly immobilized N is no more available to microorganisms and plants than the native humus N. Stabilization processes, probably involving polymerization of amino compounds and polyphenols, result in incorporation of N into humic substances with a concurrent reduction in N availability. This paper presents an account of the forms and nature of organic N in soil, emphasizing possible formation pathways, chemical characterization of humic substances through conventional and solid-state techniques, and the fate and composition of newly immobilized N in soil.

Introduction

It has been well documented that over 90% of the N in most surface soils occurs in organic forms. The forms of organic N in soil can be divided into two broad categories: (i) organic residues, consisting of undecayed plant and animal residues and partial decomposition products, and (ii) soil organic matter or humus. The humus consists of nonhumic, or chemically recognizable substances (e.g. amino acids, carbohydrates, nucleic acids, etc.), and humic substances, consisting of high molecular weight, amorphous, partly aromatic substances formed by secondary reactions. The importance of humus in terms of maintaining or improving soil fertility has been widely recognized and can be attributed to positive effects of humus on physical, chemical, and biological properties of soil.

Organic soil N plays a key role in terms of plant nutrition through direct and indirect effects on microbial activity and nutrient availability. The significance of humic substances to the N fertility of soil arises from the fact that much of the organic N resists attack by microorganisms and is thereby relatively unavailable to plants. Also, as a consequence of mineralization-immobilization turnover by microorganisms, a portion of the fertilizer N applied to soil becomes stabilized by incorporation into humic substances.

Results of field trials using the stable isotope, ^{15}N, have shown that about one-third of the fertilizer N remains behind in organic forms after the first growing season, only a small fraction (<15%) of which becomes available to plants during the second growing season. Stabilization through linkages to or incorporation into the structures of humic substances appears likely. Thus, a knowledge of the chemical nature of N in humic and nonhumic substances will not only provide a better understanding of factors affecting the availability of indigenous soil N but may contribute to the development of improved management practices for the efficient and environmentally acceptable use of fertilizer N.

In this paper, the chemical nature of N in soil humus is discussed, with emphasis being given to incorporation, stabilization, and availability of immobilized fertilizer N in soil. Reviews of soil N availability and of indices of N availability have been compiled [34, 77]; recent reviews of humic substances as related to soil fertility include those of Schnitzer [61, 62], Stevenson [80, 81, 83, 84], and Tate [88].

Formation of organic N complexes in soil

Multiple pathways probably exist for the formation of humic substances and organic N complexes in soil. Early concepts, such as the lignin-protein theory of Waksman [91], theorized that humic materials represent condensation products resulting from reactions involving partially decomposed lignin and microbially synthesized amino compounds. Another popular theory, termed the polyphenol theory, postulates that humic materials are formed through condensation reactions involving amino compounds and polyphenols.

A popular current concept is that humic substances in soil are formed by a multiple stage process that includes: (i) decomposition of all plant polymers, including lignin, into simple monomers, (ii) metabolism of the monomers by microorganisms with an accompanying increase in the soil microbial biomass, (iii) repeated recycling of the biomass C and N with synthesis of new cells, and (iv) concurrent polymerization of reactive monomers into high molecular weight substances. The consensus is that polyphenols, derived from lignin together with those synthesized by microorganisms, are converted to quinones through the catalytic action of polyphenol oxidase enzymes or clay minerals. The quinones then polymerize in the presence or absence of amino compounds (amino acids, NH_3, etc.) to form brown-colored macromolecules. The net effect of this series of reactions is that the N of amino acids is incorporated into complex structures, such as in heterocyclic rings or as a bridge between quinone and aromatic rings. Whereas the relative importance of lignin or microbial synthesis as sources of polyphenols may vary, depending upon soil and climatic conditions, random abiotic polymerization reactions appear to be a central element in the formation of highly complex and heterogenous humic substances in soil.

Principal humification pathways operating during early stages of pedogenesis were evaluated in a recent study of humus formation [43]. The study was conducted using samples collected from an open-cast lignite mine site in which low organic matter (1.9 g C/kg) mine spoil material was used to reclaim mined areas. Reclamation involved liming (1 t/ha), fertilizing (23 kg N, P, K/ha), mulching (300 kg cellulose/ha), and hydroseeding with ryegrass (*Lolium perenne*), fescue (*Festuca rubra*), blue grass (*Poa pratensis*), and white clover (*Trifolium repens*). Soil samples were collected from nearby native pastures and from mined areas 1, 2, 3, 4, 5, and 7 years after application of the reclamation treatments. This provided a series of mine spoils developed from the same parent material and receiving identical management for various periods of time. The C and N contents increased from 1.9 g C/kg and 0.1 g N/kg (raw spoil material) to 37 and 2.2 g/kg, respectively, after 7 years, representing about 49 and 39% of the C and N levels in the nearby undisturbed pasture soils. Conclusions, based on analyses for molecular weight distribution, aromaticity, organometallic complexes, and clay-adsorbed and stabilized humic substances, indicated that humification processes operating in the mine spoil material were similar to those occurring in the native pasture soils. Their findings support current theories of humus formation in that the dominate humification mechanism appeared to involve abiotic polymerization reactions of small organic molecules producing fulvic acids as precursors to humic acids.

Stevenson [78] reviewed several hypothetical structures for humic and fulvic acids and pointed out that humic substances consist of a complex, heterogeneous mixture of organic compounds that cannot be adequately described by a single structural model. Several of the proposed structures contained similar features, however, including N contained in amino acids, peptides, or proteins attached to a central humic core by H-bonding or covalent linkages. Stevenson concluded that significant amounts of the N associated with humic substances cannot be accounted for in known compounds and that this N may occur as: (i) free amino ($-NH_2$) groups, (ii) open chain (-N-, =N-) groups, (iii) part of a heterocyclic ring, such as an -NH- of indole and pyrrole or the -N= of pyridine, (iv) a bridge constituent linking quinone rings, and (v) amino acids attached to aromatic rings in forms that resist hydrolysis in hot mineral acids.

Characterization of organic N by conventional techniques

The most widely used approach for characterizing organic N in soil or humic substances is by acid hydrolysis. Methods for determining the forms of N in hydrolysates of soil or soil organic matter preparations (i.e. humic acid, fulvic acid, and humin) have been described [79]. In a typical procedure, the sample is heated with 3 *N* or 6 *N* HCl for 12 to 24 hours, after which the N is separated into the following fractions:

- Acid insoluble-N: Nitrogen remaining in residue following hydrolysis; usually obtained by difference (total N − hydrolyzable N).
- NH_3-N: Ammonia recovered from the hydrolysate by steam distillation with MgO.
- Amino acid-N: Usually determined by the ninhydrin-NH_3 or ninhydrin-CO_2 methods.
- Amino sugar-N: Steam distillation with phosphate-borate buffer at pH 11.2 and correction for NH_3-N; colorimetric methods are also available.
- Hydrolyzable unknown-N (HUN fraction): Hydrolyzable N not accounted for as NH_3-N, amino acid-N, or amino sugar-N; part of this N occurs as non-α-amino N in arginine, tryptophan, lysine, and proline.

Only from one-third to one-half of the organic N in soils can be accounted for in known compounds, i.e. amino acid-N and amino sugar-N. Soils also contain trace quantities of nucleic acids and other known nitrogenous biochemicals, but specialized techniques are required for their separation and identification.

A large amount of soil N, usually about 25 to 35%, is recovered as acid insoluble-N. At one time, this fraction was thought to be the result of condensation reactions during hydrolysis, but it is now believed that part of this N occurs as structural components of humic substances.

A large proportion of the soil N, about 20 to 25% for surface soils, is recovered as NH_3-N by distillation with MgO. In the older literature, this form of N was referred to as the 'amide-N' of proteins, but it is now known that very little of the NH_3-N is derived from the amino acid amides, asparagine and glutamine. Some of the NH_3-N is derived from indigenous fixed NH_4^+; part comes from partial destruction of amino sugars. It is also known that NH_3 can arise from the breakdown of certain amino acids during hydrolysis. Tryptophan is lost completely. Other amino acids, such as serine and threonine, are partially destroyed. An accounting of all known potential sources of NH_3 in soil hydrolysates shows that about one-half of the NH_3-N, equivalent to 10 to 12% of the total organic N, is still obscure [80].

From 10 to 20% of the soil N is accounted for in the HUN fraction, of which from one-fourth to one-half has been reported to occur as the non-α-amino N of such amino acids as arginine, tryptophan, lysine, histidine, and proline [21]. The possibility that most of the HUN occurs as non-α-amino acid N is remote.

As one might expect, humic and fulvic acids, obtained by classical extraction techniques, contain the same forms of N that are obtained when soils are subjected to acid hydrolysis [2, 7, 14, 37, 56, 89]. However, the N distribution patterns vary somewhat. In comparison with unfractionated soil, lower percentages of the N in humic acids occur as NH_3-N and HUN; higher percentages occur as amino acid-N and as acid insoluble-N.

Part of the amino acid-N of humic acids may exist as peptides or proteins linked to a central core by H-bonding [24]. Peptide-like substances have been determined in humic acids by chromatographic assay of proteinaceous constituents liberated by cold hydrolysis with concentrated HCl [15, 54]. It has also been demonstrated that amino acids are released through the action of proteolytic enzymes [41]. Infrared spectra of some, but not all, humic acids show absorption bands typical of the peptide linkage [51, 82]. Evidence for N in loosely bound forms has come from the observation that the N content of humic acids can be lowered considerably by passage through a cation exchange resin in the H-form [73, 76]. Protein-rich fractions have been obtained from humic acids by extraction with phenol [12, 47, 48].

A major proportion of the N of humic acids, ranging from 30 to 60%, is recovered as acid insoluble-N. This N may occur as a structural component of the molecule, such as a bridge constituent linking quinone groups together. Additional quantities of amino acids can be recovered from humic acids by subjecting the residues from the initial acid hydrolysate to a second hydrolysis with 2.5 *N* NaOH [22, 54]. Sodium amalgam reduction of the alkali-treated residue has been shown to lead to a further release of amino acids [54]. Acid hydrolysis would be expected to remove amino acids bound by peptide bonds as well as those linked to quinone rings. On the other hand, amino acids bonded directly to phenolic rings may not be released without subsequent alkaline hydrolysis. The effect of alkaline hydrolysis was believed to be due to oxidation of the amino phenol to the quinoneimine form, with subsequent hydrolysis of the imine and release of the amino acid.

An increase in amino acid-N at the expense of the HUN fraction was reported when an acid hydrolysate of humic acids was further heated with 6 *N* HCl containing 3% H_2O_2 [2]. The increased release of amino acids was attributed to the presence of phenolic-amino acid addition products in the hydrolysate. Hydrolysis of the initial acid-insoluble residue with 3% H_2O_2-6 *N* HCl led to a further release of amino acids, as well as other N forms. It seems likely, therefore, that part of the acid insoluble-N in humic substances may occur in the form of N-phenyl amino acids resulting from the bonding between amino groups and aromatic rings. For peptides, amino acids directly attached to an aromatic ring may be less available to plants and microorganisms than amino acids of the peripheral peptide chain.

In summary, the N of humic substances exists in several forms, some being labile and readily utilized by microorganisms (e.g. H-bonded peptides and proteins) and some being recalcitrant, or nearly so. Significant amounts of the N associated with humic acids and fulvic acids cannot be accounted for in known compounds.

The assumption is sometimes made that the structural N of humic substances represents the more stable component of soil organic N. On this basis, one would expect major changes to occur in the distribution of the forms of N when soils are subjected to intensive cultivation, namely, large losses of N from amino acids and other more readily available forms at the expense of the acid insoluble-N fraction. In practice, this has not been shown to be the case [80]. Surprisingly, neither long-term cropping nor the addition of organic amendments to the soil greatly affects the relative distribution of the forms of N. All forms, including the acid insoluble-N fraction, appear to be biodegradable [30, 46]. Results of these and other studies show rather convincingly that humic substances are not completely inert but are constantly changing as a consequence of the activities of microorganisms. Under steady-state conditions, mineralization of native humus is compensated for by synthesis of new humus. During humification, the N of amino acids and other amino compounds is incorporated into the structures of humic and fulvic acids and is not readily available to plants.

Characterization of organic soil N by NMR techniques

The development of techniques for characterizing organic matter in intact soil samples represents a significant advance in efforts to further our understanding of the forms and nature of organic N in soil. These techniques, which include ^{13}C and ^{15}N nuclear magnetic resonance (NMR) spectroscopy and analytical pyrolysis (discussed later), allow for structural characterization of organic matter without chemical extraction or fractionation.

The utility of ^{13}C NMR in studies on organic N arises from the fact that one of the main 'chemical shift zones' (i.e. 50 to 60 ppm) in spectra of soil organic matter, and its subfractions, has been assigned to the C of N-alkyl compounds (e.g. amino acids, peptides, and proteins). Results obtained thus far indicate that as little as 2% to as much as 15% of the C in samples analyzed could be accounted for as proteinaceous constituents. It should be noted that the C of methoxyl groups also produces shifts in the 50 to 60 ppm range, but the methoxyl content of humic constituents and well-decomposed organic matter is normally very low. No attempt seems to have been made to interpret the results of ^{13}C NMR studies in terms of organic N composition.

^{13}C NMR spectroscopy has been widely used in studies of humus chemistry. This technique has been applied to liquid and solid-state samples, including humic acids, fulvic acids, and whole soils. The usefulness of ^{13}C NMR for characterizing complex solid-state samples is limited by line broadening and a poor signal-to-noise ratio. Cross polarization and magic angle spinning techniques (CPMAS) have been applied in conjunction with NMR spectroscopy to improve spectral quality and enhance the utility of this approach for analysis of solid-state samples.

CPMAS ^{13}C NMR has been applied in studies covering a wide range of applications and research objectives; specific references can be obtained from comprehensive reviews [25, 44, 45, 92, 93]. Examples of recent applications include: characterization of humic and fulvic acids extracted from soil by different mechanisms (charge repulsion, ligand exchange, or H-bond disruption) [52], relationships between structural characteristics of humic acids and nitrate uptake and growth regulation in plants [53], formation of humic substances in forest soils [11, 38], effects of cultivation on particle size fractions and stability of humic substances [29, 55], effects of vegetation type on structural characteristics of humic acids, fulvic acids, and humin [39, 40], degradation of humic acids by fungi and actinomycetes [17], chemical characterization of fungal melanins [59, 63], modifications and incorporation of straw-derived lignin into humic substances [4], and incorporation of ^{13}C-labeled substrates into humic fractions [6].

Malcolm and MacCarthy [45] recently summarized findings from ^{13}C NMR studies of humic substances and pointed out that the findings give evidence for higher aliphatic contents than previously thought. They also noted that results of ^{13}C NMR studies indicate that substantial differences exist in the reactivity and composition of humic and fulvic acids, that humic acids are more aromatic than fulvic acids, and that distinct differences occur among fulvic acids from diverse environments.

Solid-state ^{13}C NMR analysis is limited by the low C content and low natural abundance of ^{13}C (1.1%) in mineral soils. Baldock *et al.* [6] recently summarized studies in which they added ^{13}C-labeled glucose to a coarse-textured mineral soil to overcome these limitations. After 34 days of incubation, the soil was fractionated on the basis of particle size and density, and the various fractions were analyzed by CPMAS ^{13}C NMR. Over 72% of the residual ^{13}C was found in the clay fraction. Comparisons between ^{13}C NMR spectra of the residual glucose C and spectra of bacteria and fungi, isolated from the same soil, indicated that the glucose was predominately utilized by soil fungi with little or no incorporation into aromatic or phenolic structures.

Of the NMR spectroscopy techniques, CPMAS ^{15}N NMR has the most direct applicability for characterizing the forms and nature of organic N in soil. This technique has been applied in studies involving ^{15}N enrichment and analysis of humic substances in soil [10, 85, 86] and studies of the fate of ^{15}N-labeled glycine [9], ^{15}N-labeled melanoidins [10], and ^{15}N-labeled ammonium sulphate, urea, or wheat straw [8]. Incubation of ^{15}N-labeled glycine in sterile and nonsterile peat led to incorporation of glycine-derived N into amines, secondary amides, and pyrrole-type N forms, possibly due to condensation between sugars and amino compounds through the Mallaird reaction [9]. Further evidence for involvement of the Mallaird reaction was provided by ^{15}N NMR studies in which melanoidins, synthesized from reducing sugars and ^{15}N-labeled amino acids, were found to be quite similar to ^{15}N-labeled humic acids obtained from a mineral soil [10].

Studies involving ^{15}N NMR analysis of ^{15}N-labeled humic substances have been reported recently [85, 86]. Labeling of the humic substances was accomplished by addition of ^{15}N-labeled ammonium sulfate to a low organic matter soil followed by cropping and aerobic incubation for a period of three years. Humic and fulvic acids, extracted by conventional techniques, and humic acids recovered from the humin fraction following treatment with an HF-HCl mixture to dissolve silicate minerals, were isolated and analyzed by CPMAS ^{15}N NMR. Results indicated that most of the N occurred in amide forms (80, 86, and 87% for the humic acid, fulvic acid, and humic acid-from-humin fractions, respectively); from 9 to 12% and from 4 to 9% of the N occurred in amine and pyrrole-type forms, respectively. In contrast, much lower proportions of the N were accounted for in known forms by conventional acid hydrolysis (amino acids and amino sugars accounted for 16 to 52% of the N). Lower recoveries in known forms by acid hydrolysis were attributed to the occurrence of amide N in nonhydrolyzable or acid-resistant forms and to the release and/or reactions of amide N during hydrolysis. In a follow-up study to characterize the nonhydrolyzable (6 *N* HCl) residue of humic acids, ^{15}N NMR analysis indicated that most of the 'amide-N' had been liberated as amino acid-N or NH_3-N. All of the pyrrole N was accounted for in the HUN fraction, while the N of aromatic amines was accounted for in the HUN or NH_3-N fractions [86]. Similarities between the spectroscopic and chemical characteristics of the humic acid and humic acid-from-humin fractions indicated that part of the humin N occurs as humic acids intimately associated with soil colloids.

Characterization of organic soil N by analytical pyrolysis

Analytical pyrolysis is a thermal degradative method that can be applied to solid-state samples in soil organic matter studies. Schulten [66] pioneered a technique combining pyrolysis and soft ionization mass spectrometry in which the sample is pyrolyzed in the mass spectrometer and volatile molecular fragments, or pyrolysis products, are analyzed by field ionization mass spectrometry (Py-FIMS). The technique can be applied to whole soil samples and presents several important advantages over chemical degradative procedures. This technique eliminates the need for extraction, fractionation, and purification, provides for rapid analysis and information on a molecular level for complex organic materials, and is suitable for analysis of mineral soils [70].

Py-FIMS has been applied to the characterization of C compounds in a variety of materials; the reader is referred to recent reviews for additional references [13, 60, 62, 67]. Recent applications include comparisons of whole soils to the humic acid, fulvic acid, and humin fractions [65], characterization of morphologically differentiated horizons in a forest soil [28], humification of primary organic matter inputs (straw and farmyard manures) and dynamics of soil organic matter in agricultural soils [42], characteristics of particle-size fractions as affected by long-term fertilization with farmyard manures [69], effects of soil and crop management on humus properties [23, 68, 70], and influences and limitations due to the composition of the mineral soil matrix [70].

Although this technique has primarily been used to characterize organic C groups, Py-FIMS also offers potential for advancing our knowledge of the structural aspects and forms of organic N in soil. For example, Hempfling *et al.* [28] reported evidence for amines, nitriles, and heterocyclic N fragments released by pyrolysis of a forest soil. They assigned N-containing structural formulas to over 70 of the pyrolysis fragments but did not speculate as to the original structure of the organic N complexes. The structural assignments of the N-containing fragments, ranging in molecular weight from 45 to 410, were based on previous Py-gas chromatography/mass spectrometry (Py-GCMS) studies of humic substances, plant materials, and microbial biomass.

In a recent study involving Py-FIMS, the effects of long-term (> 100 years) fertilization with farmyard manure (FYM) on characteristics and N compounds associated with various particle-size fractions were reported [69]. Results indicated that levels of N compounds increased with decreasing particle size. The fine- and medium-clay fractions and the fine- and medium-silt fractions of the FYM-amended soil were enriched in amino-N and amides. The clay fraction of the intensively managed (unfertilized) soil showed a high relative abundance of pyrroles, pyridines, and indoles, supporting the conclusion that reduced inputs of primary organic matter result in an enrichment of refractory heterocyclic N compounds [42, 68].

The applicability of Py-FIMS for identifying N compounds in humic substances or soil organic matter was recently extended in an investigation involving analysis of 23 amino acid standards [75]. The thermal fragmentation pattern for each amino acid was delineated, and a series of peak signals were selected as markers to identify amino acids in humic substances. The intensities of the amino-marker signals were determined for 27 agricultural soils and found to be correlated with α-amino acid N ($r=0.76^{***}$) and total N ($r=0.65^{***}$).

Schulten [67] pointed out that structural investigations by Py-FIMS should include complementary destructive and/or nondestructive techniques. Combinations of Py-FIMS and other analytical approaches have been reported in studies of humic substances and organic N complexes, including ^{13}C NMR [6, 11, 23, 28), Fourier-transformed IR [4, 28, 85], Py-GCMS [75], and classical chemical techniques [68]. Schulten and Schnitzer [71] recently proposed a structural concept for humic acids based on the literature and previous investigations utilizing various combinations of analytical pyrolysis, spectroscopic, chemical, oxidative/reductive degradation, and colloid-chemical and electron microscope techniques. Their model did not include linkages or structures for proteinaceous materials, however.

The application of solid-state techniques appears to offer real potential to advance our knowledge of the structural and functional aspects of organic N complexes in soil. It is not possible to draw inferences as to the original structure of organic N complexes in soil at this time, however, due to our limited knowledge of the forms and nature of organic N in soil.

Fate and chemical nature of newly immobilized N in soil

As noted earlier, a significant fraction of the fertilizer N applied to temperate-zone soils, of the order of 20 to 35%, remains behind in organic forms after the first growing season. No more than 15% of this residual N becomes available to plants during the second growing season, and availability decreases even further for succeeding crops [81, 84]. A key factor affecting the efficiency with which fertilizer N is consumed by crops is the progressive stabilization of N by conversion to more resistant humus forms. The net result is a decrease in the 'labile' pool of potentially available N in the soil. Although a number of explanations can be given for the low availability of residual fertilizer N in soils, there is little doubt that the stability is due in part to incorporation of N into the complex structures of humic substances, for which two mechanisms can be given: (i) the turnover of N through mineralization-immobilization leads to incorporation of N into the melanins of fungi, and (ii) the N of amino acids, peptides, and proteins is stabilized through reactions involving quinones and carbonyl-containing substances.

Several approaches using the stable isotope, ^{15}N, have been utilized to characterize immobilized N in soil, including fractionation based on acid hydrolysis and partitioning into humic and fulvic acids by classical alkali extraction. A noteworthy feature of the acid hydrolysis studies is that much of the N cannot be accounted for in known forms but is recovered in the acid insoluble-N, HUN, and NH_3-N fractions. With time, more and more of the residual N is converted to these unknown forms, and, within a matter of years, the composition of the fertilizer N is indistinguishable from that of the native humus N [3, 74].

Results of the field study of Allen *et al.* [3] are typical in that an average of one-third of the applied ^{15}N was accounted for in the surface soil after the first growing season, the remainder having been taken up by plants or lost through leaching and denitrification. Isotope-ratio analysis revealed that most of the residual N had been incorporated into organic forms. Comparison of the distribution pattern for the fertilizer-derived ^{15}N with that of the native humus N showed that a considerably higher proportion of the fertilizer N occurred in the form of amino acids (59.0 vs. 36.0%) and amino sugars (9.9 vs. 8.0%); lower proportions occurred in the NH_3-N (10.6 vs. 18.1%), acid insoluble-N (10.3 vs. 21.7%), and HUN (10.2 vs. 16.2%) fractions. When the plots were resampled four years later, the fertilizer N remaining in the soil, representing one-sixth of that initially applied, had a composition very similar to that of the native humus N. The mean residence time (MRT), or average life, for the N retained after the first season was about five years. The N retained after this period was postulated to have a MRT of 25 years; thereafter, the MRT would be the same as for the native humus, or an estimated 200 to 800 years. From this, it can be seen that a small fraction of the fertilizer N applied during any given growing season will remain in the soil for a long time, perhaps centuries.

Similar results were reported in which fertilizer N was initially incorporated into such compounds as amino acids and subsequently into more stable forms [74]. As compared to the native soil N, more of the residual fertilizer N occurred in an amino acid-containing fraction, with smaller amounts being accounted for as NH_3-N and acid insoluble-N. Equilibrium with the native soil N had not been achieved in three years.

Residual fertilizer N has also been partitioned into classical humus fractions by alkali extraction [20, 57, 94]. The relative amounts of N recovered in humic and fulvic acids were found to depend on several factors, including form of applied N [94]. Mc Gill *et al.* [48] isolated humic and fulvic acids from ^{15}N-labeled soils and observed a higher degree of labeling in the fulvic acid fraction.

It should be noted that results obtained with ^{15}N-labeled substrates complement those for ^{14}C. From 30 to 37% retention of applied C has been observed at the end of the first growing season, depending on soil and climatic conditions [19, 31, 50, 72]. Experiments with ^{14}C-labeled ryegrass straw showed that approximately 33% of the applied C remained behind in the soil after the first year [31]. This residual C had a MRT of about four years; thereafter, the MRT approached that for the native humus. Much of the N required to maintain a constant C/N ratio will come from applied fertilizer N; part may come from the soil N pool.

Additional information regarding the biological conversion of N to stable humus forms has been provided by studies in which the organic N has been labeled with ^{15}N by short-term incubation of the soil with inorganic ^{15}N and a suitable C substrate. Results of these studies have confirmed that stabilization of N occurs very rapidly and that a significant portion of the immobilized N occurs in forms not readily available to plants or microorganisms. In an early study, soil was incubated for periods of up to 20 weeks with $^{15}NO_3$ and three C substrates (glucose, straw, and cellulose) [32]. For all C substrates, an initial net immobilization of ^{15}N was followed by a period of net mineralization. At the point of maximum incorporation of N into the biomass, there was a distinct difference in the percentage distribution of the forms of organic N between the newly immobilized ^{15}N and the native humus N, as determined by acid hydrolysis. Higher percentages of the immobilized N occurred as amino acids and in the HUN fraction; a lower percentage was found as acid insoluble-N. With time, the percentages of the organic N as acid insoluble-N and NH_3-N increased while the percentages in the amino acid-N and HUN fractions decreased. Similar trends have been observed by others [1, 36].

Studies involving characterization of newly immobilized ^{15}N in a typical Illinois mollisol have also been reported [26, 27, 35, 36]. The microbial biomass of a Flanagan silt loam (Aquic Argiudoll) was labeled by short-term incubation with glucose and ^{15}N. The labeled soil, collected at the point of maximum conversion of glucose C into microbial biomass, was analyzed by chemical extraction, acid hydrolysis, and alkali fractionation techniques. Utilization of the glucose was accompanied by a rapid immobilization of applied ^{15}N and conversion to organic forms. Several extractants, used as chemical indices of N availability and ranging in severity from hot water to acidified permanganate, were tested to determine the effectiveness by which the newly immobilized ^{15}N could be separated from the native humus N [35]. An inverse relationship was observed between the amount of N extracted and the proportion of the soluble N derived from the labeled biomass, indicating that milder procedures were more selective in extracting the newly immobilized ^{15}N. Distinct differences were also noted between the chemical distribution of the immobilized ^{15}N and the native humus N. Lower proportions of the immobilized N were recovered in the acid insoluble-N and NH_3-N fractions; higher proportions were recovered in the amino acid-N and HUN fractions.

The ^{15}N-labeled soil was also extracted sequentially with 0.15 *M* $Na_4P_2O_7$ and 0.1 *N* KOH, following which the N was partitioned into classical humic fractions [26]. Higher percentages of the applied ^{15}N, as compared to the native soil N, were accounted for in the two extracts (22 vs. 16% by 0.15 *M* $Na_4P_2O_7$ and 27 vs. 22% by 0.1 *N* KOH, respectively). About 49% of the applied ^{15}N was removed by sequential extraction, as compared to 38% for the native soil N. Although somewhat less than for the native soil N (59%), the percentage accounted for as humin was still surprisingly high (46%). One explanation for this result is that much of the newly immobilized N existed as insoluble cellular components of microorganisms, including fungal melanins that were not extractable under the conditions used. Short-term incubations, as carried out in the study, are known to lead to an initial increase in bacterial numbers, followed by an increase in fungal organisms. A subsequent decline in the fungal population would be expected to be followed by a second increase in bacterial numbers [27]. On a weight basis, the amount of fungal tissue greatly exceeds that for bacteria. Evidence for the presence of humic acid-like substances in alkaline extracts of fungal tissue has been reported [18, 33, 49, 58, 59, 64, 87]. Fungal melanins are not completely solubilized by dilute alkali reagents, and the insoluble residues have properties similar to those of the humin fraction of soil organic matter [58]. The organic matter solubilized by sequential extraction with $Na_4P_2O_4$ and NaOH (extracts combined) was further partitioned into humic and fulvic acids. The percentage of the extracted ^{15}N accounted for in the humic acid fraction (16%) was somewhat less than for the native soil N (24%). These results definitely show that the immobilized ^{15}N was partitioned into all humus fractions and that a significant fraction of the newly immobilized N was indistinguishable from the native humus N.

Results similar to those noted above have been obtained in other studies [5, 16, 48]. It is of interest that distribution patterns of immobilized ^{15}N in the classical humic fractions are very similar to that for the ^{14}C of labeled C substrates [90], where labeling in all fractions has been observed. Chichester [16] fractionated ^{15}N-labeled soil based on soil particle size and then extracted each particle fraction with 0.5 *M* $Na_4P_2O_7$ at 100°C. The extracted organic N was further fractionated by acid hydrolysis. Distribution patterns for the fertilizer N and the native soil N were very similar, a result that was attributed to conversion of the fertilizer-derived N into complexes similar in composition and properties to the native humus N. In a study to fractionate immobilized ^{15}N, McGill *et al.* [48] reported that at the time when the microbial population reached maximum (incubation period of five days), the added ^{15}N extracted by $Na_4P_2O_7$ represented about 20% of the total added ^{15}N and the ratio of the added ^{15}N in fulvic acids relative to that in the humic acids was greater than 2:1. Up to 10% of the total ^{15}N was accounted for in the humic acid fraction, about 50% of which was removed by phenol extraction and assumed to consist of proteinaceous material held to humic acids through H-bonding. He *et al.* [27] found that aqueous phenol was more selective in removing recently immobilized ^{15}N from soil than reagents typically used for extraction of soil organic matter (i.e. 0.15 *M* $Na_4P_2O_7$ and 0.1 *M* NaOH).

Summary and conclusions

Results of ^{15}N studies have clearly demonstrated that from 20 to 40% of the fertilizer N added to agricultural crops of temperate-zone soils is typically incorporated into organic forms during the first growing season. In comparison to the native humus N, more of the newly immobilized N occurs as amino acids and amino sugars and less occurs in unidentified forms. The newly immobilized N is more susceptible to mineralization or extraction, but a significant portion is no more available than the native humus N. Stabilization processes, including polymerization reactions between amino compounds and polyphenols, lead to the incorporation of N into humic structures and a gradual reduction in N availability.

Despite the excellent progress that has been made in recent years, much more needs to be accomplished to advance our knowledge and understanding of the forms and nature of organic N in soil. Numerous studies involving a broad range of conventional and solid-state techniques have been applied to the characterization of organic N complexes, but as much as one-half of the N of humic substances cannot been accounted for in known forms. The nature of this unknown N will remain obscure until more is known about the chemical structures of humic and fulvic acids and the mechanisms whereby N is stabilized by incorporation into humic substances.

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