Humic substances (HS) are the major components of the mixture of materials that comprise soil organic matter, and these substances, which are by far the most abundant organic materials in the environment, are themselves complicated mixtures of biologically transformed organic debris. However, it is likely that many of the solubilization properties of the mixtures arise from the presence of nonhumic components that are intimately associated with the HS and that cannot be separated effectively from these components. Separation and fractionation techniques are improving, and most of the instruments needed to advance awareness of the composition and aspects of structures are now in place. There is a need, however, to review the operational definitions that now apply and to put in place a classification system that will take into account origins and some compositional characteristics. The broad based definition of humin is especially unsatisfactory. This umbrella term covers a mixture of materials that are insoluble in aqueous systems and that contain nonhumic components such as long chain hydrocarbons, esters, acids, and even relatively polar structures of microbial origin, such as polysaccharides and glomalin, that can be associated with the nonpolar moieties and with soil minerals, as well as plant components that are highly resistant to decomposition. Advances in the humic sciences in recent times have been impressive, and the questioning of ‘ingrained’ theories and concepts is opening vistas through which we are seeing new concepts of size, shape, and association; the remarkable developments in nuclear magnetic resonance are also allowing better interpretations of compositions and of aspects of structures. From these advances will emerge a more fundamental understanding of HS functions in such important roles as the stabilization of soil aggregates, the binding of anthropogenic organic chemicals, and the sequestration of C from atmospheric CO₂. The latter function is especially important at this time because we need to know why some soils sequester more C than others and why the qualities (or composition and structure) of HS in some soils are different from those in others. (Soil Science 2001;166:723–737)

Key words: Soil and water humic substances, humin, humic structures, carbon sequestration, humic substances fractionation, humic compositions, humic acid, fulvic acid, glomalin.
HS was all that was needed to explain the role of HS in the environment”. Such sentiments, which have certainly diverted support from, and hence delayed advancement in, the humic sciences are no longer accepted as being accurate scientifically or constructive in terms of environmental considerations. The faith of the authors, like that of many colleagues, has been sustained by the vision of the few who have continued to promote the essential role of HS in soil and water systems. Progress was pedantic even 25 years ago, but events alter approaches and change preconceived notions, and it is events that have brought about the galloping pace of the advances that are now being experienced in the humic sciences.

Because HS are found in all soils, it is inevitable that they will also be found in all waters. The amount and composition of HS in any water will depend on the soils of the watershed and especially the soil mineral compositions, exchangeable cations, and pH values of the soils. Thus, arguably, one of the most significant events leading to the advancement of the humic sciences was the discovery by Rook (1974) that mutagenic substances are produced when aquatic fulvic acids (FAs) are chlorinated. This caused water scientists to focus attention on various aspects of aquatic humic chemistry, and a new breed of humic scientists was introduced. When the International Humic Substances Society (IHSS) was founded at the USGS Water Research Laboratories in Denver, Colorado, in September 1981, humic scientists came together from the soil and water fields aiming to promote and advance awareness in all aspects of the humic sciences. It was evident from the first scientific meeting of IHSS in Estes Park, Colorado, in August 1983, that many water scientists were not aware of what had been achieved in soil humic studies, and the soil humic scientists could see clearly that approaches and techniques introduced by the water scientists were immensely valuable for their studies. The lessons learned from Estes Park were clearly evident at the second meeting of IHSS at the University of Birmingham (England) in July, 1984. From the invited papers presented at that meeting (Hayes et al., 1989a), as well as those from Estes Park (Aiken et al., 1985a), there have emerged two books which have formed the nucleus for sustained advances in the humic sciences.

Considerations of global warming, arising from the ever increasing concentrations of CO2 in the atmosphere, comprise a second event that will focus even more interest on the humic sciences than Rook’s discovery. It is understood that soil organic carbon (SOC) reserves (estimated to be ca 1500 Pg, where 1 Pg = 10^{15} g) are almost three times those in all living matter on earth (Eswaran et al., 1993; Schlesinger, 1995; Lal et al., 1998). However, the carbon (C) emissions from soil (arising from microbial transformations of soil organic matter (SOM)) are considered to be about 60 Pg, which is about 10 times more than the emissions from fossil fuels. Based on the agreements reached in Kyoto in 1997 (Conference of the Parties, 1997), account can be taken of the performance of a nation in sequestering additional C when deciding on the cutbacks required in the uses of fossil fuels as sources of energy (Swift, 2001). The U.S., for example, is expected to decrease its consumption of fossil fuels by 2010 to 93% of that used in 1990.

On the basis of these considerations, it seems logical to combat increases in atmospheric C by seeking ways to lower CO2 emissions from soil and to make soil a better sink for C. Estimates of the total C sequestration potential through improved management of U.S. cropland have been placed at 75 to 208 Tg (1 Tg = 10^{12} g), and this is thought to be possible through land conservation and restoration, intensification of prime agricultural land (which would involve conservation tillage and residue management), irrigation water management, and improved cropping systems (Lal et al., 1998, Ch. 10). It is heartening to realize that C can be sequestered in soil even where intensive agricultural practices are followed. There is also room for improvement of C sequestration in range and forest soils.

A special issue of Soil Science (Vol. 164, No. 11, 1999) was devoted primarily to considerations of the sizes and shapes of HS; attention was focused on concepts of the macromolecularity of HS, their colloidal properties, and molecular aggregation patterns, and considerable emphasis was placed on the ways in which HS behave in size exclusion chromatography. In this issue some new and highly pertinent topics are considered. There is an in-depth look at the principles of HS (MacCarthy, 2001) that define their molecular nature and ecological role, the traditional concepts of structures are questioned (Burdon, 2001; Rice, 2001), and due emphasis is given to the newly emerging concepts of supramolecular structures. Humin, a component of HS in the classical definitions, has not been studied extensively, but we now have concepts presented in one Journal article (Derenne and Largeau, 2001) that will give hope to those who have shied away from this area of study because of the perceived intractable nature of the materials. As the result of this in-depth examination, however, the question...
arises as to whether humin should be classed as a humic substance.

The striking advances being made in HS studies can be attributed to the range of highly sophisticated instrumentation now available that allows studies of compositions and structure to be made as never before. These advances owe much to applications of state-of-the-art nuclear magnetic resonance (NMR) instrumentation and to other spectroscopic techniques (Hatcher et al., 2001; Simpson, 2001).

It is, of course, the contributions of HS to agriculture and to the environment that merit their study. There is increasing interest in the role of HS in the environment, and it is our hope that such interest will generate the support that is needed for their study in depth. It is only when their compositions, aspects of their structures, and the nature of their associations are understood that it will be possible to predict relatively accurately how different HS will react and interact in different soil environments.

It is our role to blend concepts pertinent to the various themes. We have gone beyond the blending process, however, to outline how modern procedures for the isolation and fractionation of HS can help studies of composition and structure. We have outlined the types of evidence clarifying aspects of the molecules that comprise HS. As yet, we cannot see clear forms of structure emerging, but the fog is beginning to clear, and significant advances can be expected in this area in the near future. Our views and interpretations may not agree totally with those of the other authors, but we do not claim the final word on any of the topics discussed.

**HUMIC SUBSTANCES: DEFINITIONS, ISOLATION, FRACTIONATION**

It is important to convey to fellow scientists and to the community at large succinct indications of what it is we are dealing with. Definitions of soil HS are based on fractions of SOM and are based on the modes of their isolation and fractionation.

**Definitions**

Invariably, soil and water scientists have clear interpretations of what HS are. As pointed out by MacCarthy (2001) in this issue, “The term humic substances refers to a category of naturally occurring materials found in or extracted from soils, sediments, and natural waters. They result from the decomposition of plant and animal residues.” In recent years many products have been marketed as humic acids (HAs) and FAs for improving plant growth and crop yields. Lignite materials are the source of many of these materials, and lignite is a legitimate source of HS. However, some of the so-called humic materials in the market place have their origins in chemically treated wood products that have not been subjected to biological transformations. Thus, such materials, although humic-like on the basis of the operational definitions (HAs are precipitated at pH 2 (accepted by water scientists) or pH 1 (by soil scientists); FAs remain in solution at all pH values; and humin materials are not soluble in aqueous acid or base), do not satisfy the basic criteria (found in or extracted from soils, sediments, and natural waters) for HS. There are many organic materials that are soluble in base and precipitated in acid that are not HAs. In the definition by Aiken et al. (1985b), also provided by MacCarthy (2001), HS are “a category of naturally occurring, biogenic, heterogeneous organic substances that can generally be characterized as being yellow-to-black in color, of high molecular weight (MW), and refractory.” There is agreement with regard to color. However, there is no longer universal acceptance that HS are of high MW, and Piccolo (2001) has put forward views in this issue that would suggest that HS are not of high MW.

MacCarthy makes clear in his article in this issue (MacCarthy, 2001) that it is difficult to provide a cryptic definition that will convey the essential features of HS. Today, HS scientists have an innate grasp of what these are, but it will be necessary to provide a series of criteria that must be met if materials are to be marketed that claim to have HS components.

The definitions of the fractions of HS are based on solubility characteristics in aqueous systems. Inevitably, it is necessary to isolate the fractions in order to characterize these. Some will argue that where chemical processes are used to isolate HS, the chemistries and compositions of the substances are altered, and the products are artifacts that are not relevant to soil processes. That argument is the same as saying that nucleic acids will be altered when isolated and cannot function outside of the living cell as they will within it. But, of course, the structures of nucleic acids could not have been determined without isolating, and their biochemistries could not have been understood without structural awareness. We suggest that where relatively mild isolation procedures are used, the composition of the components of HS will be much the same as when in the soil environment, although their associations may well be different.
**Isolation**

Hayes (1985) has discussed the principles and procedures for the isolation of HS from soils, and Hayes and Malcolm (2001) have recently briefly reviewed these principles and procedures. Traditionally, aqueous solvents are used, and as mentioned above, the definitions of humic fractions are based on solubilities in water at different pH values. Some organic solvents can have applications, and Hayes (1985) concluded that the good organic solvents have electrostatic factor (or the product of the relative permittivity and dipole moment) values \(>140\) and \(pK_{HB}\) (a measure of the strength of the solvent as an acceptor in hydrogen bonding) values \(>2\). Dimethylformamide (DMF) and dimethylsulfoxide (DMSO) meet these requirements.

Hayes et al. (1996) carried out exhaustive and sequential extractions using distilled water, sodium pyrophosphate (Pyro) at pH 7, Pyro at pH 10.6, and Pyro + 0.1 \(M\) NaOH. The diluted extracts were passed on to XAD-8 ([poly]-methylmethacrylate) and XAD-4 (styrenedivinylbenzene) resins in tandem, and HAs, FAs, and XAD-4 acids were isolated as outlined below. Cross polarization magic angle spinning (CPMAS) \(^{13}\)C–NMR spectra indicated significant compositional differences between the humic fractions isolated at the different pH values. Clapp and Hayes (1996, 1999) had earlier carried out a similar exhaustive sequential extraction process and followed that sequence using a DMSO/HCl medium. Because DMSO, a dipolar aprotic solvent, is a poor solvent for anions, but a good solvent for cations (Martin and Hauthal, 1975), it is necessary to \(H^+\)–exchange the humates causing these to behave like polar molecules capable of forming hydrogen bonds and solvating in DMSO. The results of Clapp and Hayes (1996) indicate that the materials that they isolated in DMSO/HCl, which would be classified as humin in the conventional definitions, had the characteristics of HAs and FAs.

An alternative to the exhaustive sequential extraction method using a series of solvents would be to extract with the NaOH/Pyro system and, subsequently, to fractionate by eluting at different pH values from XAD resins. Extraction with DMSO/HCl would follow exhaustive extraction with base.

The solvent-soluble substances in the foregoing sequence of extracts would normally be classified as humin. Because the materials isolated in DMSO were similar to those of HAs/FAs, Clapp and Hayes (1996) concluded that the materials did not dissolve in the aqueous media because the polar faces of the molecules had intimate associations with the inorganic colloids and exposed to the exterior the hydrophobic moieties of the molecules. Another possible interpretation is that associations between the polar moieties and non-polar molecules exposed to the outside hydrophobic faces which were not solvated in the polar aqueous media. Such associations may or may not involve the inorganic colloids. Nonpolar (and nonhumic) materials co-extracted in the DMSO/HCl solvent system would be retained on the XAD-8 (used to sorb the HS and allow removal of the DMSO/HCl) and would not be removed in the back-elution (with NaOH).

Rice and MacCarthy (1989, 1990) have described the use of methylisobutylketone (MIBK) to isolate a nominal humin fraction from soil (after exhaustive extractions with base were completed), and their observations indicate that the isolates were composed of lipid and HA-type materials (see MacCarthy, 2001, and Rice, 2001, this issue). Consideration might be given to the use of MIBK after DMSO/HCl in the sequential extraction system.

The procedure used to isolate the standards of the IHSS employs a 0.1 \(M\) HCl/0.3 \(M\) HF treatment to remove finely divided inorganic soil colloids and, thereby, provide materials with acceptable ash contents (Swift, 1996). The HCl/HF treatment is repeated as often as necessary to lower the ash content to <1%. This treatment, followed by dialysis, leads to considerable humic fraction losses. Clapp and Hayes (1996, 1999) and Hayes et al. (1996) found that filtration through partially clogged 0.45 \(\mu m\) or 0.2 \(\mu m\) filters lowers the ash content of HS fractions very effectively, and this procedure also avoids degradation of the humic materials.

**Fractionation**

Swift (1985) has described methods for the fractionation of HS, and Hayes and Malcolm (2001) have referred to fractionation procedures used before and since that time.

We see value in a procedure based on that of Malcolm and MacCarthy (1992) and used by Ping et al. (1995). In that procedure, summarized by Hayes and Malcolm (2001), SOM extracts are diluted sufficiently so that the dissolved organic matter (DOM) stays in solution when the pH is adjusted to 2. (We like to dilute to <25 mg L\(^{-1}\), although successful results can be obtained from concentrations of 50–100 mg L\(^{-1}\)). This solution is passed on to XAD-8 and XAD-4 resins in tan-
dem (packed in 0.01 M acid). As referred to above, the resin procedure was used to fractionate the HS in the sequential extraction process used by Hayes et al. (1996). The fractionations in such processes are based on charge density differences. After back elution with dilute NaOH from XAD-8, the HAs are precipitated at pH 1 and separated from the FAs.

Recent work involving the authors suggests a new approach that allows HAs to be further fractionated on the basis of charge density differences. Hayes et al. (1996) observed that a humic fraction was precipitated between pH 2.5 and 2 when dilute extracts from soil were being adjusted to 2 prior to application to XAD-8 resins. This material was compositionally significantly different from those of the HAs isolated at the different pH values and had significant amino acid and sugar contents. During the course of isolating a new IHSS standard from a fibric Minnesota peat, we observed that very fine coagulates formed when the pH values of dilute HA solutions from a fibric peat were adjusted to 2. The fine coagulates were retained on the XAD-8 resin at pH 2, but they were eluted when distilled water was passed through during the desalting process. However, when the eluate was redissolved under basic conditions, and the pH was adjusted to a value above but close to that at which coagulation had occurred, the HAs were retained on the resin until the salt was removed. This procedure offers a new approach for the fractionation of HAs.

Leenheer (1981) has described the materials retained on XAD-8 (including HAs and FAs) as the hydrophobic acids. He refers to hydrophobic neutrals as those retained on the resin after back eluting with base, and these are recovered in organic solvents, usually by soxhlet extraction using ethanol. Similar soxhlet extracts from the XAD-4 resin are classed as hydrophilic neutrals. Hot ethanolic NaOH can be used for the final resin extraction. However, this harsh treatment can lead to degradation of the resin material.

The authors do not consider that the materials retained by XAD-4 (the so-called XAD-4 acids) are valid components of HS. These would be components of the HA and FA fractions in the classical definitions, but they might be better regarded as nonhumic materials in associations with the true humic components. The authors have observed that hydrocarbon-, fatty acid-, wax-type materials are sorbed to the cellulose acetate membrane during the filtration process, and these would normally also be included with the HA fractions in the classical operational definitions.

In theory, should HS be polydisperse with respect to size and shape, as well as charge, it is logical that fractionation should also be achieved on the basis of molecular size differences. Gel chromatography, or gel permeation chromatography (GPC), has been applied extensively for the fractionation of HS on the basis of molecular size differences.

Swift (1996) has reviewed fractionation procedures that largely involve uses of gel chromatography, and more recently Perminova (1999), Swift (1999), and De Nobili and Chen (1999) have discussed applications of gel chromatography procedures for the fractionation of HS. Tombácz (1999) has shown how fractions of different compositions are obtained when dissolved HAs are filtered using graded porosity membranes.

On the basis of his communication in this issue, it is clear that Piccolo (2001) is not a disciple of the macromolecular concept with respect to HS. Whether or not the reader agrees with his viewpoints, it must be clear to all that high performance liquid chromatography (HPLC) techniques have merit for the fractionation of HS.

Fractionations on the basis of charge density and size differences (even if the size differences are based on homo- or heteromolecular associations) offer approaches for decreasing the polydispersities of humic fractions. It is desirable to have a reasonable degree of molecular homogeneity for studies of compositions and structure.

SIZES, SHAPES, COMPOSITIONS, AND STRUCTURES

Sizes and Shapes

Major emphasis was placed on the sizes and shapes of HS in Vol. 164, No. 11 of Soil Science (1999). The work of Cameron et al. (1972) has influenced concepts of sizes and shapes greatly. On the basis of ultracentrifugation studies of extensively fractionated (using gel chromatography and pressure filtration through graded porosity membranes) HAs isolated from a Sapric Histosol, they concluded that the MW values of the fractions ranged from about 2000 to 1,300,000 Da. A linear relationship was obtained for MW values up to about 400,000 Da when frictional ratios, obtained from the ultracentrifugation data were plotted against the log of the MW values (see Swift, 1989). Approximately 75% of the HAs had MW values <100,000 Da, and 25% had values <10,000 Da. Less than 20% fell into the very high MW category. The linearity fitted the model for a random coil structure. The deviation
for the higher MW values was attributed to branching. However it was found subsequently that the high MW components contained small amounts of colloidal sodium or aluminum silicates, and that could account, in part, for the high MW values (Swift, 1999).

The concept of ‘pseudomacromolecularity’ is gaining adherents. This concept suggests that the macromolecular–like properties arise from associations of smaller molecular species in micellar or pseudomicellar structures or through other forms of molecular associations involving, perhaps, nonpolar molecules such as long chain hydrocarbons, fatty acids/esters/suberin-type components. This viewpoint is treated in detail in this issue by Piccolo (2001). Swift (1999) has defended the concept of macromolecularity. He argues that should lignin modification and degradation be major processes in the formation of HS, the products could then be expected to have a range of MW values because of the different extents of cleavage of the altered lignin parent materials. He also argues that if microbial synthesis processes are taken into account, it is not reasonable to conclude that the formation of high MW products is not possible in the absence of genetic control, and he refers to evidence of synthesis of HS from plant precursors, but in the absence of lignin (Haider et al., 1975; De Nobili et al., 1989).

The fractionation procedure used by Cameron et al. (1972) required extensive applications of the HAs to gel columns until the fractions applied were contained within discrete bands. On that basis, Swift (1999) has argued that if macro-molecular species should form from associations of smaller molecules, it would be extraordinary if the associations always provided materials of the same molecular sizes (or had the same elution volumes) during the course of the later stages of the elution processes from the gel columns. The possibility cannot be dismissed, based on the compositional differences between the different sized fractions observed by Tombácz (1999), that molecules that had (after several reprocessing gel chromatography runs) relatively discrete sizes were combinations or associations of molecules of relatively similar compositions. Also, the data of Cameron et al. (1972) and their interpretations cannot be rejected until applications of the most modern procedures for the fractionation of HS are applied to extracts from the soil used in the study by Cameron et al. or to extracts from Sapric Histosol soils of similar compositions and properties.

Engebreton and von Wandruszka (1994) and Gueltzoff and Rice (1994) have suggested that HS form micellar structures, and considerations of micelle-type associations of humic molecules have considerable support. Wershaw (1994, 1999) has proposed that hydrophobic bonding can cause humic molecules to associate in micelle-like aggregates. At low concentrations, amphiphilic molecules exist solely as single unit species. At concentrations greater than the critical micelle concentration (CMC, the concentration above which a surfactant molecule in solution will form an ordered aggregate or micelle) micelles will form. In this, the hydrophobic groups orientate towards each other and the interior, and the hydrophilic functionalities are on the outside. Polar groups can be on the inside, and in such cases, interactions such as hydrogen bonding will decrease the hydrophilic character of the polar groups. Also, the hydrophilic groups (inside or outside) in the micelle structures can interact through hydrogen bonding and dipole–dipole interactions with the solvent (water). Entropy increases as water structure is disrupted and decreases with micelle formation.

Materials soluble in organic solvents were obtained when natural OM was silylated (Hertkorn et al., 1997) and the products were soluble in organic solvents. Silylation decreased hydrogen bonding. The fact that the products then dissolved in organic solvents may be indicative of relatively small molecules. Further indications that HS molecules are not highly macromolecular can be provided by fast-atom bombardment mass spectrometry (FABS), laser desorption mass spectrometry (LDMS), and matrix-assisted laser desorption mass spectrometry (MALDI). (See Hatcher et al., 2001, this issue for descriptions of some of these techniques.) The limited applications of these new techniques suggest that the MW values of HS range from several hundred to several thousand Da.

The micelle and hydrogen bonding concepts are, of course, forms of molecular associations. The micelle concept would also include hydrophobic bonding through associations of the nonpolar functionalities. Tombácz (1999) has shown that HAs from different sources had different CMC values, and the largest values corresponded to the fractions having the highest hydrocarbon contents (as determined by NMR) and
the largest sizes. That could indicate that associations of humic molecules with fatty acids/esters/suberins may have conferred micelle-forming properties on the humates.

**Compositions**

In this issue, MacCarthy (2001) has emphasized the supermixture nature of HS. He is right in assuming that it will not be possible to isolate a true humic component that satisfies all the criteria for purity that characterize discrete chemical compounds. It is clear that most of the studies in the past dealt, usually unwittingly, with so-called humic fractions that contained much non-humic material. These materials included carbohydrates, peptides, fatty acids, hydrocarbons, and various materials that were residues of nonhumified plant and microbial remains. We believe that it is these materials with which the HS are associated that present the problems, not necessarily the HS per se. It is important to continue to develop procedures that will free HS from their associated materials. When that is achieved, it may then be possible to get improved fractionations of the HS, and the fractions will yield much compositional and structural information. We would regard the fractionation approaches we have outlined above merely as a start.

Chemical degradation procedures and NMR have arguably provided the most interpretable data with regard to the components of HS. However, there have recently been significant improvements in pyrolysis procedures, and Hatcher et al. (2001) have described the types of information that can be obtained using pyrolysis gas chromatography mass spectrometry (Py-GC/MS) techniques. Except in the cases of hydrocarbon structures and aliphatic carboxylic acids and esters (likely to be associated with the humic molecules and not parts of these), it is important to be aware of the types of structures that would give rise to the compounds identified in the pyrograms.

The information from the chemical degradation reactions suggests that the component molecules of HS are relatively simple. Most likely, the long chain hydrocarbon, fatty acid, and ester structures are of plant and microbial origins and are merely associated with the true humic molecules. Regardless of the size of the humic molecules, the problems of degrading the core or backbone structures are the same. The linkages between the component molecules in the structures are not labile in mild degradative processes such as hydrolysis and require high inputs of energy to achieve release of the molecules that make up the structures. This means that the molecules released have varying residence times in the degradative digests and will undergo chemical modifications during that time.

Even hydrolysis in acid can lead to significant changes. Up to 50% of the masses of soil HAs can be lost as CO₂ (Parsons, 1989), especially from activated carboxyl groups, such as β-keto acids, and from hydroxybenzenecarboxylic acids, and as soluble molecules (e.g., sugars, amino acids, small amounts of purine and pyrimidine bases, and phenolic substances). These estimates were made for HA fractions, which had saccharide and peptide materials, especially, associated with the HAs. Strangely, the total acidities of soil HAs are usually not decreased by the hydrolysis because other acid groups are formed (e.g., from esters and lactones).

An appropriate treatise is given by several authors in Part 1 (Degradative Studies of the Structures of Humic Substances) of *Humic Substances II. In Search of Structure* (Hayes et al., 1989a, b). Hayes and Swift (1978, 1990) compiled a list of more than 100 products that had been identified in the digests of a variety of oxidative degradations of HS. Many additional products have been identified in the digests of reductive degradation procedures and in the pyrograms from pyrolysis applications. The digest products were once considered building blocks of humic molecules. Hayes and Swift looked at the mechanisms involved in the degradations by the different chemical reagents and showed that one parent compound in the humic molecule could give rise to several digest products. The products depend on the type and concentration of the reagent, the temperature, the reaction time, and so on. Most of the compounds identified in oxidative degradations are acids, as would be expected on the basis of mechanistic considerations. The aromatic acids range from benzenedi- to benzenhexacarboxylic acids, and there is abundant evidence for mono- to trihydroxy (or methoxy) and hydrocarbon substituents on the aromatic rings.

Carbonylation reactions could result in benzene-carboxylic acids, and this possibility has not been resolved for alkaline degradations of HS.

Fused aromatic structures are major digest products in Zn dust distillation and fusion reactions, and some of these have heterocyclic N [digest products are listed by Hayes and Swift (1978) and by Stevenson (1989)]. It is very unlikely that fused aromatic structures will be components of biologically transformed organic materials, and it is probable that the fused structures were formed in the elevated temperatures used in these degra-
In general, it is unlikely that more than one carboxyl substituent will reside on a single aromatic unit in humic molecules. Invariably products identified in digests of reductive degradation using mild procedures such as sodium amalgam have only one carboxyl group (see reviews by Hayes and Swift, 1978; Stevenson, 1989). A variety of noncarboxyl bearing hydroxy/methoxy benzene structures have been identified in the amalgam digests, and such activating substituents would promote the decarboxylation.

Sodium sulfide (Na$_2$S) at elevated temperatures is used for the delignification of wood, and Hayes and Swift (1978) described a procedure for degradations of HS using a saturated solution (10%) of Na$_2$S at 250 °C in an autoclave. Refer to Hayes and O’Callaghan (1989) for mechanisms for the degradation processes. They considered quinone methide structures to be key intermediates leading to the degradation products. The aromatic compounds identified variously had one or two methoxy substituents (hydroxyl would have been converted to methoxyl in the methylation process needed for analysis by GC/MS), one or two methyl or other aliphatic substituents (formed from the quinone methide intermediates, although methyl substituents could be artifacts formed in the methylation process), and, with the exception of phthalic acid, there was never more than one carboxyl group attached to the benzene nucleus. That contrasts with the benzenepolycarboxylic acids found in the digests in which permanganate and alkaline cupric oxide were used. A more complete explanation of the origins of the digest products is given by Hayes and Malcolm (2001).

Hayes and O’Callaghan (1989) pointed out that many of the products identified in their digests could have origins in phenylpropane structures of the types associated with lignins, in which two or three hydroxy or ether substituents were present in the propyl side chain.

Hayes and O’Callaghan (1989) have also outlined some mechanisms for degradations of HAs in phenol under reflux conditions. Examination of the digest products and evaluations of the mechanisms that could operate suggest hydrocarbon linkages between aromatic nuclei, and some of the chains could well be long. However, the HAs were isolated from a Sapric Histosol, and the hydrocarbons could have been components of the mixture, and not linked covalently to the HAs.

It is well to keep in mind that the yields of identifiable products in the different chemical degradations of HS are rarely more than 40% of the masses of the starting materials. It is likely, for example, that alkaline degradation processes create new (synthetic) macromolecules from components released in the digests from the parent HS. Aldol condensation is just one of the possible macromolecularization processes that can take place. Thus some of the residual materials in the digests that were not solubilized in the organic extractants could well be very different from the HS subjected to the degradation process. We need to learn more about such “recalcitrant” fractions. Sequential degradation procedures, as suggested by Hayes and Swift (1978), could give indications of the extent to which macromolecular residues of previous degradations would be “demacromolecularized” (or degraded to identifiable products) when a succession of chemicals and procedures are used that provide different energy inputs and mechanisms of degradation. An appropriate sequence might employ boron trifluoride-methanol, followed by sodium amalgam, then phenol under reflux conditions, then Na$_2$S, and finally alkaline permanganate or alkaline CuO. Such an approach was used by Simpson (1999) and the results await presentation in the scientific literature.

Nuclear magnetic resonance has made very significant contributions to our awareness of the compositions of HS. Hatcher et al. (2001) and Simpson (2001) have discussed in this issue some principles and applications in the humic sciences of solid state CP/MAS $^{13}$C–NMR (Hatcher) and multidimensional solution state NMR (Simpson). We will refer briefly to limited examples of applications of solid state NMR that have highlighted compositional differences in humic fractions.

Solid state NMR is an excellent fingerprint for comparing aspects of the functionalities in humic fractions. Hayes and Malcolm (2001) have presented CPMAS $^{13}$C–NMR spectra of HAs, FAs and XAD–4 acids isolated by the sequential extraction method, referred to above, from the 0– to 15-cm layer (4.1% organic C) of a long-term grassland soil from Devon, England (Hayes et al., 1996). These spectra showed clear differences between the same operationally defined humic fractions isolated at the different pH values, and
the differences were reflected in the integrated areas (Hayes et al., 1996) for the resonance bands in the 10–45 (alkyl C), 45–65 (O-alkyl), 65–110 (ethers, hydroxyls, sugars), 110–140 (aromatic C), 140–160 (phenols, O-aryls), 160–190 (carboxyl, esters, amides), and 190–220 ppm (carbonyl of aldehydes, ketones) bands. The HA fraction isolated at pH 7 was the most highly aromatic (22%), and its aromaticity was about double that for the HAs isolated at pH 10.6 (10%) and 12.6 (13%). The integrated areas for the resonances at 160–190 ppm (carboxyl, ester, amide) were 16%, 14%, and 12%, respectively, for these HA fractions, reflecting the expected decreases in carboxyl functionalities as the pH values of the extractants were raised. The contributions of amide functionalities to this resonance should not be overlooked because, for example, the contribution of amino acids to the overall composition of the HAs isolated at pH 12.6 was 16.3% (Hayes, 1996). The anomeric C resonances also increased as the pH of the extractant increased, indicating greater contributions of sugars to the compositions of the extracts. There was evidence for greater O-aromatic functionalities as the pH of the extractant increased, and this was substantiated by a sharp resonance (OCH₃) at 56 ppm. It was concluded that the materials isolated at the higher pH values were less humified than those isolated at the lower pH values, and they had characteristics that suggested origins in plants.

The spectra for the material that precipitated from dilute solution in the pH range 2.5–2.0 (referred to above) was very different from the spectra of the HAs, FAs, and XAD-4 acids. There was only a comparatively small resonance in the aromatic region, and the major resonances were in the aliphatic hydrocarbon functionality region (10–45 ppm) and in the 65–110 ppm band. Evidence for anomeric C was weak, and the neutral sugars (NS) content (6.7%) was similar to that for the HAs isolated at pH 7. The amino acid (AA) content was, however, high (14%), and it is likely that peptide functionalities contributed significantly to the resonances at 160–190, and 65–90 ppm. The composition of the precipitate isolated in a similar way (formed between pH 2.5 and 2) from the pH 12.6 isolate was very similar (Hayes, 1996; Hayes et al., 1996). This material may be similar to glomalin (Franzluebbers et al., 2000; Wright and Anderson, 2000).

The spectra for the FAs isolated at pH 7 and at pH 12.6 were also significantly different from each other. Both had features in common with the HAs isolated at the same pH values, which supports the view that HAs and FAs are part of a continuum. Both showed evidence for significant aromaticity, and the anomeric C signal was strong for the FAs isolated at pH 12.6. The NS and AA analyses indicated that these were likely to be the major contributors to the resonance at 65 to 110 ppm. There are clear indications for methoxyl in both samples, which is unusual for FAs.

The spectra for the XAD-4 acids were different from those for the FAs and HAs, and there were distinct similarities between the spectra of these acids isolated at the different pH values. Aromaticity was low for each sample (ca 4–6%), and the major resonances were in the 60–110 ppm region (53%, 58%, and 72%, respectively, for the samples isolated at pH 7, 10.6, and 12.6) and with strong resonances centered around 105 ppm (anomeric C). The AA contents (3.9%, 7.4%, and 5.8%) were relatively similar to those for the HAs and FAs. Therefore, it is likely that the XAD-4 acids were composed largely of polysaccharide/alter polysaccharide materials that had significant uronic acid contents (not measured). It is possible that mucopolysaccharides, or mucopolysaccharide-derived substances, contributed to the compositions.

Watt et al. (1996) used CPMAS ¹³C–NMR to compare the humic fractions isolated from waters from different sources. The spectra indicated that the differences observed in these could be related to the soils of the watersheds.

Concepts of Structures

According to Hayes and Swift (1990), if humic molecules are composed of numerous different chemical building blocks, and if these are assembled at random (in the absence of genetic control of synthesis), it is unlikely that there would exist anywhere two molecules that are exactly the same should their MW values be greater than 20,000, or even less. The same sentiments were expressed by Hayes et al. (1989b). On the basis of what we know now, these considerations, hypothetical though they may have been, seem naïve. First, it is very unlikely that there are vast numbers of component building blocks in any single humic molecule; second, it is unlikely that these would combine randomly because not all would have the functionalities needed to react with all of the other molecules; and third, it now seems unlikely that there are any humic molecules of MW values as high as 20,000 that would have formed solely as the result of molecular interactions in the soil environment.

Nevertheless, we cannot escape from the
mixture concept stressed by MacCarthy (2001) in this issue, and we should keep in mind the likely randomness of the synthesis or transformation processes. Suppose we accept that a major mechanism in the genesis process involves the alteration of relatively intractable plant residues, especially lignins. If we accept this, then it is relatively easy to see how the polydispersities arise from random cleavages of the macromolecules and from varying extents of oxidation of component functionalities (involving, perhaps, the C3 side chains, and ring opening). Demethylation of methoxy substituents would give rise to phenolic substituents.

However, should HS arise from lignins only, we would by now have a good awareness of their compositions and structures. It is, of course, the mixture phenomenon that has hindered our progress in putting structures on the humic components derived from lignins. It might well be asked, “If the soil microbial population has obtained energy from transformations of lignin, why do some of the HS of lignin origins persist?” The answer must be that there is some degree of protection for these products. They do, of course, decompose, albeit relatively slowly. Based on the comments of Jenkinson (1981), should such decompositions not take place, we would be buried deeply in HS. That protection can be attributed to the associations that HS have in the soil environment. Of particular importance in this regard are the associations with the inorganic soil colloids. We will discuss below the protection provided for humin by the mineral colloids, but, of course, these colloids can also be the basis for the protection of HAs and FAs. The evidence that is emerging suggests that the associations between HS and nonpolar molecules such as long chain hydrocarbon, fatty acid/ester, and suberin structures can provide major mechanisms for protection.

For the last century and beyond, there has been a compulsion among humic scientists to suggest structures, especially structures for HAs. Hayes et al. (1989c) suggested that there were not sufficient structural data to attempt to depict structures more than a decade ago. They concluded that there had emerged from the treatises in Humic Substances II (Hayes et al., 1989a) “generalized forms based on shapes and sizes, but nothing which can be interpreted in terms of discrete structure”. On the basis of what has emerged in the last decade (see Piccolo, 2001, this issue), we can no longer be sure that concepts that we held with regard to sizes and shapes have stood the tests of time and new experimentation. Certainly, the concepts of associations have dominated thinking in recent times.

In this issue, Burdon (2001) has presented a number of paper structures that have been proposed during the last century, and he has convinced us that these structures contribute nothing to the science or to realistic concepts and are best forgotten. He also discusses, realistically, the notion of chemical synthesis processes in the soil environment, and he has rid us of any lingering notions of browning reactions as major contributors to humic genesis. (Such processes may well contribute to the formation of humic-type substances in manure heaps and in compost piles where the interacting species are in close proximity and the temperatures can become elevated.) There have been some plausible suggestions put forward in the past, however, and that of the ligno-protein complex proposed by Waksman and Iyer (1933) was so widely accepted at the time that many were convinced for almost a generation that humic structures had been resolved. When a research student in the late 1950s, Hayes (1960) formed an oxidized lignin-casein complex in the manner described by Waksman and Iyer. The product did have many of the properties of HAs from a Sapric Histosol soil. However, when subjected to thermal analysis, it was clear that the complex retained all of the thermal properties of the starting materials, and these were entirely different from those of the HAs.

There is little point in seeking definitive structures of humic molecules, but it is important that we have an awareness of the types of structures in the mixtures and of the types of associations that these can form. The contribution in this issue by Simpson (2001) shows clearly that multidimensional solution state NMR provides unique tools for the studies needed. Simpson et al. (2001a) have shown how applications of a range of two-dimensional (2-D) NMR techniques have identified component structures and connectivities in a FA isolated from a podzol soil at pH 12.6 after the soil had previously been extracted exhaustively at pH 7 and at pH 10.6. However, there are much more exciting prospects ahead from applications of Diffusion Ordered SpectroscopY (DOSY) and Liquid Chromatography (LC)-NMR. When dealing with unfractionated HS materials, employing DOSY, Simpson et al. (2001b) observed distinct diffusion coefficients characteristic of the different components of the mixture, and the MW values of the components were relatively low (<2000). Thus, each of the materials had independent existences.
and were not covalently linked to other molecules in the mixture. LC-NMR showed that the components could be separated readily. These observations strengthen the evidence that is emerging that indicates HS are not macromolecular with, possibly, some degree of cross linking or branching, but are aggregates of mixtures. They consider that it will soon be possible to resolve the structures of at least some of the simpler molecules in humic mixtures.

**HUMIN**

The papers by Derenne and Largeau (2001) and by Rice (2001) in this issue are welcome contributions that emphasize the nature of humin materials. We often forget that humin materials are major components of the NOM in soils and sediments. Because humin is, by definition, insoluble in aqueous solvents, there have been few studies of compositions, structures, and properties of soil humin materials.

Rice (2001) has outlined the various concepts that soil scientists have held with regard to the compositions of humin. Most of these consider the components of humin to be closely associated with the inorganic soil colloids. However, not enough consideration has been given to the binding mechanisms involved.

As a result of applications of their methyl isobutylketone (MIBK) procedure, Rice and MacCarthy (1989, 1990) deduced that humin is a mixture of lipid-type and humic-type substances. After Clapp and Hayes (1996) extracted exhaustively with aqueous solvents, they then carried out one extraction with DMSO/HCl. The material extracted was classified as humin. It was found that HS, isolated after the DMSO had been removed using XAD-8, had properties similar to HAs and FAs. Surprisingly, the AA and NS contents were of the order of 5–6% and 6–7%, respectively, and these components (assuming that these were not covalently linked to the humic structures) would have been expected to be removed by the XAD-8 treatment. Long chain fatty acids/esters would have remained sorbed to the column during the course of back elution with base. Again, the significant NS and AA contents alert us to the possibilities of the presence of glomalin in the humin fraction (Franzluebbers et al., 2000; Wright and Anderson, 2000).

A mild boron trifluoride-methanol transesterification procedure was introduced by Almendros and Sans (1991) for studies of component molecules of HAs and humins. In this procedure, ester functionalities linked to the humic core would be cleaved to set labile (derivatized) structures free. Their yields of identifiable products were similar to those for oxidative degradation procedures and on the order of 30 to 35% of the mass of the starting material. They identified in the digests of humins a variety of monobasic straight chain and branched fatty acids, long chain dicarboxylic acids, di-hydroxymonobasic acids, trihydroxymonobasic acids, methoxybenzenecarboxylic acids, di-, tri-, tetra-methoxybenzenecarboxylic acids, and a variety of other miscellaneous acids. Although it may well be that these acids were present as esters in the humins, many (and especially the long chain aliphatic acids) are characteristic of cutins and suberins and of degradation products of plants. The suggestions made by Rice and MacCarthy (1989, 1990) following their isolation of components of humin indicate that the long chain acids released from the humin in the transesterification process may not be linked covalently to humic components in the humin.

Derenne and Largeau (2001) have introduced us to a range of substances that are rarely mentioned in studies of soil HS. If, as referred to under the Definitions heading, “humic substances result from the decomposition of plant and animal residues,” then many of the components of humin satisfy that definition only if it is considered that they represent plant and animal residues that have resisted the biotransformations of the other components of the source materials. Again, these recalcitrant components would loosely fit the definition of Aiken et al. (1985b) should biogenic be interpreted as indicating synthesis by biological processes (but distinct from the humification process) and not resulting from biological transformations of some, at least, of the compounds contained in the humin fraction. Thus, it will be appropriate to consider whether humin should be classed as a humic substance. It might be appropriate to call it the humin fraction, composed of humic substances entrained in recalcitrant organic materials of plant and microbial origins. The hydrocarbon structures and the fatty acids, esters, suberins, and kerogen materials would not be in the HS classifications.

It is likely that the nonpolar, hydrophobic molecules aid in the binding of some HS to the inorganic colloids.

We are surprised that concentrated sulfuric acid has not been used in the isolation of soil humin materials. Some sulfonation would be expected to take place, which would give rise to solvatable functionalities. Should the materials in the
HUMIC SUBSTANCES AND CARBON SEQUESTRATION

When soils are in long-term cultivation, soil C is lost, soil structure is degraded, soil is eroded, and soil fertility is depleted. Such damage is avoided when sufficient crop residues or organic manures are added. Nearly 30 years ago Larson et al. (1972) reported experiments that indicated that SOC was not decreased when 6 Mg ha$^{-1} \cdot yr^{-1}$ of cornstalks was added to a Typic Hapludoll Corn Belt silt loam (36% clay, 56% silt, and 8% sand) that was continuously cropped to corn (Zea mays L.) during the 1953–1964 period. The crop roots (presumably ca 2 Mg ha$^{-1} \cdot yr^{-1}$) will have contributed to the C sequestration. When 16 Mg ha$^{-1} \cdot yr^{-1}$ of cornstalks was added, SOC was increased by 30%.

International concerns about greenhouse gas emissions and global warming are focusing attention on the potential of soils to sequester CO$_2$ from the atmosphere. Cropland management procedures have progressed considerably since the studies of Larson et al., and it is encouraging to note from the information provided by Lal et al. (1998) that the improved practices that operate at this time are enabling C to be sequestered to such an extent that the SOM levels are increasing in croplands in the U.S. Swift (2001) has reviewed the advances that are being made in this area and has indicated how Australia proposes to meet the C sequestration targets as set out in the Kyoto Agreement (Conference of the Parties, 1997). Australia is allowed to increase its CO$_2$ inputs to the atmosphere by 2010 to 108% of what these were in 1990. In return, Australia must sequester an amount of C equivalent to the excess emitted.

That type of initiative should be part of a global project. Based on the data in Lal et al. (1998), there is an enormous potential to sequester C in the world’s agricultural soils, and it should be possible to sequester even more in grasslands and forest soils by following improved management practices. Increased SOM levels will also improve the soil fertility.

Based on the reservations we have expressed above with regard to the classification of humin as a HS, it would be wrong to attribute to HS all of the C sequestered by SOM. For example, glomalin, a glycoprotein in the hyphae of arbuscular mycorrhizal fungi (AMF) was found at levels of more than 60 mg cm$^{-3}$ in tropical soils (Rillig et al., 2001a). Based on C dating data, glomalin takes several years to decades to turn over in the soil, whereas the turnover times of the AMF hyphae were of the order of days to weeks. Furthermore, glomalin content can be correlated with aggregate stability (Wright et al., 1999; Franzluebbers et al., 2000; Wright and Anderson, 2000; Rillig et al., 2001b), and that indicates that it is sorbed to the soil (colloidal) surfaces, which would explain its resistance to decomposition. Thus glomalin would likely be included in the humin fraction, and would not be a HS in the same way as we have considered that other products such as long chain hydrocarbons, fatty acids, esters, and suberins are not HS.

GENERAL CONCLUSIONS

In our Introduction we alluded to the traditional apathy on the part of many soil scientists toward studies of the compositions and aspects of the structures of HS. This is somewhat understandable because HS, the most abundant and perhaps the most complicated of the organic molecules in the natural environment, fulfill a remarkable role in a relatively uncomplicated way in the soil environment. What we must accept is that we will not understand how HS achieve their roles in the stabilization of soil aggregates, the promotion of plant growth, their interactions with anthropogenic organic chemicals in soil and water environments, and in resisting decomposition and thereby sequestering C until we know more about their compositions, about aspects of their structures, and about their associations with other organic molecules and with the inorganic colloids in the soil environment.

Much emphasis is placed on the fact that HS are complicated mixtures of molecules, and it will be appropriate to give further consideration to the mixtures concepts. In situations where HS are derived from plant debris, for example, we can expect mixtures of products, of course, but many of the components in the mixture will not satisfy our concepts of HS. There will be in that mixture a range of molecules of plant and soil biotic origins, and these will be associated to varying extents in the mixtures. One of the challenges we face is to extract the true humic components from the nonhumic components in the mixtures. If, for example, lignin alteration products should be the major contributors to the HAs and FAs in any batch, interpretations of their compositions and aspects of their structures should only be moderately challenging when these are freed
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