# Humic Substances in the Suwannee River, Georgia: Interactions, Properties, and Proposed Structures

United States Geological Survey Water-Supply Paper 2373

# Humic Substances in the Suwannee River, Georgia: Interactions, Properties, and Proposed Structures

Edited by R.C. AVERETT, J.A. LEENHEER, D.M. McKNIGHT, and K.A. THORN

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# FOREWORD

Humic substances as a collective term and humic and fulvic acids as specific terms are not household words. For about a century, these terms belonged to the domain of the soil scientist. Even though their chemical structures remained elusive, they were recognized as important entities in soil. During the past decade or so, there has been a renewed interest in humic substances in soil and water. Such interest has resulted from improved analytical instrumentation and by a need to understand the structure and function of natural organic substances in water.

A responsibility of the U.S. Geological Survey is to assess the Nation's water resources; this includes assessing water quality, which is the study of material in water. Such material may be suspended, colloidal, or in true solution. Because humic substances are a major carbon source in water, they have received attention by Geological Survey scientists. This attention has been a major focus by members of the Geological Survey's organic chemistry group. For more than a decade, this group has collected samples, made analyses, and worked toward determining the structure and function of humic substances in water. Their work has brought worldwide recognition to the field and, through Geological Survey support, in 1981 they helped organize the International Humic Substances Society, which held its first meeting in Estes Park, Colorado, in August 1983.

At the second meeting of the Society in Birmingham, England, in August 1984, it became apparent that Geological Survey scientists were rapidly advancing the study of the chemistry of humic substances. It is appropriate, therefore, to publish this Water-Supply Paper on humic and fulvic acids from the Suwannee River in Georgia because these results represent the Survey's most definitive findings to date (1986). Though this work is not conclusive, it is state-of-the-science. Hopefully, the reporting on this work will aid in advancing the science of humic substances.

Philip Cohen
Chief Hydrologist

# PREFACE

This Water-Supply Paper is concerned with state-of-the-science chemistry of humic and fulvic acids from the Suwannee River in Georgia. Humic and fulvic acids are specific entities in the broad category of humic substances. Although the broad subject in the chapters that follow concerns humic substances, the primary focus will be on fulvic acids from the Suwannee River. After a decade of study, there is an improved understanding of the interactions, properties, and structures of fulvic acids that comprise the major fraction of dissolved organic substances in water.

Most of the authors of the chapters that follow are research hydrologists with the U.S. Geological Survey located in Denver, Colorado. As project personnel, they work on specific facets of organic chemistry, primarily natural organic matter in water. As a group, they devote some of their time and skill to the study of humic substances in water. This group focus on humic substances is not accidental: it is an organized effort to understand the structure and to improve definition of the function of humic substances in nature.

The study of humic substances is not a new scientific activity with regard to soil chemistry; it is, however, a relatively new field with regard to aquatic systems. Soil textbooks written before the turn of the 20th century discuss humic substances and fulvic and humic acids. These discussions are brief: descriptions of chemical properties are limited to color and solubility. Only during the past decade or so has the study of humic substances provided a much clearer understanding of the chemistry and economic and ecological importance of these substances.

The authors of the chapters in this Water-Supply Paper have contributed extensively to the understanding of humic substances in water. To further expand the understanding of humic substances in water and soil, the International Humic Substances Society was established with the support of the U.S. Geological Survey.

Why do we study humic substances in water? That question would have been simple to answer a decade or so ago because we knew then only that they were important components of the soil. Today, as our knowledge expands, there are numerous reasons to study humic substances in water. Perhaps most importantly, humic substances constitute a large proportion of the organic matter (carbon) in aquatic systems. It is for this reason that the study of the Suwannee River began—it is rich in organic matter as a result of flowing from the Okefenokee Swamp. Organic-matter production is the driving force for all ecosystems; it controls, through bacterial decomposition, dissolved-oxygen resources in aquatic systems. But there are other reasons to study humic substances in water. Humic substances interact with contaminants, such as pesticides. Moreover, humic substances chelate and transport trace metals and are a source or sink for these metals. Humic substances also are a source or sink for atmospheric carbon dioxide, which is a buffer against acidic precipitation. Finally, they have great effect on the fertility and moisture-holding capacity of soil. Modern instrumentation, especially nuclear-magnetic-resonance spectrometers, gas chromatographs, and mass spectrometers, has aided in advancing the determination of structural models of humic substances. Proposed structural models are presented in this Water-Supply Paper. As the "black box" of humic-substance chemistry is opened, our understanding of the interactions, properties, and structures of humic substances increases—this aids our understanding of water chemistry and, ultimately, of aquatic ecosystems.

However, science continues to evolve, and the findings in this Water-Supply Paper are part of the evolution of humic-substance chemistry. Because the authors of the following chapters are at the forefront of the science, it is time to share the information they have gathered and the knowledge they have gained—share, that is, with the full realization that, in this rapidly changing field, the ideas and concepts presented here may one day may be outdated or determined to be incorrect. But that, too, is a characteristic of science. If this Water-Supply Paper serves only as a historical benchmark, it will have served its purpose. If it helps the science of humic-substance chemistry to progress by stimulating other scientists, or if it provides others with a greater appreciation of this field of science, it will have accomplished more than we had planned.

R.C. Averett
J.A. Leenheer
D.M. McKnight
K.A. Thorn
Editors

# ACKNOWLEDGMENTS

Professor Michael A. Mikita, California State University at Bakersfield, deserves credit for stressing the importance of spatial relations of functional groups in structural models of fulvic acid from the Suwannee River. His participation with the research group of authors in this volume and his technical review of several reports in this volume were invaluable. Professor Jacob A. Marinsky, State University of New York, Buffalo, contributed his understanding of the protonation and metal-ion complexation equilibria of fulvic acid from the Suwannee River. Professors Stephen Cabaniss, Harvard University, and E. Michael Perdue, Georgia Institute of Technology, deserve special thanks for the difficult task of reviewing the summary chapter of this volume (chapter P).

# CONTENTS

[Letters designate chapters]

## CONVERSION FACTORS, VERTICAL DATUM, and ABBREVIATED WATER-QUALITY UNITS

For readers who prefer to use inch-pound units rather than the metric (SI) units used in these chapters, values may be converted using the following factors:

| Multiply metric unit | By | To obtain inch-pound unit |
|---|---|---|
| | **Length** | |
| angstrom (Å) | $3.937\times10^{-7}$ | inch |
| centimeter (cm) | $3.937\times10^{-1}$ | inch |
| kilometer (km) | 0.6214 | mile |
| meter (m) | 3.281 | foot |
| micrometer (µm) | $3.937\times10^{-5}$ | inch |
| millimeter (mm) | $3.937\times10^{-2}$ | inch |
| nanometer (nm) | $3.937\times10^{-8}$ | inch |
| | **Volume** | |
| cubic centimeter ($cm^3$) | $6.102\times10^{-2}$ | cubic inch |
| liter (L) | $2.642\times10^{-1}$ | gallon |
| microliter (µL) | $3.38\times10^{-5}$ | ounce, fluid |
| milliliter (mL) | $3.38\times10^{-2}$ | ounce, fluid |
| | **Mass** | |
| milligram (mg) | $3.527\times10^{-5}$ | ounce, avoirdupois |
| gram (g) | $3.527\times10^{-2}$ | ounce, avoirdupois |
| microgram (µg) | $3.527\times10^{-8}$ | ounce, avoirdupois |
| | **Energy Content** | |
| kilocalorie per gram (kcal/g) | $5.55\times10^{-4}$ | British Thermal Units per pound |
| kilocalorie per mole (kcal/mol) | 3.986 | British Thermal Units per mole |
| | **Pressure** | |
| kilopascal (kPa) | 0.145 | pound-force per inch squared |

Degree Celsius (°C) may be converted to degree Fahrenheit (°F) by using the equation:

$$1.8(°C)+32=°F$$

The following terms and abbreviations also were used in this report:

| **Time** | |
|---|---|
| day (d) | month (mo) |
| minute (min) | nanosecond (ns) |
| hour (hr) | second (s) |
| microsecond (µs) | year (yr) |
| millisecond (ms) | |

| Concentration | |
|---|---|
| gram per cubic centimeter ($g/cm^3$) | milliequivalent per liter (meq/L) |
| gram per milliliter (g/mL) | milligram per liter (mg/L) |
| microequivalent per milligram (μeq/mg) | milligram per milliliter (mg/mL) |
| microgram per gram (μg/g) | millimole per gram (mmol/g) |
| microgram per liter (μg/L) | molar (M) |
| micromole per liter (μmol/L) | mole per milligram (mol/mg) |
| milliequivalent (meq) | nanomole per milligram (nmol/mg) |
| milliequivalent per gram (meq/g) | normal (N) |

| Frequency | |
|---|---|
| kilocycles (kc) | megahertz (MHz) |
| hertz (Hz) | gigahertz (GHz) |

| Other Units | |
|---|---|
| before present (B.P.) | millimole (mmol) |
| gram per square meter per year [$(g/m^2)/yr$] | millivolt (mV) |
| liter per minute (L/min) | millivolt per hour (mV/h) |
| microequivalent (μeq) | part per million (ppm) |
| microliter per minute (μL/min) | reciprocal centimeter ($cm^{-1}$) |
| microsiemens per centimeter (μS/cm) | relative centrifugal force (*g*) |
| microsiemens per centimeter at 25 degrees Celsius (μS/cm at 25°C) | square kilometer ($km^2$) |
| milliliter per minute (mL/min) | gauss (G) |

**Sea level:** In this report "sea level" refers to the National Geodetic Vertical Datum of 1929 (NGVD of 1929)—a geodetic datum derived from a general adjustment of the first-order level nets of both the United States and Canada, formerly called Sea Level Datum of 1929.

Chapter A

# History and Description of the Okefenokee Swamp—Origin of the Suwannee River

*By* R.L. Malcolm, D.M. McKnight, *and* R.C. Averett

# CONTENTS



## PLATE

[Plate is in pocket]



## FIGURES

## Abstract

The Okefenokee Swamp is an extensive wetland in southeastern Georgia and is the source of the Suwannee River. Reference fulvic and humic acids from the Suwannee River were isolated from water collected at a sill where the Suwannee River leaves the Okefenokee Swamp. Surface water in the swamp is acidic and darkly colored. Although there are extensive peat deposits in the swamp, the source of dissolved humic substances probably is recent decomposition of swamp vegetation. The acidity and small concentrations of inorganic constituents in the surface water indicate that there is minimal contact with underlying calcareous deposits. The Okefenokee Swamp is a complex and dynamic mosaic of different habitats, including forested upland, forested wetland, scrub-shrub wetland, and prairie. Important successional processes and disturbances in the Okefenokee Swamp include: the formation of tree islands in prairies, episodic fires during droughts, and logging that occurred during the last century. The wetland is home for a diverse fauna that includes the American alligator. This chapter provides information on the physical, chemical, and biological characteristics of the Okefenokee Swamp as background for the following chapters that are concerned with fulvic and humic acids.

## INTRODUCTION

The Okefenokee Swamp long has been recognized as a unique natural area; its picturesque beauty has been preserved as a National Wildlife Refuge. Except for afternoon boat explorers and occasional overnight campers, the wetland is presently uninhabited by man. The area is now, as it was in the past, home only to alligators, turkey vultures, turtles, deer, raccoon, bears, and other wildlife.

The wetland has not always been an uninhabited wilderness. The very name of the wetland, Okefenokee, is a Seminole Indian word meaning, "the land of the quaking earth" (Cohen, Casagrande, and others, 1984), which reflects the nature of floating peat "batteries," floating and anchored "tree houses," and the unstable condition of peat deposits throughout the wetland. There are numerous indications that the wetlands were the home for several different Indian peoples for several thousand years (Trowell, 1984; Wright, 1984). The last of the Indian inhabitants were driven from the Okefenokee Swamp by pioneer settlers in the early to middle part of the 19th century. The wetland then was sparsely settled by these settlers (known as "swampers" or "Georgia crackers") who lived a very simple, secluded, and primitive lifestyle for almost a century. Soon after the beginning of the 20th century, there was a large influx of lumberjacks and railroad workers who, under the direction of the Hebard Lumber Company, began harvesting timber from the wetland, especially cypress. Some 2 million board feet of timber, representing a large percentage of merchantable timber in the swamp, was harvested from 1909 to 1927 (Izlar, 1984). In 1936, after a 20-yr effort by early environmentalists and naturalists in the area and throughout the Eastern United States, the wetland was repurchased by the Federal Government after approximately 50 yr in private ownership and was established as a National Wildlife Refuge.

The distinctive folk culture of the swampers included colorful idioms to describe important activities in their lives (Presley, 1984). When swampers talked about a "progue about in the swamp" or "a good muzog in the swamp," they were talking about exploring the Okefenokee Swamp. After spending only a few days in the wetland, or simply from looking at a map of the mosaic of different wetland habitats that comprise the Okefenokee Swamp, one can easily imagine that exploring the wetland was a lifelong activity for the swampers.

Unfortunately, all of the researchers who study the Suwannee River fulvic and humic acids will not have an opportunity to "progue about" on their own in the Okefenokee Swamp.

This chapter presents an overview of the geology, peat deposits, hydrology, water chemistry, and environment of the Okefenokee Swamp. This overview is primarily based on a comprehensive text on the Okefenokee Swamp (Cohen, Casagrande, and others, 1984). The details in the following chapters will facilitate interpretation of current and future chemical characterizations of fulvic and humic acids in the Suwannee River.

The authors acknowledge Gregor Auble and David Hamilton, U.S. Fish and Wildlife Service, for their valuable comments and assistance with this chapter.

## PHYSICAL AND CHEMICAL CHARACTERISTICS OF THE OKEFENOKEE SWAMP

The Okefenokee Swamp, which comprises about 50 percent of the Okefenokee watershed, is an extensive freshwater, peat-forming wetland occupying approximately 1,700 km$^2$ of the Atlantic Coastal Plain in the southeastern part of Georgia. The wetland is ellipsoidal in shape (about 40 km wide and 60 km long). Although the wetland appears to be perfectly flat, there are measured elevation changes of approximately 2 to 3 m across 50 km of wetland. The eastern border is approximately 70 km from the Atlantic Ocean, and the Florida State line is its southern border (fig. 1). The Okefenokee Swamp is a mixed and multiple wetland environment, the extreme diversity of which must be seen to be truly appreciated. Dispersed throughout the forested wetland ("swamp") are extensive unforested wetlands (marshes that are locally called "prairies"); large, water-saturated, floating or anchored islands of peat covered with grass, shrubs, and (or) trees (locally called "tree houses"); large and small, water-saturated, floating islands of peat covered with grass (locally called "batteries"); small crescent-shaped islands of well-drained sand deposits; and small to moderate areas of open water (locally called "lakes") that have water depths sometimes in excess of 7 m (Cohen, Casagrande, and others, 1984).

The remainder of the Okefenokee watershed is pine woodland to the west of the wetland. The pine woodland is a gently sloping sandy area with elevations ranging from 45 to 55 m above sea level that borders the Tifton Upland.

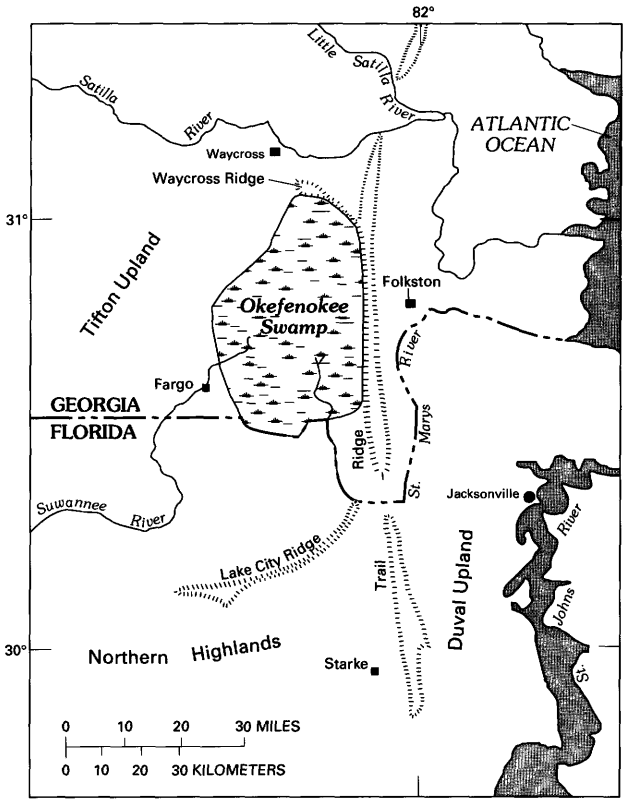


**Figure 1.** Location of the Okefenokee Swamp, Suwannee River, St. Marys River, Trail Ridge, and other physiographic features of southeastern Georgia and northeastern Florida (modified from Pirkle and others, 1977).

### Geology

Sediments on which the Okefenokee Swamp was formed were deposited on the Wicomico surface, an erosional surface that was formed approximately 220,000 to 420,000 yr B.P. (Hoyt and Hails, 1974). During late Pleistocene interglacial periods (approximately 10,000 to 250,000 yr B.P. when several sea-level changes occurred (Cohen, 1984)), sediments from the Piedmont and the inland part of the Coastal Plain were transported back and forth—the establishment of each new sea level resulted in a series of beach-sand dunes, barrier-island sediments, and lagoon marsh deposits. In southeastern Georgia and northeastern Florida, the area from the Atlantic Ocean inland for approximately 90 km is referred to as the coastal terraces province of the Coastal Plain. The terraces are a series of step-like, relatively flat surfaces (fig. 2) at successively lower elevations that generally parallel the coast. The eastern margins of each terrace are elongated beach-sand deposits or barrier islands. The Okefenokee Swamp is on the Wicomico terrace at an elevation of 30 to 45 m above sea level; its eastern margin is an ancient beach-sand deposit called Trail Ridge. The terrace is underlain by the impermeable, calcareous Hawthorne Formation, a confining unit of Miocene age. With the lowering of sea level during a period of several thousand years, the saltwater lagoon inland from Trail Ridge gradually became a freshwater marsh and then a freshwater swamp.

The geographic features contributing to the existence of the swamp are the broad, somewhat flat terrace, the Trail Ridge sand barrier, and the generally impermeable Hawthorne Formation underlying the

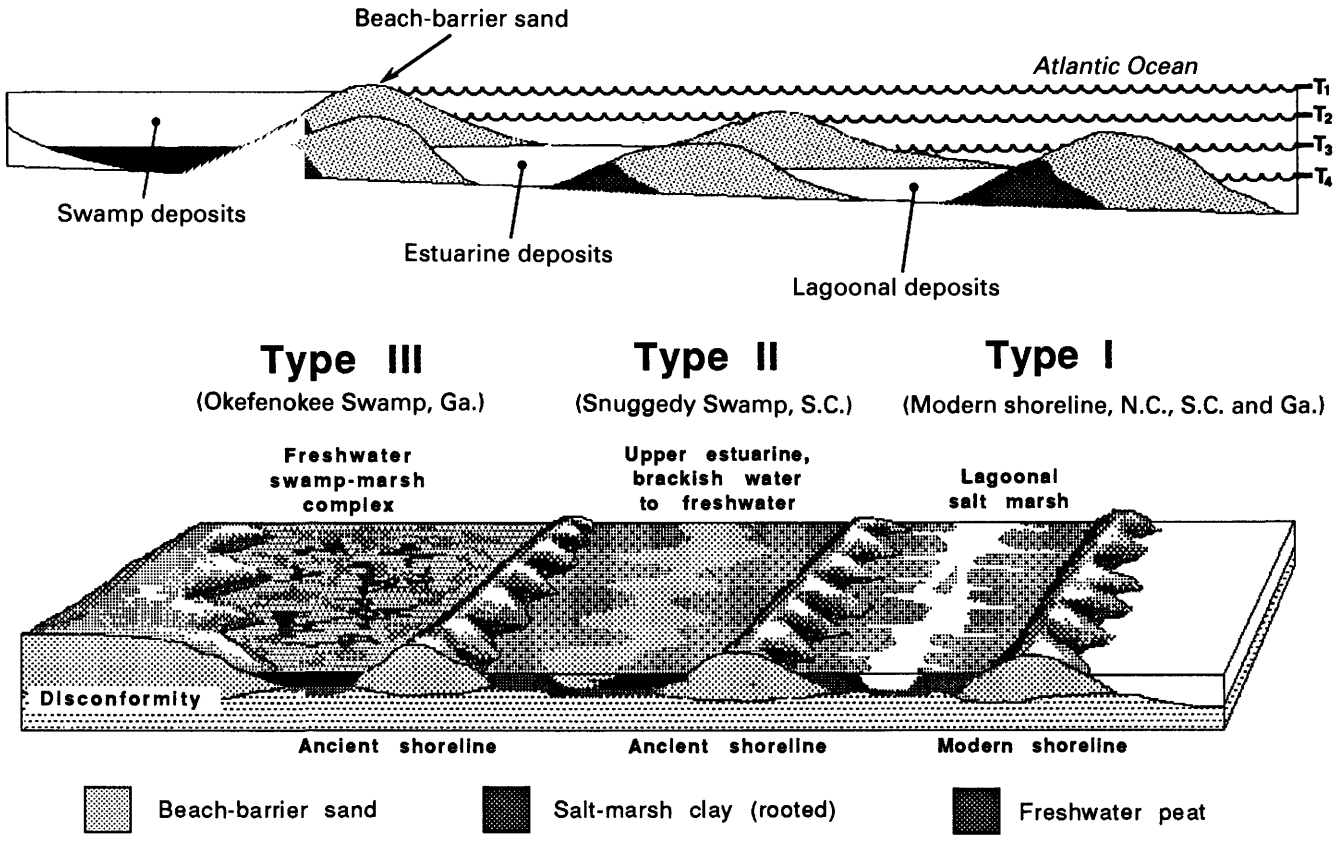


**Figure 2.** Top, stacking of back-barrier sequences; bottom, shoreline-related peat-forming environments (warm climate). Modified from Cohen, 1984; reprinted with permission of Wetlands Survey. See Cohen (1984) for additional explanation.

swamp. Additional geologic features that augment the existence of the swamp are shallow clay lenses and fossiliferous limestone within the sand deposits and at the contact of the sand deposits with the Hawthorne Formation. These geologic features limit infiltration and percolation into the subsurface.

Because of slow percolation, dissolution of calcareous sediments in surface sand deposits and dissolution of the materials in the Hawthorne Formation has been a slow and continuous process (Cohen, Casagrande, and others, 1984). This dissolution depends on the rate of percolation of acidic surface water and the susceptibility of sediments to solubilization. Dissolution has resulted in an overall slight deepening of the wetland and in local formation of deep holes or solution pits that give the swamp "floor" an uneven, karst-like surface.

A typical section of surficial and subsurface features penetrated by a hypothetical well in the swamp would be as follows: depths from 0 to 3 m, water and dense plant growth; from 3 to 6 m, peat; from 6 to 55 m, interbedded and discontinuous zones of quartz sand, shell beds, clay, and fossiliferous limestone; from 55 to 135 m, Hawthorne Formation; and depths greater than 135 m, Ocala Limestone.

## Peat Deposits

The inorganic sediments underlying the Okefenokee Swamp are covered by a continuous layer of peat that varies in thickness from 1 to 4.5 m (Cohen, Andrejko, and others, 1984). The frequency and extent of fires that occur during periodic droughts (every 25 to 50 yr) is believed to be the major factor that determines thickness of the peat. Other factors are the type and amount of vegetation, differential preservation of plant litter, underlying topography, reworking by organisms, erosion, and redeposition. The thickness of the peat deposits is extremely variable. Peats from different areas of the wet-land appear somewhat similar, but six different peat types are distinguishable by microscopic examination. The

two most common types are *Nymphaea* peat and *Taxodium* peat, and they comprise more than 80 percent of the total. The *Nymphaea* peat is formed primarily from *Nymphaea odorata*, which grows profusely in open-marsh environments; *Taxodium* peat is composed predominantly of the cypress tree of the genus *Taxodium*. According to results of carbon dating, the oldest basal peats are 6,000 to 7,000 yr old. Therefore, at least 3,000 yr elapsed between deposition of the saline marine sediments and the formation of the oldest peat deposits. Peat deposits also may have formed in the late Pleistocene, previous to the present Holocene peat deposits. The older peat deposits were either eroded during interglacial periods, decomposed in place during warmer periods, or burned upon drying. Plant growth during the last 7,000 yr has been sufficient to produce a moderate annual accumulation of plant litter. Because a major part of the wetland is saturated for most of the year, the incomplete decomposition of plant litter has resulted in an almost continuous deposition of peat at an average rate of about 1 cm/20 yr (Auble, 1982).

Litterfall peaks in the spring and the fall in the major habitats of the Okefenokee Swamp and ranges from 230 to 470 $(g/m^2)/yr$ (Auble, 1982). The rate of loss of dry weight for litter is rapid during the first year after litterfall (38 to 85 percent for herbaceous litter and 53 to 62 percent for tree-leaf litter); these rates are comparable to rapid rates in streams and other aquatic environments. This initial rapid decomposition is the major pathway for nutrient cycling in the wetland (Schlesinger, 1978). The subsequent rates of decomposition of the less readily leached organic material is much slower; for example, Auble (1982) determined no loss in dry weight of litter during the last 4 mo of a 14-mo study. These slower long-term rates are a result of anaerobic conditions and are consistent with observed peat accrual.

The elemental composition of decaying plant litter rapidly approaches the average elemental composition of peat (Auble, 1982). The average ash-free, elemental analysis of more than 100 peat samples is 60 percent carbon, 5.5 percent hydrogen, 29 percent oxygen, 3 percent nitrogen, and 0.4 percent sulfur (Cohen, Andrejko, and others, 1984). Dry peat has an average bulk *density* of 1.10 $g/cm^3$. Wet peat has a rather uniform acidic pH of 4. Most of the peat is almost pure and has an ash content that is generally less than 15 percent. The major ash component (80 percent) is silica with small quantities of kaolinite clay, iron oxides, and aluminum oxides. Silica impurities are present in the form of sponge spicules and diatoms, quartz sand, and organically complexed silica.

## Hydrology

The Okefenokee Swamp is drained by two major rivers: the Suwannee River and the St. Marys River, which carry approximately 75 percent and 25 percent, respectively, of the surface-water discharge (Cohen, 1984). The Suwannee River flows southwesterly from the wetland into the Gulf of Mexico. The flow path of St. Marys River has several geographic controls that markedly increase its distance to the ocean. The river flows south from the wetland along the western edge of Trail Ridge, then eastward through a gap in Trail Ridge (fig. 1). The river then flows almost directly north for 50 km along the eastern edge of Trail Ridge before flowing east to the Atlantic Ocean. The Satilla, Little Satilla, and Altamaha Rivers north of and adjacent to the wetland have cut directly through Trail Ridge in their course to the ocean.

The 1984 average water depth for the entire Okefenokee Swamp has been estimated to be 0.5 m (Rykiel, 1984); however, water depth varies greatly within the wetland. During 1960–62, an earthen dam or sill was constructed at the southwestern edge of the swamp where the Suwannee River flows from the wetland (pl. 1). The maximum water level of the wetland at the sill during zero outflow conditions has risen by 12 cm and extends across the wetland from the sill for a distance of 60 km. Although the sill was constructed for the purpose of decreasing fire potential within the wetland during droughts, the increased water level may have a pronounced effect on the overall hydrology, flora, and geochemical processes within the Okefenokee Swamp.

Rainfall is virtually the only source of water for the Okefenokee watershed (Patten and Matis, 1984); the average annual rainfall is 1,300 mm. Approximately 10 percent of the rainfall on the sandy, pine woods upland enters the shallow ground-water system and emerges as shallow ground-water flow into the wetland. This source of water accounts for less than 0.1 percent of the water in the wetland. Surface-water flow from the uplands accounts for only 15 percent of the water entering the wetland.

Estimates of the hydrologic budget for the Okefenokee Swamp indicate that evaporative loss

accounts for 80 percent of water losses; surface-water flow accounts for 15 percent of the loss; and seepage or recharge to deep-seated ground waters is estimated to account for 1 to 4 percent of water losses (Hyatt and Brook, 1984; Patten and Matis, 1984). These percentages are estimates and illustrate that little is known of the water budget of the Okefenokee Swamp.

On the basis of water-budget estimates, the average turnover or residence time of water in the wetland is calculated to be 3 to 4 mo (Patten and Matis, 1984). However, as with water depth, there is likely to be great variation in residence time of water in particular locations in the wetland. For example, in some of the waterways, plant leaves and stems lean with the current, and plant debris floats with the current indicating relatively rapid flow compared to that in other parts of wetland, where residence times may be much longer than several months. The relatively rapid flow rate through some areas of the wetland partly causes the relative openness and fresh air of the Okefenokee Swamp. Odors of decaying vegetation are rarely noticed.

The combined low permeability of peat deposits, clay lenses and beds in the surficial sand aquifer, and the Hawthorne Formation restrict the movement of acidic surface water of the Okefenokee Swamp to the deep ground-water system, thereby preventing extensive reaction of water with underlying calcareous sediments. The percolation or recharge of acidic surface water into deeply buried limestone must be minimal, because, if it were substantial, acidic waters would rapidly dissolve calcareous limestones, resulting in land subsidence or a substantial deepening of the water level within the wetland.

## Water Chemistry

Three distinctive features of the water quality of the Okefenokee Swamp and the Suwannee and St. Marys Rivers are the acidity (indicated by pH of 4), the small concentration of dissolved inorganic solids (indicated by specific conductance that ranges from 30 to 60 µS/cm), and the large concentration of dissolved organic substances, especially *fulvic acids*. The chemistry of these two rivers is similar to that of the nearby Satilla and Altamaha Rivers, which have been studied by Beck and others (1974). The acidity of the water results from organic acids produced during anaerobic decomposition of plant litter and peat. The water in the wetland is supersaturated with carbon dioxide and methane—both gaseous products of decomposition. Fluxes of these gases to the atmosphere are dependent on temperature and water depth and vary seasonally (Flebbe, 1984). Although mosses, such as sphagnum, are much less abundant in the Okefenokee Swamp than in many Northern United States and European wetlands, sphagnum, which releases an unusually large concentration of organic acids during decomposition and increases acidity by cation exchange, is also a possible contributor to the acidity of the water flowing from the wetland. The acidity and small concentrations of inorganic species of all surface water in the wetland are further indications that underlying calcareous sediment has little or no effect on water chemistry.

The average concentration of inorganic constituents in swamp water are: calcium, 0.60 mg/L (0.030 meq/L); magnesium, 0.45 mg/L (0.037 meq/L); sodium, 3 mg/L (0.130 meq/L); potassium, 0.2 mg/L (0.005 meq/L); and chloride, 6 mg/L (0.17 meq/L) (Auble, 1984). On the basis of evaporative losses, rainfall accounts for 74 percent of the calcium concentrations and 68 percent of the potassium concentrations.

Average concentrations of the same elements in shallow ground water from the sands and shell beds below the peat and above the limestone deposits are: calcium, 4.1 mg/L; magnesium, 1.2 mg/L; sodium, 3 mg/L; potassium, 0.54 mg/L; and chloride, 6.5 mg/L (Hyatt and Brook, 1984). These concentrations are about 7 times greater for calcium, about 2.5 times greater for magnesium, and about 2.5 times greater for potassium than the corresponding concentrations in surface water. Average concentrations of four of the five elements in ground water at depths of 150 to 200 m are: calcium, 60 mg/L; magnesium, 23 mg/L; sodium, 23 mg/L; and potassium, 2.1 mg/L.

The surface water of the Okefenokee Swamp is darkly colored; a common misconception is that the color is caused by tannins or tannic acids. In actuality, the color is caused by dissolved humic substances. The concentration of *dissolved organic carbon* (DOC) of wetland water usually is about 50 mg/L; 75 percent of the DOC is comprised of humic substances (fulvic-acid to humic-acid ratios generally are 9:1); 15 percent of the DOC consists of hydrophilic acids; and 10 percent of the DOC is composed of hydrophobic and hydrophilic neutral species (McKnight and others, 1985). Basic organic compounds generally occur in concentrations that are

less than reliable detection limits (less than 1 percent of the DOC).

One common misconception is that humic substances in waters of the Okefenokee Swamp are derived primarily from extensive peat deposits in the watershed. This theory is apparently false because the average carbon age of the peat is several thousand years old, but the radiocarbon age of humic substances isolated from the Suwannee River is 0 to 25 yr—this cannot be statistically distinguished from zero age (E.M. Thurman and R.L. Malcolm, written commun., 1987). The recent age of the dissolved humic substances is consistent with the observed rapid leaching of litterfall (G.T. Auble, University of Georgia, written commun., 1986). The sources or precursors of surface-water humic substances are apparently fresh litter, leaf, and root exudates, and leaf leachates; there are only small contributions from older, slowly decaying peat.

## MAJOR HABITATS IN THE OKEFENOKEE SWAMP

According to the National Wetland Inventory classification system (Cowardin and others, 1979) the Okefenokee Swamp is more than a large, complex wetland—it is a palustrine, acidic, freshwater wetland with organic soil and an intermittently exposed hydrologic regime. There are four major habitat classes that form the wetland mosaic (pl. 1): forested upland, forested wetland, scrub-shrub wetland, and prairie. The vegetation-distribution map presented on plate 1 has been adapted from the more detailed map by McCaffrey and Hamilton (1984) that was prepared using infrared aerial photography and on-site verification. Habitat subclasses presented on plate 1 include (1) the five subclasses of the forested wetland habitat: needle-leaved evergreen, broad-leaved evergreen, broad-leaved deciduous, needle-leaved deciduous (cypress), and mixed broad leaved and needle leaved, and (2) the two subclasses of prairie habitat: aquatic macrophyte and herbaceous. Within the scrub-shrub wetland class, which covers a large area of the wetland, there is a great diversity of subclasses (nine altogether)—thus, the wetland is actually even more of a complex mosaic than is apparent from plate 1.

All habitat classes are interrelated hydrologically through the network of canals, lakes, and prairies and through surface-sheet flow and the ground-water flow system. The following section summarizes the description of the four major habitat classes by McCaffrey and Hamilton (1984), beginning with the forested upland and ending with the open water of the prairies. Then, the dynamic processes by which the mosaic of habitats in the wetland is formed and maintained will be described.

### Forested Upland

Slash pine (*Pinus elliottii*) is the dominant vegetation of upland areas that surround the Okefenokee Swamp and of the raised sand islands within the wetland itself. These islands are accessible by boat or canoe and were important locations for swampers and logging operations in the early 1900's. Billys Island, for example, was a town site during the logging period and had a barbershop and two saloons.

Most of the uplands along the wetland borders currently (1988) are managed for timber production. However, there has been no logging on the islands within the Okefenokee Swamp since the establishment of the National Wildlife Refuge in 1936.

### Forested Wetland

The needle-leaved evergreen wetland subclass corresponds to the extension of slash pine from the uplands into adjacent wetlands. Periodic flooding and a shrub understory characteristic of the shrub wetlands distinguish this subclass from the forested upland.

The broad-leaved evergreen wetland subclass is composed of bay forest with some sphagnum-moss ground cover, small patches of shrubs, and a few cypress and pine trees. The bay trees are of medium height; common species are loblolly-bay (*Gordonia lasianthus*), swampbay (*Persea palustris*), sweetbay (*Magnolia virginiana*), large gallberry (*Ilex coriacea*) and dahoon (*I. cassine*).

Large cyprus stands dominated by pondcypress (*Taxodium ascendens*) comprise the needle-leaved deciduous wetland subclass. The cypress stands generally have a subcanopy of bay trees, a scrub-shrub understory, and some sphagnum moss ground cover. Prior to the logging operations during the early 1900's, the coverage of the Okefenokee Swamp by large cypress stands was more extensive. Based on

knowledge of the life history of the pondcypress, it seems unlikely that the pondcypress will rapidly reclaim its previous coverage from the blackgum (*Nyssa sylvatica* var. *biflora*) forests that grew after logging (Hamilton, 1984).

The dominant vegetation of the broad-leaved deciduous wetland subclass is the blackgum. This subclass occurs mainly in the western part of the wetland (pl. 1) and is present as mature stands where cypress trees were previously logged. The subcanopy of this subclass may include bay, red maple (*Acer rubrum*), and shrubs; sphagnum moss may be found as a ground cover.

The mixed forested wetland has four different dominance categories: bay-cypress, mixed cypress, cypress-shrub-prairie, and mixed pine. The inclusion of all of these in one subclass in plate 1 further minimizes the actual complexity of the distribution of vegetation in Okefenokee Swamp.

### Scrub-Shrub Wetland

The scrub-shrub wetland class includes three subclasses based on vegetational dominance: (1) broad-leaved shrubs (evergreen and deciduous)—fetterbush (*Lyonia lucida*), large gallberry (*Ilex coriacea*), dahoon (*I. cassine*), inkberry (*I. glabra*), titi (*Cyrilla racemiflora*), and others; (2) deciduous scrub (needle-leaved and broad-leaved)—young cypress and black gum trees, and (3) mixed scrub-shrub. The mixed scrub-shrub subclass has seven dominance categories.

### Prairie

The prairie includes two classes according to the classification method of McCaffrey and Hamilton (1984): emergent herbaceous prairie and aquatic macrophyte prairie. In the context of the swampers of the Okefenokee Swamp, prairie refers to shallow marshes where the vegetation is either floating or submerged to some extent, and this usage has been continued in the scientific literature. These prairies commonly have small floating or anchored islands of shrubs and trees that are called tree houses. The emergent herbaceous prairie generally is dominated by sedge (*Carex* spp.) and panic grass (*Panicum* sp.); it includes other species such as pitcher plant (*Sarracenia* sp.) and water lily (*Nymphaea* sp.). The aquatic macrophyte prairies include a variety of rooted and floating vascular plants, nonperennial emergent plants, and algae. Common species are white water lily (*Nymphaea odorata*), cow lily (*Nuphar luteum*), golden club (*Orontium aquaticum*), and bladderwort (*Utricularia* sp.). The productivity and composition of the *Utricularia*-periphyton microecosystems follow well-defined cycles related to fluctuations in temperature, water depth, pH, and other environmental characteristics (Bosserman, 1983).

## SUCCESSIONAL PROCESSES AND DISTURBANCES IN THE OKEFENOKEE SWAMP

Other than islands of forested uplands within the wetland, the current mosaic of different habitats that comprise the Okefenokee Swamp is the result of both incremental, gradual processes that occur continually in the wetland and of major events or episodes, such as fire and logging. The general successional sequence begins with prairies. Cypress and a variety of shrubs typically invade prairies and, once established, function as nuclei for further colonization. This process eventually converts an area of prairie into a patch of cypress swamp. At this point, blackgums and bays typically invade. In the absence of fire, bays eventually will replace cypress on drier sites and blackgum will become dominant on wetter sites.

### Formation of Tree Islands

A conspicuous feature of the prairie habitats is the presence of tree houses—small to large, discrete clusters of shrubs and trees appearing abruptly from the prairie. Tree houses may be completely or partially detached from surrounding peat; they may be bulges in the peat mat; or they may form by the aggregation of many small clumps of peat. Cypert (1972) hypothesized that tree houses are formed by plant colonization of floating masses of peat that are brought to the water surface by the buildup of gases in submerged anaerobic peat. Gas bubbles collected from the wetland have large concentrations of methane; it is hypothesized that gas bubbles form from a methane nucleus when methane production is sufficiently rapid (King, 1984). Islands of floating peat (without shrubs or trees) are called batteries.

Cypert (1972) and Rich (1976) describe the plant species succession that occurs once a battery has risen

to the water surface. First, water lilies die and are replaced by such species as sundew (*Drosera* sp.), orchids, or bladderwort (*Utricularia* sp.); these species are rapidly crowded by sedge. After several years, shrubs and trees begin to grow among the sedge, and the battery becomes a rooted tree house. A variety of shrubs, such as fetterbush (*Leucothoe axillaris*), buttonbush (*Cephalanthus occidentalis*), and cypress trees commonly are found on tree houses. The colonization of batteries by cypress is dependent on a nearby seed source (Hamilton, 1984). Tree houses also may be formed by plant colonization of peat accumulations or by spreading lateral root systems around established cypress trees; subsequent succession is similar to that described for batteries. This successional sequence has been confirmed by palynological and petrographic analyses (Rich, 1984). Tree-ring analysis also has shown that, as a tree house matures, large trees in the center of the house shade the shrubs and smaller trees of the subcanopy; it has also shown that the ground surface becomes higher and drier because of the accumulation of litter and roots (Duever and Riopelle, 1984).

Palynological and petrographic analyses also indicate that the formation of tree houses from the floating batteries is an important process in the long-term transition from prairie to forested wetland. Examination of large tree houses indicates that they originated as small tree houses that expanded with continued colonization of the borders by woody shrubs and trees. Thus, formation of tree houses is a nucleation process for the invasion and expansion of the cypress forest in prairies. In turn, the major restraints on this expansion are episodic fires during droughts.

### Episodic Fires

Before the installation of the large sill across the Suwannee River at the southwestern edge of the wetland, the water level in the wetland would decrease as much as 1 m during drought. The dry, exposed peat (moisture content less than 30 percent) was readily ignited by lightning, and large areas frequently would burn for weeks or months. Major fires involving large parts of the wetland are known to have occurred in 1844, 1860, 1910, 1932, and 1954–55 (Hamilton, 1984). Fire generally maintains the prairie habitats by inhibiting the expansion of forested islands. Fires, therefore, correspond to periodic local disturbances that are important in maintaining habitat mosaics in many different environments (Hamilton, 1984).

Fires in the wetland vary in their extent and severity. For less severe fires, the vegetation may return to its pre-fire condition within several years. Severe, intense fires can burn 30 cm or more of the peat and kill all mature shrubs and trees. It was reported that, during the fire in 1844, the peat was burned completely in numerous places, forming the currently existing lakes in the Okefenokee Swamp (Hamilton, 1984).

### Logging

Mature stands of cypress were logged by the Suwannee Canal Company prior to 1897 and by the Hebard Lumber Company from 1909 to 1927. The primary areas that were logged were between Suwannee Creek and Billys Island and Floyds Island (Izlar, 1984). It has been estimated that during the latter period most of the remaining merchantable cypress trees in the swamp were harvested (Hopkins, 1947).

Analysis of aerial photographs of the swamp indicate that the area that was logged most recently (1984) is dominated by dense growth of shrubs and either black gum sprouting from stumps or new growth of broad-leaved evergreen trees (Izlar, 1984). Although cypress can sprout from stumps that are as much as 200 yr old, most of the logged cypress trees ranged from 400 to 900 yr old. Stump sprouting, therefore, has not been a means of reestablishment of the cypress forest (Duever and Riopelle, 1984). Further, natural seeding as a reestablishment process has been limited because: (1) almost all cypress trees were cut in the logged areas, and (2) cypress seeds, which are dispersed by flowing water, have limited spatial distribution and will only germinate under very limited environmental conditions. Hamilton (1984) concluded that the cypress forest will not regenerate in most of the area that was logged and that the process will take hundreds of years in the limited areas where the cypress forest is returning.

## ANIMAL LIFE IN THE OKEFENOKEE SWAMP

The Okefenokee Swamp is a diverse and unique ecosystem for supporting animal life. Not only do the prairies, wetlands, and lakes provide diverse habitats,

but numerous upland islands, shorelines, small batteries, and houses also provide terrestrial habitats. Cypress and pine trees in and around the wetland provide a rich arboreal habitat. The following summary of animal life in the Okefenokee Swamp is based on works of Russel (1973) and Laerm and others (1984).

Strictly aquatic organisms include the numerous species of warmwater fish. Among these are the largemouth bass (*Micropterus salmoides*), Florida gar (*Lepisosteus platyrhincus*), several species of suckers (*Erimyzon sucetta* and *Minytrema melanops*), pirate perch (*Aphredoderus sayanus*), bluegill (*Lepomis* spp.), bowfin (*Amia calva*), brook silverside (*Labidesthes sicculus*), Eastern swamp darter (*Etheostoma fusiforme*), pickerel (*Esox* spp.), and bullhead (*Ictalurus* spp.). Their importance to the swamp ecosystem is substantial because they are a major food source for birds and mammals. Moreover, some of the fish directly convert plant tissue to animal tissue.

Major amphibians in the wetland are several species of frogs of the genera *Hyla* and *Rana*. At least 10 species of the family Hylidae and seven species of the family Ranidae have been identified.

The largest reptile in the Okefenokee Swamp is the American alligator (*Alligator mississippiansis*). Russel (1973) stated that, during the late 1960's and early 1970's, there were an estimated 10,000 alligators in the swamp. He stated that this estimate was much less than estimates for earlier times. Although the alligator population probably is much smaller than in the past, it is common for a visitor to observe several alligators during an afternoon boat trip in the wetland. The average length of an alligator from the Okefenokee Swamp is about 2.5 m, although earlier measurements have recorded them to be as long as about 7 m.

The reptiles next in size to the alligator are the numerous species of snakes. The Eastern coachwhip (*Masticophis flagellum flagellum*) and the North Florida black snake (*Seminatrix pygaea pygaea*) are representative of nonpoisonous types from the family Colubridae. The poisonous species inhabiting the swamp are the Florida cottonmouth (*Agkistrodon piscivorous conanti*), three species of rattlesnakes (two of which are the common *Crotalus* spp. and *Sistrurus miliarius barbouri*), and the uncommon Eastern coral snake (*Micrurus fulvius fulvius*). About 40 species of snakes have been observed in the swamp (Laerm and others, 1984). Although snakes are numerous, it is rare for a visitor to the Okefenokee Swamp to encounter a snake.

In contrast, visitors probably will see several turtles resting on logs protruding from the waterways and warming themselves in the sunshine. The common snapping turtle (*Chelydra serpentina serpentina*) and the alligator snapping turtle (*Macroclemys temminiki*) are found in the wetland. Also present are seven species of the family Emydidae, such as the Florida box turtle (*Terrapene carolina carolina*) and four species of the family Kinostemidae, such as the striped mud turtle (*Kinosternon bauri palmarum*).

Semiaquatic mammals include the river otter (*Lontra canadensis vaga*) and the round-tailed muskrat (*Neofiber alleni exoristus*). Visitors commonly will see otters swimming in the open waterways. In the terrestrial habitat, the whitetail deer (*Odocoileus virginianus virginianus*) and black bear (*Ursus amenicanus floridanus*) are the largest mammals in the Okefenokee Swamp. In decreasing size, there is the cougar (*Felis concolor coryi*), boar (*Sus scrofa*), bobcat (*Lynx rufus floridanus*), gray fox (*Urocyon cinereoargentus floridanus*), raccoon (*Procyon lotor elucus*), opossum (*Didelphis virginiana pigra*), several species of rabbit (*Sylvilagus* spp.), and squirrel (*Sciurus* spp.). Visitors probably will see many of the smaller terrestrial mammals, but sightings of the larger mammals are more rare.

The abundant vegetation provides a variety of arboreal habitat types, and the variety of bird life ranges from the common robin (*Turdus migratorius*) to reported sightings of the reportedly extinct ivory-billed woodpecker (*Campephilus principalis*). The pileated woodpecker (*Dryocopus pileatus*) is a common species in the wetland and is often mistaken for the ivory-billed woodpecker. The Okefenokee Swamp is used as a resting and nesting area by waterfowl, including the green-winged teal (*Anas crecca*), mallard (*Anas platyrhynchos*), American widgeon (*A. americana*), scaup (*Aythya affinis*), and wood duck (*A. clypeata*). Large birds include the turkey (*Meleagris gallopavo*), found on the shore and islands, as well as the great blue heron (*Ardea herodias*) and white ibis (*Eudocimus albus*), found along shallow-water areas. Predatory birds include the osprey (*Pandion haliaetus*), which feeds on fish, the peregrine falcon (*Falco peregrinus*), and the red-shouldered hawk (*Buteo lineatus*). On boat trips, turkey vultures (*Cathartes aura*) are commonly observed sitting in the upper branches of cyprus trees. Smaller birds include thrushes (*Catharus* spp.), the tufted titmouse (*Parus bicolor*), and the eastern wood pewee (*Contopus viren*).

The Okefenokee Swamp is a complex ecosystem. Majestic cypress trees and ominous alligators are the most easily observed and most vividly remembered of the flora and fauna of the wetland. Although the myriad of smaller animals and plants living in the water, soil, and vegetation are not as readily observed by the casual visitor, these organisms are of equal or greater importance because they are the primary producers, herbivores, carnivores, or decomposers. Their function in the swamp ecosystem, therefore, is paramount to the dynamic ecosystem structure of the Okefenokee Swamp.

## SUMMARY

The Okefenokee Swamp is a complex mosaic of plant and animal habitats, with vistas overlooking aquatic prairies, flowing canals, and majestic stands of pine, cypress, and black gum. The large concentration of dissolved humic substances in the wetland is the cumulative result of the geology, hydrology, and ecology of the swamp. Rainfall is the primary water source, and evaporation is the primary water loss. The average water depth is estimated to be 0.5 m, and the average residence time of water is estimated to be 3 to 4 mo. In the past, episodic fires have been a natural interruption to the gradual succession from prairie to forested wetland and, therefore, have created the complexity of habitats and open areas found in the wetland. The Okefenokee Swamp remains a refuge for a variety of animals, including the American alligator. This brief description of the Okefenokee Swamp illustrates that the swamp is a mosaic of life and, as subsequent chapters will show, a producer of fulvic and humic acids.

## REFERENCES CITED

Auble, G.T., 1982, Biogeochemistry of Okefenokee Swamp—Litterfall, litter decomposition, and surface water dissolved cation concentrations: Athens, University of Georgia, Ph.D. dissertation, 311 p.

———1984, Dissolved cation concentrations in Okefenokee Swamp surface water: Spatial and temporal variation, *in* Cohen, A.D., Casagrande, D.J., Andrejko, M.J., and Best, G.R., eds., The Okefenokee Swamp—Its Natural History, Geology, and Geochemistry: Los Alamos, New Mexico, Wetland Surveys, p. 320–332.

Beck, K.C., Reuter, J.H., and Perdue, E.M., 1974, Organic and inorganic geochemistry of some coastal plain rivers of the Southeastern United States: Geochimica et Cosmochimica Acta, v. 38, p. 341–364.

Bosserman, R.W., 1983, Elemental composition of *Utricularia*-periphyton ecosystems from Okefenokee Swamp: Ecology, v. 64, p. 1637–1645.

Cohen, A.D., 1984, The Okefenokee Swamp—A low-sulfur end-member of a shoreline-related depositional model for coastal plain coals: Los Alamos, New Mexico, Wetland Surveys, p. 668–680.

Cohen, A.D., Andrejko, M.J., Spackman, William, and Corvinus, D.A., 1984, Peat deposits of the Okefenokee Swamp, *in* Cohen, A.D., Casagrande, D.J., Andrejko, M.J., and Best, G.R., eds., The Okefenokee Swamp—Its Natural History, Geology, and Geochemistry: Los Alamos, New Mexico, Wetland Surveys, p. 493–553.

Cohen, A.D., Casagrande, D.J., Andrejko, M.J., and Best, G.R., 1984, The Okefenokee Swamp—Its Natural History, Geology, and Geochemistry: Los Alamos, New Mexico, Wetland Surveys, 709 p.

Cowardin, L.M., Carter, V., Golet, F.C., and LaRoe, E.T., 1979, Classification of wetlands and deep-water habitats of the U.S.: Washington, D.C., U.S. Fish and Wildlife Service, Biological Services Program, 103 p.

Cypert, E., 1972, The origin of houses in the Okefenokee prairies: American Midland Naturalist, v. 82, p. 448–458.

Duever, M.J., and Riopelle, L.A., 1984, Tree-ring analysis in the Okefenokee Swamp, *in* Cohen, A.D., Casagrande, D.J., Andrejko, M.J., and Best, G.R., eds., The Okefenokee Swamp—Its Natural History, Geology, and Geochemistry: Los Alamos, New Mexico, Wetland Surveys, p. 180–188.

Flebbe, P.A., 1984, Carbon dioxide and methane in selected Okefenokee Swamp sites, *in* Cohen, A.D., Casagrande, D.J., Andrejko, M.J., and Best, G.R., eds., The Okefenokee Swamp—Its Natural History, Geology, and Geochemistry: Los Alamos, New Mexico, Wetland Surveys, p. 380–390.

Hamilton, D.B., 1984, Plant succession and the influence of disturbance in the Okefenokee Swamp, *in* Cohen, A.D., Casagrande, D.J., Andrejko, M.J., and Best, G.R., eds., The Okefenokee Swamp—Its Natural History, Geology, and Geochemistry: Los Alamos, New Mexico, Wetland Surveys, p. 86–111.

Hopkins, J.M., 1947, Forty-five years with the Okefenokee Swamp: Georgia Society of Naturalists, Bulletin 4, 69 p.

Hoyt, J.H., and Hails, J.R., 1974, Pleistocene stratigraphy of southeastern Georgia, *in* Oaks, R.O., Jr., and DuBar, J.R., eds., Post-Miocene Pleistocene Stratigraphy, Central and Southern Atlantic Coastal Plain: Logan, Utah, Utah State Press, p. 191–205.

Hyatt, R.A., and Brook, G.A., 1984, Ground-water flow in the Okefenokee Swamp and hydrologic and nutrient budgets for the period August, 1981 through July, 1982, *in* Cohen, A.D., Casagrande, D.J., Andrejko, M.J., and Best, G.R., eds., The Okefenokee Swamp—Its Natural History, Geology, and Geochemistry: Los Alamos, New Mexico, Wetland Surveys, p. 229–245.

Izlar, R.L., 1984, A history of Okefenokee logging operations: A bourbon and branch water success story, *in* Cohen, A.D., Casagrande, D.J., Andrejko, M.J., and Best, G.R., eds., The Okefenokee Swamp—Its Natural History, Geology, and Geochemistry: Los Alamos, New Mexico, Wetland Surveys, p. 5–17.

King, G.M., 1984, Methane production in Okefenokee peats, *in* Cohen, A.D., Casagrande, D.J., Andrejko, M.J., and Best, G.R., eds., The Okefenokee Swamp—Its Natural History, Geology, and Geochemistry: Los Alamos, New Mexico, Wetland Surveys, p. 371–379.

Laerm, Joshua, Freeman, B.J., Vitt, L.J., and Logan, L.E., 1984, Appendix A—Checklist of vertebrates of the Okefenokee Swamp, *in* Cohen, A.D., Casagrande, D.J., Andrejko, M.J., and Best, G.R., eds., The Okefenokee Swamp—Its Natural History, Geology, and Geochemistry: Los Alamos, New Mexico, Wetland Surveys, p. 682–701.

McCaffrey, C.A., and Hamilton, D.B., 1984, Vegetation mapping of the Okefenokee ecosystem, *in* Cohen, A.D., Casagrande, D.J., Andrejko, M.J., and Best, G.R., eds., The Okefenokee Swamp—Its Natural History, Geology, and Geochemistry: Los Alamos, New Mexico, Wetland Surveys, p. 201–211.

McKnight, D.M., Thurman, E.M., Wershaw, R.L., and Hemond, H.H., 1985, Biogeochemistry of aquatic humic substances in Thoreau's Bog, Concord, Massachusetts: Ecology, v. 66, no. 4, p. 1339–1352.

Patten, B.C., and Matis, J.H., 1984, The macrohydrology of Okefenokee Swamp, *in* Cohen, A.D., Casagrande, D.J., Andrejko, M.J., and Best, G.R., eds., The Okefenokee Swamp—Its Natural History, Geology, and Geochemistry: Los Alamos, New Mexico, Wetland Surveys, p. 264–278.

Pirkle, E.C., Pirkle, W.A., and Yoho, W.H., 1977, The Highland heavy-mineral sand deposit on Trail Ridge in northern peninsular Florida: Florida Bureau of Geology, Report of Investigation 84, 50 p.

Presley, D.E., 1984, Life and lore of the swampers, *in* Cohen, A.D., Casagrande, D.J., Andrejko, M.J., and Best, G.R., eds., The Okefenokee Swamp—Its Natural History, Geology, and Geochemistry: Los Alamos, New Mexico, Wetland Surveys, p. 18–37.

Rich, F.J., 1976, The origin and development of tree islands in the Okefenokee Swamp, as determined by peat petrography and pollen stratigraphy: State College, Pennsylvania State University, Ph.D. dissertation, 301 p.

———1984, Ancient flora of the eastern Okefenokee Swamp as determined by palynology, *in* Cohen, A.D., Casagrande, D.J., Andrejko, M.J., and Best, G.R., eds., The Okefenokee Swamp—Its Natural History, Geology, and Geochemistry: Los Alamos, New Mexico, Wetland Surveys, p. 5–17.

Russel, Franklin, 1973, The Okefenokee Swamp: Alexandria, Virginia, Time-Life, 184 p.

Rykiel, E.J., Jr., 1984, General hydrology and mineral budgets for Okefenokee Swamp, *in* Cohen, A.D., Casagrande, D.J., Andrejko, M.J., and Best, G.R., eds., The Okefenokee Swamp—Its Natural History, Geology, and Geochemistry: Los Alamos, New Mexico, Wetland Surveys, p. 5–17.

Schlesinger, W.H., 1978, Community structure, dynamics and nutrient cycling in the Okefenokee cypress swamp-forest: Ecological Monographs, v. 48, p. 43–65.

Trowell, C.T., 1984, Indians in the Okefenokee Swamp, *in* Cohen, A.D., Casagrande, D.J., Andrejko, M.J., and Best, G.R., eds., The Okefenokee Swamp—Its Natural History, Geology, and Geochemistry: Los Alamos, New Mexico, Wetland Surveys, p. 38–57.

Wright, N.O., Jr., 1984, A cultural ecological history of the Okefenokee, *in* Cohen, A.D., Casagrande, D.J., Andrejko, M.J., and Best, G.R., eds., The Okefenokee Swamp—Its Natural History, Geology, and Geochemistry: Los Alamos, New Mexico, Wetland Surveys, p. 58–68.

Chapter B

# Isolation of Fulvic and Humic Acids from the Suwannee River

*By* R.L. Malcolm, G.R. Aiken, E.C. Bowles, *and* J.D. Malcolm

## CONTENTS



## Abstract

Dissolved fulvic and humic acids from the Suwannee River in southeastern Georgia were isolated during a period of 2 months by procedures using XAD–8 resin. During that time, approximately 17,000 liters of river water were processed, yielding 570 grams of fulvic acid and 84 grams of humic acid. The fulvic and humic acids are believed to be representative of dissolved fulvic and humic acids in blackwater streams of the United States. Fulvic and humic acids comprised approximately 75 percent of the dissolved organic carbon (average of 38 milligrams per liter during the sampling period) in the Suwannee River; these high concentrations of fulvic and humic acids enabled the maximization of fulvic- and humic-acid isolation from a minimum amount of water. The XAD–8 resin procedure of fulvic- and humic-acid isolation from stream water represents present state of the art. Advantages and disadvantages of the procedure are discussed.

## INTRODUCTION

The Suwannee River is one of many dark-brown streams of the United States. Colored streams in the Southeastern United States are different from most colored streams because they exist in a region that has a long and productive growing season, where rainfall is abundant and well distributed throughout the year, where the winters are mild and short with temperatures seldom being less than 0°C, where decomposition is relatively unhindered during the winter, and where there is no spring flush of organic

decomposition intermediates and products. The Suwannee River is unique because it contains highly colored water along its entire length—from its source in the Okefenokee Swamp to its estuary in the Gulf of Mexico. Other unusual characteristics of the river are that it generally is a large river in a very sandy area and that it usually has a constant discharge throughout the entire year.

Scientists have thought for many decades that the brown color of the water in the Suwannee River was due to the presence of organic matter. The water has been studied by several hydrologists and chemists—one of the most notable was Professor A.P. Black and his coworkers of the University of Florida during the 1950's. He precipitated colored organic components by *alum* ($Fe^{3+}$ and $Al^{3+}$ sulfates) coagulation. The colored matter from whole-river-water samples was also isolated by freeze-concentration and freeze-drying methods (Black and Christman, 1963a, 1963b). Organo-metallic interactions in the Suwannee River were studied by Casagrande and Erchull (1976), Giesy and Briese (1977), and Alberts and Giesy (1983). Both *colloidal* and dissolved organic constituents were associated with numerous metal ions and oxides. J.H. Reuter and K.C. Beck (Georgia Institute of Technology, written commun., 1975) stated that dissolved organic matter in the Suwannee River was the major control of its water chemistry.

## REASONS FOR SELECTING THE SUWANNEE RIVER SAMPLING SITE

The Suwannee River at its source at the Okefenokee Swamp was selected as the site for large-scale sampling of stream humic substances for several reasons:

1. It was determined that humic substances in the Suwannee River were generally representative of humic substances in blackwater streams of the United States.
2. The concentration of humic substances in the Suwannee River is about an order of magnitude greater than that in most natural stream waters; dissolved organic carbon (DOC) concentrations are in excess of 25 mg/L.
3. Humic substances in the Suwannee River have been sampled on a reconnaissance basis during the past 20 yr by the U.S. Geological Survey, which has long-term water-quality and water-discharge measurements for the river.
4. The headwaters of the Suwannee River occur in the remote, sparsely populated Okefenokee Swamp of southeastern Georgia; therefore, the stream is generally free of anthropogenic inputs.
5. The Suwannee River contains relatively low concentrations of dissolved inorganic constituents and suspended sediment; specific conductance usually is below 50 µS/cm, and *suspended sediment organic carbon* (SOC) concentrations usually are 2 to 4 mg/L. The low concentrations of dissolved organic constituents and SOC facilitated relatively fast filtration of the water and resulted in low ash contents in isolated humic substances.
6. There is easy access to the river for sampling equipment, and convenient space for temporary laboratory and trailer facilities exists. Electrical power also is available.
7. Long-term water-quality records for the Suwannee River document that only moderate variations in both organic and inorganic water quality exist either throughout the year or from year to year. Therefore, the planning and execution of large-scale sampling was not limited to any particular period of the year.

Even though the stream DOC varied from 35 to 50 mg/L during the 2-mo sampling period, we feel that the composition of humic substances remained relatively constant. This conclusion is based upon the similarity of previous reconnaissance data from the Suwannee River and the relatively constant percentage of DOC breakthrough from the XAD–8 isolation columns. Most streams have a tendency to increase in DOC concentration with increased discharge that accompanies rainfall during all periods of the year. The minor changes in DOC during the sampling period were attributed to this phenomenon. Many streams in colder regions of the United States have a pronounced spring flush upon snowmelt and soil thawing during which organic constituents such as DOC are two to three times higher than during other periods of the year. This type of spring flush is not observed in the Suwannee River or other local streams.

During the period of development of XAD–8 resin technology for the isolation of humic substances from natural water, an experiment was conducted during 1978 to determine the efficiency of isolation of humic substances from the Suwannee River and from other rivers. The XAD–8 resin isolation method removed 95 percent or more of colored humic substances from natural waters.

The humic and fulvic acids were characterized by elemental analyses, solid-state carbon-13 nuclear-magnetic-resonance ($^{13}$C-NMR) spectrometry, liquid-state $^{13}$C-NMR spectrometry, $^{1}$H *nuclear-magnetic-resonance spectrometry*, molecular-size analyses, molecular-weight distribution, $E_4/E_6$ ratios, titrimetry data, and functional group analyses. The results of these characterizations of Suwannee River humic and fulvic isolates were compared with a number of stream humic substances that were isolated from several streams throughout the United States by the same XAD–8 resin techniques. The humic isolates from the Suwannee River were similar to isolates from the other rivers in most of the characterizations. Because of these similarities in chemical nature, it may be that humic substances from the Suwannee River are generally representative of stream humic substances throughout the United States.

## SAMPLING OF RIVER WATER

An earthen sill with a concrete spillway across the Suwannee River at the south end of the Okefenokee Swamp was constructed in 1967 to maintain a relatively constant water level in the swamp. Earlier efforts to drain parts of the swamp during logging operations had rendered it vulnerable to fire during infrequent dry periods. The water level in the swamp is maintained by removable wooden planks in a concrete spillway near the midpoint of the sill that was in the natural channel of the upper Suwannee River. Water samples were pumped at a point above the concrete spillway from a depth of 3 to 4 m into 36-L glass jugs. The electric pump was lubricated with water and used a stainless-steel impeller. Electrical power was supplied by a small portable electric generator. Pump tubing consisted of well-leached 2.5-cm diameter polyethylene tubing. Some water samples were scooped from the surface with a plastic container and then transferred to glass containers.

The water sampling site was approximately 10 miles from the field laboratory where the water was processed. The site was accessed by truck over a limited-access State Park road. Water samples were collected daily in amounts dictated by the rate of water filtration and processing. This ensured that the maximum period from water sampling to organic matter concentration on the XAD–8 resin was no longer than 2 days.

## FILTRATION OF THE WATER SAMPLE

Unfiltered river water was transferred from the glass sampling jugs to Millipore stainless-steel pressure-filtration canisters (20-L capacity). Each canister was connected in parallel to two Millipore stainless-steel plate filter holders (142 mm in diameter) by separate, flexible, stainless-steel hoses fitted with quick-disconnect joints. Each plate filter holder held a replaceable Osmonics silver membrane filter of 0.45-µm pore size. Each canister was pressurized by a small tank of nitrogen (high-purity grade). The filtration pressure was increased in 34-kPa increments from 0 to 413 kPa during a 2-day period. In less than 1 hour, the filtration rate was reduced to a dropwise flow. This dropwise flow rate slowly diminished during a 3-day period at which point the membrane filters were changed. The filtered water was collected in 36-L glass jugs. Fifty of the 142-mm stainless-steel plate filter holders were used in the filtration process. The pressure canisters were monitored and refilled periodically before they became empty so that DOC leakage resulting from crushing of algae and bacterial cells was prevented.

Four Millipore stainless-steel plate filters (293 mm in diameter) were also used to accelerate the filtration process. Silver-membrane filters were not available in this large size; therefore, Gelman vinyl-metricel filters of 0.45-µm pore size were used. These four plate-filter units were connected in series to a stainless-steel reservoir that had a capacity of 220 L. The reservoir was pressurized with a large, Q-size container of nitrogen.

The entire water sample was pressure filtered. The parts of the sample filtered through the two different membrane filters were kept separate and were processed independently: the part filtered through the silver-membrane filters was designated as the standard sample, and the part filtered through the vinyl-metricel filters was designated as the reference sample. After a number of tests, it was determined that the silver-membrane filter neither contaminated the filtered water with DOC nor sorbed any of the numerous organic solutes found in natural water. In contrast, the vinyl-metricel membrane filters had two limitations. These filters contained a detergent coating to facilitate wetting and initial water flow through the filter. These filters also sorb small amounts of humic substances. These two limitations in the use of vinyl-metricel

membrane filters were overcome by leaching the filters at a slow flow rate for a 12-hour period with Suwannee River water. During this period, the detergent was completely leached from the filters, and the sorptive capacity for humic substances was saturated. The water filtered during this leaching was discarded.

## CONCENTRATION OF HUMIC SUBSTANCES

At the time of the sampling, the XAD–8 resin was the most suitable resin for the concentration and isolation of humic substances from water (Malcolm and others, 1977; Aiken and others, 1979). Other resins such as Duolite A–7 (Leenheer and Noyes, 1984; Aiken, 1985) may be considered for use at the present time. The XAD–1, XAD–2, and XAD–4 resins were determined to have a lower capacity for humic substances than did the XAD–8 resin. Due to the small pores of the XAD–2 and XAD–4 resins, humic substances were excluded, and part of the humic substances sorbed were irreversibly fixed by the resins and could not be recovered. The XAD–7 resin could not be used due to excessive breakdown and resin bleed in basic solution. The XAD–8 resin did not exhibit any of the limitations.

XAD–8 resin is an uncharged but slightly polar resin composed of polymerized methyl ester of polyacrylic acid. The polymer has a limited cross linkage to give an effective hydrated-pore size that enables all the macroporous network of the resin to be available for sorption of natural humic solutes that are much larger in size than simple specific organic solutes such as benzoic acid, phthalates, and so forth. The resin was extensively cleaned of unpolymerized material and impurities by repeated and sequential Soxhlet extraction with diethyl ether, acetonitrile, and methanol (Thurman and Malcolm, 1981).

After filtration, water in the 36-L glass jugs was acidified to pH 1.95±0.05 with 6 N HCl. The pH of water in each jug was measured several minutes after mixing to ensure that the pH was slightly below 2.0. Each acidified sample was pumped onto a 9-L column of XAD–8 resin at a flow rate of 1 L/min. The tubing to and from the peristaltic pump consisted of Teflon, except for the 15-cm polyvinyl tubing in contact with the pump roller. The flow rate of 1 L/min corresponded to 5 bed volumes per hour, which is considerably less than the critical flow rate of 10 bed volumes per hour; therefore, time was adequate for complete equilibration of the humic solutes with the resin sorbent. A volume of 272 L of water was pumped onto the XAD–8 resin column before elution. This volume of water results in a theoretical *column distribution coefficient* (k′) of 62. At a k′ of 50, approximately 95 percent of the colored humic substances contained in the 272-L water sample was sorbed by the resin. The real k′ was believed to be near 50, or a reduction of 20 to 25 percent in sorptive capacity due to the high DOC of 30 to 40 mg/L, which is 5 to 10 times higher than the DOC concentration in most surface waters. For a complete discussion of k′ and the chemical quantification of sorption, refer to Leenheer (1981).

The adsorbed humic substances were back-eluted from the XAD–8 resin column using three column void volumes (approximately 15 L) of 0.1 N NaOH at a flow rate of 350 mL/min. The basic solution was followed by 0.1 N HCl solution until the column eluate became acidic in preparation for the next batch of filtered water. The column flow was reversed, and the next part of the filtered water sample was pumped onto the column.

This elution technique enabled the collection of a highly concentrated center part or "center cut" of humic substances elution in a 1-L volume. The humic substances in this part were in excess of 1,000 mg/L as carbon and required no further concentration. This part of the eluate was acidified immediately to pH 2 and stored on ice for transport to the laboratory in Denver, Colorado. The 1-L part of the eluate prior to the "center cut" and the 4-L part after the "center cut" were combined in a separate container and placed in an ice bath for later reconcentration. The void volumes during elution prior to the retained parts and until the eluate became acidic were combined, acidified to pH 2, and added to the filtered river sample for the next column run. After 10 column runs, the accumulated "other than center cut" concentrated organic eluates were reconcentrated as a separate run on the large XAD–8 resin column. The final reconcentrations were conducted on smaller columns of XAD–8 resin. Virtually all of the humic substances were adsorbed upon reconcentration with minimal losses.

## SEPARATION OF FULVIC AND HUMIC ACIDS

All of the elution "center cuts" and the reconcentrated humic substances were mixed in a 36-L glass container and acidified to pH 1.0 with concentrated HCl. In the well-homogenized concentrate, the carbon concentration was approximately 1,000 mg/L. A carbon concentration of 500 mg/L is the minimum value for rapid and complete precipitation of humic acids. The concentrated sample was chilled to 2°C in an ice bath; the precipitated humic acids were resuspended several times over a 2-day period and then allowed to settle. The precipitated humic acids were separated from the soluble fulvic acids by centrifugation.

## DESALTING, HYDROGEN SATURATION, AND FREEZE-DRYING

The fulvic acid solution contained high concentrations of sodium and HCl. The major portion of these ions and salts was removed by desalting on an XAD–8 resin column. Fulvic acids at pH 1.0 were pumped slowly onto a large column of XAD–8 resin until the observed color of the sorbed fulvic acids extended approximately one-third down the length of the column. At this time, pumping of fulvic acid ceased and pumping of deionized water began. Specific-conductance monitoring of the effluent was initiated at this time using a flow-through cell. During leaching of the acidic salt solution from the void volume of the column, the specific conductance decreased rapidly. Concurrent with acid removal, the pH increased, and the fulvic acid began to move slowly down the column. The column was rinsed with deionized water until the specific conductance was decreased to 250 µS/cm. The XAD–8 resin column was then back-eluted with five column volumes of 0.1 N NaOH. Color due to fulvic acids was normally observed to elute from the column during rinsing when the specific conductance decreased to less than 700 to 800 µS/cm. These colored washings, collected until the specific conductance decreased to 250 µS/cm, were acidified and added to the next desalting run.

The XAD–8 resin column was back-eluted rapidly with 0.1 N NaOH, the dilute first part of the back eluate was mixed with the initial, concentrated, basic part of the elution, and the solution was passed rapidly through a small column of hydrogen-saturated exchange resin ("precolumn"). This procedure minimized the contact time of fulvic acid with base. The last part of the base eluate of fulvic acid was introduced directly into the cation-exchange resin column. The purpose of the precolumn was not to completely hydrogen saturate the fulvic acid but to remove most of the sodium and to neutralize the basic solution. The solution of fulvic acid was below pH 4.5 after the pretreatment. The solution of fulvic acid then was passed at a fast, dropwise rate through another hydrogen-saturated resin exchange column for complete hydrogen saturation. After complete hydrogen saturation, the sodium concentration was less than 0.02 mg/L.

The precipitated humic acid was kept moist with deionized water until it was desalted and hydrogen saturated. To desalt the humic acid, it was solubilized in dilute NaOH, and the DOC concentration was adjusted to 500 mg/L or less as carbon. The solution then was adjusted to pH 2.0 and pumped slowly onto a large XAD–8 resin column. At this point, the procedure used for desalting the humic acid is the same as that for fulvic acid. To accomplish hydrogen saturation of the humic acid without precipitation in the cation-exchange resin column, it is imperative that the humic-acid concentration in solution not exceed 500 mg/L as carbon during passage through the cation exchange resin. Concentrations of humic acid in excess of 500 mg/L frequently precipitate and clog the cation exchange resin, necessitating a repeat of the desalting procedure. The hydrogen-saturated fulvic and humic acids were freeze-dried, homogenized, and stored in glass vials for future characterization and use.

## ADVANTAGES AND DISADVANTAGES OF THE ISOLATION PROCEDURE

Major advantages of this isolation procedure for stream humic substances are:

1. Over 95 percent of the colored humic substances in water, as defined by the XAD–8 procedure, are concentrated and recovered.
2. Humic substances are not excluded from the XAD–8 resin nor are they irreversibly sorbed by the XAD–8 resin.
3. Other classes of organic compounds such as *polysaccharides*, simple sugars, and low-molecular-weight organic acids are not isolated with the humic substances.

4. Inorganic acids and salts are not included with humic substances by the isolation procedure, nor is dialysis (with associated losses of humic substances) required to remove inorganic salts.
5. Inorganic cations are reduced to low concentration in fulvic and humic acids by the ion-exchange method.
6. Humic substances are rendered relatively free of mineral and particulate constituents by filtration.
7. The XAD–8 procedure is one of the mildest and most effective quantitative methods for the isolation of dissolved humic substances and for separation of humic substances from other organic and inorganic constituents in water. This procedure was the major method of preparation that was available in 1982 when the isolation was accomplished.

Possible disadvantages of the procedure are:

1. Acidification to pH 2 changes the humic association with silicon and metal ions.
2. Exposure to acid and base conditions and freeze-drying may cause chemical alteration of the original humic substance.

The possible disadvantages tested for this procedure are probably of minor consequence when compared to the advantages, especially if the freeze-dried sample is redissolved in water before experimental use. Many of the reactions that occur upon drying are readily reversible upon rewetting. A short contact time with base was employed to minimize hydrolytic reactions of the humic substances (Bowles and others, chap. L, this volume).

## CONCLUSIONS

The XAD–8 resin procedure was successful in isolating a large quantity of humic substances because:

1. DOC concentrations in the water were high (average of 38 mg/L).
2. SOC concentrations were low (less than 2 mg/L); this facilitated fast filtration of the sample and resulted in minimal ash content in the isolated humic substances.
3. Humic substances constituted a high percentage of the DOC (ranging from 75 to 85 percent) during the sampling period.
4. Concentrations of inorganic constituents in the water were low (specific conductance was less than 50 µS/cm).

Even though the stream DOC concentrations varied from 35 to 50 mg/L during the 2-month sampling period, the chemical composition of humic substances was almost constant. This conclusion is based on the similarity of data collected for this experiment with previously collected data from the Suwannee River and on the almost constant percentage of DOC breakthrough from the XAD–8 resin columns. Minor changes in DOC concentrations during the sampling period were attributed to this phenomenon.

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## REFERENCES CITED

Aiken, G.R., 1985, Isolation and concentration techniques for aquatic humic substances, *in* Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediment, and Water: New York, John Wiley, p. 363–385.

Aiken, G.R., Thurman, E.M., Malcolm, R.L., and Walton, Harold, 1979, Comparison of XAD macroporous resins for the concentration of fulvic acid from aqueous solution: Analytical Chemistry, v. 52, p. 1799–1803.

Alberts, J.J., and Giesy, J.P., 1983, Conditional stability constants of trace metals and naturally occurring humic materials—Application and verification with field data, *in* Christman, R.F., and Gjessing, E.T., eds., Aquatic and Terrestrial Humic Materials: Ann Arbor, Michigan, Ann Arbor Science, p. 333–348.

Black, A.P., and Christman, R.F., 1963a, Characteristics of colored surface waters: American Water Works Association Journal, v. 55, p. 753–770.

———1963b, Chemical characteristics of fulvic acids: American Water Works Association Journal, v. 55, p. 897–912.

Casagrande, D.J., and Erchull, L.D., 1976, Metals in Okefenokee peat-forming environments—Relation to constituents found in coal: Geochimica et Cosmochimica Acta, v. 40, p. 387–393.

Giesy, J.P., and Briese, L.A., 1977, Metals associated with organic carbon extracted from Okefenokee Swamp water: Chemical Geology, v. 20, p. 109–120.

Leenheer, J.A., 1981, Comprehensive approach to preparative isolation and fractionation of dissolved organic carbon from natural waters and wastewaters: Environmental Science and Technology, v. 15, p. 578–587.

Leenheer, J.A., and Noyes, T.I., 1984, A filtration and column-adsorption system for onsite concentration

and fractionation of organic substances from large volumes of water: U.S. Geological Survey Water-Supply Paper 2230, 16 p.

Malcolm, R.L., Thurman, E.M., and Aiken, G.R., 1977, The concentration and fractionation of trace organic solutes from natural and polluted waters using XAD–8, a methylmethacrylate resin: 11th Annual Conference of Trace Substances in Environmental Health Proceedings, Columbia, Missouri, p. 307–314.

Thurman, E.M., and Malcolm, R.L., 1981, Preparative isolation of aquatic humic substances: Environmental Science and Technology, v. 15, p. 463–466.

Chapter C

# Interactions of Organic Contaminants with Fulvic and Humic Acids from the Suwannee River and with Other Humic Substances in Aqueous Systems—With Inferences Pertaining to the Structure of Humic Molecules

*By* D.E. Kile, C.T. Chiou, *and* T.I. Brinton

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## Abstract

Water *solubility enhancements* of nonionic organic solutes by dissolved Suwannee River fulvic acid, by other humic substances, and by synthetic organic polymers have been determined. The interaction between a solute and dissolved organic matter is accounted for by a partition model that is compatible with observed solute solubility enhancements and with the proposed molecular weights and structures of humic substances. The apparent water solubility of nonionic organic solutes increases with increasing dissolved-organic-matter concentration and shows no competitive effect between solutes in a binary-solute solution. The partition coefficients between dissolved organic matter and water ($K_{dom}$), as determined for solutes with Suwannee River fulvic acid, are comparable to those determined with Suwannee River humic acid and are approximately 5 to 7 times less than those for a soil-derived humic acid. The magnitude of this solubility enhancement is controlled by the molecular size, polarity, and concentration of the dissolved organic matter and by the inherent water solubility of the solute. At a given dissolved organic matter concentration, those solutes that are least soluble in water show the greatest enhancement. Important structural features in Suwannee River fulvic acid (and in other humic macromolecules that promote a partition interaction) are low polarity, high *aromatic* content, and a molecular configuration and conformation that permits the formation of a sizable intramolecular nonpolar organic environment.

## INTRODUCTION

An important consideration in assessing the transport and fate of an organic pollutant in an aqueous system is its interaction with naturally occurring organic matter. A fundamental knowledge of the mechanism of interaction between anthropogenic compounds and dissolved organic matter, such as fulvic acid and other humic substances, in relation to the structure and chemical composition of dissolved organic matter is important for a better understanding of the transport and fate of contaminants within an aquatic system. Because aquatic fulvic acids are recognized to be the predominant component of intermediate-molecular-weight organic matter in natural water (Aiken and others, chap. J, this volume; Malcolm, 1985), a detailed study of the effect of aquatic fulvic acids on the behavior of organic pollutants is of theoretical and practical interest.

Dissolved organic matter may modify the behavior of certain organic contaminants by specific or nonspecific interactions if these interactions result in improved stability of the compounds. From a hydrodynamic standpoint, this effect would increase the apparent water solubility of the contaminants and, therefore, promote their transport in the water body. From a molecular standpoint, this interaction decreases the fraction of free contaminant species that may be susceptible to degradation and distribution to other aquatic compartments. It has been reported, for example, that dissolved organic matter at relatively low concentrations in water sorbs a significant amount of the 1-octyl ester of (2,4-dichlorophenoxy) acetic acid and, hence, decreases its apparent rate of hydrolysis relative to that in water containing no dissolved organic matter (Perdue and Wolfe, 1982). Similarly, the volatilization rate of 2,2′,5,5′–tetrachlorobiphenyl was decreased by dissolved organic matter because of an apparent increase in the solubility of the compound (Hassett and Milicic, 1985); an increased rate of *photolysis* was observed for certain organic compounds in the presence of dissolved organic matter (Zepp and others, 1985). Other studies demonstrated that dissolved organic matter reduced the bioavailability of some polynuclear aromatic hydrocarbons (Leversee and others, 1983; McCarthy, 1983; McCarthy and Jimenez, 1985) and decreased the apparent sorption coefficients of dichlorodiphenyltrichloroethane (DDT) (Caron and others, 1985) and some polychlorinated biphenyls (PCB) (Gschwend and Wu, 1985) with sediments.

Earlier studies indicated that the magnitude of solubility enhancement of a nonionic organic solute depends on the concentration and source of the dissolved organic matter as well as the type of organic compound. Wershaw and others (1969) found that the presence of 0.5 percent soil-derived sodium humate in water increased the apparent solubility of DDT to

more than 200 times that in pure water; Poirrier and others (1972) reported that DDT was concentrated by a factor of 15,800 into an organic colloid isolated from a natural surface water. On the other hand, Boehm and Quinn (1973) reported that fulvic acid in a marine sediment increased the apparent solubility of some higher alkanes but did not increase the apparent solubility of the more water-soluble aromatic compounds. Carter and Suffet (1982) found that the magnitude of the DDT sorption coefficient with dissolved organic matter varied with the source of dissolved organic matter as well as the pH. Caron and others (1985) subsequently illustrated that a low concentration of dissolved sediment-derived humic acid strongly influenced the sorption coefficient of DDT but had little effect on the sorption coefficient of the more water-soluble lindane. Similarly, Haas and Kaplan (1985) demonstrated that humic material has only a small effect on the solubility of toluene, which also has a high water solubility. Thus, from a fundamental point of view and from the results of previous studies, one would expect that water solubility enhancement by dissolved organic matter would be most significant for those solutes that are relatively insoluble in water.

Given that solubility enhancement is most significant for the least water-soluble organic solutes, it is of interest to consider which of the properties of dissolved organic matter are most important to solubility enhancement. In principle, a dissolved macromolecule (i.e., natural organic matter) can produce an enhancing effect on solute solubility either by changing the solvency of the solution or by a direct solute interaction via adsorption or partitioning. It is unlikely that dissolved organic matter at the relatively low concentrations that are typically found in natural aquatic systems would have a strong effect on water solvency. It is also doubtful that the solubility enhancement of a nonionic organic solute by dissolved organic matter could result from a complexation or other specific interaction because the hydrophilic functional groups of a humic molecule would be preferentially associated with highly polar water molecules.

Considering the fact that humic substances are species of intermediate molecular weight that contain nonpolar moieties within their molecules, Chiou and others (1986) suggested that a partition-like interaction of the nonionic organic solute with the dissolved humic substance is responsible for solute solubility enhancement. The effectiveness of such an interaction is considered to be a function of the size and polarity of dissolved organic matter. Thus, humic molecules of sufficiently large size with a relatively nonpolar intramolecular environment would effectively promote solute partitioning, whereas an organic substance of low molecular weight or a highly polar organic polymer would not effectively promote such a partition interaction. For the solute, the important properties that promote partitioning are low water solubility and significant compatibility with the organic phase (as characterized by solutes having high partition coefficients between an organic solvent, i.e., octanol, and water).

If one assumes that the water solubility enhancement of an organic solute by fulvic acid from the Suwannee River or by other dissolved organic matter is due to a partition-like interaction between the solute and macromolecule, then the magnitude of this effect with respect to both the solute and a specific dissolved-organic-matter molecule may be defined as:

$$S_w^* = S_w + XC_o \quad (1)$$

where

- $S_w^*$ is the apparent solute solubility in water containing dissolved organic matter at concentration X,
- $S_w$ is the solute solubility in pure water, and
- $C_o$ is the mass of solute partitioned into a unit mass of dissolved organic matter.

In accordance with this model, a solute partition coefficient ($K_{dom}$) between dissolved organic matter and pure water may be defined as:

$$K_{dom} = \frac{C_o}{S_w} \quad (2)$$

Substituting equation 2 into equation 1 gives:

$$S_w^* = S_w\ (1 + XK_{dom}) \quad (3)$$

If $C_o$ and X are defined in terms of the organic carbon content, equation 3 may also be written as:

$$S_w^* = S_w\ (1 + XK_{doc}) \quad (4)$$

where

$K_{doc}$ is the corresponding partition coefficient based on the dissolved organic carbon content.

According to equation 3, the magnitude of solubility enhancement is determined by the $XK_{dom}$ term, which, in turn, is related to the concentration of the dissolved organic matter (X) and the solute partition coefficient ($K_{dom}$). The magnitude of $K_{dom}$ depends on the type of solute and the nature of the organic macromolecule. For a given solute–dissolved-organic-matter system, the $K_{dom}$ can be determined experimentally by measuring the apparent solute solubility over a range of dissolved organic matter concentrations. A plot of $S_w$* versus X should yield a straight line: the slope equals $S_wK_{dom}$, and the intercept equals $S_w$.

In a study by Chiou and others (1986), experiments were conducted to determine solubility enhancements of some environmentally significant pollutants by Suwannee River fulvic acid and other soil and aquatic humic acids as well as by high-molecular-weight polyacrylic acids over a range of concentrations that are typical in natural aquatic systems. These studies were conducted with highly purified humic substances to eliminate possible complications by inorganic components (i.e., ash) in the samples. Since humic substances from different sources vary significantly in composition and structure, a comparison of a solute's solubility enhancement (for example, the magnitude of $K_{dom}$) by different dissolved-organic-matter samples and a study of the variation of the $K_{dom}$ values for different solutes with a given dissolved organic matter facilitates an evaluation of the important structural features of Suwannee River fulvic acid and other humic substances that promote solute solubility in water. These structural considerations are substantiated in this study by a further investigation of the effect of an alkaline pH on the solubility enhancement of selected organic solutes by natural dissolved-organic-matter species and by an investigation of the solubility enhancement of selected organic compounds using high-molecular-weight polyethylene glycol as the dissolved organic matter. Results are summarized in this chapter in relation to the properties of the solutes and to the composition, polarity, configuration, and conformation of the organic macromolecules.

## EXPERIMENTAL METHODS

Test solutes (p,p′-DDT, 2,4,5,2′,5′-PCB, 2,4,4′-PCB, lindane, and 1,2,3-trichlorobenzene) and cosolutes used as dissolved organic matter (phenylacetic acid and polyacrylic acid) were reagent grade or analytical standards purchased from commercial sources (Aldrich and Analabs) and were used as received. The humic and fulvic acids used in this study were in a freeze-dried, hydrogen-saturated form with a low ash content. The fulvic acid and humic acid were concentrated from water collected from the Suwannee River, near Fargo, Georgia, using techniques incorporating XAD–8 resin chromatography. The sample was subsequently fractionated into humic and fulvic acids at a pH of 1.0 and then purified by the method of Thurman and Malcolm (1981) and Malcolm and others (chap. B, this volume).

The pH of the reconstituted Suwannee River fulvic acid varied from about 3.9 at a concentration of 94 mg/L to 5.7 at 9.4 mg/L, and the pH of the humic-acid solution from the Suwannee River ranged from approximately 4.1 at a concentration of 91 mg/L to 5.5 at 9.0 mg/L. The solution pH was not adjusted for most solute-solubility-enhancement experiments conducted with fulvic and humic acids from the Suwannee River because the dissolved organic matter remained completely soluble at all concentrations. However, in a separate study of DDT solubility enhancement, the pH of the fulvic-acid and humic-acid solutions from the Suwannee River was increased to 6.5 in order to compare the result with the enhancement effect by the soil-derived humic acid at pH 6.5.

In addition to the aquatic fulvic and humic acids described above, a soil-derived humic acid was used in this study to supplement the evaluation of the mechanism of solute–dissolved-organic-matter interactions. The soil humic acid (provided by R.L. Malcolm, U.S. Geological Survey) was obtained by alkaline extraction of the surface horizon (A1) of the Sanhedrin soil series collected in the Mattole River Valley in Mendocino County of northern California. Subsequent fractionation and purification of the Sanhedrin soil humic acid followed established procedures (Malcolm, 1976). Complete solubilization of the lyophilized Sanhedrin soil humic acid was accomplished by adjusting the hydrated sample to a pH of approximately 10 and

subsequently converting it to a hydrogen form by passing the solution through a hydrogen ion-exchange column (Bio-Rad AD-50W-X8, 20–50 mesh). The pH of the 100 mg/L Sanhedrin soil humic-acid solution thus prepared was approximately 4.0; this pH was adjusted to 6.5 to prevent any precipitation of the humic acid at higher concentrations during centrifugation.

In other experiments, phenylacetic acid, polyacrylic acid, and polyethylene glycol were used as reference models of dissolved organic matter; solutions containing these organic compounds were prepared without pH adjustment. The concentration of phenylacetic acid was extended to more than 600 mg/L to facilitate a more accurate assessment of the enhancement effect. The pH of this solution varied from 3.7 at a concentration of 720 mg/L to 4.1 at 100 mg/L. The pH of a polyacrylic acid solution, with an average molecular weight of 90,000 *daltons,* varied from about 4.4 at a concentration of 100 mg/L to 5.3 at 10 mg/L, while the pH of a solution of the same compound with an average molecular weight of 2,000 daltons ranged from approximately 4.1 at a concentration of 100 mg/L to 4.7 at 10 mg/L.

The solubility enhancement of a solute by dissolved organic matter was determined by equilibrating an excess quantity of the solute in a series of solutions containing increasing concentrations of dissolved organic matter, ranging from 0 to 100 mg/L (0 to 600 mg/L for phenylacetic acid). In addition, the solubility enhancements of 2,4,4′-PCB and 2,4,5,2′,5′-PCB as binary solutes in water containing Sanhedrin soil humic acid were determined. The effect of alkaline pH on the solubility enhancement of organic solutes by Suwannee River fulvic acid and other humic substances was determined by measuring solute solubility in a series of parallel solutions where the pH was adjusted to 8.5.

Duplicate samples were equilibrated for 12 to 18 hours on a reciprocating shaker at 24°±1°C. Excess solute was separated by a series of centrifugation steps (1 hour at 2,987 *g*) followed by removal of the residual solute from the solution meniscus. A 2-mL sample was withdrawn and extracted with an equal volume of n-hexane (Baker, reagent grade) and subsequently analyzed by packed-column gas chromatography (3 percent OV–1 on 100/120 Supelcoport) using either an electron capture detector (for DDT and PCB) or a flame ionization detector (for lindane and trichlorobenzene). The recovery efficiency for this extraction procedure was greater than 95 percent for all solutes tested. A more complete account of this procedure is given by Chiou and others (1986).

Elemental analyses of the humic substances used in this study were obtained to give an indication of their respective compositions (table 1). These analyses were performed by Huffman Laboratories Inc., Golden, Colorado.

## RESULTS OF SOLUBILITY ENHANCEMENT STUDIES

The dependence of the apparent water solubility ($S_w$*) of p,p′-DDT, 2,4,5,2′,5′-PCB, and 2,4,4′-PCB (as single solutes) on the concentration of Suwannee River fulvic acid is shown in figure 1, with the fulvic acid concentration ranging from 0 to about 94 mg/L. Concentrations of the humic substances presented in figure 1 as well as in figures 2 through 4 have been corrected for the ash and moisture content of the lyophilized samples. The apparent water-solubility values shown in figures 1 through 4 represent averages of duplicate determinations; the error bars indicate the range of variation. The results in figure 1 represent solute solubility determined at the original Suwannee River fulvic acid solution pH of 3.9 to 5.7. No significant difference in solute solubility was observed when the solution pH was adjusted to 6.5 (data not shown), indicating that the magnitude of enhancement was not pH sensitive throughout this range. A linear increase in apparent water solubility with increasing concentrations of dissolved fulvic acid is shown in figure 1. The relative solubility enhancement factors ($S_w$*/$S_w$) for these three solutes follow the order of p,p′-DDT > 2,4,5,2′,5′-PCB > 2,4,4′-PCB. Solubility of selected organic solutes in pure water ($S_w$) is presented in table 2.

The apparent solubility of lindane in water containing Suwannee River fulvic acid is included in figure 1. There is no discernible solubility enhancement for lindane in the same concentration range of Suwannee River fulvic acid.

The apparent water solubility of p,p′-DDT, 2,4,5,2′,5′-PCB and 2,4,4′-PCB at pH 8.5 and in the same concentration range of Suwannee River fulvic acid is shown in figure 2. The solubility enhancement of these solutes at this pH is less than that at pH 3.9 to 5.7.

**Table 1.** Ash-free elemental analysis of soil and stream organic-matter extracts

[Values in percent, on moisture-free basis]

| Humic substance | C | H | O | N | S | P | Total | Ash |
|---|---|---|---|---|---|---|---|---|
| Suwannee River fulvic acid | 53.78 | 4.24 | 40.28 | 0.65 | 0.60 | 0.01 | 99.56 | 0.68 |
| Suwannee River humic acid | 54.22 | 4.14 | 39.00 | 1.21 | 0.82 | 0.01 | 99.40 | 3.18 |
| Sanhedrin soil humic acid | 58.03 | 3.64 | 33.59 | 3.26 | 0.47 | 0.10 | 99.09 | 1.19 |

Water solubility enhancement of the same three solutes is shown in figure 3 for Suwannee River humic acid at pH 4.1 to 5.5, and in figure 4 for Sanhedrin soil humic acid at pH 6.5. The magnitude of solubility enhancement for these solutes with Suwannee River humic acid is essentially the same as that obtained with Suwannee River fulvic acid, while that obtained with Sanhedrin soil humic acid is five to seven times greater than with either Suwannee River fulvic or humic acid.

Solubility enhancement data for 2,4,5,2′,5′-PCB and 2,4,4′-PCB as binary solutes in solutions containing Sanhedrin soil humic acid are included in figure 4. Sanhedrin soil humic acid, rather than Suwannee River fulvic acid, was used for this study because the substantial solubility enhancement caused by this humic acid makes a comparison with the single solute solubility data less ambiguous. In this binary-solute system, the solubility enhancement for each solute is virtually identical to that found in the single-solute

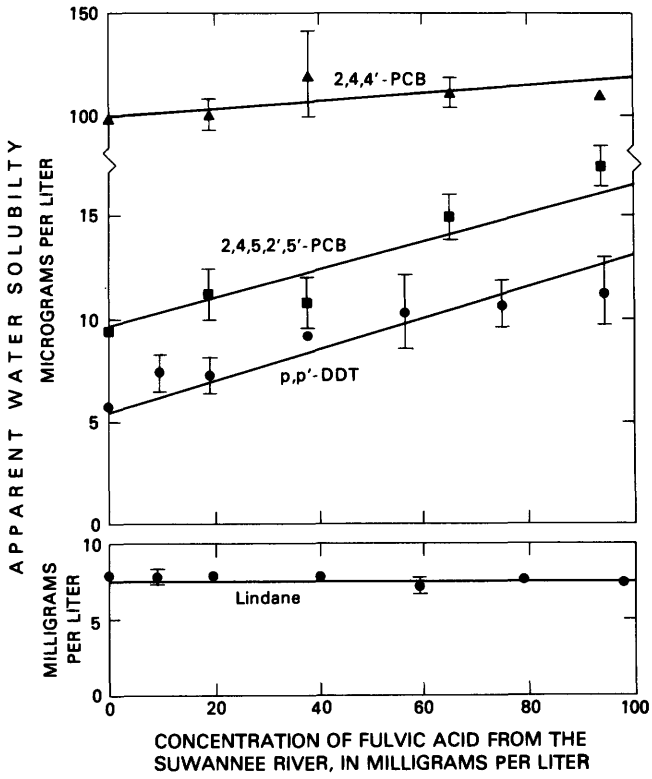


**Figure 1.** Dependence of apparent water solubility of p,p′-DDT, 2,4,5,2′,5′-PCB, 2,4,4′-PCB, and lindane at 25 degrees Celsius on concentration of Suwannee River fulvic acid (pH 3.9 to 5.7). Error bars indicate range of replicate analyses.

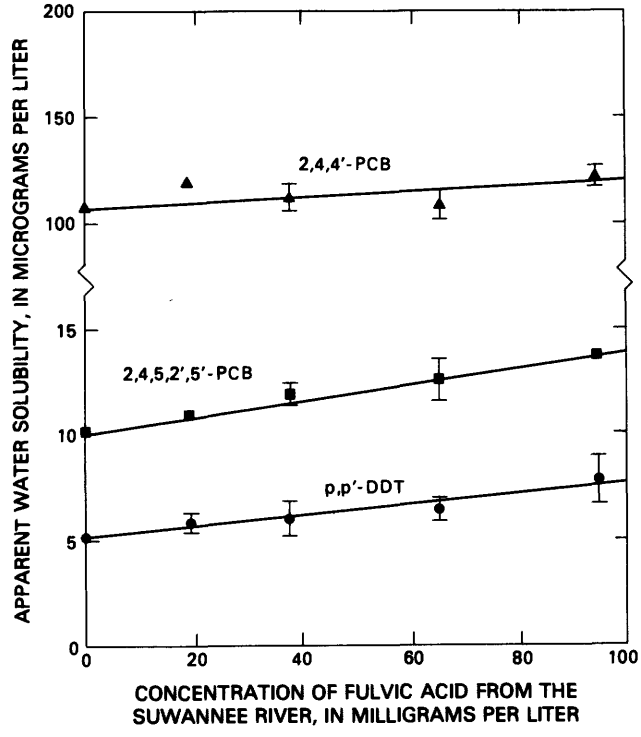


**Figure 2.** Dependence of apparent water solubility of p,p′-DDT, 2,4,5,2′,5′-PCB, and 2,4,4′-PCB at 25 degrees Celsius on concentration of Suwannee River fulvic acid (pH 8.5). Error bars indicate the range of replicate analyses.

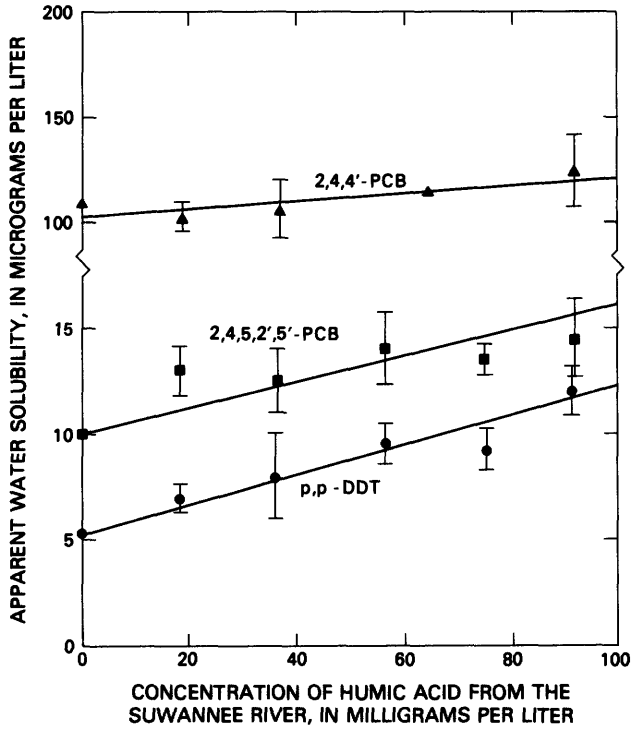


**Figure 3.** Dependence of apparent water solubility of p, p'-DDT, 2,4,5,2',5'-PCB, and 2,4,4'-PCB at 24±1 degrees Celsius on concentration of Suwannee River humic acid (pH 4.1 to 5.5). Error bars indicate range of replicate analyses.

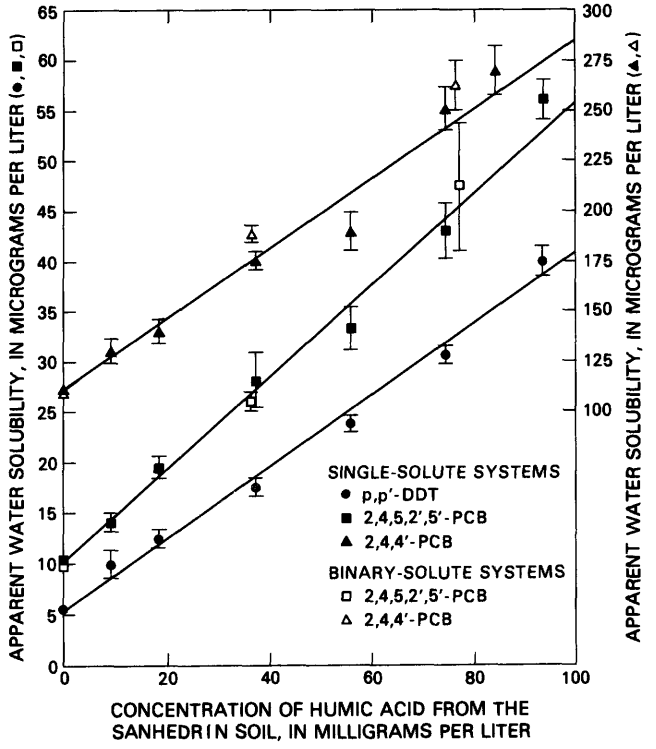


**Figure 4.** Dependence of apparent water solubility of p,p'-DDT, 2,4,5,2',5'-PCB, and 2,4,4'-PCB at 24±1 degrees Celsius on concentration of Sanhedrin soil humic acid (pH 6.5). Error bars indicate range of replicate analyses.

systems, showing that there is no competitive interference between these solutes. The solubility of lindane and 1,2,3-trichlorobenzene was not affected by Suwannee River humic acid or Sanhedrin soil humic acid with the concentration of these dissolved humic substances extending to about 90 mg/L (data not shown).

The effect of phenylacetic acid (as low-molecular-weight dissolved organic matter) on the apparent solubility of p,p'-DDT, lindane and 1,2,3-trichlorobenzene, with the concentration of phenylacetic acid exceeding 600 mg/L, is shown in figure 5. The p,p'-DDT data shows a slight solubility enhancement, the magnitude of which is substantially smaller (per unit mass of dissolved organic matter) than that determined with the fulvic or humic acids. Lindane and 1,2,3-trichlorobenzene data indicate no apparent solubility effects in the presence of phenylacetic acid.

**Table 2.** Pure-water solubility ($S_w$) of selected organic solutes

| Compound | Water solubility, $S_w$, in milligrams per liter | |
|---|---|---|
| | From literature[1] | This study |
| p,p'-DDT | $5.5 \times 10^{-3}$ (25 °C) | $5.4 \times 10^{-3}$ (24 °C) |
| 2,4,5,2',5'-PCB | $1.0 \times 10^{-2}$ (24 °C) | $1.1 \times 10^{-2}$ (25 °C) |
| 2,4,4'-PCB | 0.115 (20 °C) | 0.116 (25 °C) |
| 1,2,3-Trichlorobenzene | 16.3 (23 °C) | 18.0 (25 °C) |
| Lindane | 7.80 (25 °C) | 7.87 (24 °C) |

[1]Cited in Chiou and others (1986).

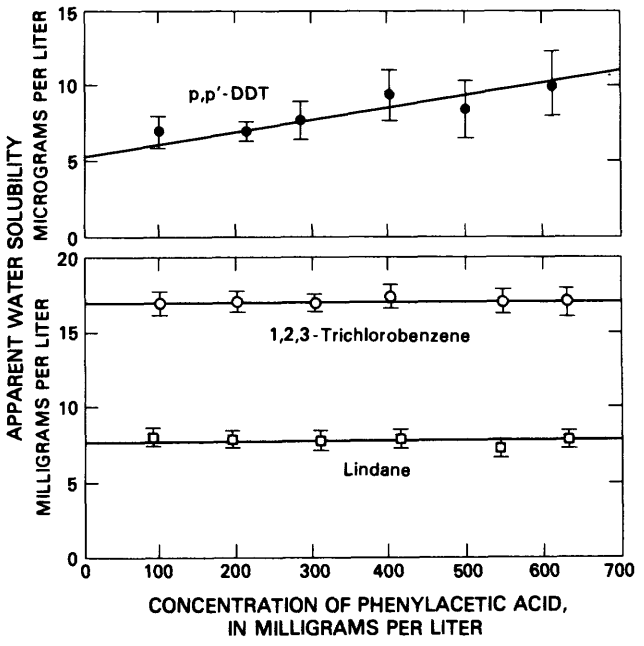


**Figure 5.** Effect of phenylacetic acid on apparent water solubility of p,p'-DDT, lindane, and 1,2,3-trichlorobenzene at 24±1 degrees Celsius. Error bars indicate range of replicate analyses.

The inability of polyacrylic acid (molecular weight = 2,000 and 90,000 daltons; data from higher molecular weight not shown) as dissolved organic matter to affect the solubility of p,p'-DDT, 2,4,5,2',5'-PCB, 2,4,4'-PCB and 1,2,3-trichlorobenzene is shown in figure 6. Similarly, polyethylene glycol, having molecular weights ranging from 200 to 3,400 daltons, exhibited no noticeable solubility enhancement of p,p'-DDT, when the concentration of polyethylene glycol was extended to 100 mg/L (data not shown).

## FACTORS AFFECTING APPARENT WATER SOLUBILITY OF ORGANIC SOLUTES

A comparison of figures 1 through 4 shows that Sanhedrin soil humic acid is most effective in enhancing the apparent water solubility of organic solutes and that p,p'-DDT is the solute most sensitive to dissolved organic matter. The absence of interference between the binary solutes, the linear relation between apparent solute solubility and dissolved-organic-matter concentration, and the dependence of solubility enhancement on the solute's pure-water solubility (as well as the polarity and molecular weight of the dissolved organic matter) are consistent with the proposed partition interaction between a solute and dissolved organic matter.

As previously noted, the magnitude of solubility enhancement for the solute is related to its pure-water solubility ($S_w$); those compounds that are least soluble in water are most strongly affected by dissolved organic matter. Thus, the greater enhancement effect for p,p'-DDT and 2,4,5,2',5'-PCB relative to 2,4,4'-PCB is attributable to their lower water solubilities (table 2). Lindane and 1,2,3-trichlorobenzene, which have relatively high water solubilities, show little solubility enhancement in the same concentration range of dissolved organic matter. Because the water solubility of the solutes is inversely proportional to the corresponding *octanol-water partition coefficients* ($K_{ow}$) (Mackay and others, 1980; Chiou and others, 1982), the log $K_{dom}$ values calculated from equation 3 are essentially linear with the respective log $K_{ow}$ values (table 3).

As indicated earlier, the molecular size of dissolved organic matter is one of the important factors in the partition interaction of the solute with dissolved organic matter. This is evidenced by the observation that the small-sized phenylacetic acid (which would

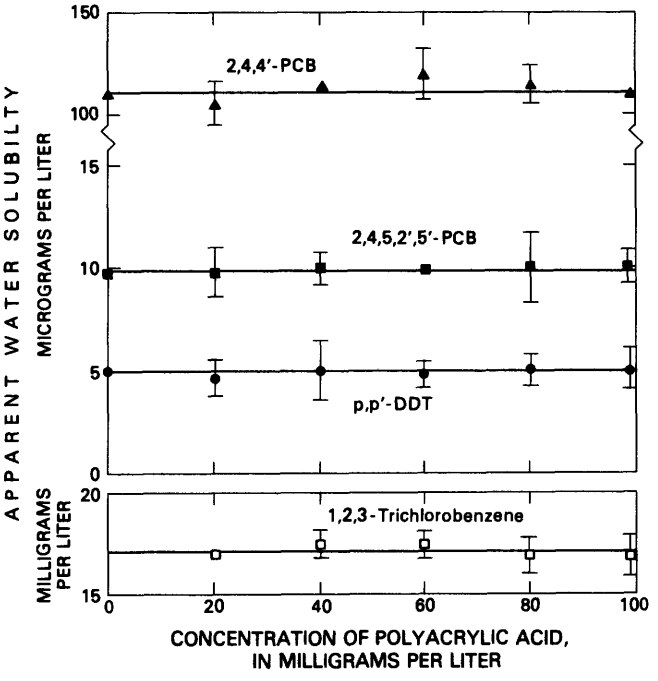


**Figure 6.** Effect of polyacrylic acid (molecular weight = 2,000 daltons) on apparent water solubility of p,p'-DDT, 2,4,5,2',5'-PCB, 2,4,4'-PCB, and 1,2,3-trichlorobenzene at 24±1 degrees Celsius. Error bars indicate range of replicate analyses.

**Table 3.** Log $K_{dom}$ and log $K_{doc}$ values of selected organic solutes with Suwannee River fulvic acid, Suwannee River humic acid, and Sanhedrin soil humic acid

[Solute octanol-water partition coefficients (log $K_{ow}$) are shown for each solute]

| Compound | log $K_{ow}$[1] | log $K_{dom}$[2] | | |
|---|---|---|---|---|
| | | Suwannee River fulvic acid (pH 3.9-5.7) | Suwannee River humic acid (pH 4.1-5.5) | Sanhedrin soil humic acid (pH 6.5) |
| p,p'-DDT---------- | 6.36 | 4.13(4.40) | 4.12(4.39) | 4.82(5.06) |
| 2,4,5,2',5'-PCB--- | 6.11 | 3.83(4.10) | 3.80(4.07) | 4.63(4.87) |
| 2,4,4'-PCB-------- | 5.62 | 3.30(3.57) | 3.17(3.54) | 4.16(4.40) |
| 1,2,3-trichloro-benzene[3]-------- | 4.14 | ~1.7 (2.0) | ~1.7 (2.0) | ~2.8 (3.0) |
| Lindane[3]---------- | 3.70 | ~1.2 (1.5) | ~1.2 (1.5) | ~1.2 (1.5) |

[1]Values from Chiou and others, 1986.

[2]Numbers in parentheses are log $K_{doc}$ values.

[3]Approximate log $K_{dom}$ and log $K_{doc}$ values for 1,2,3-trichlorobenzene and lindane were obtained by linear extrapolations of log $K_{dom}$ and log $K_{doc}$ values against log $K_{ow}$ values for p,p'-DDT, 2,4,5,2'5'-PCB, and 2,4,4'-PCB.

otherwise be a strong enhancer if the solubility enhancement of the solute were caused by a specific interaction) did not have a significant effect on solute solubility. The small solubility increase observed with p,p′-DDT is possibly caused by a change in the solvency of water, similar to the effect noted in a mixed solvent system. The relatively large solubility enhancement for p,p′-DDT and 2,4,5,2′,5′-PCB with Sanhedrin soil humic acid (relative to Suwannee River fulvic and humic acids) may be attributed in part to the greater molecular weight of Sanhedrin soil humic acid. The range of molecular weight for soil-derived humic acids is considered to be 2,000 to 20,000 daltons depending on the source of the sample and extraction procedure used (Schnitzer and Kahn, 1972); whereas, the currently proposed molecular weight (number average) of Suwannee River fulvic acid is approximately 800 daltons (Aiken and others, chap. J, this volume). Based on these values and on the elemental analyses data, it may be assumed that dissolved soil humic acids should provide a relatively larger nonpolar intramolecular environment to more effectively promote a partition-like interaction with nonionic organic solutes.

Even though the molecular size of the dissolved organic matter is important, the polarity and molecular configuration of the dissolved organic matter are also significant factors in partition interactions. This is illustrated by a comparison of the solubility enhancement data obtained with Suwannee River fulvic and humic acids with their respective molecular size and polarity. The molecular weights of aquatic humic acids are considered to range from 1,000 to 10,000 daltons (Thurman and others, 1982), which suggests that humic acid from the Suwannee River is larger in size than fulvic acid from the Suwannee River (Aiken and others, chap. J, this volume). Nevertheless, the enhancement effects with Suwannee River fulvic and humic acids are comparable despite this difference in molecular weight. The data thus suggest that the molecular size of dissolved organic matter is a controlling factor for solubility enhancement only to a certain point, beyond which the polarity and molecular configuration become the predominate factors controlling the partition interaction. Therefore, Suwannee River fulvic and humic acids, which have comparable polarity (as indicated by their carbon-to-oxygen ratios) and possibly similar molecular configuration, show comparable solubility enhancement. The greater effect with Sanhedrin soil humic acid appears to result more from its lower polarity than from its greater size.

The importance of molecular structure and configuration is exemplified by the ineffectiveness of

polyacrylic acid (molecular weight = 2,000 and 90,000 daltons), as dissolved organic matter, to enhance nonionic organic solute solubility. Even though the molecular weight of this linear *polyelectrolyte* is sufficiently large and its carbon (50 percent), oxygen (44.4 percent), and hydrogen (5.6 percent) contents are quite comparable to those of humic substances, the extended chain configuration of this molecule in addition to the frequent and orderly attachment of hydrophilic functional groups to the carbon chain apparently precludes the formation of a sizable intramolecular nonpolar environment. This point is further illustrated by the inability of polyethylene glycol (with a molecular weight ranging from 300 to 3,400 daltons) to enhance the solubility of the solutes tested.

The effect of pH on the solubility enhancement of a nonionic solute by dissolved organic matter can be rationalized in terms of the effect of pH on the dissolved organic matter configuration and conformation. The degree of ionization of the acidic groups in humic molecules, as controlled by pH, would affect either the size or the compactness of the molecular nonpolar organic environment of the dissolved humic substance. At an alkaline pH, the hydration of the dissociated acidic groups and the concomitant repulsion between the ionized functional groups would result in a more extended molecular configuration/conformation, thus reducing the intensity of the nonpolar environment. Data in figure 2 indicate a noticeable decrease of solute solubility enhancement by dissolved Suwannee River fulvic acid at pH = 8.5 in comparison to the enhancement effect at pH 3.9 to 5.7 (fig. 1); the calculated $K_{dom}$ values at pH 8.5 for p,p′-DDT, 2,4,5,2′,5′-PCB and 2,4,4′-PCB are 31, 61, and 35 percent, respectively, of the $K_{dom}$ values obtained at the lower pH.

## INFERENCES ABOUT CHARACTERISTICS OF SUWANNEE RIVER FULVIC ACID

Data presented in this chapter lead to several suppositions as to the molecular weight, composition, configuration, and conformation of Suwannee River fulvic acid. For example, it may be assumed that Suwannee River fulvic acid has a sufficiently large molecular size (and, hence, molecular weight) to promote solute partitioning in order to account for observed solute solubility enhancement. Secondly, in aqueous solution, dissolved Suwannee River fulvic acid may be considered to exist either in a truly dissolved state or possibly in an aggregate form, with the configuration/conformation changing from an acidic to alkaline pH. This conclusion agrees with current measurements of molecular weights of Suwannee River fulvic acid. Whether or not the aquatic fulvic acid exists in an aggregate form in water needs to be verified by an independent study.

Finally, a general structural model for Suwannee River fulvic acid can be hypothesized based on elemental analysis data and the observed solute solubility enhancement effect. That Suwannee River fulvic acid is moderately effective in enhancing the solubility of nonionic organic solutes indicates that there should be substantial fractions of both polar and nonpolar functional groups present within the molecular structure. Therefore, any proposed structural model needs to account for the presence of a sufficient nonpolar moiety within the molecule (or within the molecular aggregate) to promote effective partitioning. The magnitude of the enhancement effect by Suwannee River fulvic acid relative to that by polyacrylic acid (which has a higher molecular weight and carbon-to-oxygen ratio) indicates that the fulvic acid has structure(s) far from being linear and has a less orderly attachment of hydrophilic functional groups to the molecular network. In addition, fulvic acid from the Suwannee River likely contains a certain proportion of nonpolar aromatic components that contribute to its nonpolar molecular moiety; these aromatic groups could be linked to the molecular framework to form a relatively flexible, liquid-like intramolecular environment. A predominately *aliphatic* macromolecule having the same number of polar functional groups as is in the fulvic acid would tend to make the molecule form an extended configuration/conformation that would not be as effective for solute partitioning. This inferred distribution of the aliphatic-aromatic carbons in the proposed molecular structure of the Suwannee River fulvic acid appears to be compatible with nuclear-magnetic-resonance data (Malcolm and MacCarthy, 1986; Thorn, chap. N, this volume).

## CONCLUSIONS

An understanding of the mechanism of interaction between dissolved organic matter, such as Suwannee River fulvic acid and an organic solute, in relation to

the molecular properties of the dissolved organic matter and solute, is essential for an accurate assessment of the transport and fate of organic contaminants in a natural aquatic system. This interaction, as manifested by an enhancement of the water solubility of the organic solute, can be accounted for by a partition-like interaction between the solute and the organic macromolecule. The dependence of the enhancement effect on a solute's pure-water solubility, the molecular size and polarity of the organic macromolecule, the linear relation between apparent water solubility and concentration of dissolved organic matter, and the absence of solute competition in a binary system are observations supporting this concept.

At concentrations less than or equal to 100 mg/L, the effect of Suwannee River fulvic acid and other humic substances on solute solubility is practically insignificant for relatively water-soluble compounds such as lindane and 1,2,3-trichlorobenzene. For relatively water-insoluble solutes such as DDT and certain PCB's, the effect can be appreciable, even at moderate concentrations of dissolved organic matter such as may be present in a natural aquatic system.

The partition coefficients for given solutes between dissolved organic matter and water ($K_{dom}$) are approximately five to seven times greater for Sanhedrin soil humic acid than for Suwannee River fulvic or humic acids. The $K_{dom}$ value increases with a decrease of the solute's water solubility (or with an increase of the octanol-water partition coefficient) and is inversely related to the polarity (as indicated by elemental analysis) of dissolved organic matter. This information provides a basis for estimating the impact of dissolved organic matter on solute behavior in natural waters.

Certain general structural features of Suwannee River fulvic acid may be inferred that are compatible with the solubility enhancement data and consistent with the partition concept. These results indicate that the molecular framework of Suwannee River fulvic acid contains a sizable, nonpolar moiety that contributes to partition interactions with organic pollutants. Such a relatively nonpolar moiety may result from the aromatic components present in the fulvic acid molecule. These structural features are in agreement with the proposed models based on independent nuclear-magnetic-resonance data and molecular weight determinations, and they reinforce the validity of the partition concept of interaction between an organic solute and dissolved organic matter.

## ACKNOWLEDGMENT

The humic and fulvic acids and elemental analysis data used in this study were provided by R.L. Malcolm, U.S. Geological Survey, Denver, Colorado.

## REFERENCES CITED

Boehm, P.D., and Quinn, J.G., 1973, Solubilization of hydrocarbons by the dissolved organic matter in sea water: Geochimica et Cosmochimica Acta, v. 37, p. 2459–2477.

Caron, G., Suffet, I.H., and Belton, T., 1985, Effect of dissolved organic carbon on the environmental distribution of nonpolar organic compounds: Chemosphere, v. 14, p. 993–1000.

Carter, C.W., and Suffet, I.H., 1982, Binding of DDT to dissolved humic materials: Environmental Science and Technology, v. 16, p. 735–740.

Chiou, C.T., Malcolm, R.L., Brinton, T.I., and Kile, D.E., 1986, Water solubility enhancement of some organic pollutants and pesticides by dissolved humic and fulvic acids: Environmental Science and Technology, v. 20, p. 502–508.

Chiou, C.T., Schmedding, D.W., and Manes, M., 1982, Partitioning of organic compounds in octanol-water systems: Environmental Science and Technology, v. 16, p. 4–10.

Gschwend, P.M., and Wu, S-C., 1985, On the constancy of sediment-water partition coefficients of hydrophobic organic pollutants: Environmental Science and Technology, v. 19, p. 90–96.

Haas, C.M., and Kaplan, B.M., 1985, Toluene–humic acid association equilibria: Isopiestic measurements: Environmental Science and Technology, v. 19, p. 643–645.

Hassett, J.P., and Milicic, E., 1985, Determination of equilibrium and rate constants for binding of a polychlorinated biphenyl congener by dissolved humic substances: Environmental Science and Technology, v. 19, p. 638–643.

Leversee, G.J., Landrum, P.F., Giesy, J.P., and Fannin, T., 1983, Humic acids reduce bioaccumulation of some polycyclic aromatic hydrocarbons: Canadian Journal of Fisheries and Aquatic Sciences, v. 40 (supplement 2), p. 63–69.

Mackay, D., Bobra, A., Shiu, W.Y., and Yalkowsky, S.H., 1980, Relationships between aqueous solubility and octanol-water partition coefficients: Chemosphere, v. 9, p. 701–711.

Malcolm, R.L., 1976, Method and importance of obtaining humic and fulvic acids of high purity: U.S. Geological Survey, Journal of Research, v. 4, p. 37–40.

———1985, Geochemistry of stream fulvic and humic substances, *in* Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediment, and Water: New York, John Wiley, p. 181–209.

Malcolm, R.L., and MacCarthy, P., 1986, Limitations in the use of commercial humic acids in water and soil research: Environmental Science and Technology, v. 20, p. 904–911.

McCarthy, J.F., 1983, Roles of particulate organic matter in decreasing accumulation of polynuclear aromatic hydrocarbons by *Daphnia magna*: Archives of Environmental Contamination and Toxicology, v. 12, p. 559–568.

McCarthy, J.F., and Jimenez, B.D., 1985, Interactions between polycyclic aromatic hydrocarbons and dissolved humic material—Binding and dissociation: Environmental Science and Technology, v. 19, p. 1072–1076.

Perdue, E.M., and Wolfe, N.L., 1982, Modification of pollutant hydrolysis kinetics in the presence of humic substances: Environmental Science and Technology, v. 16, p. 847–852.

Poirrier, M.A., Bordelon, B.R., and Laseter, J.L., 1972, Adsorption and concentration of dissolved carbon-14 DDT by coloring colloids in surface waters: Environmental Science and Technology, v. 6, p. 1033–1035.

Schnitzer, M., and Khan, S.U., 1972, Humic Substances in the Environment: New York, Marcel Dekker, 317 p.

Thurman, E.M., and Malcolm, R.L., 1981, Preparative isolation of aquatic humic substances: Environmental Science and Technology, v. 15, p. 463–466.

Thurman, E.M., Wershaw, R.L., Malcolm, R.L., and Pinckney, D.J., 1982, Molecular size of aquatic humic substances: Organic Geochemistry, v. 4, p. 27–35.

Wershaw, R.L., Burcar, P.J., and Goldberg, M.C., 1969, Interaction of pesticides with natural organic material: Environmental Science and Technology, v. 3, p. 271–273.

Zepp, R.G., Schlotzhauer, P.F., and Sink, R.M., 1985, Photosensitized transformations involving electronic energy transfer in natural waters—Role of humic substances: Environmental Science and Technology, v. 19, p. 74–81.

Chapter D

# Complexation of Copper by Fulvic Acid from the Suwannee River—Effect of Counter-Ion Concentration

*By* D.M. McKnight *and* R.L. Wershaw

## CONTENTS



### FIGURES



### TABLES

## Abstract

Manning's theory on counter-ion condensation was used to develop two hypotheses for the effect of counter-ion concentration on copper complexation by fulvic acid from the Suwannee River. These hypotheses were tested experimentally by potentiometric titrations of fulvic acid solutions with variable concentrations of potassium and calcium. The hypothesis of territorial binding of counter ions by fulvic acid was supported by the determination that activity coefficients were substantially less than unity (0.4–0.6) for copper ions in fulvic-acid solutions. The other hypothesis (that territorial binding of counter ions would affect the apparent formation constants of site-specific copper binding) was not supported by the experimental results. Potassium concentration was determined to have no detectable effect on copper complexation. The effects of calcium concentration were contrary to predictions of the original hypothesis and could be explained by competition between copper and calcium for some part of the binding sites and no competition for other sites. This result is evidence of the importance of structural heterogeneity, as well as electrostatic effects, in copper complexation by fulvic acid from the Suwannee River.

## INTRODUCTION

One impetus behind this study of naturally occurring fulvic acids from aquatic environments has been the demonstrated importance of fulvic acids in controlling the chemical speciation of trace metals in aquatic environments. The chemical speciation of trace metals, in turn, controls the biological effect of trace metals and their transport and fate in the environment.

One trace metal that has received a great deal of study in this context is copper. There are three primary reasons for this emphasis on copper. First, numerous analytical techniques are available for studying the complexation of copper by humic substances (Shuman and others, in press); they include: *anodic-stripping voltammetry* (Shuman and Woodward, 1973, 1977); potentiometric titration using a cupric-ion selective electrode (Saar and Weber, 1980; McKnight and others, 1983); fluorescence-quenching titration (Ryan and Weber, 1982); dialysis titration (Truitt and Weber, 1981); and various chromatographic methods using gels (Mantoura and others, 1978), anionic exchange resins (Matsunaga and others, 1980), or manganese oxides (Van den Berg and Kramer, 1979). Secondly, copper is ubiquitous in the environment; it is present in measurable concentrations in both unpolluted and polluted waters. Thirdly, copper is both a required micronutrient and a potential toxicant for aquatic organisms. In general, we conclude that, in many natural waters, the copper/fulvic-acid complex is a major species of copper (Mantoura and others, 1978; Matsunaga and others, 1980; Ryan and Weber, 1982; and McKnight and others, 1983).

Another area of active research has been the application and development of various models for the complexation of copper by fulvic acid. These models range from simple two-ligand and Scatchard-type empirical models that are used for analysis of analytical data (for example, McKnight and others, 1983) to more mechanistic models that are intended to include critical aspects of the interactions between copper and fulvic acid. These mechanistic models have focused on either: (1) The diversity of specific copper-binding sites present in the complex heterogeneous mixture of organic compounds that comprise aquatic fulvic acid (MacCarthy and Smith, 1979; Perdue and Lytle, 1983), or (2) the electrostatic interactions between binding sites present on the fulvic acid macromolecules (Wilson and Kinney, 1977; Gamble and others, 1980; and Marinsky and others, 1982). As was discussed by Cabaniss and others (1984), it is not possible to discriminate, based on ability to fit experimental data, between a simple two-ligand model and possibly more mechanistically correct models. Further, a model that included both mixture and electrostatic effects is unworkable because it would include a myriad of unmeasurable parameters. In addition to mixture and electrostatic effects, the conformation of fulvic-acid molecules also may affect copper complexation. The future progress in the quest for a true, mechanistically correct model for metal-ion complexation by fulvic acid depends on a better understanding of the structure or structures of fulvic acid and of the nature of the binding mechanism or mechanisms between metal ions and fulvic acid.

Although the goal of a true, mechanistically correct model may be unattainable at this time, current (1988) models may be used to generate new and testable hypotheses concerning copper complexation by

fulvic acid. The process of developing and testing these new hypotheses may lead to a better understanding of complexation of copper by fulvic acid. In this chapter, the polyelectrolyte theory developed by Manning (1979, 1981) is used to develop a hypothesis for the effect of counter-ion concentration on copper complexation by fulvic acid. This hypothesis then is tested experimentally with both *ion-selective electrode potentiometry* and spectrophotometric measurement of a copper/fulvic-acid charge-transfer band. The results of these experiments indicate that both structural heterogeneity and electrostatic interactions need to be addressed to explain the effect of counter-ion concentration on copper complexation by fulvic acid. Because of the considerable range in concentration of major cations in freshwater, the results also have geochemical significance.

## EXPERIMENTAL METHODS

### Potentiometric Titrations

Potentiometric titrations were performed using a Radiometer "Selectrode" cupric-ion selective electrode, an Orion pH electrode, an Orion double-junction reference electrode, and Orion 701 and 801 pH meters. The Nernstian response of the cupric-ion-selective electrode was verified in freshly prepared solutions of $10^{-5}$ and $10^{-6}$ M $Cu(NO_3)_2$ in $10^{-3}$ M $KNO_3$ at pH 4.0 immediately before each titration. The response of the electrode was considered Nernstian if it was 29±2 mV per decade. The measured potential in the $10^{-5}$ M $Cu^{2+}$ standard solution was 232±6 mV.

Freshly prepared 50-mL solutions of fulvic acid from the Suwannee River (with $Ca(NO_3)_2$ or $KNO_3$ at various concentrations as a background electrolyte) were titrated with copper at pH 6.0 over a total copper concentration ranging from $10^{-6}$ to $4\times10^{-4}$ M. Copper additions were made from stock solutions of $10^{-1}$, $10^{-2}$, and $10^{-3}$ M $Cu(NO_3)_2$. During preparation of the fulvic-acid solutions for titration, the goal was to have the carbon concentration in the fulvic-acid solutions be 25 mg/L—the actual concentration was measured with a Beckman 915 C carbon analyzer subsequent to each titration. The fulvic-acid solutions were continuously bubbled with $N_2$ (gas) at a constant temperature of 25°C; the pH was adjusted to 6.0±0.05; and additions of dilute, carbonate-free NaOH were made at the beginning of each titration and after each copper addition. The equilibration of the cupric-ion-selective electrode was monitored after each copper addition (using a strip-chart recorder) until the change in potential with time was less than 4 mV/h.

In order to analyze the potentiometric data, cupric-ion activities were calculated from measured potentials using the *Nernst equation* and potentials measured in standard copper solutions immediately prior to each titration. Formation constants and binding-site concentrations were calculated from the potentiometric data using the computer program *FITEQL* (Westall, 1982) in a manner similar to that described by McKnight and others (1983). For analysis of these titrations using FITEQL, the optimized parameters used were the activity coefficient of free ionic copper and the concentrations and formation constants of two copper-binding ligands. This two-ligand model is not a mechanistically correct model; it is, however, a means to quantitatively compare potentiometric titrations.

Further interpretation of the potentiometric titrations was facilitated by using a chemical-equilibrium computer program MINEQL (Westall and others, 1976) to generate copper titration curves using different assumptions regarding competitive interactions between copper and calcium in the binding of these cations by fulvic acid from the Suwannee River.

### Spectrophotometric Titrations

The fulvic-acid solutions used for spectrophotometric titrations were analogous to the solutions used for potentiometric titrations. The concentrations of fulvic acid were the same; the pH was adjusted to 6.0±0.05 using dilute NaOH; and the solutions were bubbled with $N_2$ (gas) before and after spectrophotometric measurement. The differences between the two titration procedures were: the lesser sensitivity for the spectrophotometric detection of copper/fulvic-acid charge-transfer band restricted the range of the titration to $10^{-5}$ to $4\times10^{-4}$ M copper; KCl and $CaCl_2$ were used as background electrolytes; and copper was added as $CuCl_2$ because of the substantial absorbance of $NO_3$ in the ultraviolet region.

The spectrophotometric titrations were performed using a Cary 118 spectrophotometer with 1-cm-path-length quartz cells containing 3.5 mL of fulvic-acid solution. The experimental cell was balanced against

a cell containing the same fulvic-acid and background electrolyte solution adjusted to pH 6.0±0.05 and initially purged with $N_2$ (gas). Copper additions were made directly in the 3.5-mL cell. After each addition, the pH was measured with a small Orion combination pH electrode and adjusted to pH 6.0±0.05, and the solution was purged with $N_2$ (gas). The absorbance was scanned from 210 to 300 nm. The spectra were corrected for the absorbance of free ionic copper by subtraction of spectra of $CuCl_2$-KCl solutions weighted according to the free cupric-ion concentration at each total-copper concentration determined in the potentiometric titration. This correction became significant at copper concentrations greater than $10^{-4}$ M.

## HYPOTHESES DEVELOPMENT BASED ON MANNING'S THEORY OF COUNTER-ION CONDENSATION IN POLYELECTROLYTE SOLUTIONS

Manning (1979, 1981) has developed a theory for the behavior of counter ions in polyelectrolyte solutions. This theory considers two types of counter-ion binding: site-specific binding (such as in a charge-transfer complex) and territorial binding (where there are intervening water molecules between counter ions and the polyion). The other major feature of Manning's theory of counter-ion condensation is that, for pure solutions of a known polyelectrolyte, the model parameters are derived directly from the geometry of the polyion (described as an infinitely long, linear lattice) and the intrinsic properties of the acidic functional groups. Given the currently unknown structure of aquatic fulvic acid, it is not possible at this time to evaluate the applicability of this feature of Manning's theory.

In solutions of simple electrolytes, counter ions will be distributed uniformly in solution. In solutions of polyelectrolytes, counter ions will be more abundant in the vicinity of the charged polyelectrolyte than in the bulk solution. In Manning's theory, territorial binding or counter-ion condensation refers to this nonuniform distribution of counter ions and can be interpreted as a decrease in the activity coefficient of the counter ion (Manning, 1979). Activity coefficients of about 0.2 are not unusual in polyelectrolyte solutions (Manning and Zimm, 1965). The magnitude of the decrease in activity coefficient will be dependent on the relative concentrations of polyelectrolytes and counter ions.

Territorial binding of counter ions, in turn, will affect the binding of metal ions at specific binding sites on the polyion (Manning, 1981). The apparent enhancement of intrinsic formation constants for these specific binding sites through neutralization of charge on the charged polyion will decrease with increasing counter-ion condensation. Manning (1981, equation 60) developed the following equation for the effect of monovalent, territorially bound counter ions on the dissociation of site-bound protons from a polyelectrolyte:

$$pK_a = pK_a^0 + 0.434 - \log A^2 + 2\log(\alpha\bar{\xi}) - 0.434(\alpha\bar{\xi}) - \log C_s \quad (5)$$

where

$K_a$ is the apparent equilibrium constant for site-specific binding,

$K_a^0$ is the intrinsic equilibrium constant (at $\alpha=0$),

$A^2=5.58$ in water at 25°C,

$\alpha$ is the ratio of ionized functional groups to the total number of acidic functional groups,

$\bar{\xi}$ is the charge-density parameter for the completely ionized polyelectrolyte ($\alpha=1$), and

$C_s$ is the molarity of the counter-ion salt.

Although this equation was developed and tested for proton binding by a polyelectrolyte, it can be applied directly to site-specific binding of monovalent metals by a polyelectrolyte in the presence of monovalent counter ions (G.S. Manning, Rutgers University, oral commun., 1986). For a true polyelectrolyte, that is, one that conforms to linear lattice geometry, $\bar{\xi}$ is calculated from the charge spacing of the completely ionized polyelectrolyte. This equation is valid for values of larger than the critical value at which counter-ion condensation begins, and it predicts that $pK_a$ minus $pK_a^0$, should vary linearly with the logarithm of the salt concentration.

This equation will not be applicable directly to the effect of counter-ion concentration on site-specific binding of copper by fulvic acid for several reasons. First, the model was developed for monovalent counter ions and site-specific binding of monovalent metals—however, copper ions are divalent, and major cations in natural water include both monovalent ($K^+$ and $Na^+$) and divalent ($Ca^{2+}$ and $Mg^{2+}$) cations. Secondly, aquatic fulvic acid probably does not meet the geometric criterion of an infinitely long,

linear lattice on which the model was developed. With a molecular weight of about 1,000 daltons and less than 10 carboxylic-acid groups per molecule, aquatic fulvic acid is more accurately described by the term "oligoelectrolyte," rather than "polyelectrolyte." For an oligoelectrolyte, the nonuniform distribution of charge in the molecule (referred to as "end effects") will cause deviation from equation 1 (G.S. Manning, Rutgers University, oral commun., 1983). Thirdly, there are at least two, and probably many more, different types of copper-binding sites in fulvic acid, although only a few may be present in any one fulvic-acid molecule. A fourth reason for deviation from equation 1 is that divalent counter ions, such as $Ca^{2+}$ or $Mg^{2+}$, also may compete with $Cu^{2+}$ for specific binding sites as described by the following reactions:

$$\begin{array}{ll} Ca^{2+} + L^{2-} \Leftrightarrow CaL & K_2 \\ Cu^{2+} + L^{2-} \Leftrightarrow CuL & K_1 \\ \hline Cu^{2+} + CaL \Leftrightarrow CuL + Ca^{2+} & K_3 = K_1/K_2 \end{array} \qquad (2)$$

In view of these factors, one would not expect equation 1 to apply directly to copper/fulvic-acid complexation. However, more general hypotheses can be developed from equation 1 as follows: (1) Electrostatic enhancement of site-specific binding decreases with increasing counter-ion concentration, and (2) cupric ions comprise a fraction of the territorially bound ions in proportion to their fraction of the cations in the bulk solution. These hypotheses predict that: (1) The *apparent formation constants* will decrease with increasing counter-ion concentration, and (2) the activity coefficient for copper ions will be less than unity. Although general, these hypotheses can be tested experimentally and have interesting implications for trace-metal complexation in different aquatic environments.

## TESTING OF HYPOTHESES

Potassium and calcium were chosen as a monovalent and a divalent counter ion for testing the two hypotheses developed from Manning's (1979, 1981) theory. With calcium, a possibility exists of competition between copper and calcium for the strong metal-binding sites. At greater calcium concentrations, however, it should be possible to differentiate between an effect from counter-ion condensation and a competitive effect.

The results of the potentiometric titrations of solutions of fulvic acid from the Suwannee River with $Ca(NO_3)_2$ as a background electrolyte at concentrations ranging from $10^{-2}$ to $10^{-5}$ M and a titration with no added background electrolyte are presented in figure 1. Several conclusions can be made about these results. All titration curves indicate the presence of some strong copper-binding sites in the fulvic acid. This is indicated by the cupric-ion activity being several orders of magnitude less than the total copper concentration for the smaller concentrations of added copper. At the greatest additions of copper, all titration curves converge to a line below and parallel to the line defined by $p\{Cu^{2+}\} = pCu_T$; the curves also remain below the solubility limit for $Cu(OH)_2(s)$. The titration curves with no added $Ca(NO_3)_2$ and with $10^{-5}$ M $Ca(NO_3)_2$ are virtually identical and indicate the greatest degree of copper complexation. The titration curves with $10^{-2}$ and $10^{-3}$ M $Ca(NO_3)_2$ as a background electrolyte also are similar, indicating a lesser degree of copper complexation. The titration curve for $10^{-4}$ M $Ca(NO_3)_2$ is intermediate between the above-mentioned two pairs of titration curves.

In contrast to the titration curves for a range of $Ca(NO_3)_2$ concentrations, the titration curves for $10^{-2}$ and $10^{-3}$ M $KNO_3$ are not significantly different from the titration curve with no background electrolyte (fig. 2). In general, these results indicate that, for fulvic acid from the Suwannee River, there is no loss in copper complexation capacity with increasing $KNO_3$ concentrations up to $10^{-2}$ M.

To facilitate interpretation of these results, the titration curves for all concentrations of background electrolyte were fit to a two-ligand model with a non-ideal activity coefficient for the free cupric ion using the computer program FITEQL (Westall, 1982). As discussed by Dzombak and others (1986), one of the difficulties in modeling metal/humic-substance complexation is that there is no independent knowledge of ligand concentration, $L_T$. Therefore, the ligand concentrations were calculated for each titration. The results of this curve-fitting exercise are presented in table 1 for the example of five fitting parameters; i.e., the concentrations and formation constants of the two ligands and the activity coefficient of $Cu^{2+}$. Ligand concentrations are expressed as moles of ligand per milligram of carbon of fulvic acid from the Suwannee River to correct for small differences in fulvic-acid concentration of the titrations. In the FITEQL program, the value of V is a measure of the quality of fit (the weighted sum of squares divided by the degrees

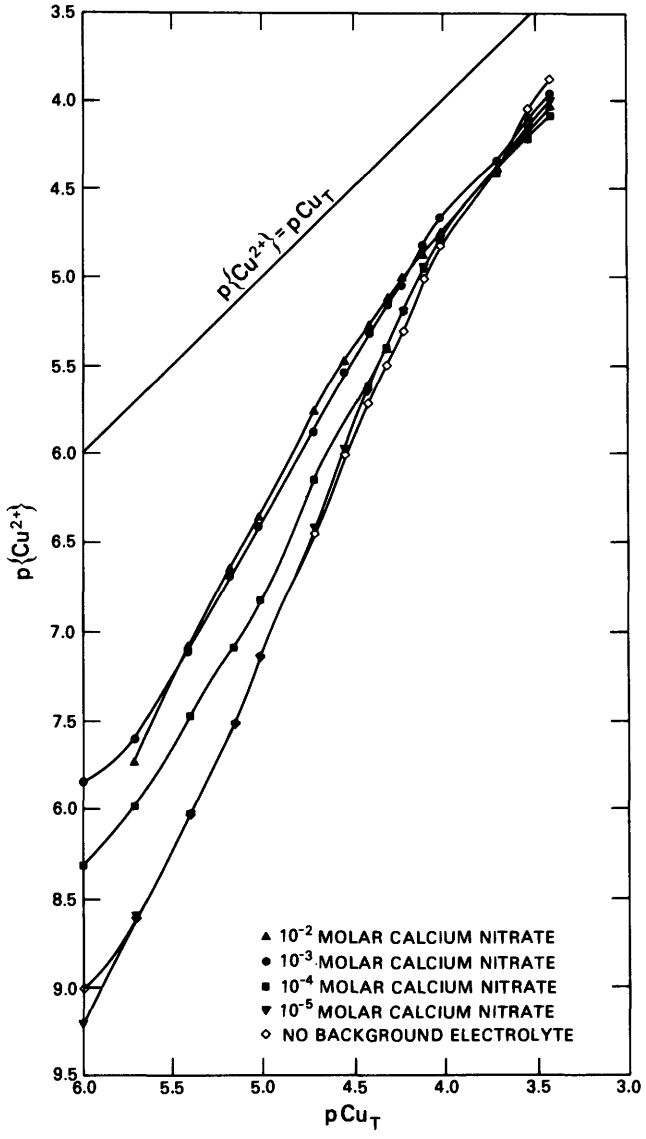


**Figure 1.** Potentiometric titration curves for solutions of fulvic acid from the Suwannee River with variable concentrations of calcium nitrate as background electrolyte.

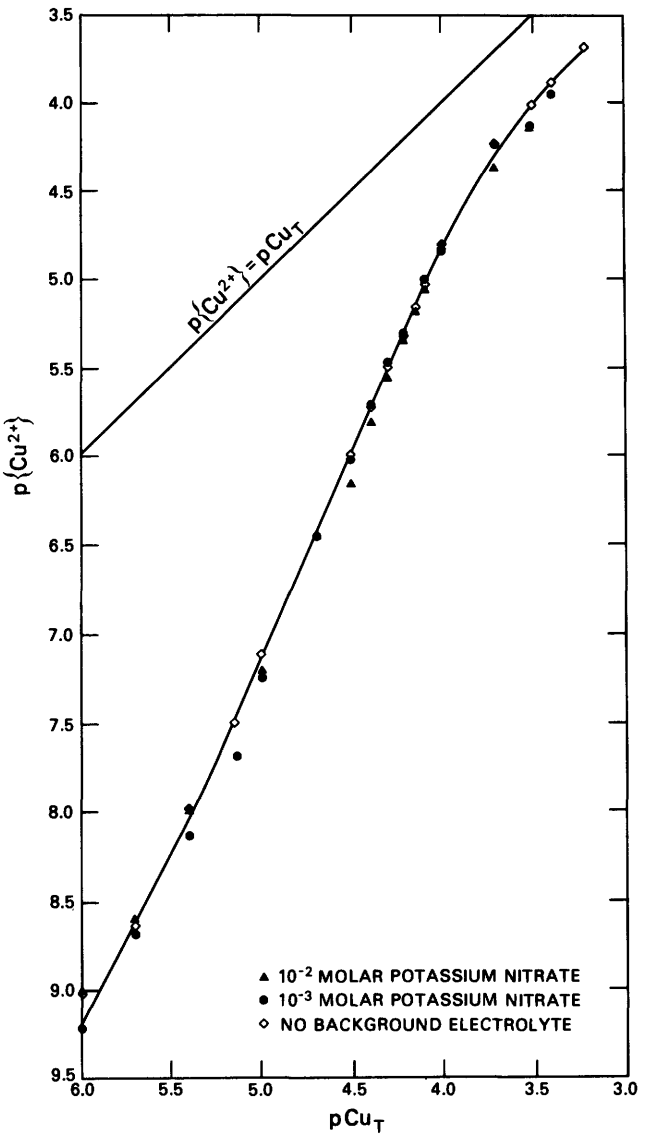


**Figure 2.** Potentiometric titration curves for solutions of fulvic acid from the Suwannee River with variable concentrations of potassium nitrate as background electrolyte.

of freedom), and acceptable values range from 0.1 to 10. The two-ligand, one-activity-coefficient model yields an acceptable fit with all titration curves.

The values for the ligand concentrations and the formation constants are all within the range reported by McKnight and others (1983) for a set of 18 aquatic fulvic acids using $10^{-3}$ M $KNO_3$ as the background electrolyte. The fulvic acid from the Suwannee River was included in this earlier study and, as presented in table 1, the parameters describing the two ligands obtained in that study are very similar to those obtained in this study for $10^{-3}$ M $KNO_3$. In the study by McKnight and others (1983), the carbon concentrations of the fulvic acid in the titration solutions were smaller than those in this study (5 to 15 mg/L versus 25 mg/L), and the conversion of the potential measurements to free cupric-ion activities was performed differently, such that calculation of an activity coefficient in the FITEQL fitting procedure was not used in the previous study.

In the context of this fitting procedure, the differences in the titration curves for the range of $Ca(NO_3)_2$ concentrations appear as differences in ligand concentration ($L_1$ and $L_2$). The magnitude of

**Table 1.** Effect of background electrolyte concentration on copper complexation by fulvic acid from the Suwannee River

[V, quality of fit measurement; $\gamma_{Cu^{2+}}$, activity coefficient of cupric ion; mol/mgC, moles per milligram of carbon in fulvic acid from the Suwannee River; M, molar concentration. See text for definition of other terms]

| Background electrolyte concentration (M) | V | $\gamma_{Cu^{2+}}$ | $L_1$ (mol/mgC) | Log $K_1$ | $L_2$ (mol/mgC) | Log $K_2$ |
|---|---|---|---|---|---|---|
| | | | NO BACKGROUND ELECTROLYTE | | | |
| 0 | 11.3 | 0.40 | $1.9\times10^{-6}$ | 5.9 | $2.7\times10^{-7}$ | 8.1 |
| | | | CALCIUM AS BACKGROUND ELECTROLYTE | | | |
| $10^{-2}$ | 10.0 | 0.62 | $7.3\times10^{-7}$ | 5.9 | $1.4\times10^{-7}$ | 7.65 |
| $10^{-3}$ | 5.1 | .52 | $9.7\times10^{-7}$ | 5.6 | $1.9\times10^{-7}$ | 7.2 |
| $10^{-4}$ | 9.3 | .60 | $1.1\times10^{-6}$ | 6.0 | $1.8\times10^{-7}$ | 7.6 |
| $10^{-5}$ | 11.7 | .57 | $1.4\times10^{-6}$ | 6.3 | $2.5\times10^{-7}$ | 8.3 |
| | | | POTASSIUM AS BACKGROUND ELECTROLYTE | | | |
| $10^{-3}$ | 12.7 | 0.51 | $1.4\times10^{-6}$ | 5.9 | $3.1\times10^{-7}$ | 8.1 |
| [1]$10^{-3}$ | 5.6 | ([2]) | $1.2\times10^{-6}$ | 5.9 | $2.7\times10^{-7}$ | 7.8 |

[1]McKnight and others (1983), using a smaller dissolved organic carbon concentration (12 milligrams per liter as carbon).
[2]Activity coefficient assumed to be unity.

the decrease in $L_1$ concentrations between the no-background electrolyte and the $10^{-2}$ M $Ca(NO_3)_2$ titrations is at least twice as great as the differences in $L_1$ concentration calculated for similar titration curves (for example, $10^{-2}$ M and $10^{-3}$ M $Ca(NO_3)_2$). In actuality, the total concentration of ligands cannot have changed between titrations because the concentrations of fulvic acid from the Suwannee River were the same. Therefore the change in the calculated values of $L_1$ and $L_2$ may be a result of competition between calcium and copper (see equation 2). There is no trend in the associated formation constants ($K_1$ and $K_2$). Further, the fitted activity coefficients range from 0.4 to 0.6 and also did not vary with increasing $Ca(NO_3)_2$ concentration.

To better resolve a possible trend of decreasing ligand concentration, the titration curves for different $Ca(NO_3)_2$ concentrations were fitted with a two-ligand activity-coefficient model and the formation constant for the most abundant binding site ($K_1$) was fixed at $10^{5.9}$. This value for $K_1$ was chosen because it was intermediate between $K_1$ values obtained in the initial curve-fitting procedure for the titrations with $Ca(NO_3)_2$ as background electrolyte and because it was also the $K_1$ value obtained in the curve fitting of all other titrations (table 1). With $K_1$ so constrained, the decrease in $L_1$ from the $10^{-5}$ M $Ca(NO_3)_2$ and no background electrolyte titrations to the $10^{-3}$ and $10^{-2}$ M $Ca(NO_3)_2$ titrations is about twofold (table 2). With the exception of the $10^{-5}$ M $Ca(NO_3)_2$ titration, minimal change occurred in the quality of the curve fit associated with fixing $K_1$ at $10^{5.9}$.

## SPECTROPHOTOMETRIC STUDIES

Additional evidence for an effect of calcium concentration on copper complexation was obtained by measuring the absorbance in the ultraviolet region of the spectra of solutions of copper and fulvic acid from the Suwannee River with $CaCl_2$ concentrations of as much as $10^{-2}$ M. As shown by Wershaw and others (1983) for fulvic acid from the Suwannee River and by Piotrowicz and others (1984) for fulvic acid from marine sources, solutions of copper and fulvic acid have a peak within the wavelength range from 240 to 245 nm that is associated with a copper/carboxylate charge-transfer band. Although this peak

**Table 2.** Effect of calcium nitrate as background electrolyte on copper complexation by fulvic acid from the Suwannee River

[V, quality of fit measurement; $\gamma_{Cu^{2+}}$, activity coefficient of cupric ion; mol/mgC, moles per milligram of carbon in fulvic acid from the Suwannee River. See text for definition of other terms]

| Molar calcium concentration | V | $\gamma_{Cu^{2+}}$ | $L_1$ (mol/mgC) | $^1K_1$ | $L_2$ (mol/mgC) | $K_2$ |
|---|---|---|---|---|---|---|
| $10^{-2}$ | 9.1 | 0.62 | $7.2\times10^{-7}$ | 5.9 | $1.3\times10^{-7}$ | 7.7 |
| $10^{-3}$ | 6.2 | .53 | $8.7\times10^{-7}$ | 5.9 | $1.2\times10^{-7}$ | 7.4 |
| $10^{-4}$ | 8.7 | .59 | $1.2\times10^{-6}$ | 5.9 | $2.1\times10^{-7}$ | 7.5 |
| $10^{-5}$ | 20 | .53 | $1.6\times10^{-6}$ | 5.9 | $3.8\times10^{-7}$ | 8.0 |

$^1K_1$ was fixed at $10^{5.9}$ for all curve-fitting calculations.

is present consistently in solutions of copper and fulvic acid, it is difficult to analyze the spectrophotometric data quantitatively because aggregation of fulvic acid causes scattering interference in the spectra (Wershaw and others, 1983). The extent of aggregation appears to increase with the concentration of added copper and with the time after copper addition.

Despite these potential difficulties, similar spectra generally were obtained for differing KCl concentrations. However, comparison of spectra for solutions with different $CaCl_2$ concentrations and constant copper and fulvic-acid concentrations indicates a trend of decreasing peak height with increasing calcium concentration for copper concentrations less than or equal to the apparent equivalence point of the potentiometric titrations. An example of this trend is shown in figure 3 for a total copper concentration of $5\times10^{-5}$ M in solutions of fulvic acid from the Suwannee River containing carbon concentrations of 25 mg/L.

This result is consistent with the results of the potentiometric titrations. However, the decrease in peak height may indicate a change in absorptivity or energetics of the copper/fulvic-acid charge-transfer complex (change in $K_1$) or a competition between calcium and copper for fulvic-acid binding sites (equation 2) with a lesser concentration of CuL at greater calcium concentrations.

## COMPARISON OF EXPERIMENTAL RESULTS TO HYPOTHESES

The experiments presented in this study were designed to test two hypotheses: (1) The apparent formation constant for site-specific binding of copper by fulvic acid from the Suwannee River decreases with increasing counter-ion concentration, and (2) the activity coefficient for copper is less than unity because of territorial binding of copper by fulvic acid. The concentration of ionized, acidic functional groups in solution can be estimated from the concentration of carboxylic-acid functional groups for the fulvic acid from the Suwannee River (about 6.0 mmol/g) (Thurman and Malcolm, 1983; Bowles and

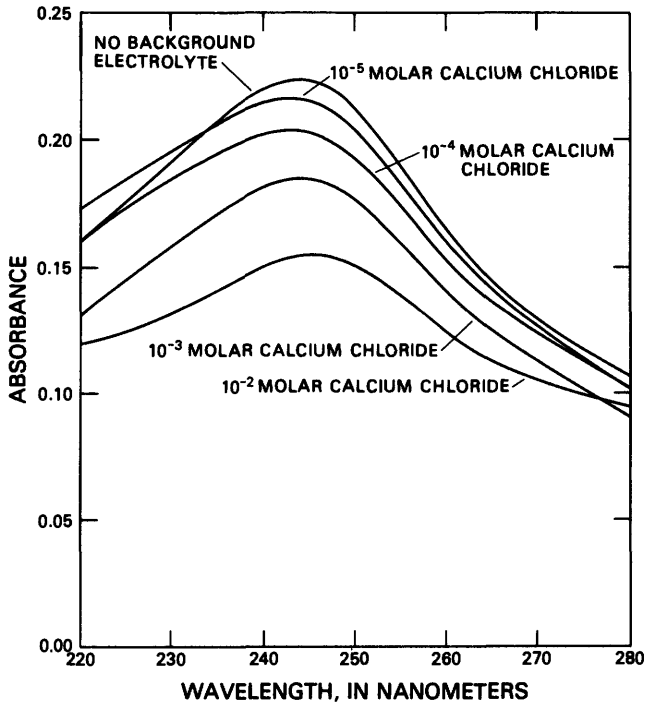


**Figure 3.** Ultraviolet spectra for solutions of fulvic acid from the Suwannee River with $5\times10^{-5}$ molar copper concentration and variable concentrations of calcium chloride as background electrolyte.

others, chap. L, this volume) and the dissolved-organic-carbon concentrations (25 mg/L as carbon): a value of $3\times10^{-4}$ M is thus obtained. This value is comparable to the total copper concentrations at the end of the titrations, where the displacement of titration curve relative to the line defined by $p\{Cu^{2+}\} = pCu_T$ is readily observed. Because the concentration of ionized carboxylic-acid functional groups is greater than the difference between the total copper added and the cupric-ion activity for this upper part of the titration curve, the data can be interpreted as being consistent with the second hypothesis. Analysis of the potentiometric data using FITEQL indicates that the activity coefficient for territorial binding of copper under these experimental conditions ($\gamma_{Cu2+}$) is in the range of 0.4 to 0.6. In summary, we conclude that the behavior of fulvic acid from the Suwannee River is consistent with the non-site-specific territorial-binding aspect of Manning's (1979, 1981) theory.

In terms of the first hypothesis, however, the experimental results are more ambiguous. For potassium as the counter ion, there is no evidence of a decrease in apparent formation constant with increasing counter-ion concentration, and there is no evidence of any other effect on copper complexation. For calcium as the counter ion, there is an overall decrease in copper complexation with increasing calcium concentration (fig. 1). However, as will be discussed later in this section, the nature of the effect is not well described by Manning's (1979, 1981) theory or by simple direct competition of calcium for the binding sites.

In equation 1, from Manning's (1979, 1981) theory, if $\alpha$ is assumed to be independent of $C_s$ (the counter-ion concentration), a one-to-one, log-linear relation is predicted between $C_s$ and the apparent formation constant. However, the results of curve-fitting calculations (table 1) do not indicate any changes in the apparent constant ($K_1$) of the most abundant ligand ($L_1$) that are comparable in magnitude to the changes in $C_s$.

Therefore, the results for both potassium and calcium lead to the conclusion that, although there is evidence for territorial binding, territorial binding does not affect site-specific binding as it would if the fulvic acid from the Suwannee River were a true polyelectrolyte and not an oligoelectrolyte. The major reason for such a deviation probably is that the infinite linear-lattice model is inappropriate, given the mole cular weight range of 700 to 1,000 daltons for fulvic acid from the Suwannee River. In that range, it is likely that "end effects"—the effects of the actual position of the charged functional groups within the molecule—would determine the extent to which the presence of territorially bound counter ions affected site-specific metal binding (G.S. Manning, Rutgers University, oral commun., 1983). Further, for such an oligoelectrolyte that has only 10 or fewer ionized functional groups per molecule, the relation between the position of the ionized groups and the ionic radius of the counter ion may also become a critical factor. The differences in the effects of potassium and calcium may be related to the greater ionic radius of potassium as well as the differences in valency. It is also probable that $\alpha$ (the degree of ionization of the oligoelectrolyte) may have changed with changing calcium concentration.

The effects of calcium on copper complexation are not consistent with simple competition between calcium and copper for the specific metal-binding sites. Two series of titration curves calculated using MINEQL with two assumed values ($10^{3.6}$ and $10^{4.6}$) for the formation constant of the calcium/fulvic-acid complex (Mantoura and others, 1978) are presented in figure 4. The results of these calculations indicate that, for a simple competitive interaction, no measurable copper complexation will occur at a calcium concentration of $10^{-2}$ M for $\log K_{CaL} = 3.6$, or at calcium concentrations of $10^{-2}$ and $10^{-3}$ M for $\log K_{CaL} = 4.6$—however, experimental results indicated substantial copper complexation at calcium concentrations of $10^{-2}$ M and did not show a substantial change between calcium concentrations of $10^{-3}$ and $10^{-2}$ M (fig. 1).

When calculating the curves presented in figure 4, it was assumed that all metal-binding sites were identical: the formation constant for copper binding ($\log K_{CuL}$) was set to 6.0 in both parts *A* and *B* of the figure, whereas the formation constant for calcium binding ($\log K_{CaL}$) was set to 3.6 in figure 4*A* and to 4.6 in figure 4*B*. By assuming instead that the binding sites are not identical (i.e., one-half of the sites can bind copper and calcium and the other one-half can bind only copper), it is possible to reproduce the general aspects of the experimental effect of calcium on copper complexation as indicated by calculated titration curves in figure 5. These calculations indicate that the relative position of the $10^{-3}$ and $10^{-4}$ M

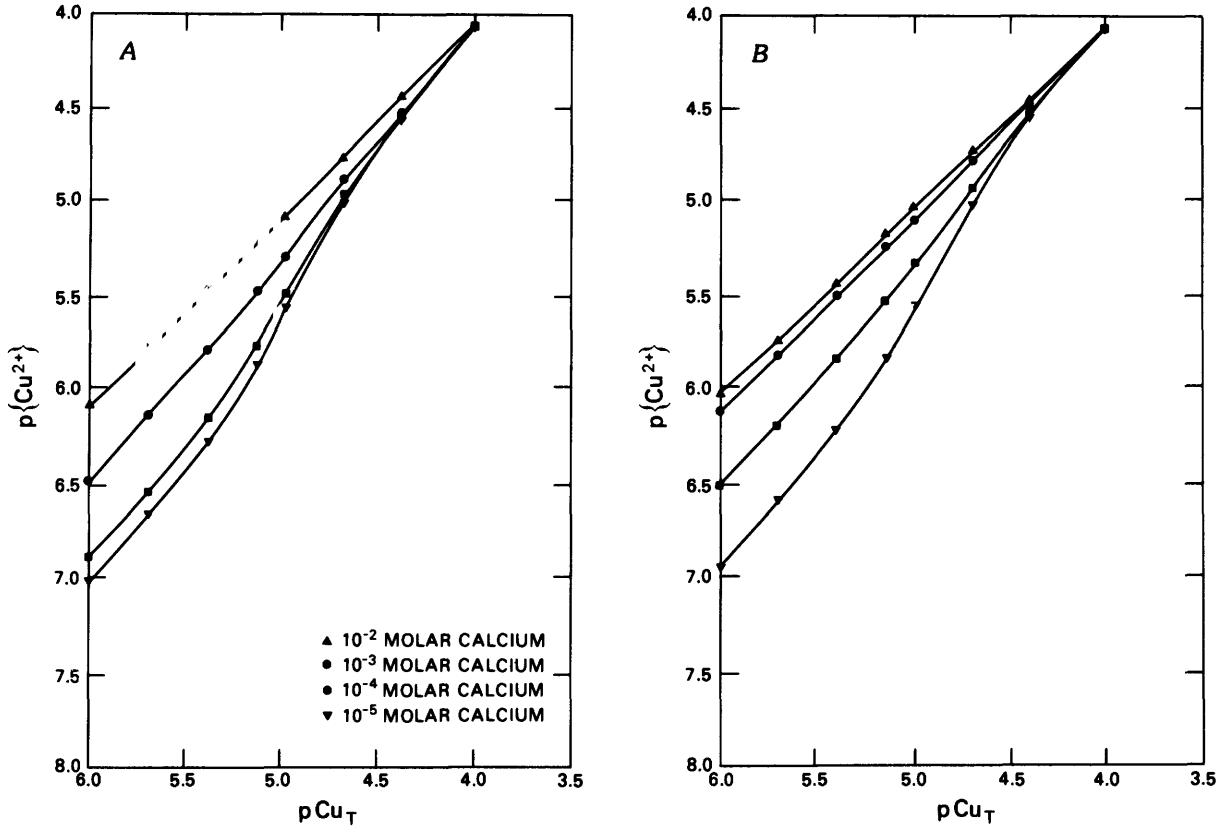


**Figure 4.** Calculated titration curves for solutions of fulvic acid showing competitive interaction between calcium and copper for a total ligand concentration, $L_T$, of $10^{-5}$ molar; log $K_{CuL}$ = 6.0. *A*, log $K_{CaL}$ = 3.6; *B*, log $K_{CaL}$ = 4.6.

curves for calcium is dependent on the assumption for log $K_{CaL}$.

The general similarity between the experimental data in figure 1 and the calculated data in figure 5 is only an indication that differences in calcium affinity among sites and competition between calcium and copper are responsible for the experimental results—it is not a verification of the exact assumptions used in the calculations for figure 5. It is likely that a closer fit to the experimental data could be obtained by assuming a continuous distribution of binding constants for calcium binding. It may be that differences in the secondary structure (i.e., the conformation or spatial arrangement) of fulvic-acid molecules result in differences in calcium affinity. Calcium has a larger ionic radius than copper, and it may be that binding sites exist that are not accessible to calcium ions but are accessible to copper ions. Conversely, it may be that the experimental results are a reflection of differences in the primary structure of the metal-binding sites (i.e., those comprised of different functional groups). Calcium forms strong complexes with functional groups containing oxygen, whereas copper forms strong complexes with functional groups containing oxygen, sulfur, and nitrogen (Nieboer and Richardson, 1980). It may be that the copper-binding sites with little calcium affinity involve functional groups containing sulfur or nitrogen. In either instance, results for the effect of calcium on copper complexation are evidence for the importance of structural heterogeneity within the fulvic-acid molecular mixture. Although several models for complexation of metal ions and fulvic acid have been developed based on the assumption of structural heterogeneity, little direct experimental evidence for its importance has been presented previously.

## CONCLUSIONS

The experiments presented in this study were designed to test hypotheses developed from the theory of counter-ion condensation for polyelectrolytes that has been proposed by Manning (1979, 1981).

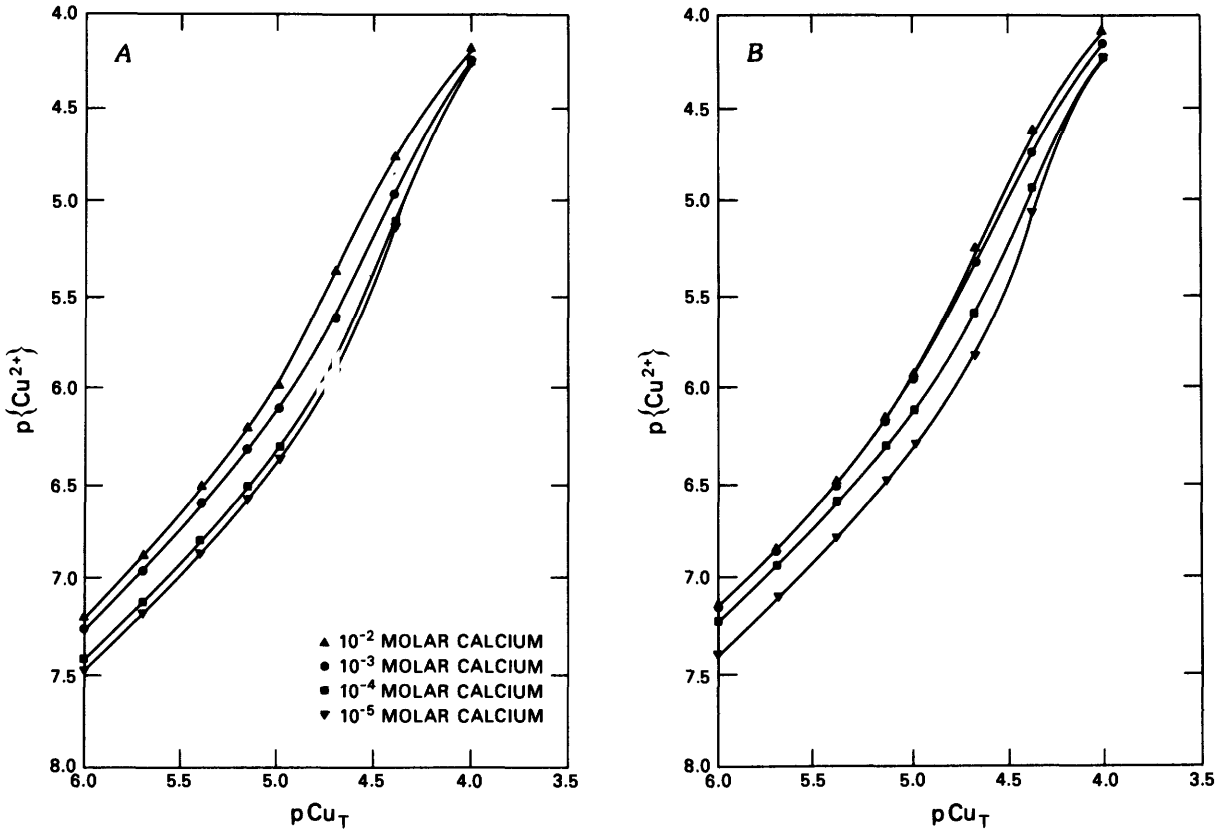


**Figure 5.** Calculated titration curves for solutions of fulvic acid showing competitive interaction between calcium and copper assuming two types of ligands (log $K_{CuL_a}$ = log $K_{CuL_b}$ = 5.9) and a ligand concentration of $1.8 \times 10^{-5}$ molar. No competitive interaction with calcium for $L_a$ is assumed. *A*, log $K_{CaL_b}$ = 3.6; *B*, log $K_{CaL_b}$ = 4.6.

The experimental results provide support for the hypothesis of territorial binding of counter ions by fulvic acid from the Suwannee River by indicating that the activity coefficient for copper ions in solution with fulvic acid is less than unity (it is in the range of $\gamma_{Cu^{2+}}$ = 0.4 to 0.6). However, the experimental results do not provide support for the hypothesis that increased counter-ion concentrations result in a decrease in apparent formation constants. Instead, experimental results indicate that the effects or lack of effects of counter-ion concentration on site-specific binding of copper by fulvic acid are due to primary or secondary structural aspects of the fulvic-acid molecular mixture. Therefore, a more general conclusion of this experimental work is that advances in the understanding of the structure or structures of aquatic fulvic acid are critical to the understanding of geochemical processes involving aquatic fulvic acid. A "black box" structural model is not sufficient to interpret the results presented in this study.

## REFERENCES CITED

Cabaniss, S., Shuman, M.S., and Collins, B.J., 1984, Metal-organic binding—A comparison of models, *in* Kramer, C.J.M., ed., Complexation of Trace Metals in Natural Waters: Amsterdam, The Hague, Martinus Nijhoff/W. Junk, p. 165–179.

Dzombak, D.A., Fish, William, and Morel, F.M.M., 1986, Metal-humate interactions. 1. Discrete ligand and continuous distribution models: Environmental Science and Technology, v. 20, no. 7, p. 669–675.

Gamble, D.S., Underdown, A.W., and Langford, C.H., 1980, Copper (II) titration of fulvic acid ligand sites with theoretical potentiometric and spectrophotometric analysis: Analytical Chemistry, v. 52, no. 12, p. 1901–1908.

MacCarthy, Patrick, and Smith, G.C., 1979, Stability surface concept—A quantitative model for complexation in multiligand mixtures, *in* Jenne, E.A., ed., Chemical Modeling in Aqueous Systems: American Chemical Society, p. 201–222.

Manning, G.S., 1979, Counterion binding in polyelectrolyte theory: Accounts of Chemical Research, v. 12, p. 413–449.

———1981, Limiting laws and counterion condensation in polyectrolyte solutions. 6. Theory of the titration curve: Journal of Physical Chemistry, v. 85, no. 7, p. 870–877.

Manning, G.S., and Zimm, B.H., 1965, Cluster theory of polyelectrolyte solutions. I. Activity coefficients of the mobile ions: Journal of Physical Chemistry, v. 43, no. 12, p. 4250–4259.

Mantoura, R.F.C., Dickson, A., and Riley, J.P., 1978, The complexation of metals with humic materials in natural waters: Estuarine Coastal Marine Science, v. 6, p. 387–408.

Marinsky, J.A., Gupta, S., and Schindler, P., 1982, A unified physicochemical description of the equilibria encountered in humic acid gels: Journal of Colloidal and Interface Science, v. 89, no. 2, p. 412–426.

Matsunaga, K., Negushi, M., and Fukase, S., 1980, Chemical species of copper in the River Shukunohe and the Lake Ohnuma waters in Japan: Geochimica et Cosmochimica Acta, v. 44, p. 1615–1619.

McKnight, D.M., Feder, G.L., Thurman, E.M., Wershaw, R.L., and Westall, J.C., 1983, Complexation of copper by aquatic humic substances from different environments: Science of the Total Environment, v. 28, p. 65–76.

Nieboer, E., and Richardson, D.H., 1980, The replacement of the nondescript term "heavy metals" by a biologically and chemically significant classification of metal ions: Environmental Pollution Series B, v. 1, p. 3–26.

Perdue, E.M., and Lytle, C.R., 1983, A critical examination of metal-ligand complexation models—Application to define multi-ligand mixtures, *in* Christman, R.F., and Gjessing, E.T., eds., Aquatic and Terrestrial Humic Materials: Ann Arbor, Michigan, Ann Arbor Press, p. 295–313.

Piotrowicz, S.R., Harvey, G.R., Boran, D.A., Weisel, C.P., and Springer-Young, M., 1984, Cadmium, copper, and zinc interactions with marine humus as a function of ligand structure: Marine Chemistry, v. 14, p. 333–346.

Ryan, D.K., and Weber, J.H., 1982, Copper (II) complexing capacities of natural waters by fluorescence quenching: Environmental Science and Technology, v. 16, no. 6, p. 866–872.

Saar, R.A., and Weber, J.H., 1980, Comparison of spectrofluorometry and ion-selective electrode potentiometry for determination of complexes between fulvic acid and heavy-metal ions: Analytical Chemistry, v. 52, no. 13, p. 2095–2100.

Shuman, M.S., Weber, J.H., and Cabaniss, S.E., in press, Experimental methods for studying metal-humic coordination, *in* MacCarthy, Patrick, Hayes, M.H.B., Swift, R.S., and Malcolm, R.L., Humic Substances. III. Interactions with Metals, Minerals, and Organic Chemicals: Chichester, England, John Wiley.

Shuman, M.S., and Woodward, G.P., Jr., 1973, Chemical constants of metal complexes from a complexometric titration followed with anodic stripping voltametry: Analytical Chemistry, v. 45, no. 12, p. 2032–2035.

———1977, Stability constants of copper-organic chelates in aquatic samples: Environmental Science and Technology, v. 11, no. 8, p. 809–813.

Thurman, E.M., and Malcolm, R.L., 1983, Structural study of humic substances—New approaches and methods, *in* Christman, R.F., and Griessing, E.T., eds., Aquatic and Terrestrial Humic Materials: Ann Arbor, Michigan, Ann Arbor Science, p. 1–25.

Truitt, R.E., and Weber, J.H., 1981, Determination of complexing capacity of fulvic acid for copper (II) and cadmium (II) by dialysis titration: Analytical Chemistry, v. 53, no. 2, p. 337–342.

Van den Berg, C.M.G., and Kramer, J.R., 1979, Determination of complexing capacities of ligands in natural waters and conditional stability constants of the copper complexes by means of manganese dioxide: Analytica Chimica Acta, v. 106, p. 113–120.

Wershaw, R.L., McKnight, D.M., and Pinckney, D.J., 1983, The speciation of copper in the natural water systems. I. Evidence of the presence of a copper (II)-fulvic acid charge transfer complex, *in* Schallinger, K.M., ed., Peat in Agriculture and Horticulture: Jerusalem, Hebrew University, Proceedings of Second International Symposium, p. 205–221.

Westall, J.C., 1982, FITEQL, a program for the determination of chemical equilibrium constants from experimental data: Corvallis, Oregon State University, Department of Chemistry, Technical Report 18, 101 p.

Westall, J.C., Zachary, J.L., Morel, F.M.M., 1976, MINEQL, a computer based program for the calculation of chemical equilibrium composition of aqueous systems, *in* Parsons, R.M., laboratory technician, Water Quality Note 18: Cambridge, Massachusetts Institute of Technology, 91 p.

Wilson, D.E., and Kinney, P., 1977, Effects of polymeric charge variations on the proton–metal ion equilibria of humic materials: Limnology and Oceanography, v. 22, no. 2, p. 281–289.

Chapter E

# Occurrence and Distribution of Selected Trace Metals in the International Humic Substances Society's Standard and Reference Fulvic and Humic Acids Isolated from the Suwannee River

*By* H.E. Taylor *and* J.R. Garbarino

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## Abstract

The occurrence and distribution of trace metals in the International Humic Substances Society's standard and reference fulvic and humic acids isolated from the Suwannee River were determined by using *inductively coupled plasma–mass spectrometry*. The determination of trace metals in these fulvic and humic acids was based on mass-spectral scans from 5 to 240 daltons. The concentrations of all identified trace metals were estimated semiquantitatively by using a single-standard calibration. Trace metals having semiquantitative concentrations greater than 5 micrograms per gram in at least three of the four samples were: Ag, Al, B, Ca, Fe, Mo, Na, Sn, Th, and Zr. Isotope-dilution analysis was used to accurately determine the concentrations of Ba, Cd, Cu, Ni, Pb, and Sr. Trace-metal concentrations in the humic acids generally were greater than those in the fulvic acids. Results of this study may be important in experiments that use these humic substances. However, the presence of trace metals in these substances may be a result of processing procedures and does not reflect trace-metal/humic-substance interactions associated with natural aquatic systems.

## INTRODUCTION

Naturally occurring trace organic compounds have been isolated from samples collected from the Suwannee River, Georgia, by adsorption on XAD–8 acrylic-ester resins after filtration to remove particulate matter (Thurman and Malcolm, 1981; Aiken, 1985; Malcolm and others, chap. B, this volume). These compounds were eluted from the XAD–8 resin by using sodium hydroxide and separated into fulvic- and humic-acid fractions by precipitating humic acid under acidic conditions (Thurman and Malcolm, 1981; Leenheer and Noyes, 1985; Malcolm and others, chap. B, this volume). After the separation, the fulvic and humic acids were concentrated by freeze-drying techniques.

The trace-metal composition of the fulvic and humic acids is an important aspect of their total chemical characterization. Even though metals associated with these fulvic and humic acids do not reflect the natural aquatic system, their identification and quantification may be important in future studies that use these materials. Because the concentration of these fulvic and humic acids in natural surface waters is small (1 to 30 mg/L), it is difficult to isolate each fraction in quantities greater than 1 milligram—this requires that all trace-metal analyses be performed on a micro scale. Inductively coupled plasma–mass spectrometry was selected as the analytical method of choice because of its operating characteristics, which include: (1) rapid, multielement analysis capability using only microliter quantities of sample, (2) high sensitivity (Gray, 1985), (3) the ability to detect almost all trace metals and to quantify their concentration (Date and Gray, 1983), and (4) the ability to perform multiple isotope-dilution quantitation of several, selected trace metals (Garbarino and Taylor, 1987).

## EXPERIMENTAL CONDITIONS

### Sample Preparation

Samples of fulvic and humic acids that were obtained by filtration of untreated surface water through both silver-membrane and vinyl-metricel-membrane filters were identified as "standard stream" and "reference stream" samples, respectively, by the International Humic Substances Society. Carefully weighed 50-mg quantities of each freeze-dried sample were placed into nitric-acid-leached, 1-cm by 10-cm quartz test tubes. Two milliliters of double-deionized water and 100 µL of ultra-high-purity nitric acid were added to the samples.

Acid oxidation alone did not completely dissolve all of the organic matter in the fulvic- and humic-acid samples. Therefore, each sample was exposed overnight to 253.7-nm ultraviolet radiation. Acid oxidation combined with photodecomposition completely dissolved both fulvic-acid samples; however, substantial quantities of undissolved organic matter remained in the humic-acid samples. This residue was dissolved completely after adding five drops of 30-percent hydrogen peroxide and heating the solution for a short time. After total dissolution, each solution was quantitatively transferred to a 5-mL volumetric flask and diluted to volume. Two reagent blanks also were prepared using processes identical to those used in preparation of the fulvic- and humic-acid samples.

**Table 1.** Inductively coupled plasma–mass spectrometric operating conditions

| | |
|---|---|
| **Inductively coupled plasma** | |
| Incident power | 1.2 kilowatts |
| Plasma flowrate | 13 liters per minute |
| Nebulizer flowrate | 0.55 liter per minute |
| Auxillary flowrate | 1.4 liters per minute |
| **Quadrupole mass spectrometer** | |
| **Ion Optics** | **Voltage (direct current)** |
| Barrel lens | 6.3 |
| Einzel lenses | -14.4 |
| Plate lens | -13.8 |
| Stop lens | -2.6 |

| **Data acquisition parameters** | Isotope-dilution analysis | Semiquantitative analysis |
|---|---|---|
| Resolution | Low | Low |
| Measurements per peak | 3 | 10 |
| Measurement time (seconds) | 0.5 | 0.1 |
| Replicates per block | 5 | 1 |

## Instrumentation and Analytical Procedures

A SCIEX model 250 ELAN inductively coupled plasma–mass spectrometer fitted with a fritted-disk pneumatic nebulizer was used to perform all analytical measurements. The fritted-disk nebulizer was selected to allow slow sample uptake rates (20 µL/min) for sample conservation while maintaining sensitivity (Layman and Lichte, 1982). Simplex optimization techniques were used to establish optimum operating conditions (Taylor and Garbarino, 1985; Garbarino and Taylor, 1987). Instrument operating conditions are specified in table 1.

Two modes of instrument operation were utilized: (1) Multielement analysis using elemental mass–peak intensities calibrated with standard mixed-element solutions to quantify concentrations (Taylor, 1986), and (2) isotope-ratio measurements of selected elemental isotopes used to perform isotope-dilution determinations (Taylor and Garbarino, 1985; Taylor, 1989). Differences in operating conditions between each mode of operation are outlined in table 1. All calibration standards were prepared gravimetrically from high-purity metals or metal salts dissolved in double-deionized water and acidified with high-purity nitric acid. Isotopically enriched, pure elements and compounds were obtained from Oak Ridge National Laboratory, Oak Ridge, Tennessee, and were prepared in a similar fashion. The natural and enriched abundance ratios for the isotopes used are listed in table 2.

Initially, each sample was analyzed by using a rapid mass-spectral scan for the following ranges: 5–12, 23–28, 43–78, and 82–240 daltons. In less than 4 minutes, a scan of these ranges permits determination of most of the elements in the periodic chart. Discontinuities in the mass range correspond to regions in the spectra where there are intense molecular-ion interferences. The mass-spectral scan was used to semiquantitatively determine the concentration of selected elements by analyzing a multi-element standard immediately after the sample. The multielement standard contained each of the elements listed in table 3 at concentrations based on preliminary analyses of the fulvic- and humic-acid samples. Concentrations of the elements in the fulvic- and humic-acid samples were calculated on a proportional basis.

The second analytical approach was based on multielement *isotope-dilution analysis*. Isotope-dilution analysis was used to accurately determine

**Table 2.** Enriched isotopes used for isotope-dilution measurements

| Isotopes | Natural abundance ratio | Enriched abundance ratio |
|---|---|---|
| $^{135}Ba/^{138}Ba$ | 0.092 | 3.4 |
| $^{116}Cd/^{114}Cd$ | .27 | 45.1 |
| $^{63}Cu/^{65}Cu$ | .45 | 321 |
| $^{61}Ni/^{60}Ni$ | .048 | 14.5 |
| $^{86}Sr/^{88}Sr$ | .12 | 45 |
| $^{206}Pb/^{208}Pb$ | .48 | 3,325 |

the concentrations of Ba, Cd, Cu, Pb, and Sr in all fulvic- and humic-acid samples. The selection of these analytes was based on preliminary semiquantitative analysis of the fulvic and humic acids and the availability of stable, enriched isotopes. The technique is based on the spike addition of a known quantity of isotopically enriched standard and the determination of three isotope-ratio measurements. The isotope ratios for the analyte in the original sample, in the isotopically enriched standard, and in the spiked sample are the only measurements needed. The ratio for the isotopically enriched standard was determined only once. The data obtained from the mass-spectral scans were used to verify that analyte ratios for the samples were consistent with natural abundances. Therefore, only a single isotope-ratio

**Table 3.** Semiquantitative concentrations of selected elements determined by standard calibration

[Values in micrograms per gram]

| Element | Standard fulvic acid | Reference fulvic acid | Standard humic acid | Reference humic acid |
|---|---|---|---|---|
| Ag | 9 | 3 | 200 | 20 |
| Al | 30 | 20 | 200 | 100 |
| B | 200 | 80 | 300 | 200 |
| Ba | .3 | .4 | .1 | .2 |
| Ca | 100 | 90 | 90 | 200 |
| Cd | <.1 | <.1 | <.1 | <.1 |
| Cr | 4 | 2 | 10 | 10 |
| Cu | 3 | 2 | 10 | 4 |
| Fe | 20 | ND[1] | 400 | 200 |
| Hg | 1 | <.1 | 20 | 20 |
| Mg | 2 | 6 | 4 | 4 |
| Mo | 5 | 2 | 30 | 20 |
| Na | 50 | 60 | 40 | 100 |
| Ni | 1 | 2 | 10 | 8 |
| Pb | 4 | 5 | 2 | 3 |
| Sc | 3 | 1 | 2 | 3 |
| Sn | 10 | 20 | 20 | 20 |
| Sr | .4 | .4 | .1 | .1 |
| Th | 10 | 20 | 7 | 6 |
| Ti | 1 | 1 | 10 | 10 |
| Zn | 3 | 3 | 4 | 4 |
| Zr | 7 | 6 | 90 | 80 |

[1]Not determined, less than blank concentration.

measurement was required for each sample—that corresponding to the spiked sample itself. The isotope ratios measured were: $^{135}Ba/^{138}Ba$, $^{116}Cd/^{114}Cd$, $^{65}Cu/^{63}Cu$, $^{61}Ni/^{60}Ni$, $^{206}Pb/^{208}Pb$, and $^{86}Sr/^{88}Sr$. Isobaric corrections were made for isotopic contributions of La and Ce on Ba, Sn on Cd, and Kr on Sr. Analyte concentrations are determined most accurately when the spike addition to the sample results in an isotope ratio of unity. Therefore, the addition is based on previous estimates of the concentration in the sample, which, in this case, are the semiquantitative results obtained from mass-spectral scans. The equation used to calculate analyte concentrations (C, in nanograms per milliliter), based on isotope dilution is:

$$C = (R_e - R_m)/(R_m - R_n) \cdot (\Sigma R_n/\Sigma R_e) \cdot A_n/A_e \cdot W/V \quad (1)$$

where

$R_e$ is the isotope ratio for isotopically enriched standard,

$R_m$ is the isotope ratio for spiked sample,

$R_n$ is the isotope ratio for original sample,

$\Sigma R_n$ is the ratio sum of all isotopes for original sample,

$\Sigma R_e$ is the ratio sum of all isotopes for isotopically enriched standard,

$A_n$ is the atomic weight of element in original sample,

$A_e$ is the atomic weight of element in isotopically enriched standard,

W is the weight of spike addition (in nanograms), and

V is the volume of original sample (in milliliters).

The value used for $R_m$ was based on the mean of three blocks of data, each based on five replicate isotope-ratio measurements. The corresponding precision data are the standard deviation at the 95-percent confidence level.

## OCCURRENCE AND DISTRIBUTION OF TRACE METALS

Representative mass-spectral scans for the standard fulvic and humic acids are shown in figures 1 through 6. A scan of the standard fulvic acid from 43 to 78 daltons is shown in figure 1, from 82 to 130 daltons in figure 2, and from 130 to 240 daltons in figure 3. A scan of the standard humic acid from 43 to 78 daltons is shown in figure 4, from 82 to 130 daltons in figure 5, and from 130 to 240 daltons in figure 6. Intensity scales on the spectra are adjusted to provide a clear illustration of the differences in the concentrations of individual isotopes in the standard fulvic and humic acids. Isotope and molecular-ion identifications for all prominent mass-spectral peaks are provided in figures 1 through 3; only the isotopes used for quantitation are labeled in figures 4 through 6. Isotopes and molecular ions are listed in order of their contribution to the total ion intensity; those contributing most are listed at the top and those contributing the least at the bottom. Spectra for masses less than 43 daltons are not shown due to the confusing nature of the many molecular-ion peaks present from 5 to 43 daltons. Although isotopes of Ta and W were observed in the spectrum at masses of 181, 182, 183, 184, and 186 daltons, they were not quantified because of their presence at comparable concentrations in the reagent blank. All other elements are listed in table 3 as blank-subtracted concentrations. The accuracy of these concentrations is within ±20 percent of the concentration reported because of the method of standardization. It should be noted that appreciable quantities of Ag are present in the samples that were processed with the silver-membrane filters (table 3).

Interestingly, both the fulvic- and humic-acid samples contained measurable concentrations of Hf and Zr. These trace metals invariably occur together in nature; however, their presence in these samples has yet to be explained. Contamination through the digestion procedure was eliminated as a possible source because the reagent blanks showed no detectable concentrations of either element. One possible source of contamination is the stainless-steel apparatus employed in processing the samples—some forms of stainless steel contain a significant quantity of Zr. Further investigations are needed to evaluate other possible sources of contamination before these elements are definitely associated with the fulvic and humic acids.

The apparent concentrations of Hf in the fulvic and humic acids were estimated using other elemental standard intensities obtained during the analysis (standards for Hf were unavailable at the time of analysis). The intensities were adjusted appropriately for differences in isotopic abundances and were used to calculate the corresponding concentrations. Based on this method, the estimated concentration of Hf in

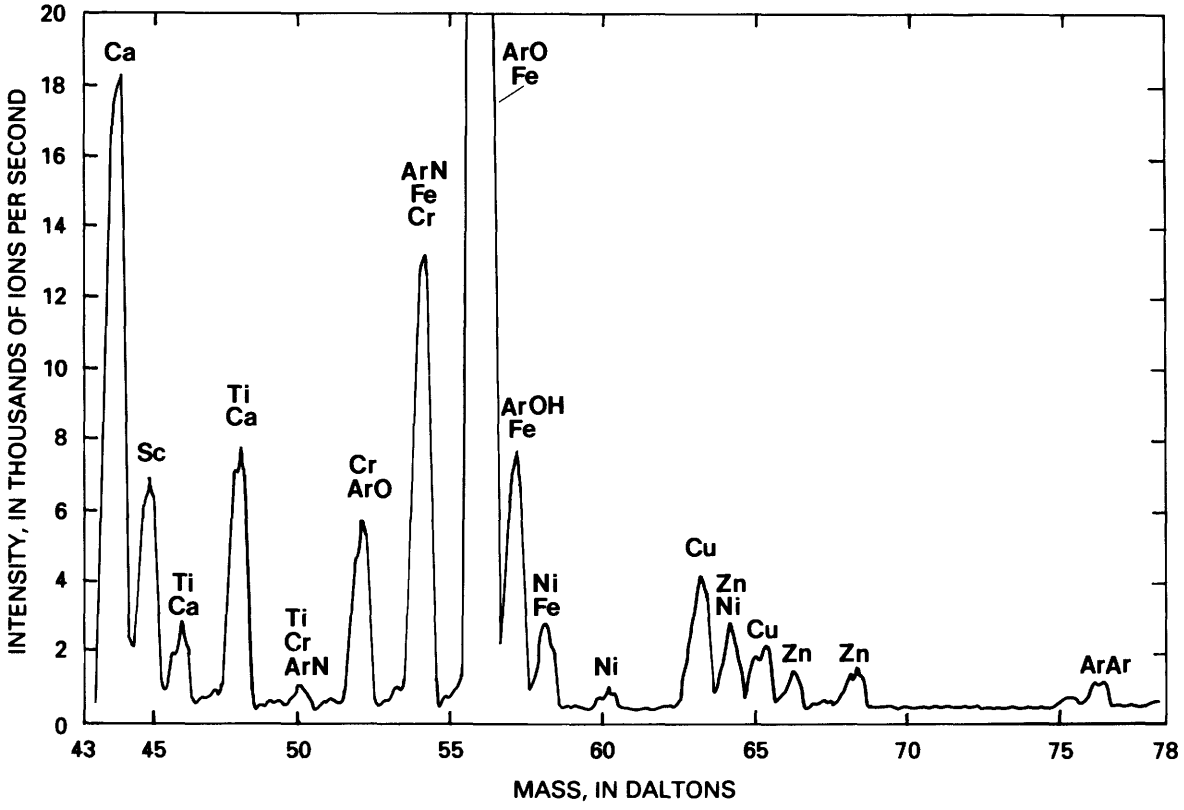


**Figure 1.** Mass-spectral scan for standard fulvic acid, range 43 to 78 daltons.

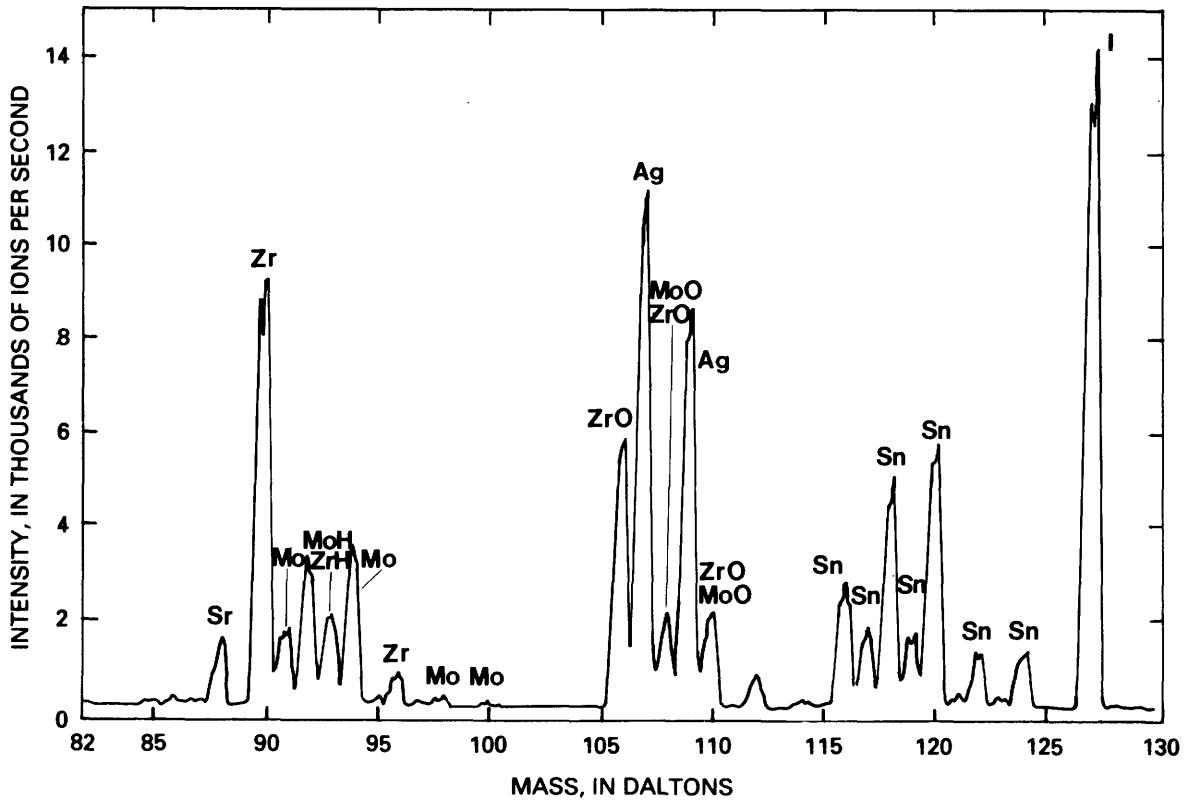


**Figure 2.** Mass-spectral scan for standard fulvic acid, range 82 to 130 daltons.

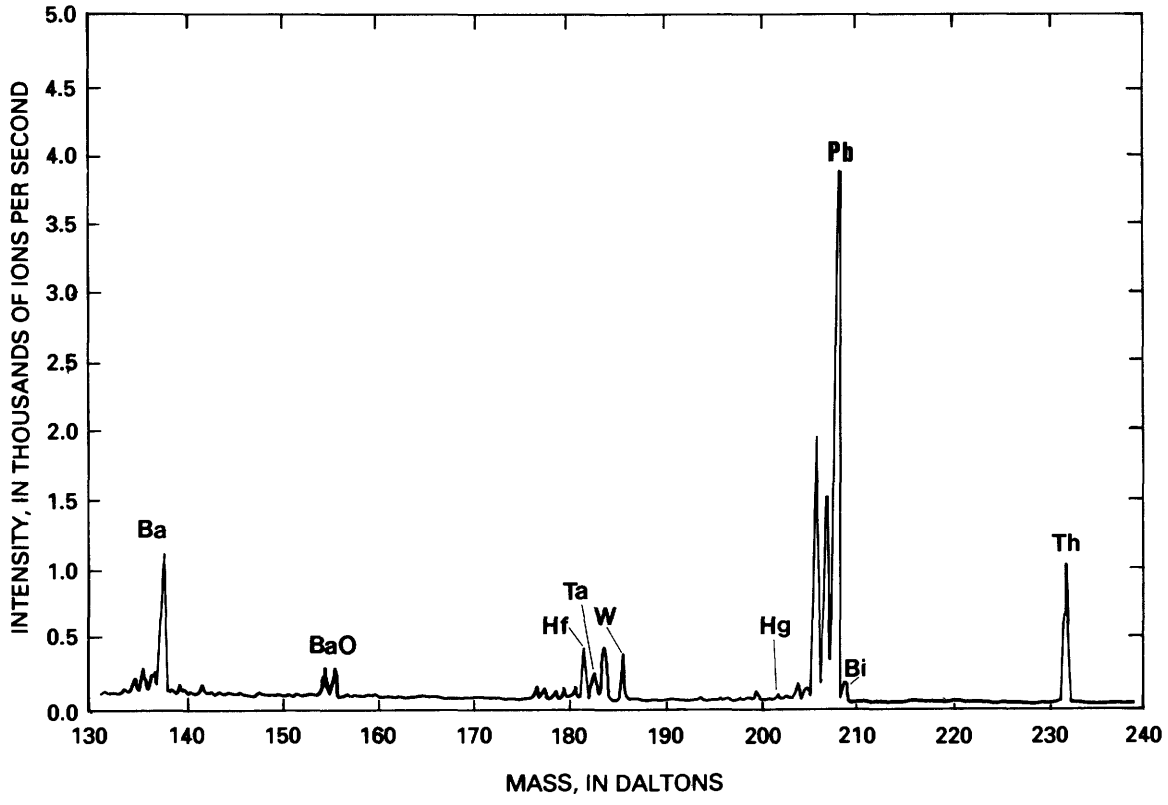


**Figure 3.** Mass-spectral scan for standard fulvic acid, range 130 to 240 daltons.

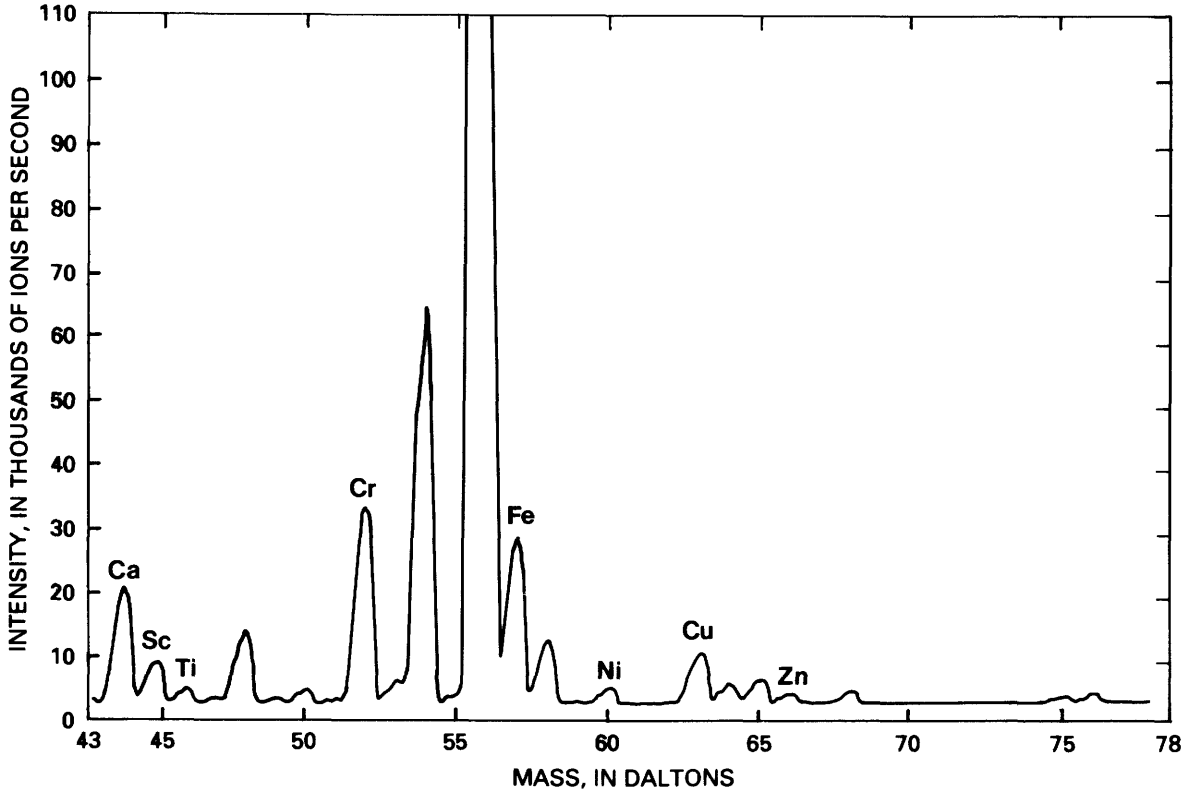


**Figure 4.** Mass-spectral scan for standard humic acid, range 43 to 78 daltons.

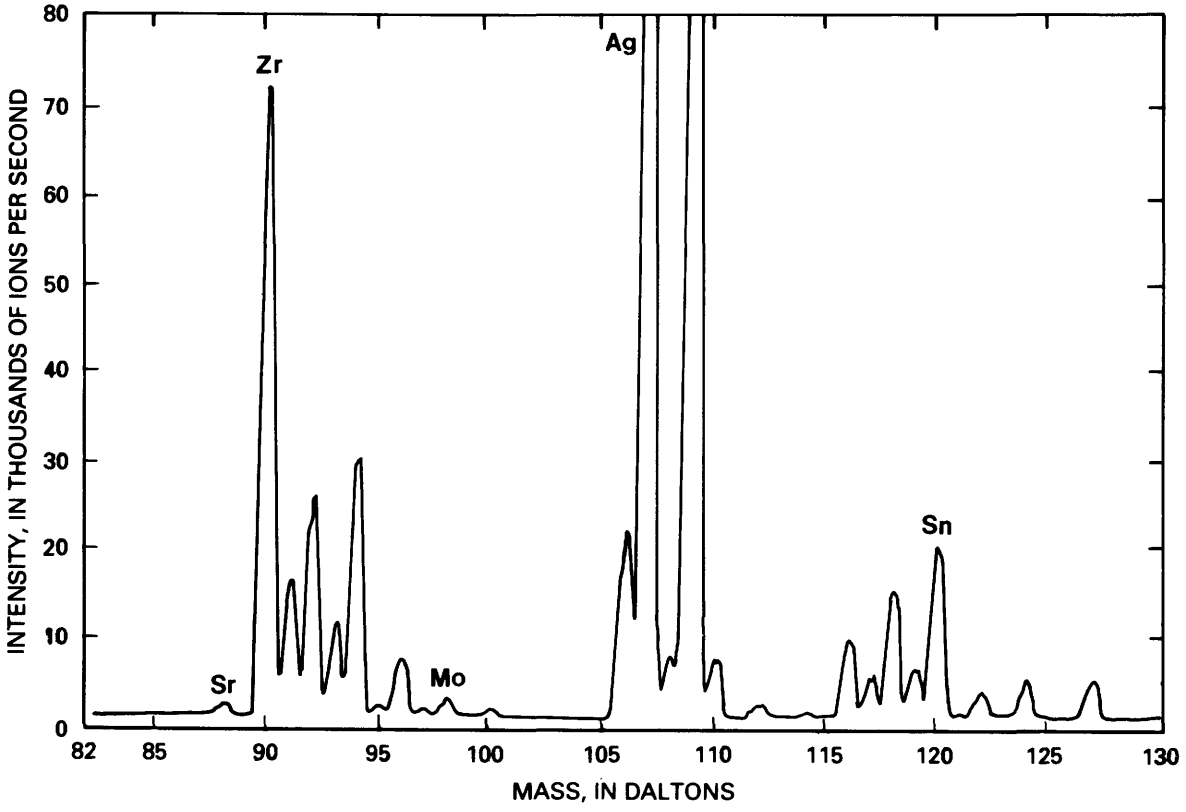


**Figure 5.** Mass-spectral scan for standard humic acid, range 82 to 130 daltons.

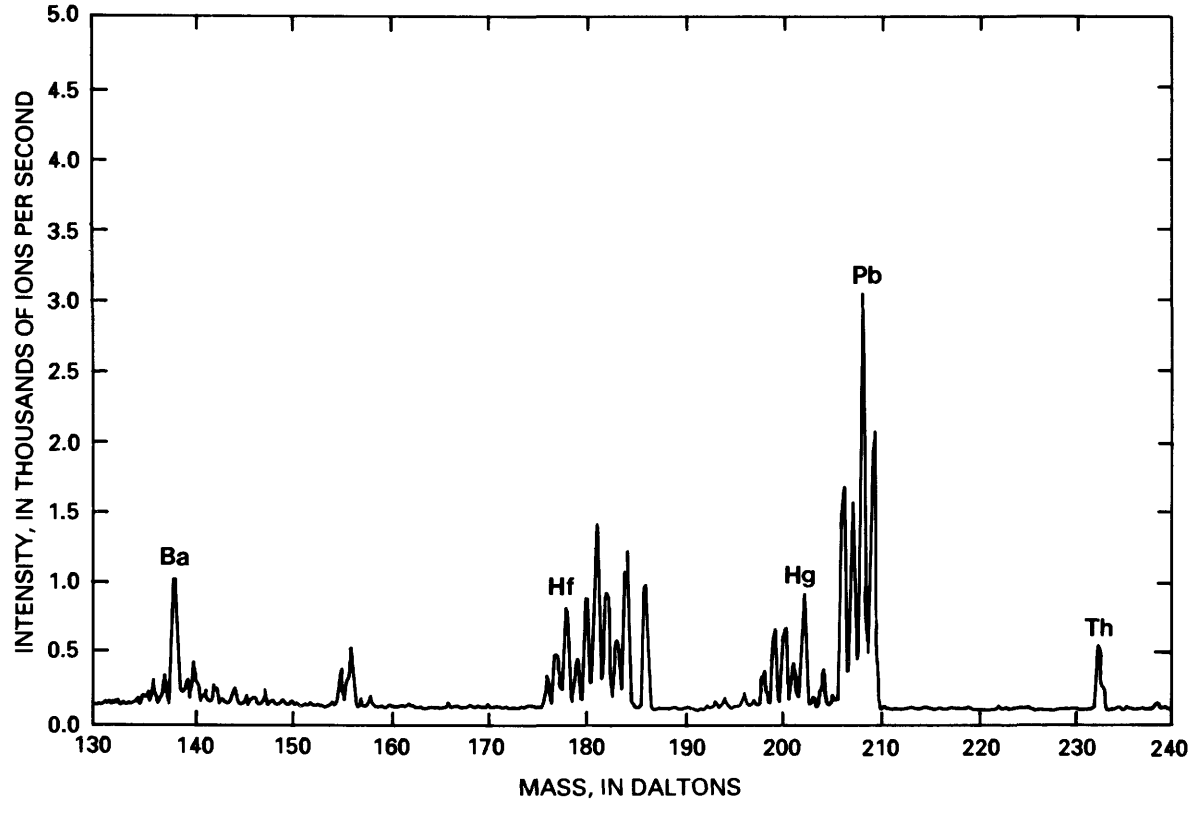


**Figure 6.** Mass-spectral scan for standard humic acid, range 130 to 240 daltons.

**Table 4.** Concentrations of selected trace elements determined by isotope-dilution analysis

[Values in micrograms per gram[1]]

| Element | Standard fulvic acid | Reference fulvic acid | Standard humic acid | Reference humic acid |
|---|---|---|---|---|
| Ba | 6.8 ± 0.8 | 7.8 ± 0.4 | 10.5 ± 2 | 3.9 ± 0.4 |
| Cd | 0.06 ± 0.02 | 0.09 ± 0.1 | 0.07 ± 0.04 | 0.14 ± 0.02 |
| Cu | 1.7 ± 0.08 | 1.7 ± 0.2 | 9.6 ± 0.8 | 5.0 ± 0.4 |
| Ni | 0.92 ± 0.04 | 1.6 ± 0.1 | 7.2 ± 0.2 | 5.4 ± 0.04 |
| Pb | 1.2 ± 0.06 | 1.8 ± 0.06 | 1.4 ± 0.04 | 1.6 ± 0.6 |
| Sr | 0.12 ± 0.002 | 0.21 ± 0.002 | 0.26 ± 0.04 | 0.83 ± 0.1 |

[1]Standard errors computed at 95-percent confidence level.

the fulvic acids was 0.1 µg/g and 2 µg/g for the humic acids. The semiquantitative concentrations of Zr averaged 6.5 µg/g in the fulvic-acid samples and 85 µg/g in the humic-acid samples.

Results of isotope-dilution experiments used for the measurement of concentrations of selected trace metals are listed in table 4. Measurement precisions based on isotope-ratio counting statistics are tabulated as standard errors computed at the 95-percent confidence level.

## CONCLUSIONS

The data presented in this chapter represent the trace-metal characterization of a limited quantity of standard and reference fulvic and humic acids. The confidence level of the analytical results could have been increased by using larger quantities of fulvic and humic acids. Because the occurrence of many of the trace metals probably are related to the collection, isolation, and concentration procedures used to prepare the samples and because they are dependent on the contaminant concentrations in the reagents used in these steps (for example, acids, base, and *XAD resins*) and the extensive use of stainless-steel holding tanks and filtration apparatus (Malcolm and others, chap. B, this volume), interpretation of the significance of the presence and concentration of these trace metals must be made with caution. Unfortunately, experimental blanks processed through the entire sampling, isolation, preconcentration, and analysis-preparation steps were not available for evaluation. Data indicate that trace-metal concentrations in the humic acids are generally greater than those in the fulvic acids. These variations could result from differences in the separation procedures after the XAD–8 resin concentration procedure or because the humic acid is chemically different from the fulvic acid and may have stronger affinity for trace metals.

Results of this work show that inductively coupled plasma–mass spectrometry is useful to characterize the trace element composition of small quantities of environmentally related materials. The technique is especially powerful when only a small sample is available and when accurate and precise data are desired.Isotope-dilution techniques are extremely useful when trace metal concentrations must be quantified in sample matrices of unknown complexity.

## REFERENCES CITED


Aiken, G.R., 1985, Isolation and concentration techniques for aquatic humic substances, *in* Aiken, G.R., MacCarthy, Patrick, McKnight, D.M., and Wershaw, R.L., eds., Humic Substances in Soil, Sediment, and Water: New York, John Wiley, p. 363–385.

Date, A.R., and Gray, A.L., 1983, Progress in plasma source mass spectrometry: Spectrochimica Acta, v. 38B, p. 29–37.

Garbarino, J.R., and Taylor, H.E., 1987, Stable isotope dilution analysis of hydrologic samples by inductively coupled plasma-mass spectrometry: Analytical Chemistry, v. 59, no. 11, p. 1568–1575.

Gray, A.L., 1985, The ICP as an ion source—Origins, achievements, and prospects: Spectrochimica Acta, v. 40B, p. 1525–1537.

Layman, L.R., and Lichte, F.E., 1982, Glass frit nebulizer for atomic spectrometry: Analytical Chemistry, v. 54, no. 4, p. 638–642.

Leenheer, J.A., and Noyes, T.I., 1985, A filtration and column adsorption system for onsite concentration and fractionation of organic substances from large

volumes of water: U.S. Geological Survey Water-Supply Paper 2230, 16 p.
Taylor, H.E., 1986, Inductively coupled plasma-mass spectrometry—An introduction: Spectroscopy, v. 1, no. 11, p. 20–22.
Taylor, H.E., 1989, Water resources, *in* Date, A.R., and Gray, A.L., eds., Applications of Inductively Coupled Plasma Mass Spectrometry: Blackie, p. 71–89.
Taylor, H.E., and Garbarino, J.R., 1985, Inductively coupled plasma-mass spectrometry for the quantitative analysis of natural waters: Rocky Mountain Conference on Analytical Chemistry, 27th, Denver, Colorado, 1985, Proceedings, p. 25.
Thurman, E.M., and Malcolm, R.L., 1981, Preparative isolation of aquatic humic substances: Environmental Science and Technology, v. 15, no. 4, p. 463–466.

Chapter F

# Nitrogen and Amino Acids in Fulvic and Humic Acids from the Suwannee River

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CONTENTS



FIGURES



TABLES



## Abstract

The *amino-acid* residues in fulvic and humic acids from the Suwannee River were measured in hydrolyzed and unhydrolyzed samples. The fulvic acid contained 34 nanomoles per milligram of combined amino acids, and the humic acid contained 110 nanomoles per milligram of combined amino acids. No free (associated) amino acids were detected. The fulvic-acid fraction was enriched in neutral and acidic amino-acid residues. The humic-acid fraction was enriched in basic, hydroxy-imino, and aromatic amino-acid residues. These differences in composition most likely were related to the separation of humic acid from fulvic acid by precipitation at

pH 1.0—this procedure aggregates both basic and aromatic amino acids. The low nitrogen content of the fulvic acid (0.56 percent) and the low amino-acid content of the fulvic acid (8.73 percent of the nitrogen and 0.28 percent of the carbon) indicate that amino acids are not significant in the structure of fulvic-acid molecules (on the average, fulvic acid contains one amino-acid residue per 10 molecules). In contrast, humic acid is larger in molecular weight and contains at least one amino-acid residue per molecule. Although amino acids account for a significant proportion of the nitrogen in humic acid (26 percent), they account for only 1.01 percent of the carbon. The points of attachment of amino-acid residues to humic substances are unknown.

## INTRODUCTION

Nitrogen is a trace constituent in aquatic humic substances and comprises 0.5 to 2 percent of the elemental analysis (Thurman, 1985a). For instance, the nitrogen content of fulvic acid from river water varies from 0.5 to 1.5 percent (Thurman, 1985a, chap. 10; Malcolm, 1985); in ground water, it varies from 0.5 to 1.8 percent (Thurman, 1985b); and in lake water, it varies from 0.7 to 2.2 percent (Eberle and Feuerstein, 1979; Wilson and others, 1981; McKnight and others, 1984; Steinberg and Muenster, 1985). Only aquatic humic substances from seawater contain greater nitrogen concentrations: from 1 to 6.5 percent (Stuermer and Payne, 1976; Harvey and Boran, 1985)—however, these anomalously high concentrations of nitrogen may be due to contamination during elution and concentration from hydrophobic macroporous resins by ammoniacal methanol (Thurman, 1985a, p. 292).

Aquatic humic acid, in contrast, contains twice as much nitrogen as fulvic acid (Thurman, 1985a, chap. 10). For example, the concentration of nitrogen in fulvic and humic acids from the Suwannee River is 0.56 and 1.08 percent, respectively (table 1). Thus, nitrogen is a trace constituent of the molecular composition.

The nitrogen content of soil humic substances is generally greater than that of aquatic humic substances: fulvic acid from soil varies from less than 1 percent to 3 percent, and humic acid from soil varies from 2 to 6 percent (Schnitzer, 1976, 1985; Steelink, 1985). Humic substances from sediments also have greater concentrations of nitrogen than do aquatic humic substances: lake sediments contain as much as 7.6 percent nitrogen for fulvic acid and as much as 5.63 percent nitrogen for humic acid (Ishiwatari, 1985). Marine sediments contain from 4.3 to 5.1 percent nitrogen for humic acid (Hatcher and others, 1985).

A general trend for nitrogen content of humic substances, summarized from the preceding references, indicates that nitrogen content decreases as follows: lake-sediment fulvic and humic acids, marine-sediment humic acid, soil humic acid, soil fulvic acid, aquatic humic acid, and aquatic fulvic acid. Thus, aquatic humic substances contain less nitrogen than the humic substances from these other environments.

Although Stevenson (1982) and Schnitzer (1985) have summarized soil literature on the distribution of nitrogen in soil fulvic and humic acids, little is known about the distribution of nitrogen in aquatic humic substances. Thurman (1985a, chap. 6) summarized data on amino-acid distribution in aquatic humic substances from the literature. However, this summary contained only limited information on the quantity and distribution of amino acids in humic substances. One reason for the limited information is that amino acids generally are measured on the whole-water sample (Lee and Bada, 1975; Dawson and Pritchard, 1978; Dawson and Liebezeit, 1981; Thurman, 1985a, chap. 6).

Amino acids are measured in two fractions in natural waters: dissolved-free and dissolved-combined amino acids (Thurman, 1985a). The water sample is filtered and amino acids are measured before and after hydrolysis in 6 N HCl. Free amino acids are those detected before hydrolysis; total amino acids are those detected after hydrolysis; combined amino acids are the difference between free and total amino acids.

The amino acids in humic substances are combined chemically in dissolved fulvic and humic acids; however, this fraction is seldom determined. Lytle and Perdue (1981) fractionated particulate and dissolved amino acids from the Williamson River in Oregon—they separated the combined amino acids into a humic fraction and a combined fraction and determined that 96 percent of the total dissolved amino acids were associated with the humic fraction. This is the only study dealing directly with amino

**Table 1.** Elemental analysis of fulvic and humic acids

| Acid | Concentration (Percent, moisture-free basis) | | | | | | Ash | Carbon: nitrogen ratio (atomic) | Carboxyl functional-group content (milli-equivalents per gram) | Phenolic functional-group content (milli-equivalents per gram) | Aromatic carbon (percent) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Carbon | Hydrogen | Oxygen | Nitrogen | Sulfur | Phosphorus | | | | | |
| Fulvic-- | 51.3 | 4.32 | 42.9 | 0.56 | 0.2 | 0.1 | 0.05 | 107:1 | 6.0 | 1.5 | 37 |
| Humic--- | 51.2 | 4.38 | 40.9 | 1.08 | 0.6 | 0.1 | 0.05 | 55:1 | 4.6 | 2.8 | 50 |

acids in the humic fraction. DeHaan and DeBoer (1978, 1979) and Tuschall and Brezonik (1980) also determined that amino acids were associated with the humic fraction, but *Sephadex* chromatography was used to separate the amino acids and humic substances in these studies. A humic-fraction isolation was not done; therefore, these studies did not indicate structural amino acids in the humic fraction.

Because the humic fraction is implicated as an important amino-acid fraction, it is useful to study the distribution of amino acids that are structurally part of the humic fraction. Furthermore, amino acids and simple sugars are known to condense and form brown polymeric substances by the reaction known as the *Maillard reaction* or Browning reaction (Maillard, 1913). This reaction has been suggested as an important mechanism for the production of *autochthonous* humic substances in aquatic environments (Hedges, 1978) and soils (Stevenson, 1982; Schnitzer, 1985). This chapter addresses the quantity and distribution of amino acids combined in humic and fulvic acids from the Suwannee River. Specific amino-acid residues are given and their incorporation into humic substances are discussed.

## EXPERIMENTAL PROCEDURES

The humic and fulvic acids from the Suwannee River were isolated in January of 1977 by the method developed by Thurman and Malcolm (1981). The samples discussed in this chapter are not the International Humic Substances Society's (IHSS) standard or reference samples.

Samples were filtered through 0.45-μm silver filters, and humic substances were isolated by sorption onto Amberlite XAD–8 resin at pH 2.0 (HCl was used to decrease pH). Humic substances were eluted from the resin with 0.1 N NaOH, and humic acid was precipitated from fulvic acid by lowering the pH to 1 with HCl. Fulvic acid was isolated and desalted by readsorption on Amberlite XAD–8, removal of salt with a distilled-water rinse, and elution with 0.1 N NaOH, which was removed by passage of the eluate through a cation-exchange resin in hydrogen form

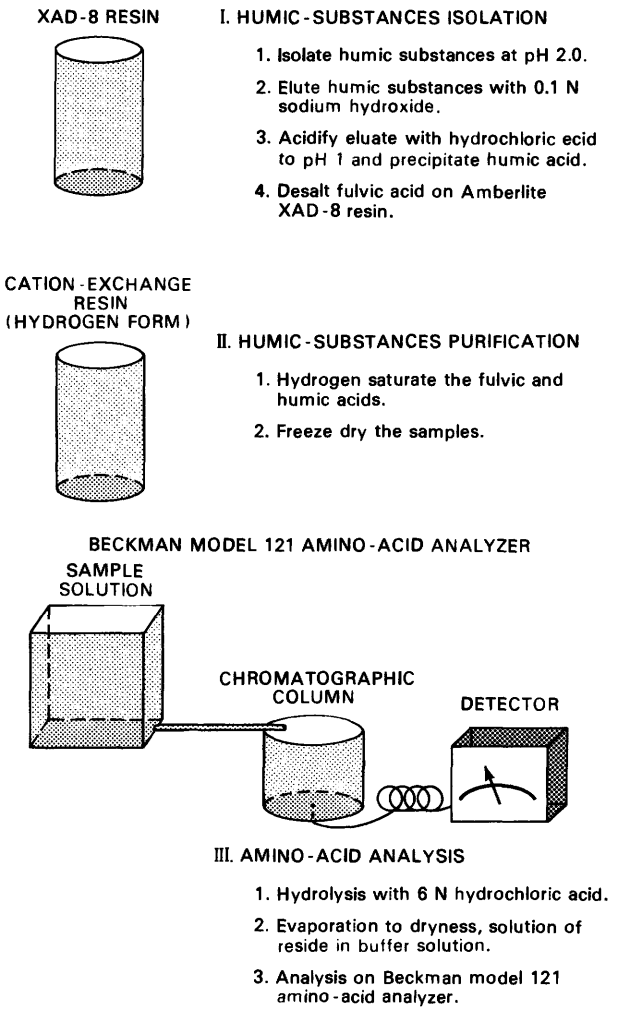



**Figure 1.** Procedures for analysis of combined amino acids in humic and fulvic acids.

(fig. 1). The cation-exchange resin was exhaustively cleaned with methanol in a Soxhlet extractor and was saturated with 1 N HCl. Amino-acid blanks were negative for amino acids coming from the cation-exchange resin. The eluate from the cation-exchange resin was freeze-dried to produce purified fulvic acid.

Ten-milligram samples of humic substances were analyzed for amino acids by hydrolysis with 6 N HCl in a sealed tube at 110°C for 12 hours. Samples were evaporated to dryness, dissolved in a lithium citrate buffer, and analyzed on a Beckman model 121 amino-acid analyzer. Ninhydrin was used for the detection of amino acids (amino-acid analyses were done by S. Goodman, University of Colorado Medical Center, Denver, Colorado). Replicate analyses were reproducible within ±2 percent for blind samples.

Elemental analyses of humic substances from Suwannee River were conducted by Huffman Laboratories, Inc., Golden, Colorado, by methods described by Huffman and Stuber (1985). Carbon and hydrogen were analyzed by combustion in oxygen (combustion gases were swept through a combustion tube and measured by volume in a manometer). Nitrogen was measured by reduction to dinitrogen gas, and volume was measured in a manometer. Oxygen was measured by a reductive pyrolysis that converts organic oxygen to carbon monoxide. Carbon monoxide was measured by gas chromatography.

Solid-state carbon-13 nuclear-magnetic-resonance ($^{13}$C-NMR) spectra were measured using 100-mg samples by the Regional NMR Center at Colorado State University (Fort Collins, Colorado) with the following conditions: cross polarization, magic-angle spinning (54.8°), 1.4-Tesla field strength, 10,000–30,000 transients, contact time of 0.1 μs, repetition rate of 3 s, and manual integration of peak areas.

Liquid-state $^{13}$C-NMR spectra were measured using 75 mg of hydrogen-saturated fulvic acid and 75 mg of hydrogen-saturated humic acid, each of which was dissolved in 0.5 g of $^{13}$C-depleted dimethylsulfoxide (DMSO). Acquisition parameters were as follows: 50,000-Hz spectral window, 0.2-s acquisition time, 45° pulse angle, 8-s pulse delay, inverse-gated *decoupling*, and line broadening of 20 Hz. Spectra were recorded at 75.4 MHz on a Varian XL–300 (for further details, see Thorn, chap. N, this volume).

## NITROGEN AND AMINO-ACID CONTENTS IN FULVIC AND HUMIC ACIDS

### Nitrogen Content

The fulvic and humic acids from the Suwannee River contain low concentrations of nitrogen (table 1). The fulvic acid contains 0.56 percent nitrogen and humic acid contains 1.08 percent. The percentage of carbon was similar for both humic acid and fulvic acid (51.2 and 51.3 percent), and these percentages were typical for aquatic humic substances (Thurman, 1985a, chap. 10). Average molecular weights of humic substances from the Suwannee River vary from 500 to 1,500 daltons for fulvic acid and from 1,000 to 8,000 daltons for humic acid (Giesy and Briese, 1977; Reuter and Perdue, 1977; Thurman and others, 1982; Aiken and others, chap. J, this volume). Our best estimates for the molecular weights of these samples are about 800 daltons (number average) for the fulvic acid and about 1,100 daltons for the humic acid (Aiken and others, chap. J, this volume).

The C/N (atomic) ratio was large (107:1 for the fulvic acid and 55:1 for the humic acid). On the average, this ratio indicates one nitrogen atom per every three fulvic-acid molecules and one nitrogen atom per humic-acid molecule.

This ratio is significant for the fulvic acid. If the cross linking of amino-acid nitrogen, or ammonia, is a significant process in the formation of aquatic fulvic acid from the Suwannee River (i.e., *melanoidin* theory of formation), then the ratio of C/N would be much smaller than 107:1. Humic acid, while only containing one atom (on the average) of nitrogen per molecule of humic acid, may contain cross-linked nitrogen either as amino-acid nitrogen or ammonia. Thus, the amino-acid/melanoidin process may be important for cross linking of humic-acid fragments, but it is not important for the majority of the fulvic-acid fraction (80 percent). Furthermore, the low nitrogen content of the fulvic acid results in minimal nutrient value for microorganisms and indicates that the fulvic-acid fraction may be more biologically degraded than the humic-acid fraction.

### Amino-Acid Content

Twenty amino-acid residues were identified in fulvic and humic acids from the Suwannee River; their

**Table 2.** Comparison of percentage of carbon and nitrogen in fulvic and humic acids

| | Fulvic acid | Humic acid |
|---|---|---|
| Percentage of sample as amino acid------ | 0.35 | 1.25 |
| Percentage of carbon as amino acid------ | 0.28 | 1.01 |
| Percentage of nitrogen as amino acid---- | 8.73 | 26.0 |

structures are shown in figure 2. These are common amino acids identified in natural waters (Thurman, 1985a, chap. 6).

The total quantity of amino-acid residues was 34 nmol/mg for fulvic acid and 110 nmol/mg for humic acid; this accounts for 8.73 percent of the nitrogen in the fulvic acid and 26.0 percent of the nitrogen in the humic acid (table 2). Amino-acid residues account for 0.28 percent of the carbon in the fulvic acid and 1.01 percent of the carbon in the humic acid. Thus, amino-acid residues are a significant component of the nitrogen pool of humic substances, but they are a minor component of the carbon pool.

This finding indicates that amino-acid residues are not a major structural component of the humic substances from the Suwannee River, but they are a major component of the nitrogen content. Amino acids may play a role in cross linking of humic acid, but, with only about 1 percent of the carbon as amino-acid residues, they are a minor component. Because the amino acids account for 26.0 percent of the nitrogen in the humic acid, the maximum melanoidin cross linking would be only 4 times greater, or 4 to 5 percent of the carbon. This calculation assumes that the other amino-acid residues may have lost their identity in the cross-linking process.

Amino acids are grouped into six different groups according to the R-side chain on the residue: basic, acidic, hydroxy-imino, neutral, sulfur, and aromatic (fig. 2). The distribution of amino-acid residues into these groups is shown in table 3. The neutral amino-acid residues account for the largest proportion (56 and 40 percent for the fulvic and humic acids), followed by acidic residues (26 percent) for fulvic acid, and hydroxy-imino residues for humic acid (32 percent). Together, these three groups account for the majority of the amino-acid residues.

This result is similar to results reported in the literature for amino-acid distribution in natural waters. These results were summarized by Thurman (1985a, chap. 6), who noted that acidic and neutral amino-acid residues are most abundant. Their abundance suggests two possibilities: First, combined amino acids in natural waters may be affected greatly by the concentration of humic substances, or second, the source of amino acids in soil, plants, and aquatic organisms does not vary significantly within acidic and neutral amino acids.

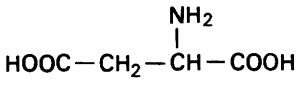
If we examine the humic-acid:fulvic-acid ratio of amino-acid residues (table 3), there are some important differences to note. The humic acid is enriched, relative to fulvic acid, in the basic fraction (2.2 times), in the hydroxy-imino fraction (2.2 times), in the sulfur fraction, and in the aromatic fraction (1.6 times). The humic acid is depleted in the acidic fraction (0.74 times) and in the neutral fraction (0.72 times) relative to fulvic acid.

**Table 3.** Comparison of amino-acid residues in fulvic and humic acids

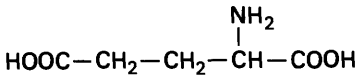
| Amino-acid residue group | Fulvic acid (parts per thousand) | Humic acid (parts per thousand) | Humic-acid:fulvic-acid ratio |
|---|---|---|---|
| Basic---------- | 20.6 | 46.2 | 2.24 |
| Acidic---------- | 255.9 | 190.4 | .74 |
| Hydroxy-imino------- | 147 | 318 | 2.16 |
| Neutral---------- | 559 | 400.7 | .72 |
| Sulfur---------- | 12.7 | 0 | ∞ |
| Aromatic---------- | 28.1 | 17.6 | 1.60 |

*A* -- AMINO-ACIDS ENRICHED IN FULVIC-ACID FRACTION

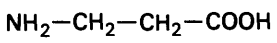
Acidic Amino Acids



Aspartic acid (asp)

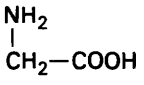


Glutamic acid (glu)

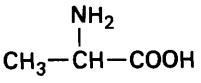


Beta-alanine ($\beta$-ala)

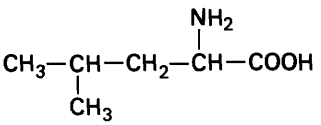
Neutral Amino Acids



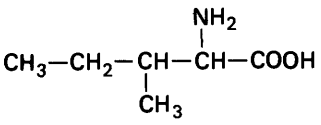
Glycine (gly)



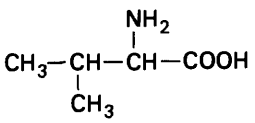
Alanine (ala)



Leucine (leu)



Isoleucine (Ile)



Valine (val)

**Figure 2** (above and on facing page). Chemical structures of amino acids in fulvic and humic acids: *A*, Amino acids enriched in fulvic-acid fraction; *B*, Amino acids enriched in humic-acid fraction.

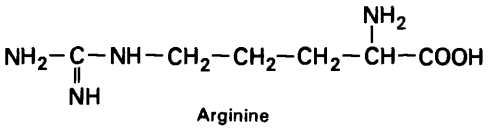
The increase in the basic fraction and the decrease in the acidic fraction may be explained by the acid precipitation that was used to fractionate fulvic and humic acids. Basic amino acids are protonated on the amino functional groups under acidic conditions and are free to interact with the carboxyl groups of humic acid (4.6 μeq/mg, or one carboxyl group per 8 carbon atoms). The interaction is more pronounced in basic amino acids because of the increased number of amino functional groups. The weaker amino nitrogens present in arginine, ornithine, and histidine could also interact with the increased *phenolic*-group content of humic acid: this would enhance precipitation of the humic-acid fraction.

The decrease in carboxyl-group acidity is commonly found in humic acid relative to fulvic acid when an acid titration is used. Decreased carboxyl-group acidity limits solubility of humic acid under acidic conditions of separation. Thus, the fulvic acid contains 10 to 30 percent more of carboxyl groups than does the humic acid (Beck and others, 1974; Perdue, 1978, 1979; Perdue and others, 1980; Wilson and others, 1981; Oliver and others, 1983; Thurman and Malcolm, 1983; Thurman, 1985a, chap. 10, p. 297). The increase in aromatic residues in the humic acid agrees with what is known of the aromatic content of humic and fulvic acids (Hatcher and others, 1981; Thurman and Malcolm, 1983; Malcolm, 1985; Thurman, 1985a, chap. 10).

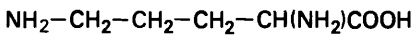
For example, the liquid- and solid-state $^{13}$C-NMR spectra for both fulvic and humic acids from the Suwannee River are shown in figure 3. The peaks from about 110 to 165 ppm are in the aromatic region of the spectrum; the humic acid contains about 1.5 to

*B*--AMINO-ACIDS ENRICHED IN HUMIC-ACID FRACTION

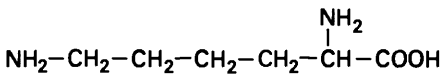
Basic Amino Acids



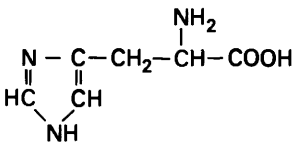
Arginine



Ornithine (orn)



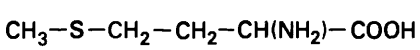
Lysine (lys)



Histidine (his)

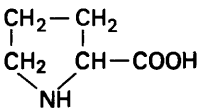
Neutral Sulfur Amino Acids

Cysteine (cys)

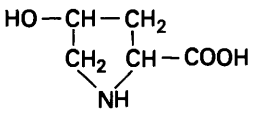


Methionine (met)

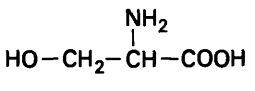
Hydroxy-imino Amino Acids



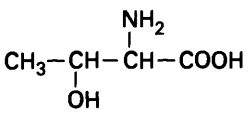
Proline (pro)



Hydroxyproline (hyp)

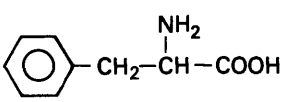


Serine (ser)

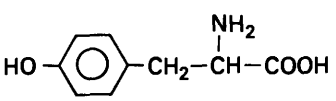


Threonine (thr)

Aromatic Amino Acids



Phenylalanine (phe)



Tyrosine (tyr)

2 times more aromatic carbon than the fulvic acid. The humic-acid:fulvic-acid ratio of aromatic amino acids is 1.6.

A detailed examination of the concentrations of amino-acid residues in the fulvic and humic acids indicates four major differences (table 4). First, the major amino-acid residues in fulvic acid were glycine and aspartic acid. These two residues accounted for 50 percent of the total in fulvic acid. In the humic acid, glycine, hyproxyproline, and aspartic acid were the major amino acids, accounting for 45 percent of the total.

Second, the abundance of hydroxyproline in humic acid and its absence in fulvic acid was noted. Although there is no simple explanation for this difference, hydroxyproline may be a biological marker for collagen precursors and may originate in organic-rich bottom sediments (Sigleo and others, 1983). Furthermore, the difference indicates that fulvic and humic acids may have separate pathways of origin. Alternatively, the fractionation of humic acid by precipitation also may be responsible for this difference.

The third major difference between fulvic and humic acids was the occurrence of β-alanine in fulvic acid and its absence in humic acid. Again, either the fractionation procedure may be responsible for this difference or a greater extent of oxidation of fulvic acid compared to humic acid may have occurred.

Fourth, the humic acid contained trace concentrations of sulfur amino acids (cysteine and methionine) and basic amino acids (arginine and ornithine) that were not present in the fulvic acid. The greater

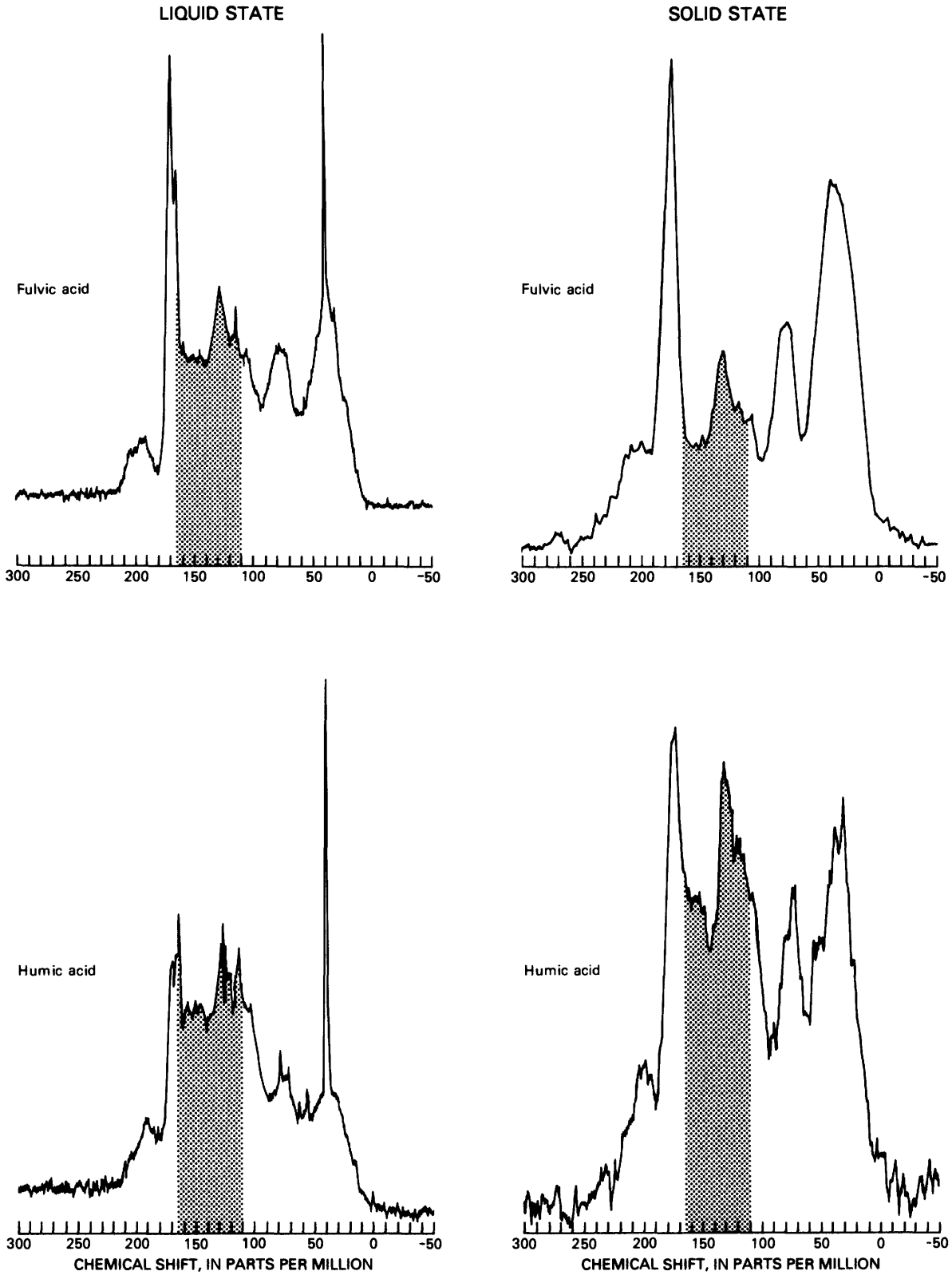



**Figure 3.** Liquid- and solid-state carbon-13 nuclear-magnetic-resonance spectra for fulvic and humic acids. Shaded areas beneath curves indicate aromatic regions of the spectra.

**Table 4.** Amino-acid residues in fulvic and humic acids

[nmol/mg, nanomole per milligram]

| Amino-acid residue | Concentration in fulvic acid (nmol/mg) | Residue per 1,000 | Concentration of humic acid (nmol/mg) | Residue per 1,000 | Humic acid: fulvic acid ratio |
|---|---|---|---|---|---|
| Basic: | | | | | |
| Arginine | 0 | 0 | 1.4 | 12.7 | ∞ |
| Ornithine | 0 | 0 | .4 | 3.6 | ∞ |
| Lysine | .5 | 14.7 | 2.4 | 21.8 | 1.5 |
| Histidine | .2 | 5.9 | 1.3 | 11.8 | 2.0 |
| Acidic: | | | | | |
| Aspartic acid | 5.7 | 167.6 | 11.9 | 107.9 | .6 |
| Glutamic acid | 3 | 88.2 | 9.1 | 82.5 | .9 |
| β-alanine | 0 | 0 | 2.9 | 85.3 | 0 |
| Hydroxy-imino: | | | | | |
| Proline | 1.8 | 52.9 | 7.8 | 70.7 | 1.3 |
| Hydroxyproline | 0 | 0 | 16.2 | 146.9 | ∞ |
| Serine | 1.5 | 44.1 | 5.3 | 48.1 | 1.1 |
| Threonine | 1.7 | 50 | 5.8 | 52.6 | 1 |
| Neutral: | | | | | |
| Glycine | 10.5 | 308.8 | 22 | 199.5 | .6 |
| Alanine | 2.8 | 82.4 | 9.7 | 87.9 | 1.1 |
| Leucine | .9 | 26.5 | 4.3 | 39 | 1.5 |
| Isoleucine | .7 | 20.6 | 2.9 | 26.3 | 1.3 |
| Valine | 1.2 | 35.3 | 5.3 | 48.1 | 1.4 |
| Sulfur: | | | | | |
| Cysteine | 0 | 0 | .7 | 6.3 | ∞ |
| Methionine | 0 | 0 | .7 | 6.3 | ∞ |
| Aromatic: | | | | | |
| Phenylalanine | .4 | 11.8 | 2 | 18.0 | 1.5 |
| Tyrosine | .2 | 5.9 | 1.1 | 10 | 1.7 |
| Sum of amino acids | 34.0 | 1,000.0 | 110.3 | 1,000.1 | 1.0 |

abundance (table 1) of sulfur in humic acid (0.6 percent) than in fulvic acid (0.2 percent) is a consistent indicator. The trace concentrations of sulfur containing amino-acid residues indicate that the humic acid may have originated in a less oxidizing environment than did the fulvic acid (such as bottom sediments and peat of the Okefenokee Swamp). This hypothesis is further substantiated by the greater abundance of phenolic carbons in the humic acid of the Suwannee River (Thurman and Malcolm, 1983), which are more stable in reducing environments. The aromatic amino-acid residue, tyrosine, contains a phenolic functional group, and it is 1.7 times more abundant in the humic-acid fraction. Again, the fractionation of humic acid from the bulk of the aquatic humic substances may be responsible for this increase in phenolic content. The humic acid is less soluble and larger in molecular weight than the fulvic acid and is removed during the precipitation process.

Other possible reasons for removal of aromatic and phenolic carbon is the interaction of aromatic rings under acidic conditions: this causes aggregation of humic-acid molecules. For example, the pi electrons of the aromatic ring may be polarized by the ring

substituents. Thus, the rings may interact with one another, much like "slices of bread in a sandwich," with the pi electrons acting as the "mayonnaise."

When humic acid is precipitated, the concentration of the humic solution is in the range of 1,000 mg/L; thus, there is the opportunity for pi-pi interactions. The carboxyl functional group also may interact with the aromatic ring by a "hydrogen bond" to the pi electrons of the aromatic ring.

### Structures of Amino Acids

The amino-acid residues that have been discussed probably are structural amino acids rather than amino acids that are weakly associated with humic substances. In previous studies (Lytle and Perdue, 1981; DeHaan and DeBoer, 1978, 1979), it was not possible to distinguish between these two possibilities.

Because we used cation exchange to hydrogen saturate the humic substances, free amino acids were probably removed before hydrolysis and analysis. In order to be sure of this separation, amino acids were analyzed before hydrolysis, and no residues were detected. It may be possible that the amino-acid analysis (which involves separation of the individual residues by ion exchange) may not break up association among the humic amino acids. However, this seems unlikely for the majority of the residues—otherwise, the amino-acid analyzer would not be a successful technique for the complex body-fluid analysis for which it is used.

The concentrations of amino-acid residues detected in fulvic and humic acids were substantially less than those reported for soil fulvic and humic acids, which ranged from 150 to 775 nmol/mg for fulvic acid and 700 to 1,800 nmol/mg for humic acid (Schnitzer, 1985; Thurman, 1985a, chap. 10, p. 328). The increased amino-acid content of soil humic substances indicates a major difference in the nitrogen distribution in soil and aquatic humic substances.

Finally, the type of covalent linkage of amino acids to humic substances is unknown. It would be useful to determine the type of covalent linkage between amino acids and humic acid because this may help to understand what effects amino acids may have on the formation of aquatic humic acid—such a study should be undertaken.

## CONCLUSIONS

The major conclusions reached as a result of this study are:

1. The concentrations of amino acids are least in fulvic acid (34 nmol/mg) for the Suwannee River and greatest in humic acid (110 nmol/mg).
2. There are major differences between the amino acids in fulvic and humic acids. Humic acid is enriched in basic, hydroxy-imino, sulfur, and aromatic amino acids and depleted in acidic and neutral amino acids compared to fulvic acid.
3. Amino acids may be involved in the cross linking of humic acid, but they are not involved in the majority of the fulvic acid from the Suwannee River.
4. Amino-acid residues account for 8.73 percent of the nitrogen in fulvic acid and 26.0 percent of the nitrogen in the humic acid from the Suwannee River. Amino-acid residues account for 0.28 percent of the carbon in fulvic acid and 1.0 percent of the carbon in humic acid from the Suwannee River.

## REFERENCES CITED


Beck, K.C., Reuter, J.H., and Perdue, E.M., 1974, Organic and inorganic geochemistry of some coastal plain rivers of the Southeastern United States: Geochimica et Cosmochimica Acta, v. 38, no. 3, p. 341–364.

Dawson, R.A., and Liebezeit, G.A., 1981, The analytical methods for the characterization of organics in seawater, *in* Duursma, E.K., and Dawson, R.A., eds., Marine Organic Chemistry: Amsterdam, Elsevier, p. 445–496.

Dawson, R.A., and Pritchard, R.G., 1978, The determination of alpha-amino acids in seawater using a fluorimetric analyser: Marine Chemistry, v. 6, no. 1, p. 27–40.

DeHaan, Hank, and DeBoer, T.D., 1978, A study of the possible interactions between fulvic acids, amino acids, and carbohydrates from Tjeukemeer, based on gel filtration: Water Research, v. 12, no. 12, p. 1035–1040.

———1979, Seasonal variations of fulvic acids, amino acids, and sugars in Tjeukemeer, The Netherlands: Archiv für Hydrobiologie, v. 85, no. 1, p. 30-40.

Eberle, S.H., and Feuerstein, W., 1979, On the pK spectrum of humic acid from natural waters: Naturwissenschaften, v. 66, p. 572–573.

Giesy, J.P., Jr., and Briese, L.A., 1977, Metals associated with organic carbon extracted from Okefenokee swamp water: Chemical Geology, v. 20, no. 1, p. 109–120.

Harvey, G.R., and Boran, D.A., 1985, Geochemistry of humic substances in seawater, *in* Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediment, and Water: New York, John Wiley, p. 233–247.

Hatcher, P.G., Breger, I.A., Maciel, G.E., and Szeverenyi, N.M., 1985, Geochemistry of humin, *in* Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediment, and Water: New York, John Wiley, p. 275–301.

Hatcher, P.G., Schnitzer, Morris, Dennis, L.W., and Maciel, G.E., 1981, Aromaticity of humic substances in soils: Soil Science Society of America Journal, v. 45, no. 6, p. 1089–1094.

Hedges, J.I., 1978, The formation and clay mineral reactions of melanoidins: Geochimica et Cosmochimica Acta, v. 42, no. 1, p. 69–76.

Huffman, E.W.D., Jr., and Stuber, H.A., 1985, Analytical methodology for elemental analysis of humic substances, *in* Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediment, and Water: New York, John Wiley, p. 433–455.

Ishiwatari, Ryoshi, 1985, Geochemistry of humic substances in lake sediments, *in* Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediment, and Water: New York, John Wiley, p. 147–179.

Lee, Cindy, and Bada, J.L., 1975, Amino acids in the equatorial Pacific Ocean water: Earth and Planetary Science Letters, v. 26, no. 1, p. 61–68.

Lytle, C.R., and Perdue, E.M., 1981, Free proteinaceous, and humic-bound amino acids in river water containing high concentrations of aquatic humus: Environmental Science and Technology, v. 15, no. 2, p. 224–228.

Maillard, L.C., 1913, Formation des matieres humiques par action des acides amines sur les sucres: Paris, Comptes Rendus Hebdomadaires des Sances de l'Academie des Sciences, v. 156, no. 3, p. 1159.

Malcolm, R.L., 1985, Geochemistry of stream fulvic and humic acids, *in* Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediment, and Water: New York, John Wiley, p. 181–209.

McKnight, D.M., Klein, J.M., and Wissmar, R.C., 1984, Changes in the organic material in lakes in the blast zone of Mount St. Helens, Washington: U.S. Geological Survey Circular 850–L, 26 p.

Oliver, B.G., Thurman, E.M., and Malcolm, R.L., 1983, The contribution of humic substances to the acidity of colored natural waters: Geochimica et Cosmochimica Acta, v. 47, no. 11, p. 2031–2035.

Perdue, E.M., 1978, Solution thermochemistry of humic substances. I. Acid-base equilibria of humic acid: Geochimica et Cosmochimica Acta, v. 42, no. 9, p. 1351–1358.

———1979, Solution thermochemistry of humic substances. II. Acid-base equilibria of river water humic substances, *in* Jenne, E., ed., Chemical Modeling in Aqueous Systems: American Chemical Society Symposium Series 93, p. 94–114.

Perdue, E.M., Reuter, J.H., and Ghosal, M., 1980, The operational nature of acidic functional group analyses and its impact on mathematical descriptions of acid-base equilibria in humic substances: Geochimica et Cosmochimica Acta, v. 44, no. 11, p. 1841–1851.

Reuter, J.H., and Perdue, E.M., 1977, Importance of heavy metal–organic matter interactions in natural waters: Geochimica et Cosmochimica Acta, v. 41, no. 2, p. 325–334.

Schnitzer, Morris, 1976, The chemistry of humic substances, *in* Nriagu, J.O., ed., Environmental Biochemistry: Ann Arbor, Michigan, Ann Arbor Science, v. 1, p. 84–107.

———1985, Nature of nitrogen in humic substances *in* Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediment, and Water: New York, John Wiley, p. 303–325.

Sigleo, A.C., Hare, P.E., and Helz, G.R., 1983, The amino acid composition of estuarine colloidal material: Estuarine, Coastal, and Shelf Science, v. 17, p. 87–96.

Steelink, Cornelius, 1985, Implications of elemental characteristics of humic substances, *in* Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediment, and Water: New York, John Wiley, p. 457–475.

Steinberg, Christian, and Muenster, Uwe, 1985, Geochemistry and ecological role of humic substances in lakewater, *in* Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediment, and Water: New York, John Wiley, p. 105–145.

Stevenson, F.J., 1982, Humus Chemistry; Genesis, Composition, Reactions: New York, Wiley-Interscience, 443 p.

Stuermer, D.H., and Payne, J.R., 1976, Investigation of seawater and terrestrial humic substances with carbon-13 and proton nuclear-magnetic-resonance: Geochimica et Cosmochimica Acta, v. 40, no. 9, p. 1109–1114.

Thurman, E.M., 1985a, Organic Geochemistry of Natural Waters: Dordrecht, The Netherlands, Martinus Nijhoff–Dr. Junk Publishers, 497 p.

———1985b, Humic substances in groundwater, *in* Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediment, and Water: New York, John Wiley, p. 87–103.

Thurman, E.M., and Malcolm, R.L., 1981, Preparative isolation of aquatic humic substances: Environmental Science and Technology, v. 15, no. 4, p. 463–466.

———1983, Structural study of humic substances—New approaches and methods, *in* Christman, R.F., and Gjessing, E.T., eds., Aquatic and Terrestrial Humic

Materials: Ann Arbor, Michigan, Ann Arbor Science, p. 1–23.

Thurman, E.M., Wershaw, R.L., Malcolm, R.L., and Pinckney, D.J., 1982, Molecular size of aquatic humic substances: Organic Geochemistry, v. 4, no. 1, p. 27–35.

Tuschall, J.R., and Brezonik, P.L., 1980, Characterization of organic nitrogen in natural waters—Its molecular size, protein content, and interactions with heavy metals: Limnology and Oceanography, v. 25, no. 3, p. 495–504.

Wilson, M.A., Barron, P.F., and Gillam, A.H., 1981, The structure of freshwater humic substances as revealed by $^{13}C$-NMR spectroscopy: Geochimica et Cosmochimica Acta, v. 45, no. 10, p. 1743–1750.

Chapter G

# Electron-Spin Resonance of Fulvic and Humic Acids from the Suwannee River

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### TABLES



## Abstract

An initial electron-spin-resonance (ESR) study was done using the International Humic Substances Society's standard and reference fulvic and humic acids from the Suwannee River in solid and alkaline-liquid phases at room temperature. Results indicate that *organic free radicals* are integral parts of fulvic-acid and humic-acid macromolecules. The ESR spectra had a single symmetrical absorption line similar to those detected for soil- and sediment-derived fulvic and humic acids. The ESR characteristics of the standard and reference fulvic and humic acids are comparable. Little structural information can be derived from the spectra. The free-radical content corresponds to concentrations of 1 to 2 radicals per thousand molecules of fulvic

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acid based on an estimated molecular weight of 1,000 daltons and may be used as an indicator of the reactivity of fulvic and humic acids toward free-radical-mediated reactions such as polymerization and photochemical degradation.

## INTRODUCTION

*Electron-spin-resonance (ESR) spectrometry* has been used in humic-substances research since 1960 (Rex, 1960; Schnitzer and Skinner, 1969; Riffaldi and Schnitzer, 1972a; Senesi and Schnitzer, 1978; Wilson and Weber, 1977). In most instances, the ESR spectra of fulvic and humic acids isolated from various types of soils and sediments had a single symmetrical absorption line that had: a spectrometric splitting factor (g) of 2.003±0.002, a line width of 2 to 8 gauss, and a spin content of $10^{16}$ to $10^{18}$ $[S]g^{-1}$. However, a few reports indicated the presence of hyperfine structure consisting of three or four lines in the ESR spectra of chemically hydrolyzed (Lisanti and others, 1977; Senesi and others, 1977) or oxidized (Steelink and Tollin, 1966, 1967) soil-derived humic substances. It is generally accepted that the free radical in humic substances is present primarily because of *semiquinone* and hydroquinone structures (Tollin and others, 1963; Steelink and Tollin, 1967). However, other free-radical structures may be present (Cheshire and others, 1977; Eltantawy and Baverez, 1978).

Several reports have appeared in the literature on effects of factors such as temperature, ultraviolet or visible irradiation, oxidation, reduction, and chemical derivatization on the ESR spectra of humic and fulvic acids (Riffaldi and Schnitzer, 1972b; Slawinska and others, 1975; Slawinska and Slawinski, 1975; Senesi and Schnitzer, 1977; Chen and others, 1978). In some instances, the results are contradictory, but the common feature is that the free radicals in humic substances have several reactions that are characteristic of organic free radicals (March, 1968), including different types of propagation and termination reactions.

Several investigators have used ESR spectrometry to study the interaction between humic substances and paramagnetic metal ions such as Cu(II), Mn(II), VO(II), (McBride, 1978, 1980, 1982; Boyd and others, 1981, 1983). Results provided structural information on the ligand binding sites and on the ligand-metal interactions. However, no coherent model of metal complexes of fulvic acid has yet been developed.

ESR has been used to study the interaction between humic substances and xenobiotics (such as herbicides and pesticides); it has also been used to study the degradation products of herbicides and pesticides (Senesi and Testini, 1982, 1983, 1984). Results have confirmed the presence of charge transfer complexes and electron donor/acceptor mechanisms.

The above review indicates that ESR has been extensively used in humic-substance research. Most of the earlier studies were based on fulvic and humic acids extracted from soils and sediments. Anomalies in the results were sometimes attributed to the lack of uniform methods and even to the definitions of fulvic and humic acids. The recent availability of the International Humic Substances Society's (IHSS) standard and reference fulvic and humic acids enables improved usage of ESR and other spectrometric methods in humic-substance research.

The purpose of this chapter is to present an overview of the scope, application, and limitation of ESR spectrometry; this is followed by initial results and discussion of an ESR study done using the IHSS standard and reference fulvic and humic acids extracted from the Suwannee River. ESR results on humic extracts from the Suwannee River will be compared with ESR results on humic extracts from soils and other waters.

## BACKGROUND

ESR spectrometry is one of the most useful techniques for investigation of the unpaired electron in radicals, molecules, or ions (Ayscough, 1967; Gordy, 1980). The phenomenon of ESR is based on the fact that an electron is a charged particle that constantly spins around its axis with a certain angular momentum. Associated with the intrinsic spin is a magnetic moment, the value of which is the Bohr magneton, $\beta$. The spinning electrons are like a magnet with its poles along the axis of rotation. The magnetic moment of an electron, $\mu$, can be written in the general form:

$$\mu=g\beta(Ms) \qquad (1)$$

where

g is the spectrometric splitting factor, with a value that is a function of the electron environment (the value is about 2 for free electrons),

$\beta$ is the Bohr magneton, with a function for converting angular momentum to magnetic moment, and

Ms is the angular-momentum quantum number, which can have a value of $+1/2$ or $-1/2$.

Radiation in the microwave frequency range induces transition of the unpaired electron between magnetic energy levels. The magnetic energy splitting is created by a static magnetic field. In the absence of a magnetic field, the free electron may exist in one of two states ($+1/2$ and $-1/2$) of equal energy (degenerate). Imposition of an external static magnetic field, H, removes degeneracy and establishes two energy levels. The lesser energy state has the spin magnetic moment aligned with the field and corresponds to Ms = $-1/2$, and:

$$\Delta E = g\beta H = h\nu \qquad (2)$$

where

$\Delta E$ is the difference in energy levels,

h is Planck's constant, and

$\nu$ is the electromagnetic radiation of frequency.

The transition from one energy state to the other can be induced by irradiation of the electron with electromagnetic radiation in the microwave frequency range. The unpaired electron not only interacts with the externally applied magnetic field but also interacts with nuclei that possess magnetic moments. This may cause further splitting of the energy levels.

A typical ESR spectrum is characterized by the position, intensity, and shape of each component line. The position of the main line, or the center of gravity of a hyperfine pattern, provides the g-value; the spacing between the lines of a hyperfine multiplet provides the hyperfine coupling constant, A. The intensity of the resonance line is proportional to the number of spins in the sample. For a single intensity, this corresponds to the integrated area under the absorption curve.

Information about the unpaired electron environment can be obtained by analyzing line width and intensity (Ingram, 1967). Factors that may affect line width and intensity include: (1) electron spin exchange between identical and nonidentical molecules, (2) chemical exchange between paramagnetic molecules and their environment, and (3) interaction with neighboring molecules having spins. From the number, intensity, and distribution of the spectral lines, one can determine how many nuclei interact with the unpaired electron.

The g-values of most organic free radicals are within 1 percent of the free-electron value, ($g_0$ = 2.0023); whereas, those of hydrocarbon free radicals are within 0.1 percent of the free-electron value (Ingram, 1967). The deviation between the measured g-value and the $g_0$-value has been attributed to spin-orbit coupling. The spin-orbit-coupling characteristics for most heteroatoms are greater than those of carbon atoms; this explains why these radicals that have spin densities localized on oxygen, nitrogen, or sulfur atoms have g-values that deviate more than those of carbon radicals. It also is reported that, for simple alkyl radicals, g-values differ only very slightly (in the fourth decimal place) from the $g_0$-value. If hydroxyl or alkoxyl groups are present at the $\alpha$-carbon atom, the g-value increases by about 0.001. For $\alpha$-aldehydic and ketonic groups, the g-value may increase by 0.002. For conjugated carbonyl groups, no such correlation exists, but, for simple radicals, the above information may aid identification (Forrester and others, 1968).

Precise measurements of the g-value for various series of substituted hydrocarbons and semiquinones have been reported (Ingram, 1967; Forrester and others, 1968). Small, but systematic, changes were determined for homologous series and between different halogen-substituted ions. There is an approximate linear relation between these increments and the spin-orbit-coupling constant. Addition of oxygen atoms to the aromatic ring, as in hydroxylation, causes a comparatively large increase in g-value (about 0.01); whereas, *methylation* causes a small decrease. For pure compounds of known molecular structures, the g-value can be used to characterize free radicals that lack hyperfine structure. However, for free radicals of undefined molecular structures, little information can be derived from the g-value.

In the solid phase, purified fulvic and humic acids are amorphous materials. The ESR spectra of such material are likely to provide a less detailed indication of their structure and electronic distribution than spectra of free radicals in single crystals (Walling, 1957). Fulvic and humic acids are soluble in polar solvents, such as water and alkaline solutions. In this instance, in addition to the generalized effects of temperature and viscosity on line width, there may be some local reorganization of the solvent molecules around the paramagnetic species. This may affect the ESR spectra.

Thus, ESR spectrometry can be a useful method in humic-substance research. However, there are some inherent problems and limitations that must be considered when the technique is applied.

## EXPERIMENTAL CONDITIONS

### Samples

IHSS standard and reference fulvic and humic acids were provided as solid, amorphous materials of 100 mg each by the U.S. Geological Survey in Denver, Colorado. The fulvic and humic acids had been extracted from the Suwannee River according to published procedures (Thurman and Malcolm, 1981). ESR spectra were recorded for solid samples and, after 30 minutes contact time, for 0.03 M NaOH solutions containing 1 percent fulvic acid or humic acid at pH 12.5.

### Instrumentation

All ESR spectra were measured on a Varian Model 4502 spectrometer at room temperature (24°±1°C). A quartz tube, 14.6 cm long with an inside diameter of 4 mm, was used for solid samples, and a quartz flat cell was used for liquid samples. The following are the conditions under which the measurements were made: frequency, 9.5 GHz; modulation frequency, 100 kc; magnetic field setting, 3,350 to 3,370 G; standard for solid state, 2,2-diphenyl-1-picrylhydrazyl (DPPH) diluted with KCl; and standard for liquid phase, DPPH in benzene.

The g-values were calculated from the relation:

$$g_{sple} = 2.0036 \times \frac{H_{std}}{H_{sple}} \times \frac{\nu_{sple}}{\nu_{std}} \qquad (3)$$

where

$H_{std}$ and $H_{sple}$ are the instrument magnetic fields with the standard and sample, and

$\nu_{std}$ and $\nu_{sple}$ are the instrument frequencies of the standard and sample.

The line width, in G, was measured in peak-to-peak distance of the first derivative (in gauss). Spin content was determined by comparing the areas of the first-derivative curves of the sample and standard under identical conditions. The area under the curves is given by: height times the square of the width.

Precision of the ESR characteristics was evaluated by replicate measurements of the solid and liquid samples. In both instances, precision was less than 0.01 percent. A limited interlaboratory study was made for comparison of ESR characteristics of the standard fulvic acid (Saleh, 1985).

## ELECTRON-SPIN RESONANCE OF FULVIC AND HUMIC ACIDS

For the liquid phases, the ESR spectrum of the standard and reference fulvic and humic acids had a single, symmetrical line devoid of hyperfine structure. The g-value is 2.00, which indicates an organic free radical. These results are in agreement with most of the previous ESR results for soil- and sediment-derived fulvic and humic acids. A symmetrical signal similar to those of fulvic and humic acids is reported for hydrocarbon free radicals in pitch and crude oil (Forrester and others, 1968). Little structural information can be obtained from such a signal other than that the spectrum indicates no measurable anisotropy.

The ESR characteristics of standard and reference fulvic and humic acids from the Suwannee River are listed in table 1. The table also includes the mean and standard deviations of ESR characteristics from the interlaboratory study of the standard fulvic acid (Saleh, 1985). A summary of published data for ESR characteristics in water-, soil-, and sediment-derived fulvic and humic acids is listed in table 2.

There is close agreement between the mean values determined by the interlaboratory study and the experimental results of this study. The g-values were identical for the solid phase and were almost identical for the liquid phase. Results of the two studies for spin content and line width are close, considering that the types of instruments used and calibration methods employed are not identical. For the solid phase, ESR characteristics of the standard and reference fulvic acids are comparable. This may indicate that the free-radical properties are not changed by the extra precautions taken in preparation of the standard fulvic and humic acids.

Some investigators have used the g-value to characterize the nature of free radicals in coal (Retcofsky and others, 1981). Steelink and Tollin (1966, 1967)

**Table 1.** Electron-spin-resonance characteristics[1] of standard and reference fulvic and humic acids in solid and liquid phases

| Type of acid | Solid phase | | | Liquid phase[2] | | | |
|---|---|---|---|---|---|---|---|
| | g-value | Spin contents per gram[1] | Line width (gauss) | g-value | Spin contents per gram[1] | Line width (gauss) | pH |
| Standard fulvic acid | 2.0040 | $5.41\times10^{17}$ | 9.80 | 1.9983 | $1.06\times10^{18}$ | 6.4 | 12.51 |
| Standard fulvic acid[3] | 2.0040 | $2.29\times10^{17}$ | 5.54 | 1.9923 | $0.87\times10^{18}$ | 3.7 | 12.50 |
| | ±0.0011 | $\pm2.203\times10^{17}$ | ±3.76 | ±0.0157 | $\pm0.22\times10^{18}$ | ±2.6 | -- |
| Reference fulvic acid | 2.0066 | $7.85\times10^{17}$ | 8.70 | 1.9968 | $1.29\times10^{18}$ | 7.6 | 12.45 |
| Standard humic acid | 1.9994 | $3.93\times10^{17}$ | 8.00 | 1.9984 | $2.62\times10^{18}$ | 7.1 | 12.50 |
| Reference humic acid | 1.9982 | $4.87\times10^{17}$ | 7.80 | 2.0041 | $2.04\times10^{18}$ | 7.1 | 12.47 |

[1]All measurements were made at 24±1 degrees Celsius.
[2]One percent fulvic or humic acid in 0.03 M NaOH, contact time 30 minutes.
[3]Average of results of the interlaboratory study, number of observations (n) = 4 (Saleh, 1985).

and Tollin and others (1963) used the g-values of fulvic and humic acids as one of the indicators of *quinone* and semiquinone structures. Other investigators (Forrester and others, 1968; Rabek and Ranby, 1977; Gordy, 1980) believe that the lack of hyperfine structure severely limits the ability to develop exact structural information. Theories relating to the molecular structure of the network associated with the free radical in fulvic and humic acids are still being developed (Thurman and Malcolm, 1983). The current study indicates that, until more information is developed about the overall structure of fulvic and humic acids, correlation of the g-values with heteroatoms or specific structures may be risky.

For the solid phases, the standard and reference humic acids had g-values of 1.9994 and 1.9982, respectively. It is difficult to rationalize g-values less than that of a free electron (2.0023). However, considering the inherent problems associated with ESR of amorphous material and the complexity of humic-acid structure, the results are not surprising. In fact, g-values less than those for a free electron or organic free radicals are sometimes reported in the literature (Rabek and Ranby, 1977). Also, calculation commonly is based on the assumption of constancy of the microwave frequency, which is not experimentally true.

In solution, the g-values of the standard and reference fulvic acids were 1.9983 and 1.9968, respectively. The mean g-value from the interlaboratory study was 1.9923. The agreement between the results eliminates doubts regarding experimental errors that may be associated with ESR measurement. As discussed in an earlier section, ESR measurements of humic substances in aqueous solutions are subject to several inherent problems that may affect the ESR spectra.

The spin content per gram ($[S]g^{-1}$) provides useful information about the content of free radicals in the sample. In the solid phases, the spin content of the standard and reference fulvic and humic acids ranged from $3.93\times10^{17}$ to $7.85\times10^{17}$ $g^{-1}$. These values are in the same order of magnitude as the reported values in table 2. In the alkaline-liquid phase, spin contents are larger by one order of magnitude. Similar results were reported by other investigators (Steelink and Tollin, 1967; Cheshire and others, 1977; Senesi and Schnitzer, 1978).

On the basis of an estimated molecular weight of 1,000 daltons for fulvic acid, the free-radical contents

**Table 2.** Comparison between electron-spin-resonance characteristics of fulvic and humic acids in water, soil, and sediment

[Leaders (--) indicate no data]

| Reference | Type of sample | g-value | Spin contents per gram | Line width (gauss) |
|---|---|---|---|---|
| This study | Water, fulvic acid | 1.9983-2.0066 | $5.4\times10^{17}$-$12.9\times10^{17}$ | 6.4-9.8 |
| | Water, humic acid | 1.9982-2.0041 | $3.9\times10^{17}$-$26.2\times10^{17}$ | 7.1-8.0 |
| Chen and others (1978) | Soil, fulvic acid | 2.0037-2.0050 | $1.14\times10^{17}$-$1.97\times10^{17}$ | 4.5-7.5 |
| | Soil, humic acid | 2.0031-2.0047 | $4.45\times10^{17}$-$16.55\times10^{17}$ | 4.8-5.2 |
| Wilson and Weber (1977) | Water, fulvic acid | 2.0038 | [1]1.00 | 4.0 |
| | Water, humic acid | 2.0038 | 3.18 | 5.6 |
| | Soil, fulvic acid | 2.0037 | 1.91 | 3.3 |
| | Soil, humic acid | 2.0038 | 5.87 | 4.0 |
| Lakatos and others (1977) | Peat, fulvic acid | 2.0009 | -- | 4.5 |
| | Peat, humic acid | 2.0032-2.0035 | -- | 2.8-3.5 |
| Hayes and others (1975) | Soil, fulvic acid | -- | $0.2\times10^{16}$-$1.4\times10^{16}$ | -- -- |
| | Soil, humic acid | -- | $3.4\times10^{16}$-$4.6\times10^{16}$ | -- -- |
| Slawinska and others (1975) | Soil, humic acid | 2.0055-2.0056 | -- -- | 5.0-5.1 |
| Ishiwatari (1974) | Sediment, humic acid | 2.0032-2.0035 | -- -- | 3.6-4.5 |

[1]Relative concentration.

range from 1 to 2 radicals per thousand molecules of fulvic acid. This is an extremely small content and represents an insignificant contribution to the molecular structure. However, even these small contents of free radicals could regulate several important reactions such as catalyzing polymerization reactions and reactions that initiate photochemical and biochemical transformations. These reactions are of extreme importance in terrestrial and aquatic environments.

## CONCLUSIONS

1. The ESR spectra of the standard and reference fulvic and humic acids from the Suwannee River had a single symmetrical absorption line devoid of hyperfine structure. The ESR spectra of replicate measurements are satisfactorily reproducible. The ESR characteristics of the standard and reference fulvic and humic acids are similar. The ESR characteristics of fulvic and humic acids from the Suwannee River are comparable to those previously reported for soil- and sediment-derived fulvic and humic acids.

2. Results of an interlaboratory study indicated acceptable agreement between several investigators. Such studies, if expanded, may be useful for detecting inherent problems, verifying the measurements, and developing standardized procedures.

3. The free-radical content can be used as an indicator of the effects of the reactivity of fulvic and humic acids toward several chemical and biochemical reactions. The free-radical contents of the standard and reference fulvic and humic acids range from 1 to 2 radicals per thousand molecules and, thus, represent a small contribution to the overall molecular structure. However, studies directed toward the investigation of the nature and identity of the free radicals in humic substances are critically important.

4. Lack of hyperfine structures in the ESR spectra of the fulvic and humic acids severely limit the ability to identify free-radical structures. An advanced technique, such as electron nuclear double resonance, or combined techniques, such as chromatography-ESR and nuclear-magnetic-resonance spectrometry, would provide more information.

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## REFERENCES CITED

Ayscough, P.B., 1967, Electron Spin Resonance in Chemistry: London, Methuen & Co., p. 1–27.

Boyd, S.A., Sommers, L.E., Nelson, D.W., and West, D.X., 1981, The mechanism of copper (II) binding by humic acid—An electron spin resonance study of a copper (II)–humic acid complex and some adducts with nitrogen donors: Soil Science Society of America Journal, v. 45, no. 4, p. 745–749.

———1983, Copper (II) binding by humic acid extracted from sewage sludge—An electron spin resonance study: Soil Science Society of America Journal, v. 47, no. 1, p. 43–46.

Chen, Y., Senesi, N., and Schnitzer, M., 1978, Chemical and physical characteristics of humic and fulvic acids extracted from soils of the Mediterranean region: Geoderma, v. 20, no. 2, p. 87–104.

Cheshire, M.V., Berrow, M.L., Goodman, B.A., and Mudie, C.M., 1977, Metal distribution and nature of some Cu, Mn, and V complexes in humic and fulvic acid fractions of soil organic matter: Geochimica Cosmochimica Acta, v. 41, no. 8, p. 1131–1138.

Eltantawy, I.M., and Baverez, M., 1978, Structural study of humic acids by X-ray, electron spin resonance, and infrared spectroscopy: Soil Science Society of America Journal, v. 42, no. 6, p. 903–905

Forrester, A.R., Hay, J.M., and Thomson, R.H., 1968, Organic Chemistry of Stable Free Radicals: New York, Academic Press, 13 p.

Gordy, W., 1980, Theory and Applications of Electron Spin Resonance: New York, John Wiley, p. 1–27.

Hayes, M.H.B., Swift, R.S., Wardle, R.E., and Brown, J.K., 1975, Humic material from organic soil—A comparison of extractants and of properties of extracts: Geoderma, v. 13, no. 3, p. 231–240.

Ingram, D.J.E., 1967, Free radicals as studied by electron spin resonance: Ann Arbor, Michigan, University Microfilm, 10 p.

Ishiwatari, R., 1974, Electron spin resonance of sedimentary humic acids in relation to their aromatic character: Geochemical Journal, v. 8, no. 2, p. 97–102.

Lakatos, B., Tibai, T., and Meigel, J., 1977, ESR spectra of humic acids and their metal complexes: Geoderma, v. 19, no. 4, p. 319–338.

Lisanti, L.E., Testini, C., and Schnitzer, M., 1977, Research on the paramagnetic properties of humic compounds. VI. Identification of radical species by ESR spectrometry: Agrochimica, v. 21, no. 1, p. 47–56.

March, J., 1968, Advances Organic Chemistry—Reactions, Mechanisms, and Structures: New York, McGraw Hill, 153 p.

McBride, M.B., 1978, Transition metal binding in humic acid—An ESR study: Soil Science, v. 126, no. 4, p. 200–209.

———1980, A comparative electron spin resonance study of $VO^{2+}$ complexation in synthetic molecules and soil organics: Soil Science Society of America Journal, v. 44, no. 3, p. 495–499.

———1982, Electron spin resonance investigation of $Mn^{2+}$ complexation in natural and synthetic organics: Soil Science Society of America Journal, v. 46, no. 6, p. 1137–1143.

Rabek, J.F., and Ranby, B.G., 1977, ESR Spectroscopy in Polymer Research: New York, Springer Verlag, 312 p.

Retcofsky, H.L., Hough, M.R., Maguire, M.M., and Clarkson, R.B., 1981, Nature of free radicals in coal, pyrolyzed coals, solvent refined coals, and coal liquification products, *in* Gorbaty, M.L., and Ouichi, K., eds., Coal's Structure: Washington, D.C., American Chemical Society, Advances in Chemistry Series 192, p. 37–58.

Rex, R.W., 1960, Electron paramagnetic resonance studies of stable free radicals in lignins and humic acids: Nature, v. 188, no. 4757, p. 1185–1186.

Riffaldi, R., and Schnitzer, M., 1972a, Electron spin resonance spectrometry of humic substances: Soil Science Society of America Proceedings, v. 36, no. 2, p. 301–305.

———1972b, Effects of diverse experimental conditions on ESR spectra of humic substances: Geoderma, v. 8, no. 1, p. 1–10.

Saleh, F.Y., 1985, Interlaboratory study for evaluation of the ESR parameter measurement: Unpublished report to the U.S. Geological Survey, 5 p.

Schnitzer, M., and Skinner, S.I.M., 1969, Free radicals in soil humic compounds: Soil Science, v. 108, no. 6, p. 383–390.

Senesi, N., Chen, Y., and Schnitzer, M., 1977, Hyperfine splitting in electron spin resonance spectra of fulvic acid: Soil Biology and Biochemistry, v. 9, no. 5, p. 371–372.

Senesi, N., and Schnitzer, M., 1977, Effects of pH, reaction time, chemical reduction, and irradiation on ESR

spectra of fulvic acid: Soil Science, v. 123, no. 4, p. 224–234.

———1978, Free radical in humic substances, *in* Krumbien, E.W., ed., Environmental Biogeochemistry and Geomicrobiology: Ann Arbor, Michigan, Ann Arbor Science, p. 467–481.

Senesi, N., and Testini, C., 1982, Physiochemical investigations of interaction mechanisms between s-triazine herbicides and soil humic acids: Geoderma, v. 28, no. 2, p. 129–146.

———1983, Spectroscopic investigations of electron donor-acceptor processes involving organic free radicals in the adsorption of substituted urea herbicides by humic acids: Pesticide Science, v. 14, no. 1, p. 78–79.

———1984, Theoretical aspects and experimental evidence of the capacity of humic substances to bind herbicides by charge-transfer mechanisms (electron donor-acceptor processes): Chemosphere, v. 13, no. 3, p. 461–468.

Slawinska, D., and Slawinski, J., 1975, Spectroscopic study on mild oxidation of humic acids. I. Oxidation with molecular oxygen: Polish Journal of Soil Science, v. 8, no. 1, p. 37–47.

Slawinska, D., Slawinski, J., and Sarna, T., 1975, The effect of light on the ESR spectra of humic acids: Journal of Soil Science, v. 26, no. 2, p. 93–99.

Steelink, C., and Tollin, G., 1966, Biological polymers related to catechol—Electron paramagnetic resonance and infrared studies of melanin, tannin, lignin, humic acid, and hydroxyquinones: Biochimica et Biophysica Acta., v. 112, no. 2, p. 377–379.

———1967, Free radicals in soil, *in* McLaren, A.D., and Peterson, G.H., eds., Soil Biochemistry: New York, Marcel Dekker, p. 147–169.

Thurman, E.M., and Malcolm, R.L., 1981, Preparative isolation of aquatic humic substances: Environmental Science and Technology, v. 15, no. 4, p. 463–466.

———1983, Structural study of humic substance, *in* Christman, R.F., and Gjessing, E.T., eds, New Approaches and Methods in Aquatic and Terrestrial Humic Materials: Ann Arbor, Michigan, Ann Arbor Science, p. 107–126.

Tollin, G., Reid, T., and Steelink, C., 1963. Structure of humic acid. IV. Electron paramagnetic resonance studies: Biochimica et Biophysica Acta, v. 66, no. 2, p. 444–447.

Walling, C., 1957, Free Radicals in Solutions: New York, John Wiley, 7 p.

Wilson, S.A., and Weber, J.H., 1977, Electron spin resonance analysis of semiquinone free radicals of aquatic and soil fulvic and humic acids: Analytical Letters, v. 10, no. 1, p. 75–84.

Chapter H

# Methods for Determination of Structural Models of Fulvic Acid from the Suwannee River by Convergent Independent Analyses

*By* J.A. Leenheer

## CONTENTS



## Abstract

Methods of constructing structural models of fulvic acid from the Suwannee River are presented. These models determined average molecular formulas and distributed carbon, hydrogen, and oxygen into various structural elements. Independent methods were used to assemble the data set. Convergence of data from independent methods may be regarded as circumstantial evidence that supports the accuracy of the data. When data used for structural models do not converge, problems in the methods used to determine carbon distribution and carboxyl-, ester-, ketone-, and hydroxyl-group contents can be identified. The structural-model approach can be used for both mixtures and pure compounds; it is designed to evaluate methods and to follow chromatographic separation of aquatic fulvic acid into more homogeneous fractions.

## INTRODUCTION

Determination of chemical structures of the various components that comprise fulvic acids has not been possible because a pure fulvic-acid component has never been isolated by chromatography for ultimate structural analysis. Another problem in the chemical analyses of fulvic acids is that the

complexity of the fulvic-acid mixture and the unknown chemistry of humic substances in general severely limits the applicability and accuracy of many analytical methods.

An approach to resolve the chromatography and chemical-analyses problems is to construct chemical structural models of fulvic-acid mixtures. These models attempt to incorporate all the analytical data in a specific data set. Discrepancies and omissions that arise in the data set during construction of the models can lead to critical evaluation of the data, generation of new data, and development of improved methods. Examination of the chemical properties of the models can lead to new chromatographic separations that exploit the differences in chemical properties of various components of the mixtures.

Models can be constructed for different levels of specificity. A single structural model that incorporates and averages all the data for a fulvic acid can be constructed. Multiple models can be constructed for subfractions of fulvic acid; these models may be based upon size differences, oxygen functional-group differences, or origin and diagenetic differences.

The objective of this chapter is to present a critical overview of methods for generation of data needed to construct structural models of fulvic acid from the Suwannee River. The emphasis is on independent methods rather than dependent methods, and direct analyses are preferred to difference analyses to minimize errors. Convergence of the data between two or more independent analyses is regarded as verification of the accuracy of the data. Subsequent chapters in this volume present detailed methods and results of structural analyses of fulvic acid from the Suwannee River; the final chapter will combine the results into structural models that represent various aspects of fulvic-acid chemistry.

## METHODS FOR DETERMINATION OF STRUCTURAL MODELS OF FULVIC ACID

### Molecular Formulas

Elemental analyses and molecular-weight measurements are needed to determine molecular formulas. A review of analytical methods for elemental analyses of humic substances was done by Huffman and Stuber (1985). They indicated that the major limitation to accurate elemental analyses of humic substances was the variable water content of fulvic- and humic-acid preparations, which affected the oxygen and hydrogen content. They recommended that the water content be determined separately on equilibrated samples by Karl Fischer titrimetry and the elemental results should then be corrected. This approach is preferable to conducting elemental analyses on rigorously dried samples that tended to regain water rapidly before analyses—samples also apparently began to decompose during rigorous drying. Other sources of error in elemental analyses result from large ash contents (which have large oxygen content because of the contribution of inorganic oxides) and from determination of oxygen by difference methods rather than by direct methods.

A review of molecular-weight measurements on humic substances (Wershaw and Aiken, 1985) included *ultracentrifugation*, *viscometry*, and *colligative-property* measurements (for example, *freezing-point lowering*, *vapor-pressure osmometry*, and membrane equilibria). Ultracentrifugation data is dependent on density measurements, and the solute must be electrostatically neutral for the method to work. Semiquantitative estimates of molecular-weight distributions can be obtained by ultracentrifugation methods. Viscometry is of limited usefulness because viscosity is related to molecular weight by two adjustable parameters and because the method needs to be calibrated using standards similar to the chemical structure of fulvic acid (which is unknown). Colligative-property measurements provide only number-average, molecular-weight data without any indication of molecular-weight distribution. Correction factors also need to be used for dissociation of organic acids, or solvents need to be used that eliminate organic-acid dissociation. The use of multiple methods for molecular-weight determination is especially important because of analytical limitations created by the *polydisperse* nature of fulvic acid and the dependence of each method on other measurements, such as density and the degree of proton dissociation.

Once molecular formulas have been determined by combining elemental analysis and number-average molecular-weight data, the number of rings or pi bonds (moles of unsaturation) in structural models containing only carbon, hydrogen, and oxygen can be determined by the equation:

$$\Phi=[(2C+2)-H]/2 \qquad (1)$$

where

Φ equals the moles of unsaturation,
C is the number of carbons in number-average molecular formula, and
H is the number of hydrogens in number-average molecular formula.

Perdue (1984) used the moles-of-unsaturation calculation to impose analytical constraints on the aromatic content of samples of humic substances with given molecular formulas, carboxyl-, and carbonyl-group contents.

The accuracy of molecular (or empirical) formulas can be evaluated by determining a *heat of combustion* in which the fulvic acid is oxidized to carbon dioxide and water. Reddy and others (chap. I, this volume) describe the confirmation of the empirical formula by *calorimetry*.

## Carbon Distribution

The only method whereby comprehensive data can be obtained about the distribution of carbon among various structural units of fulvic acid is natural abundance, carbon-13 nuclear-magnetic-resonance ($^{13}$C-NMR) spectrometry. Quantitative $^{13}$C-NMR spectrometry of humic substances is a technique that needs to be quantitatively refined to achieve the accuracy necessary for use in structural models. Thorn (chap. N, this volume) presents the conditions whereby quantitative $^{13}$C-NMR spectroscopy of humic substances can be achieved. Accurate representations of carbon distributions are critical elements of structural models, and independent methods are needed to evaluate the accuracy of $^{13}$C-NMR data.

An upper limit for the number of aromatic carbons can be calculated from the moles of unsaturation minus the moles of carboxyl plus carbonyl groups (Perdue, 1984). The presence of alicyclic rings in the fulvic-acid structure will also contribute to the moles-of-unsaturation calculation and will decrease the aromatic-carbon content. Comparison of contents of certain functional groups (such as carboxyl, ester, and hydroxyl groups determined by independent methods) with the $^{13}$C-NMR determination of these groups also can be used to verify the quantitative accuracy of $^{13}$C-NMR spectrometry. Finally, the quantitative accuracy of $^{13}$C-NMR spectrometry can be partially verified by determining hydrogen to carbon (H:C) ratios for various structural units that are also measured in the proton NMR spectrum. For example, the H:C ratio of the aliphatic hydrocarbon moiety may range between 1.6 and 2.4, that of carbohydrates and ethers between 1.0 and 1.5, and that of aromatic carbons between 0.2 and 0.4. These ratios are based on known structural elements of humic substances presented in reviews by Schnitzer (1978) and Stevenson (1982); H:C ratios not included in these ranges may indicate that the $^{13}$C-NMR data are erroneous, assuming the proton NMR data are accurate.

## Hydrogen Distribution

The number of hydrogen atoms indicated by the molecular formula must be subdivided into exchangeable and nonexchangeable hydrogen because the distribution of each type of hydrogen has different properties and is determined by different methods. Exchangeable-hydrogen distribution (mainly carboxyl and hydroxyl groups) is determined primarily by functional-group analyses, and nonexchangeable-hydrogen distribution is determined primarily by proton NMR spectrometry, although there is some cross application of methods.

### Exchangeable Hydrogen

Total exchangeable hydrogen can be estimated by measurement of the broad exchangeable-hydrogen peak in the proton NMR spectrum of fulvic acid that is dissolved in dioxane (Noyes and Leenheer, chap. M, this volume). However, the validity of these results is doubtful because of questions about the complete removal of water and intramolecular *esterification* reactions that occur during complete removal of water. Various methods (Stevenson, 1982) for determining total exchangeable hydrogen and carboxyl and hydroxyl groups were attempted: these methods yielded variable results because the esters (2 mmol/g) in fulvic acid from the Suwannee River tended to hydrolyze during analyses. Exchangeable-hydrogen distributions were determined by direct methods that were optimized to prevent side reactions (Noyes and Leenheer, chap. M, this volume).

Carboxyl groups can be estimated fairly accurately by acid-base titrimetry (Bowles and others, chap. L, this volume), but additional consumption of base caused by ester hydrolysis occurred when phenols were titrated at alkaline pH. Carboxyl groups can be determined by methylation using diazomethane, and reactive phenols can be determined by *acetylation*

during Schotten-Baumann conditions (Noyes and Leenheer, chap. M, this volume). The determination of methoxy groups (for carboxyl groups) and acetoxy groups (for phenols) is then done by proton NMR spectrometry (Noyes and Leenheer, chap. M, this volume). (Rigorous methylation or acetylation procedures that derivatized alcoholic hydroxyl groups and phenols unreactive to Schotten-Baumann acetylation also derivatized weakly acidic protons on carbon; therefore, these procedures were not specific for hydroxyl groups.) Residual alcoholic and phenolic hydroxyl groups in the methylated and acetylated derivative can be determined by quantitative infrared spectroscopy when the sample is dissolved in pyridine (Kabasakalian and others, 1959). The methylation and acetylation procedures eliminated interferences caused by the carboxyl groups and phenols in infrared-spectrometry measurements of the alcoholic hydroxyl functional groups, and the pyridine quantitatively hydrogen bonded with the alcoholic hydroxyl groups. This enabled quantitation within 10-percent accuracy, which is the variation in molar absorptivity of various alcoholic hydroxyl groups in pyridine.

Wershaw and others (1981) labeled the carboxyl, phenol, and alcohol groups with $^{13}C$-enriched methyl groups by $^{13}C$ methylation using a two-step derivatization procedure that used diazomethane followed by sodium hydride and methyl iodide. Carboxyl groups were well differentiated from phenol and alcoholic groups by $^{13}C$-NMR spectrometry of the derivative, but phenols were not differentiated from the alcoholic hydroxyl groups. Possible reduction of aryl ketones to alcohols by sodium hydride and cleavage of ester linkages are probable side reactions that would increase the carboxyl and hydroxyl group contents (Fieser and Fieser, 1967; Kovac and Anderle, 1978).

### Nonexchangeable Hydrogen

The distribution of nonexchangeable hydrogen can be determined only by quantitative proton NMR spectrometry. The relatively small nonexchangeable-hydrogen content of fulvic acid coupled with interferences of various hydroxyl groups render quantitative infrared spectrometry useless in measurement of the carbon-hydrogen (C-H) stretch band near 2,900 $cm^{-1}$. Proton NMR spectrometry of fulvic acid can be performed under conditions that give quantitative proton distributions (Noyes and Leenheer, chap. M, this volume). The four broad spectral bands observed in proton NMR spectra can be assigned to aliphatic hydrogens or carbons that are two or more carbons removed from carbonyl groups or aromatic rings, aliphatic hydrogens on carbons adjacent to carbonyl groups or aromatic rings, aliphatic hydrogens on carbons singly bonded to oxygen, and aromatic hydrogen (Noyes and Leenheer, chap. M, this volume). The presence of water and exchangeable hydrogen in fulvic acid must be eliminated by deuterium exchange or by derivatization before distributions of nonexchangeable hydrogen can be measured without interference.

## Oxygen Distribution

Oxygen contained in carboxyl, phenolic hydroxyl, and aliphatic hydroxyl groups can be accounted for by the same methods discussed in the "Exchangeable Hydrogen" section. Ester groups are determined by measuring the decrease of infrared integrated absorption intensity from 1,780 to 1,700 $cm^{-1}$ after *saponification* of the sample. The samples were dissolved as the tetrabutylammonium salt in acetonitrile, and a series of 20 different esters and *lactone* standards were used to calibrate the procedure (Leenheer and others, 1986). Infrared spectrometry is the only direct method to measure esters; methods based on ester saponification followed by titrimetry are affected by conversion of carbohydrates to saccharinic acids (Davis and Mott, 1981). Esters also can be determined as the difference between the carbonyl carbon in the 165- to 185-ppm region of the $^{13}C$-NMR spectrum and the methyl ester carbon (at 52 ppm) of the methylated sample. Such a measurement is subject to errors of the quantitative $^{13}C$-NMR spectrum discussed previously.

Total carbonyl-group content (aldehydes, ketones, acetals, and ketals) typically are measured by forming Schiff-base derivatives with hydroxylamine, methoxylamine, or phenyl hydrazine (Leenheer and Noyes, 1989); however, Thorn and others (1986) recently reported that ester groups in the fulvic-acid structure also reacted with hydroxylamine to form hydroxamic acids—these methods are, therefore, not specific for carbonyl groups.

Leenheer and others (1987) recently presented qualitative $^{13}C$-NMR- and infrared-spectroscopic evidence that the majority of the carbonyl groups in fulvic acid from the Suwannee River are aryl aliphatic and diaryl ketones. These ketones occur between 190 and 200 ppm in the $^{13}C$-NMR spectrum, and integration of the quantitative $^{13}C$-NMR spectrum can be

used to quantify the free ketones. Aldehydes and ketones that are combined into acetal and ketal linkages occur in the 95- to 105-ppm region of the $^{13}$C-NMR spectrum, but there is some overlap with the aromatic carbons in this range.

Ethers cannot be measured specifically by any spectrometric technique. Methoxy groups in humic substances can be determined by Zeisel's alkoxy method, but methoxy content is generally small in aquatic fulvic acids. Carbon-oxygen (C-O) linkages due to ethers are determined in the $^{13}$C-NMR spectrum but cannot be distinguished from C-O alcohol, phenol, or ester linkages. In the absence of any quantitative methods, ether can be estimated approximately as the deficit in oxygen accounted by direct methods of oxygen functional-group analysis or as an excess in C-O linkages indicated by the quantitative $^{13}$C-NMR spectrum after accounting for all other oxygen functional groups. Comparison of the oxygen-deficit method with the C-O excess method for ether estimate will improve the accuracy of the estimates.

Oxygen balances obtained from functional-group analyses should be compared with the oxygen balance obtained by measurement of carbons attached to oxygen in the quantitative $^{13}$C-NMR spectrum. Agreement between the two independent methods of oxygen balance confirms the accuracy of methods for oxygen analysis and also confirms the quantitation of the $^{13}$C-NMR spectrum.

## SUMMARY

The critical overview of methods applicable to structural analysis of fulvic acid from the Suwannee River stresses quantitation problems inherent in many methods. The absence of definitive quantitative methods for certain measurements, therefore, requires that circumstantial evidence be provided by convergence of semiquantitative data or by convergence of indirectly related data. Subsequent chapters in this volume present specific data on structural analysis, and the final chapter will present structural models by which convergence (or divergence) of various data can be evaluated.

## REFERENCES CITED


Davis, H., and Mott, C.J.B., 1981, Titrations of fulvic acid fractions. II. Chemical changes at high pH: Soil Science, v. 32, p. 393–397.

Fieser, L.F., and Fieser, M., 1967, Reagents for Organic Synthesis: New York, John Wiley, p. 1080.

Huffman, E.W.D., Jr., and Stuber, H.A., 1985, Analytical methodology for elemental analysis of humic substances, *in* Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediment, and Water: New York, John Wiley, p. 433–456.

Kabasakalian, P., Townley, E.R., and Yudis, M.D., 1959, Infrared determination of hydroxyl equivalents in steroids: Analytical Chemistry, v. 31, no. 3, p. 375–376.

Kovac, P., and Anderle, D., 1978, Protective alkylation, *in* Blau, K., and King, G.S., eds., Handbook of Derivatives for Chromatography: London, Heyden, p. 201–233.

Leenheer, J.A., and Noyes, T.I., 1989, Derivatization of humic substances for structural studies, *in* Hayes, M.H.B., MacCarthy, P., Malcolm, R.L., and Swift, R.S., eds., Humic Substances II: In Search of Structure: Chichester, England, John Wiley, p. 257–280.

Leenheer, J.A., Noyes, T.I., and Brown, P.A., 1986, Analysis and significance of phenolic esters in aquatic humic substances [abs.]: 8th Rocky Mountain Regional Meeting, American Chemical Society, Denver, 1986, Proceedings, p. 139.

Leenheer, J.A., Wilson, M.A., and Malcolm, R.L., 1987, Presence and potential significance of aromatic ketone groups in aquatic humic substances: Journal of Organic Geochemistry, v. 11, no. 4, p. 273–280.

Perdue, E.M., 1984, Analytical constraints on the structural features of humic substances: Geochimica et Cosmochimica Acta, v. 48, no. 7, p. 1435–1442.

Schnitzer, M., 1978, Humic substances-Chemistry and reactions, *in* Schnitzer, M., and Kahn, S.U., eds., Soil Organic Matter: New York, Elsevier, p. 1–58.

Stevenson, F.J., 1982, Reactive functional groups of humic substances, *in* Stevenson, F.J., ed., Humus Chemistry—Genesis, Composition, Reactions: New York, John Wiley, p. 221–243.

Thorn, K.A., Aterburn, J., and Mikita, M.A., 1986, $N^{15}$-NMR investigation of hydroxylamine-derivatized humic substances: 8th Rocky Mountain Regional Meeting, American Chemical Society, Denver, 1986, Proceedings, p. A–140.

Wershaw, R.L., and Aiken, G.R., 1985, Molecular size and weight measurements of humic substances, *in* Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediment, and Water: New York, John Wiley, p. 477–492.

Wershaw, R.L., Mikita, M.A., and Steelink, C., 1981, Direct $^{13}$C-NMR evidence for carbohydrate moieties in fulvic acids: Environmental Science and Technology, v. 12, no. 13, p. 1461–1463.

Chapter I

# Elemental Analysis and Heat of Combustion of Fulvic Acid from the Suwannee River

*By* M.M. Reddy, J.A. Leenheer, *and* R.L. Malcolm

## CONTENTS



### FIGURES



### TABLES



## Abstract

Elemental analysis and values of heat of combustion have been determined for a fulvic-acid sample from the Suwannee River that was collected, isolated, and characterized in conjunction with the International Humic Substances Society's reference sample program. The elemental analysis of this reference fulvic acid is consistent with a partially oxidized hydrocarbon that has small quantities of nitrogen and sulfur and is similar to a fulvic acid obtained from Coal Creek, Colorado. An empirical

formula, based on a molecular weight of 750 daltons, for the major elements in the sample is:

$$C_{34.3(\pm 0.2)}H_{33.1(\pm 0.7)}O_{19.2(\pm 0.3)}$$

Mean heat of combustion for the fulvic acid, adjusted for water content, was 3,372 kilocalories per mole with an estimated uncertainty of ±5 kilocalories per mole. This value is based on a nominal molecular weight of 750 daltons. Elemental-analysis data and the measured heat of combustion are consistent.

Hypothetical molecular structures for Suwannee River fulvic acid, based on carbon-13 nuclear-magnetic-resonance spectrometry, were assigned values for heat of combustion. The variation in the calculated heat of combustion among the proposed fulvic-acid models, calculated by assigning heat of combustion values to each bond, is insufficient to enable designation of an unambiguous molecular structure.

## INTRODUCTION

Elemental analysis has been used extensively to characterize synthetic and natural organic substances. Analytical procedures used to determine elemental composition are reliable, precise, and require relatively small quantities of sample. These procedures commonly are used to characterize fulvic and humic acids; commonly accepted analytical procedures for this purpose have been evaluated recently by Huffman and Stuber (1985).

Huffman and Stuber (1985) state that, although procedures of elemental analysis have been developed for a variety of organic substances, they have not been developed specifically for substances such as fulvic and humic acids, which are hydroscopic mixtures of related organic compounds. Another significant analytical problem associated with the determination of elemental composition of fulvic and humic acids is the reliable measurement of ash content. Ash content can have larger than expected variance in samples containing trace sodium salts (E.W.D. Huffman, Jr., Huffman Laboratories, Golden, Colorado, oral commun., 1986). Thus, elemental analyses of fulvic and humic acids may have somewhat greater uncertainties than typical elemental analyses of synthetic organic substances.

Measurements of heat of combustion can augment elemental analysis: these data may enable independent verification of the elemental composition and, in favorable circumstances, may provide some indication of molecular bonding. Several techniques (some based on the concept of bond energies, some based on functional-group contributions, and some based on both types) have been developed that enable prediction of heat of combustion within accuracies of 0.5 kcal/mol (Stull and others, 1969). Interpretation of the results of elemental analysis of fulvic and humic acids have been given by Steelink (1985), Stevenson (1982), and the references therein.

Aquatic fulvic acids are generally considered to be mixtures of related plant-decomposition products. They are characterized by a substantial degree of oxygenation and a complex functionality. Recently, the International Humic Substances Society (IHSS) began a program to prepare, characterize, and distribute reference samples of fulvic and humic acids. In this chapter, the results of elemental analyses and heat of combustion calculations for an IHSS reference sample (fulvic acid from the Suwannee River) are reported. The elemental analysis and independent estimates of molecular weight (by vapor-pressure osmometry and ultracentrifugation) and organic functional-group analysis (by nuclear magnetic resonance, NMR) have facilitated development of several molecular models of fulvic acid from the Suwannee River. These models have been examined to determine whether they are consistent with data for heat of combustion.

## EXPERIMENTAL CONDITIONS

### Elemental Analysis

Elemental analysis was performed by Huffman Laboratories, Golden, Colorado. Analytical methods, accuracy, precision, and interlaboratory comparison of the methods used are discussed in detail by Huffman and Stuber (1985). Briefly, the components analyzed and their methods of analysis were: carbon and hydrogen, modified Coulometric; oxygen, modified Coulometric Incorporated model 5060 oxygen analyzer; nitrogen, modified Dumas (Merz); sulfur, Fisher Model 475 sulfur analyzer; and ash, platinum boat inside a platinum sleeve in a tube with oxygen flow heated to 750°C to constant weight (E.W.D. Huffman Jr., Huffman Laboratories, Golden, Colorado, oral commun., 1986). Analytical accuracies are 0.3 percent absolute for elemental analysis (Huffman and Stuber, 1985).

**Table 1.** Elemental analysis of Suwannee River reference fulvic acid on an ash-free and moisture-free basis

[Results shown are for replicate analyses of two subsamples: replicates 1–3 are for subsample A, replicates 4–7 are subsample B. RSD, relative standard deviation]

| Sample | Percent | | | | | |
|---|---|---|---|---|---|---|
| | Carbon | Hydrogen | Oxygen | Nitrogen | Sulfur | Ash |
| Replicate 1 | 55.36 | 4.30 | 41.30 | 0.57 | 0.52 | 0.57 |
| Replicate 2 | 53.29 | 4.38 | 41.29 | .74 | .52 | .80 |
| Replicate 3 | 54.12 | 4.41 | 39.38 | .74 | .53 | 1.28 |
| Replicate 4 | 53.64 | 4.19 | 41.50 | .68 | .54 | 1.04 |
| Replicate 5 | 53.64 | 4.19 | 41.18 | .70 | .64 | .86 |
| Replicate 6 | 53.01 | 4.19 | 41.50 | .68 | .54 | 1.04 |
| Replicate 7 | 53.39 | 4.37 | 40.99 | .69 | .63 | .39 |
| Mean | 53.493 | 4.290 | 41.020 | .696 | .560 | .854 |
| Standard deviation | .351 | .099 | .745 | .057 | .052 | .303 |
| RSD, percent | .66 | 2.3 | 1.8 | 8.2 | 9.3 | 35.5 |

### Heat of Combustion

Determinations of heat of combustion were performed by using an automated Parr adiabatic calorimeter. Instrument calibration was monitored daily by determination of the heat of combustion of a primary standard benzoic acid obtained from the National Bureau of Standards. Instrument calibration checks agreed with the known value. A brief review of the experimental procedure and data analysis associated with measurements of heat of combustion has been presented by Shoemaker and Garland (1962).

## ELEMENTAL ANALYSIS

The elemental analysis of the fulvic acid from the Suwannee River used in this investigation is presented in table 1. Results in the table are for replicate analyses of two subsamples. Replicates 1–3 are for subsample A; replicates 4–7 are for subsample B.

Analytical precision, expressed as relative standard deviation (RSD), is best for carbon (0.66 percent). Hydrogen and oxygen have similar RSD values (2.3 percent and 1.8 percent), which may indicate variability in water content of the sample. Minor components in the sample (for example, nitrogen, sulfur, and ash) have larger RSD values than those for elements present in large concentrations. Analytical precision values in table 1 are similar to those reported by Huffman and Stuber (1985).

The elemental analysis was converted to an empirical molecular formula having a nominal molecular weight of 750 daltons (Leenheer and others, chap. P, this volume) and has the formula:

$$C_{34.3(\pm 0.2)}H_{33.1(\pm 0.7)}O_{19.2(\pm 0.3)}$$

where the values in parentheses are the standard deviations of the elemental analysis expressed in terms of the stoichiometry.

The elemental analysis for the reference fulvic acid (table 1) can be compared with a fulvic acid isolated from Coal Creek, a tributary of the Yampa River in Colorado (Huffman and Stuber, 1985). The Coal Creek sample is an aquatic fulvic acid that was prepared according to the method described by Thurman and Malcolm (1981). Sample isolation and preparation techniques were similar for the samples from the Suwannee River and Coal Creek. Analytical methods used to characterize both fulvic-acid samples have been described by Huffman and Stuber (1985). Comparison between these two samples should indicate only differences in the chemical composition.

Elemental analyses of the two fulvic acids is shown in figure 1 where the mean elemental composition (in weight percent) and the range, ±1 standard deviation, is plotted on a log scale for each element for the samples from the Suwannee River and Coal

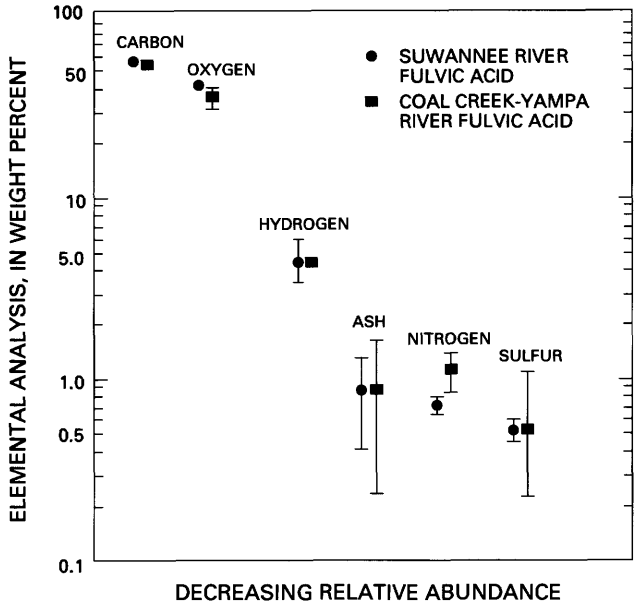


**Figure 1.** Elemental analyses of two aquatic fulvic-acid samples isolated by the procedure of Thurman and Malcolm (1981) and analyzed by the methods described by Huffman and Stuber (1985). Vertical bars indicate estimated standard deviations of each determination.

Creek. With the exception of oxygen and nitrogen, the elemental analyses for the two fulvic-acid samples are similar. The significance of the similarity of the elemental analysis of the two fulvic-acid samples is unclear at the present time.

## HEAT OF COMBUSTION

Measurements of heat of combustion are summarized in table 2. Replicate determinations were performed on samples with residual water content (Huffman and Stuber, 1985), and heat of combustion was adjusted for this water content. Uncertainties in the values of heat of combustion are due to the error in weight loss on drying and to the error in ash-content determination. Results in table 2 indicate a mean heat of combustion of 3,373 kcal/mol and an estimated uncertainty of ±5 kcal/mol.

Values of heat of combustion can also be used to obtain information concerning atomic bonding within a proposed organic structure. Indications of bond arrangement and energy can be obtained from the *heat of formation* of an organic compound. Heat of formation is calculated from the measured heat of combustion and published values for the heat of formation of the combustion products (carbon dioxide and water). An alternative approach is to assign each bond a value of heat of combustion. These values are conveniently obtained from tables of heat of combustion for simple organic compounds (Weast, 1981). This approach assumes that the heat of combustion for a bond conforms to an additive criteria. The heat of combustion of an organic compound can be calculated by summing the values of heat of combustion for individual bonds. Values of heat of combustion for various bond types anticipated to be present in the fulvic-acid sample from the Suwannee River are listed in table 3.

Bond-type contributions to the measured heat of combustion were calculated from tabulated values using an algebraic technique. Using this technique, the heat of combustion for the carbon-hydrogen (C-H) bond was determined using values of heat of combustion for n-pentane, n-hexane, n-heptane, and n-octane, each in the liquid state. A value for the C-H bond was obtained from pairwise subtraction. The subtraction involved simultaneous linear equations for each

**Table 2.** Heat of combustion for Suwannee River reference fulvic acid

[BTU/lb, British Thermal Units per pound; kcal/g, kilocalories per gram; kcal/mol, kilocalories per mole. Heat of combustion on a dry basis, heat of combustion corrected for sample ash content, and heat of combustion in units of kilocalories per gram are in columns 4, 5, and 6, respectively]

| Replicate number | Heat of combustion, wet material (BTU/lb) | Weight loss on drying (percent) | Heat of combustion | | | |
|---|---|---|---|---|---|---|
| | | | Dry material (BTU/lb) | Dry, ash-free material (BTU/lb) | kcal/g | kcal/mol |
| 1 | 7,500 | 7.29 | 8,090 | 8,157 | 4.494 | 3370.5 |
| 2 | 7,501 | 7.36 | 8,097 | 8,164 | 4.500 | 3375.0 |
| 3 | 7,500 | 7.36 | 8,096 | 8,163 | 4.500 | 3375.0 |

**Table 3.** Bond-type contribution to measured heat of combustion

[kcal/bond, kilocalories per bond]

| Bond type | Contribution to heat of combustion (kcal/bond) |
|---|---|
| Carbon-hydrogen | 54.8 ± 1.1 |
| Carbon-carbon | 45.3 ± 0.2 |
| Carbon-carbon (conjugated) | 105.4 ± 1 |
| Carbon-oxygen (aliphatic) | 8.2 ± 1 |
| Carbonyl | 14.3 ± 0.2 |
| Carboxylic | 0 |
| Hydroxyl | 0 |

alkane expressing the measured heat of combustion as a sum of the number of carbon-hydrogen (C-H) and carbon-carbon (C-C) bonds. Equations for two different alkanes were multiplied by an appropriate stoichiometric factor and then subtracted to eliminate the contribution of the C-C bond. Heat of combustion for the C-H bond was calculated directly from the result. The average value of heat of combustion for the C-H bond was 54.8 kcal/bond.

The average value for the C-H bond then was used to calculate the heat of combustion for the C-C bond in the straight-chain hydrocarbons mentioned above. The average value of heat of combustion for the C-C bond calculated in this manner was 45.3 kcal/bond. There are slight systematic deviations in the calculated values with increasing molecular weight. These trends are reflected in the standard deviations assigned to each bond-type heat of combustion in table 3. Heat of combustion for the conjugated C-C double bond was calculated from the heat of combustion for benzene using the above calculated C-H and C-C bond values. For the calculations performed here, the benzene molecule was assumed to have six C-H bonds, three C-C single bonds, and three conjugated C-C double bonds. The calculated average value for the conjugated C-C double bond was 105.4 kcal/bond. Heat of combustion for the carbon-oxygen bond was calculated using methanol, ethanol, and propanol. Heat of combustion for the carbonyl bond was calculated using acetaldehyde and propionaldehyde. Heats of combustion for the carboxylic and hydroxyl bonds were calculated using acetic, propionic, and butyric acids. The carboxylic and hydroxyl bond types have values of heat of combustion that are zero.

To test the correctness of the technique, the heat of combustion of an organic molecule (phenylacetic acid) was calculated using the data in table 3. The assignment of bond types for phenylacetic acid were: seven C-H bonds, five C-C single bonds, seven C-H bonds, three conjugated C-C double bonds, and one carboxylic bond. The calculated heat of combustion, 926.3 kcal/mol, is similar to the calorimetric value, 930.4 kcal/mol. The calculated value agrees within 0.5 percent of the calorimetric value, supporting the assigned bond-type heat of combustion.

Bond-type contributions to heat of combustion have been summed for the proposed structures of fulvic acid from the Suwannee River. The proposed structures were selected from several possible or hypothetical molecular arrangements that were indicated by carbon-13 nuclear-magnetic-resonance ($^{13}$C-NMR) spectrometry (Hatcher and others, 1980; Thorn, 1987) and elemental analysis. Representative $^{13}$C-NMR spectra used to develop the proposed structures are shown in figure 2. The liquid-state $^{13}$C-NMR spectrum in figure 2*A* was recorded at 75.4 MHz on a Varian XL–300 NMR spectrometer (for experimental details see Thorn 1987; Thorn, chap. N, this volume). Briefly, 75 mg of the hydrogen-saturated fulvic acid was dissolved in 0.5 g of deuterated and $^{13}$C-depleted dimethylsulfoxide. Acquisition parameters included: sweep width, 50,000 Hz; acquisition time, 0.2 seconds; flip angle, 45 degrees; pulse delay, 8 seconds; and line broadening, 20 Hz. The solid-state $^{13}$C-NMR spectrum in figure 2*B* was obtained using cross-polarization and magic-angle spinning (for experimental details see, for example, Hatcher and others, 1980). Briefly, samples were analyzed using a custom-built Chem-Magnetic M–100S Spectrometer. The

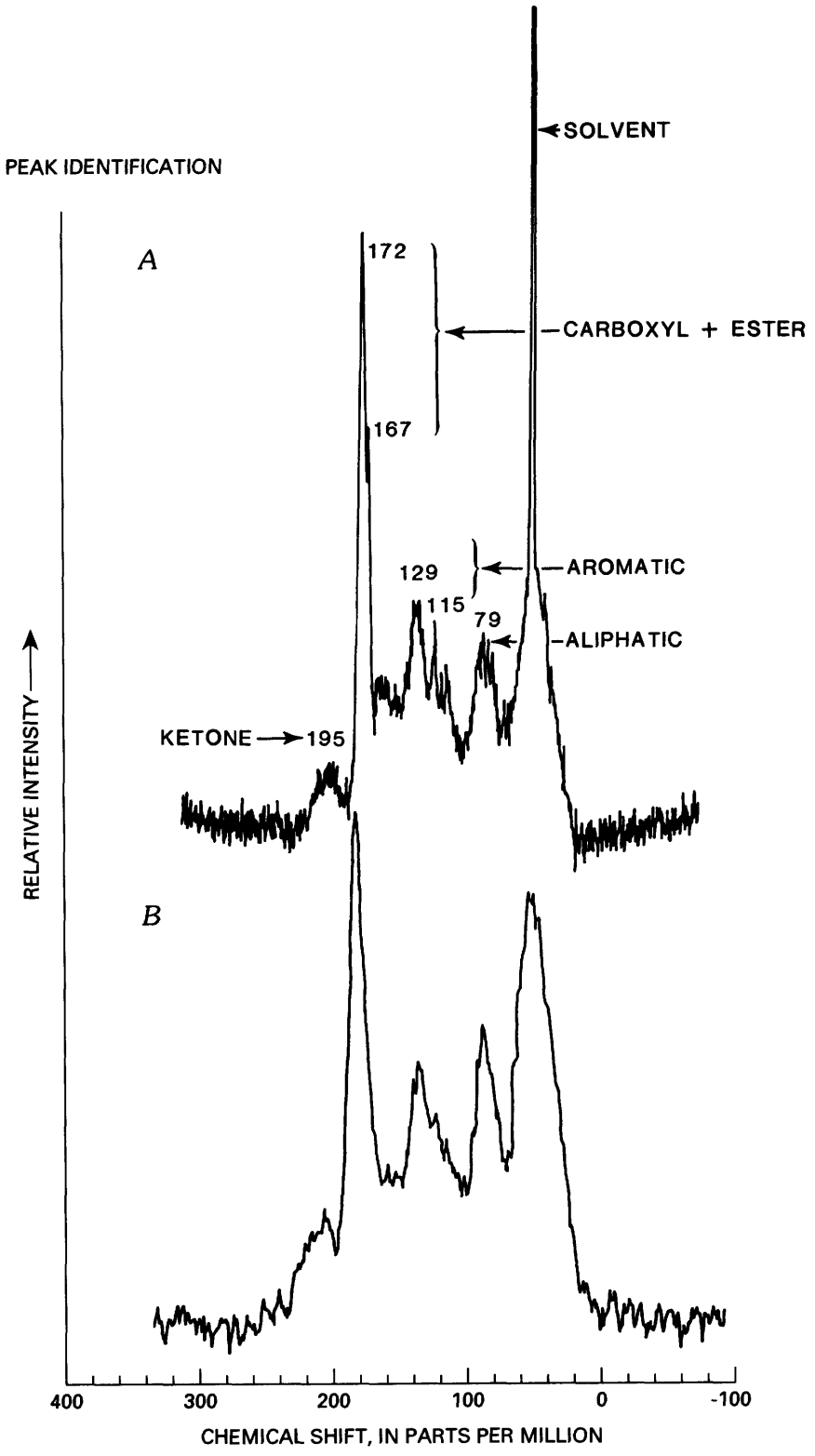


**Figure 2.** Carbon-13 nuclear-magnetic-resonance spectra of reference fulvic acid: *A*, liquid state; *B*, solid state.

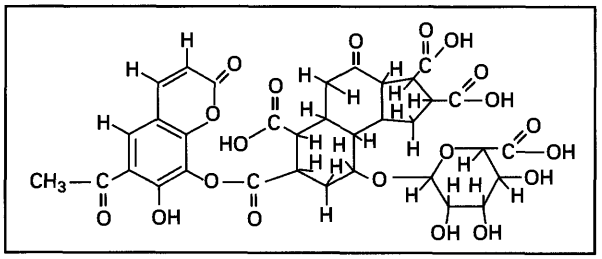

**Figure 3.** Hypothetical structural model for fulvic acid based on liquid-state carbon-13 nuclear-magnetic-resonance data.

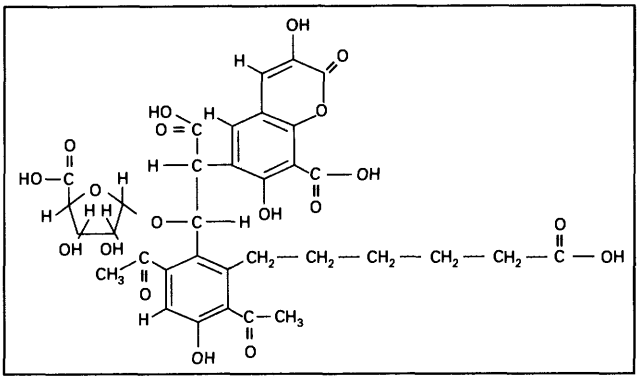

**Figure 4.** Hypothetical structural model for fulvic acid based on solid-state carbon-13 nuclear-magnetic-resonance data.

spectrum was recorded at a carbon resonate frequency of 90.1 MHz. Acquisition parameters included: spinning rate, 3 kHz; repetition rate, 1 second; and contact time, 1 millisecond. Characteristic signals for bond types in fulvic acids are also shown in figure 2.

The $^{13}$C-NMR spectra shown in figure 2 are recent spectra for the reference fulvic acid from the Suwannee River. These spectra are representative of the $^{13}$C-NMR spectral data available when the preliminary structural data first were used to develop hypothetical structural models.

The bond types and frequencies for each of the hypothetical structural models for fulvic acid from the Suwannee River (figs. 3 and 4) and their estimated contribution to the calculated heat of combustion are summarized in table 4. Results in table 4 indicate that there is not a significant difference in the estimated heat of combustion for the two hypothetical structural models. The heat of combustion appears to

**Table 4.** Summary of nominal bond-type heat of combustion to the total heat of combustion for two hypothetical structural models of fulvic acid

[kcal, kilocalories]

| Bond type | Number of bonds | Contribution to heat of combustion (kcal) |
|---|---|---|
| A. Liquid state | | |
| Carbon-hydrogen | 25 | 1,370 |
| Carbon-carbon | 27 | 1,221.8 |
| Carbon-carbon (conjugated) | 7 | 737.8 |
| Carbon-oxygen (aliphatic) | 10 | 82.0 |
| Carbonyl | 2 | 28.6 |
| Carboxylic | 5 | 0 |
| | Total | 3,440.2 |
| B. Solid state | | |
| Carbon-hydrogen | 25 | 1,370 |
| Carbon-carbon | 32 | 1,448 |
| Carbon-carbon (conjugated) | 4 | 421.6 |
| Carbon-oxygen (aliphatic) | 10 | 82 |
| Carbonyl | 2 | 28.6 |
| Carboxylic | 6 | 0 |
| | Total | 3,350.2 |

be insufficiently sensitive to identify differences in bond types in fulvic acid from the Suwannee River.

A revised molecular weight for the Suwannee River fulvic acid is 730 daltons (Leenheer and others, chap. P, this volume). This corresponds to an empirical formula of $C_{33}H_{32}O_{19}$ and a mean heat of combustion of 3,295 kcal/mol. Additional experimental data has resulted in a revised hypothetical structural model for the fulvic acid from the Suwannee River that differs from the original hypothetical structural model shown in figure 3. This revised model has been used to calculate the nominal bond-type heat of combustion. The revised hypothetical structural model, described in detail by Leenheer and others (chap. P, this volume), has a calculated heat of combustion of 3,579 kcal/mol.

## CONCLUSIONS

Elemental analyses of a reference fulvic-acid sample from the Suwannee River indicate that the fulvic acid is a partially oxidized hydrocarbon containing small quantities of nitrogen and sulfur. Heat of combustion of this reference sample is consistent with elemental analysis but does not enable differentiation among several hypothetical structural models based on independent experimental evidence.

## ACKNOWLEDGMENTS

The authors would like to express their appreciation to K.A. Thorn and R.L. Wershaw of the U.S. Geological Survey, Denver, Colorado, and to G.E. Maciel, Colorado State University, Fort Collins, Colorado, for conducting nuclear-magnetic-resonance analyses of the reference fulvic acid used in this investigation.

## REFERENCES CITED

Hatcher, P.G., VanderHart, D.L., and Earl, W.L., 1980, Use of solid-state $^{13}C$ NMR in structural studies of humic acids and humin from Holocene sediments: Organic Geochemistry, v. 2, no. 1, p. 87–92.

Huffman, E.W.D., Jr., and Stuber, H.A., 1985, Analytical methodology for elemental analysis of humic substances, *in* Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediment, and Water: New York, Wiley-Interscience, p. 433–455.

Shoemaker, D.P., and Garland, C.W., 1962, Experiments in Physical Chemistry: New York, McGraw-Hill, 471 p.

Steelink, Cornelius, 1985, Implications of elemental characteristics of humic substances, *in* Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediment, and Water: New York, Wiley-Interscience, p. 457–476.

Stevenson, F.J., 1982, Humus Chemistry: New York, John Wiley, 443 p.

Stull, D.R., Westrum, E.F., Jr., and Sinke, G.C., 1969, The Chemical Thermodynamics of Organic Compounds: New York, John Wiley, p. 140–154.

Thorn, K.A., 1987, Structural characteristics of the IHSS Suwannee River fulvic and humic acids determined by solution state C–13 NMR spectroscopy: The Science of the Total Environment, v. 62, p. 175–183.

Thurman, E.M., and Malcolm, R.L., 1981, Preparative isolation of aquatic humic substances: Environmental Science and Technology, v. 15, no. 4, p. 463–466.

Weast, R.C., ed., 1981, Heats of combustion for organic compounds, *in* CRC Handbook of Chemistry and Physics: Boca Raton, Florida, CRC Press, p. D251–D257.

Chapter J

# Molecular Size and Weight of Fulvic and Humic Acids from the Suwannee River

*By* G.R. Aiken, P.A. Brown, T.I. Noyes, *and* D.J. Pinckney

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### FIGURES



### TABLES

## Abstract

Molecular-size and molecular-weight data for aquatic fulvic and humic acids isolated from the Suwannee River are presented. Measurements were made by using small-angle X-ray scattering, vapor-pressure osmometry, equilibrium ultracentrifugation, and fast-atom bombardment mass spectrometry. The fulvic acids in three samples are shown to be relatively *monodisperse* by small-angle X-ray scattering and to have an average *radius of gyration* of 7.5 angstroms. A number-average molecular weight of about 800 daltons is a reasonable general estimate for these samples. The humic acids in two samples are shown to be more polydisperse than the fulvic acids by small-angle X-ray scattering and to have an average radius of gyration of 11.3 angstroms.

## INTRODUCTION

The purpose of this chapter is to present molecular-size and molecular-weight data obtained for fulvic- and humic-acid samples from the Suwannee River by small-angle X-ray scattering, vapor-pressure osmometry (VPO), and equilibrium ultracentrifugation. A multimethod approach is desirable because each method yields a different type of molecular-weight information about the samples. In addition, some mass-spectral data obtained by fast-atom bombardment mass spectrometry (FABMS) will be presented.

There are a number of methods (small-angle X-ray scattering, gel filtration, ultrafiltration) that measure molecular size. In these methods, model compounds of known molecular size, weight, and composition are used to estimate the molecular size and weight of humic substances. Problems can arise if the model compounds are not sufficiently similar to the humic substances of interest. Choice of appropriate model compounds is hampered by lack of detailed information about the chemical structures of humic substances.

The usual methods used to determine molecular weights yield average molecular weights, and, depending on the methods used, these averages are not directly comparable (Lansing and Kraemer, 1935). Failure to consider this fact is one source of confusion in analyzing and using molecular-weight data for humic substances. Two different types of molecular-weight averages commonly used for humic substances are shown below:

1. Number-average molecular weight, $\overline{M}_n$, is determined by physiochemical methods that determine the total number of molecules present, regardless of size. It is expressed mathematically as:

$$\overline{M}_n = \frac{\sum_i n_i M_i}{\sum_i n_i} \qquad (1)$$

where

$n_i$ is the number of molecules of molecular weight $M_i$.

Colligative-property measurements (e.g., freezing-point lowering, *vapor-pressure lowering*, *boiling-point elevation*, and osmotic-pressure change), which depend on the number of molecules in solution, are used to determine $\overline{M}_n$. In the case of a polydisperse system, $\overline{M}_n$ emphasizes the lower molecular weight species in a mixture (Moore, 1972).

2. Weight-average molecular weight, $\overline{M}_w$, is determined by methods such as light scattering and sedimentation, which depend on the masses of material in different fractions (Moore, 1972). Weight-average molecular weight is expressed as:

$$\overline{M}_w = \frac{\sum_i n_i M_i^2}{\sum_i n_i M_i} \qquad (2)$$

Weight-average molecular weight emphasizes the heavier molecular weight species in a mixture, resulting in higher molecular-weight values than ($\overline{M}_n$). For a monodisperse system, $\overline{M}_n = \overline{M}_w$; for a polydisperse system, $\overline{M}_n < \overline{M}_w$. The ratio of $\overline{M}_w$ to $\overline{M}_n$ can be used as an indication of polydispersity (Stevenson, 1982).

The molecular weight of a compound is one of the most basic and useful types of chemical information. In the study of humic substances, molecular weights are important for: (1) establishing molecular formulas in conjunction with data provided by other methods of characterization, (2) establishing stoichiometric relations between humic substances and other chemical species, and (3) comparing humic substances extracted from various environments. Despite the utility of molecular-weight data, however, the task of

determining molecular weights for humic substances is not simple. These materials comprise one of the most widely distributed classes of natural products on Earth and are a complex mixture of organic substances. It is because of the complexity of this mixture that so many important analytical methods for the characterization of humic substances, including the methods used for molecular-weight determination, yield data of limited usefulness.

## DESCRIPTION OF METHODS

A brief description of each of the methods used to obtain molecular-size and molecular-weight data for fulvic- and humic-acid samples from the Suwannee River will be presented in this section. A critical review of the methods has been written (Wershaw and Aiken, 1985), and the reader is directed to this paper for a more thorough discussion.

### Small-Angle X-Ray Scattering

The radius of gyration, $R_g$, which is defined as the root-mean-square distance of the electrons in a molecule from the center of charge, is a useful general characteristic of molecular size and is related to molecular weight (Tanford, 1961). $R_g$ is obtainable by using small-angle X-ray scattering where X-rays are impinged on a molecule. The angular distribution and the intensity of scattered X-rays of the same frequency as the impinging radiation are functions of the size and shape of the molecules. From the measured values of the intensity of scattered radiation, I(h), and the scattering angle, $2\theta$, $R_g$ is given by the following expression:

$$\ln I(h) = \frac{(-h^2) R_g^2}{3} + \text{constant} \qquad (3)$$

where

$h=2\pi(\sin 2\theta)/\lambda$, and

$\lambda$ is the wavelength of the impinging X-ray.

$R_g$ is calculated from the slope of the plot of $\ln I(h)$ versus $h^2$; this is called a *Guinier plot.* For a monodisperse system in which all of the scattering particles are of the same molecular size, the Guinier plot is a straight line; for a polydisperse system, the plot is concave upward.

Molecular-weight data can be estimated most simply from $R_g$ data by comparing the radii of standards of known molecular weight with the radii of the unknown material. Careful selection of appropriate standards that have chemical properties (such as molecular size, polarity, and functional group distribution) similar to those of the unknown material should be made. In this chapter, the radii of fulvic acids from the Suwannee River were compared with the radii of other aquatic fulvic acids for which $\overline{M}_n$ data were available in order to obtain an estimate of $\overline{M}_n$ for fulvic acids from the Suwannee River.

### Vapor-Pressure Osmometry

A colligative property is a thermodynamic property that depends on the number of particles in solution and not on the nature of these particles. The colligative properties of a solution are: vapor-pressure lowering, freezing-point lowering, boiling-point elevation, and osmotic-pressure change. VPO is a method used to determine the extent of vapor-pressure lowering of a solvent as a result of solvating a solute. The vapor-pressure difference between the solution and the pure solvent is determined by measuring the temperature increase associated with the condensation of vapor into the solution. These data then are used to calculate $\overline{M}_n$ for the solute. The VPO method is included in works by Bonnar and others (1958) and Glover (1975) that describe the determination of molecular weight by measurement of colligative properties. Application of colligative-property measurements to the study of humic substances in particular is discussed by Aiken and Gillam (1989).

VPO data for fulvic acids from the Suwannee River were obtained in two different solvent systems. The data obtained in water were corrected for dissociation according to the method of Gillam and Riley (1981). The data obtained in tetrahydrofuran (THF) did not require any correction for dissociation because THF has a low *dielectric constant.* The equation defining the relation between the instrument response, $\theta$, and the concentration of the sample, w, is given by:

$$\theta=\theta_0+aw+bw^2 \qquad (4)$$

The $\overline{M}_n$ of the solute is related to the instrument calibration constant, $K_{app}$, and the first virial coefficient, *a*, by the following equation:

$$\overline{M}_n = \frac{K_{app}}{a} \quad (5)$$

The second virial coefficient, *b*, is not directly related to the molecular weight; however, *b* does affect the value of *a*. The value for $K_{app}$ is determined for a given solvent and osmometer system by determining the first virial coefficients for standards of known molecular weight. The procedures outlined by Glover (1975) to obtain the best data fit and the most reliable molecular weight were then employed.

### Equilibrium Ultracentrifugation

Equilibrium ultracentrifugation is a method that can be used to determine $\overline{M}_w$ data. At equilibrium, the concentration gradient of a solution in a centrifugal or gravitational field is such that, at every point in the solution, the chemical potential is equal but opposite in sign to the gravitational potential (Lansing and Kraemer, 1935). The concentration of solute at any point, x, in the cell can be determined spectrophotometrically, and the $\overline{M}_w$ at point x, $\overline{M}_{w,x}$, can be calculated from the following:

$$\overline{M}_{w,x} = \left(\frac{2RT}{(1-\bar{v}\rho)\,\omega^2}\right)\left(\frac{2.303\,d\,(\log c)}{d\,(r^2)}\right) \quad (6)$$

where

R is the universal gas constant ($8.315 \times 10^7$ ergs per degree per mole),

T is the absolute temperature, in degrees Kelvin,

$\bar{v}$ is the *partial specific volume* of solute, in milliliters per gram,

$\rho$ is the density of solution, in grams per milliliter,

$\omega$ is the angular velocity, in radians per second,

c is the concentration of solute at point x, in milligrams per milliliter, and

r is the radial distance of point x, in centimeters.

In equilibrium ultracentrifugation, it is assumed that the light-absorption properties and partial specific volumes of all solutes in a mixture are independent of molecular weight and can be represented by the average values obtained for the mixture as a whole. In addition, it is assumed that there are no charge effects of any kind between molecules of solute. It is recognized that these assumptions are not rigorously met for humic materials; however, within the constraints of the method, useful information may still be obtained. The reader is referred to Swift (1989) for a more thorough discussion of the application of equilibrium ultracentrifugation to the study of humic substances.

### Fast-Atom Bombardment Mass Spectroscopy

Fast-atom bombardment mass spectroscopy is a method designed to provide mass-spectral data of underivatized polar molecules (Rinehart, 1982). In this method, the sample is dissolved in a solvent such as glycerol or thioglycerol and is bombarded by neutral argon or xenon atoms. In this collision, energy is transferred to the molecules, and the resultant ions are sputtered off the sample probe. The advantage of this method compared to other mass-spectral methods is that FABMS is a low-energy ionization method, and a greater number of molecular ions survive the ionization process than is the case with other methods.

## MOLECULAR SIZE

Radii-of-gyration data for fulvic and humic acids from the Suwannee River are given in table 1. These values are within the range of $R_g$ values for aquatic humic substances reported by Thurman and others (1982). Measurements were made at pH 6 and pH 9. Differences between the resulting $R_g$ values have been attributed to pH-dependent aggregation effects (Wershaw and Pinckney, 1973). There is good agreement of the $R_g$ data between the International Humic Substances Society's (IHSS) standard and reference fulvic acids—these were isolated from samples that were collected over the same time period. The fulvic acid in another sample collected from the Suwannee River (fulvic-acid sample 1217), however, has a larger $R_g$ value than those of the IHSS standard and reference fulvic acids. This sample was collected on a single day during the collection of the IHSS standard and reference fulvic-acid samples. The $R_g$ values for the IHSS standard and reference humic acids were larger than those for corresponding fulvic acids.

A typical Guinier plot for fulvic acid from the Suwannee River (fulvic-acid sample 1217) is shown in figure 1. The Guinier plot for the fulvic acid in each of the samples in this study is linear. Within the

**Table 1.** Radii-of-gyration data for fulvic and humic acids

[IHSS, International Humic Substances Society; leaders (--) indicate that estimates of molecular weight were not made at pH = 6 due to possible aggregation effects]

| Sample | pH | Radius of gyration (angstroms) | Estimated molecular weight (daltons) |
|---|---|---|---|
| IHSS standard fulvic acid | 6 | 6.8, 7.6 (7.2)[1] | -- |
| | 9 | 7.1, 8.3 (7.7) | 711 |
| IHSS reference fulvic acid | 6 | 7.6, 7.0 (7.3) | -- |
| | 9 | 7.3, 6.6 (7.0) | 645 |
| Fulvic-acid sample 1217 | 9 | 8.8 | 816 |
| IHSS standard humic acid | 6 | 11.1 | -- |
| | 9 | 11.4 | 1,066 |
| IHSS reference humic acid | 6 | 11.4 | -- |
| | 9 | 11.3 | 1,056 |

[1]Average values are in parentheses.

limits of the X-ray experiment, the samples appear to be monodisperse; however, this term is misleading because fulvic acid is recognized as a complex mixture of organic acids. Therefore, these samples are said to be relatively monodisperse, meaning that the distribution of particle sizes in the samples is narrow (i.e., ±0.588Å).

## MOLECULAR WEIGHT

It is possible to estimate the molecular weight of fulvic acid in these samples from $R_g$ data by comparing the $R_g$ of the fulvic acid with the $R_g$ data obtained for aquatic fulvic acids for which molecular-weight data have been obtained by other methods. The fulvic acids from the Suwannee River were compared with other aquatic fulvic acids for which $R_g$ data and $\overline{M}_n$ data were available (Aiken and Malcolm, 1987).

Estimates of molecular weight (table 1) were made at pH 9, because, at this pH, aggregation effects are minimized relative to pH 6. By comparing these estimated values with the $\overline{M}_n$ data obtained by VPO (table 2), it is apparent that the molecular-weight

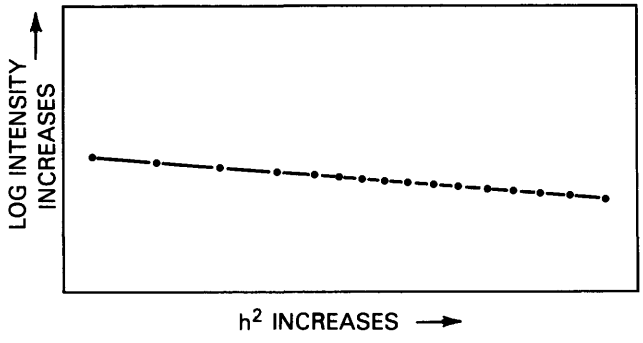


**Figure 1.** Guinier plot for a 1-percent solution of fulvic acid at pH 9.

**Table 2.** Number-average molecular weights of fulvic acids obtained by vapor-pressure osmometry

[IHSS, International Humic Substances Society; THF, tetrahydrofuran]

| Sample | Solvent | | | |
|---|---|---|---|---|
| | THF | | $H_2O$ | |
| IHSS standard fulvic acid | 766<br>746 | (756)[1] | 844<br>623 | (734) |
| IHSS reference fulvic acid | 802<br>760 | (781) | 865<br>681 | (823) |
| Fulvic-acid sample 1217 | 840<br>841 | (840) | 959<br>689 | (824) |

[1]Average values are in parentheses.

estimates that are based on $R_g$ data are lower than the $\overline{M}_n$ determined by VPO.

The $\overline{M}_n$ values obtained for these fulvic acids by VPO in THF and water are presented in table 2. The results obtained in THF are in close agreement with the results obtained in water. THF is a better solvent than water for VPO measurements because the instrument-calibration constant, $K_{app}$, obtained in THF is greater than that obtained in water, and because it is difficult to obtain accurate pH data on these samples to correct for dissociation. As a result, the data obtained in THF are more consistent and reproducible than the data obtained in water. The data obtained in water are important, however, because these values more closely reflect the molecular-weight values expected for these solutes in natural waters. On the basis of these data, a value of about 800 daltons appears to be a good average estimate for fulvic acid from the Suwannee River.

Equilibrium ultracentrifugation is a particularly useful method for the determination of molecular weight because values can be calculated for both $\overline{M}_n$ and $\overline{M}_w$, and the degree of polydispersity can be estimated from the ratio of $\overline{M}_w$ to $\overline{M}_n$. Recently, an $\overline{M}_n$ value of 655 daltons and an $\overline{M}_w$ value of 1,335 daltons have been obtained on the IHSS reference fulvic acid by equilibrium ultracentrifugation. The $\overline{M}_n$ value of 655 daltons is less than the value of 781 daltons obtained by VPO and may indicate some degree of aggregation in the VPO experiment. The $\overline{M}_w$ to $\overline{M}_n$ ratio for the IHSS reference fulvic acid is 2.04 and indicates that the sample is more polydisperse than the results indicated by small-angle X-ray scattering measurements. The molecular-weight measurements of the IHSS reference fulvic acid determined by equilibrium ultracentrifugation were made in THF to minimize charge effects and solute-solute interactions. An advantage of equilibrium ultracentrifugation compared to methods such as small-angle X-ray scattering and VPO is that the measurements are made using dilute solutions where solute-solute interactions leading to aggregation effects are minimized. Small-angle X-ray scattering and VPO require relatively large concentrations of solute (1–2 percent).

The $\overline{M}_n$ values obtained for the fulvic acids from the Suwannee River by VPO and equilibrium ultracentrifugation are in good agreement with $\overline{M}_n$ values obtained by other workers for aquatic fulvic acids from other environments that vary from dark-water systems with small specific-conductance values and large dissolved-organic-carbon concentrations to marine environments with large specific-conductance values and small dissolved-organic-carbon concentrations. The narrow range of molecular weights obtained for aquatic fulvic acids, 500 to 950 daltons, is controlled by the solubility considerations of polarity and molecular size. Compared with the fulvic-acid data in table 3, the fulvic acids from the Suwannee River are heavier than fulvic acids isolated from most surface waters and are similar in molecular weight to other fulvic acids isolated from dark-water systems (such as Lake Celyn, Wales). Because VPO measurements are made using solutions containing relatively large concentrations of solutes (1–2 percent), solute-solute interactions are possible. The resulting values are not as large as has been suggested in other

**Table 3.** Number-average molecular-weight values for aquatic fulvic acid reported in the literature

| Site | Molecular weight (daltons) | Authors |
|---|---|---|
| Suwannee River, Georgia | 829 | Aiken and Malcolm (1987)[1] |
| Coal Creek, Colorado | 743 | Aiken and Malcolm (1987)[1] |
| Ohio River, Ohio (fall) | 594 | Aiken and Malcolm (1987)[1] |
| (spring) | 618 | Aiken and Malcolm (1987)[1] |
| Missouri River, Iowa | 640 | Aiken and Malcolm (1987)[1] |
| Ogeechee River, Georgia | 714 | Aiken and Malcolm (1987)[1] |
| Oyster River, New Hampshire | 626 | Wilson and Weber (1977)[2] |
| Satilla River, Georgia | 614 | Reuter and Perdue (1981)[1] |
| A peaty stream, Cheshire, United Kingdom | 943 | Gillam and Riley (1981)[1] |
| Lake Celyn, Wales, United Kingdom | 846 | Gillam and Riley (1981)[1] |
| River Dee, Wales, United Kingdom | 761 | Gillam and Riley (1981)[1] |
| Irish Sea (marine) | 623 | Gillam and Riley (1981)[1] |
| Skaggerak (marine) | 501-792 | Gillam and Riley (1981)[1] |

[1]Values obtained by VPO.
[2]Value obtained by cryoscopy.

molecular-weight studies utilizing ultrafiltration or gel filtration; however, these other methods are subject to interactions that can lead to higher molecular-weight values (Wershaw and Aiken, 1985).

The fact that the molecular weights of aquatic fulvic acids are moderate is a major constraint on structural modeling and influences our understanding of aquatic fulvic acid chemistry. The chemical implications of moderate molecular weight can be observed for fulvic acid from the Suwannee River. From elemental-analysis data (Reddy and others, chap. I, this volume) and the molecular weight of about 800 daltons (as determined in water), it can be shown that this sample is a heterogeneous mixture. Only 1 out of every 2.5 molecules can contain a nitrogen atom, for instance. Similarly, not all molecules can possess the same chemical characteristics or trace constituents. McKnight and others (1983) report the presence of two types of Cu(II) binding sites in aquatic fulvic acids. For the fulvic acid from the Suwannee River, the concentrations of type I and II sites is $1.2\times10^{-6}$ and $2.7\times10^{-7}$ mol/mg of carbon, respectively. Only 1 out of every 2 molecules of this fulvic acid, therefore, can have a type I site, and only 1 out of every 8 molecules can have a type II site. Other structural information can be obtained by considering the distribution of functional groups in this fulvic acid. Thurman and Malcolm (1983) report that the carboxyl content of fulvic acid from the Suwannee River is 6.0 mmol/g. With a molecular weight of about 800 daltons, this sample contains about 4.8 carboxyl functional groups per molecule. These molecules, therefore, are not polyelectrolytes and cannot be modeled as such.

Relatively few attempts have been made to apply mass spectrometry to the study of fulvic and humic acids from the Suwannee River. Mass spectrometry is greatly hampered by the polydisperse nature of this material, because it is almost impossible to deconvolute mass-spectral data of mixtures. Future developments in separation science will increase the applicability of this method to the study of humic substances. Of particular interest is FABMS, which is a method designed to obtain mass-spectral data on the types of molecules present in fulvic acid. A spectrum for Suwannee River fulvic acid at three magnification factors is presented in figure 2. The absence of mass-to-charge ratios greater than 1,100 in the spectrum and the heavy distribution of mass-to-charge ratios less than 800 provides support for molecular-weight data provided by the other methods discussed in this chapter. These data are consistent for a relatively polydisperse mixture with a $\overline{M}_n$ of 800. The spectrum, however, is too complicated to provide detailed information about individual constituents of the fulvic acid. A number of experimental factors, in addition to the polydisperse nature of the fulvic acid, contribute to the overall complexity of the spectrum, and it is not possible to sort out the effects of each factor.

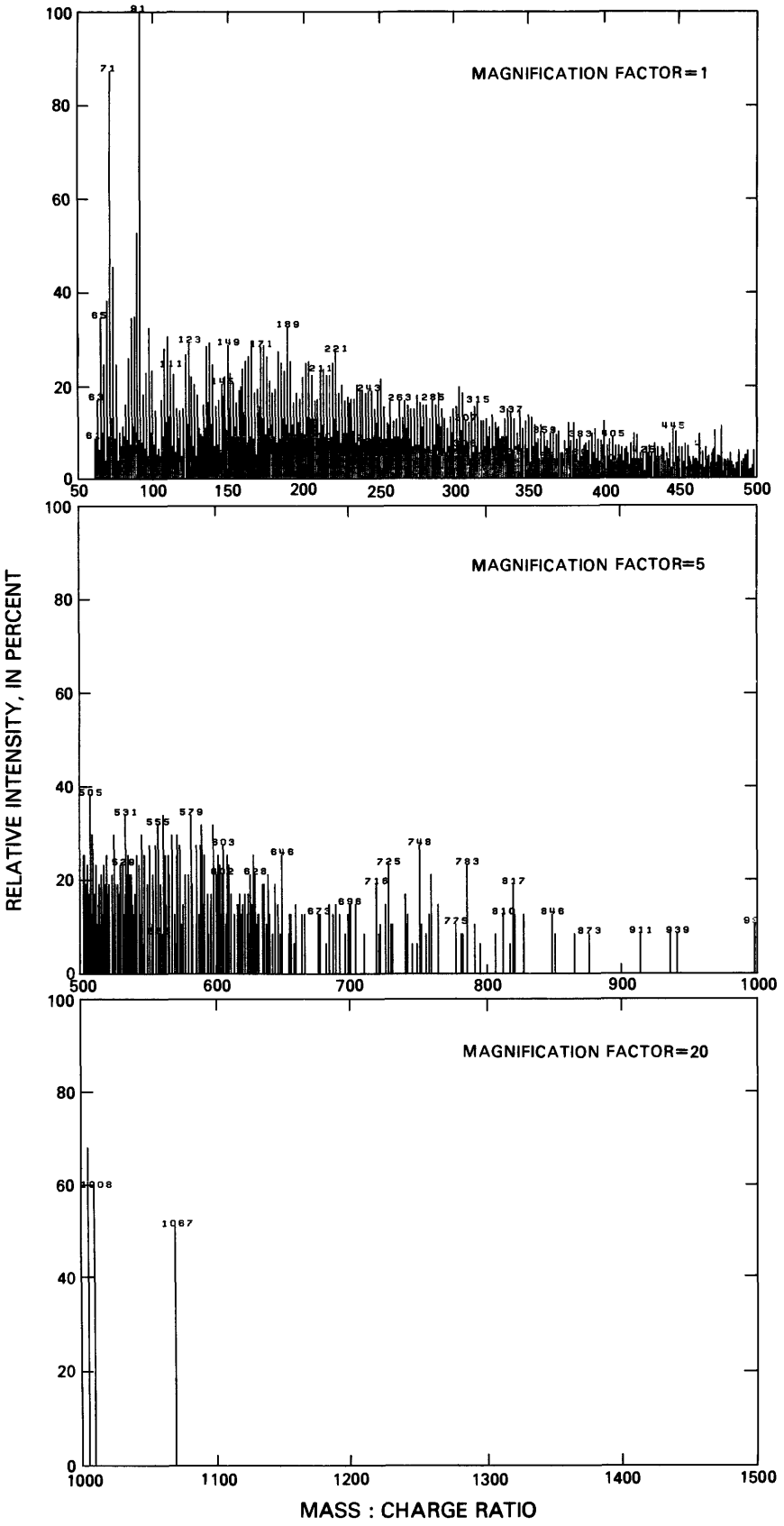


**Figure 2.** Fast-atom bombardment mass spectroscopy spectrum of fulvic acid obtained using xenon gas and 8 kilovolts accelerating voltage.

## CONCLUSIONS

To adequately define the molecular size and weight of aquatic fulvic and humic acids isolated from the Suwannee River, it is necessary to use a number of different methods of determination. In this chapter, the results obtained using small-angle X-ray scattering, VPO, equilibrium ultracentrifugation, and FABMS methods have been described. The results from each of these methods are in close agreement, and it appears that an acceptable estimate of $\overline{M}_n$ in water is about 800 daltons and that this material in water is relatively monodisperse with respect to size and weight.

The lower molecular-weight values and the small range of values obtained for fulvic acid are probably the result of solubility constraints for this class of compounds in water. Because fulvic acid represents approximately 80 percent of the dissolved organic carbon in the Suwannee River, these results indicate that the molecular-weight distribution of dissolved organic carbon in this system is less than would be estimated by size-distribution studies using gel filtration or ultrafiltration because these methods are subject to interactions that can lead to higher molecular-weight values.

## REFERENCES CITED


Aiken, G.R., and Gillam, A.H., 1989, Determination of molecular weights of humic substances by colligative property measurements, *in* Hayes, M.H.B., MacCarthy, P., Malcolm, R.L., and Swift, R.S., eds., Humic Substances II: In Search of Structure: Chichester, England, John Wiley, p. 515–544.

Aiken, G.R., and Malcolm, R.L. 1987, Molecular weights of aquatic fulvic acids by vapor pressure osmometry: Geochimica et Cosmochimica Acta, v. 51, p. 2177–2184.

Bonnar, R.V., Dimbat, M., and Stross, F.H., 1958, Number Average Molecular Weights: New York, Wiley-Interscience, 310 p.

Gillam, A.H., and Riley, J.P., 1981, Correction of osmometric number-average weights of humic substances for dissociation: Chemical Geology, v. 33, p. 355–366.

Glover, C.A., 1975, Absolute colligative property measurements, *in* Slade, P.E., Jr., ed., Polymer Molecular Weights. Part 1: New York, Marcel Dekker, p. 79–159.

Lansing, W.D., and Kraemer, E.O., 1935, Molecular weight analysis of mixtures by sedimentation equilibrium on the Svedberg ultracentrifuge: Journal of the American Chemical Society, v. 57, p. 1369–1377.

McKnight, D.M., Feder, G.L., Thurman, E.M., Wershaw, R.L., and Westall, J.G., 1983, Complexation of copper by aquatic humic substances from different environments: Science of the Total Environment, v. 28, p. 65–76.

Moore, W.J., 1972, Physical Chemistry (4th ed.): Englewood Cliffs, N.J., Prentice-Hall, 977 p.

Reuter, J.H., and Perdue, E.M., 1981, Calculation of molecular weights of humic substances from colligative data: Application to aquatic humus and its molecular size fractions: Geochimica et Cosmochimica Acta, v. 45, p. 2017–2022.

Rinehart, K.L., 1982, Fast atom bombardment mass spectrometry: Science, v. 218, p. 254–260.

Stevenson, F.J., 1982, Humus Chemistry: Genesis, Composition, Reactions: New York, Wiley-Interscience, 443 p.

Swift, R.S., 1989, Molecular weight, size and shape of humic substances by ultracentrifugation, *in* Hayes, M.H.B., MacCarthy, P., Malcolm, R.L., and Swift, R.S., eds., Humic Substances II: In Search of Structure: Chichester, England, John Wiley, p. 467–498.

Tanford, C., 1961, Physical Chemistry of Macromolecules: New York, John Wiley, 709 p.

Thurman, E.M., and Malcolm, R.L., 1983, Structural study of humic substances: New approaches and methods, *in* Christman, R.F., and Gjessing, E.T., eds., Aquatic and Terrestrial Humic Materials: Ann Arbor, Michigan, Ann Arbor Science, p. 1–23.

Thurman, E.M., Wershaw, R.L., Malcolm, R.L., and Pinckney, D.J., 1982, Molecular size of aquatic humic substances: Organic Geochemistry, v. 4, p. 27–35.

Wershaw, R.L., and Aiken, G.R., 1985, Molecular size and weight measurements of humic substances, *in* Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediment, and Water: New York, John Wiley, p. 477–492.

Wershaw, R.L., and Pinckney, D.J., 1973, Determination of the association and dissociation of humic acid fractions by small angle X-ray scattering: U.S. Geological Survey Journal of Research, v. 1, p. 702–707.

Wilson, S.A., and Weber, J.H., 1977, A comparative study of number-average dissociation corrected molecular weights of fulvic acids isolated from water and soil: Chemical Geology, v. 19, p. 285–293.

Chapter K

# Fluorescence Measurements of the Volume, Shape, and Fluorophore Composition of Fulvic Acid from the Suwannee River

*By* M.C. Goldberg *and* E.R. Weiner

## CONTENTS



### FIGURES

TABLE



## Abstract

This chapter presents results of a detailed study in which techniques of *fluorescence spectrometry* were used to measure the physical characteristics of fulvic acid. Information about the molecular conformation, volume, and shape was obtained using steady state and dynamic depolarization measurements where the ratio of temperature to solution viscosity was varied and reciprocal polarization was measured. These measurements indicate that fulvic acid rotates isotropically in water at pH 6.5, which is characteristic of a spherical shape. Measurements of reciprocal polarization as a function of pH show that the shape of fulvic acid in water remains constant throughout the pH range from 3 to 11.5 and indicates that the conformation changes outside this range. The equivalent spherical radius of fulvic acid was measured as 1.17 nanometers in water and 1.04 nanometers in dimethylsulfoxide, corresponding to *hydrodynamic molar volumes* of 3,950 cubic centimeters and 2,860 cubic centimeters, respectively. Several hypotheses concerning solvent- and pH-dependent structural changes are proposed for these results. *Phase-resolved emission spectra*, fluorescence-lifetime measurements, and three-dimensional plots of the spectral excitation-emission matrix are reported. The excitation maximum was at 348 nanometers when the excitation was scanned holding the emission constant at 460 nanometers, and the emission maximum was 460 nanometers when the emission was scanned holding the excitation constant at 350 nanometers. The phase-resolved emission spectrum was measured at constant excitation of 350 nanometers and contained emission peaks at 417 nanometers and 455 nanometers. All these data indicate that at least two different *fluorophores* are present in fulvic acid. Their fluorescence lifetimes were measured as 1.05±0.12 nanoseconds and 6.54±0.14 nanoseconds in water and 1.31±0.14 nanoseconds and 6.78±0.36 nanoseconds in dimethylsulfoxide at 30 degrees Celsius. With two or more fluorophores, the amount of depolarization depends on the individual lifetimes, making it necessary to use weighted average values for lifetime and limiting-polarization values in the calculation of molecular volume. This problem is discussed and a method for calculating the required weighted quantities is presented.

## INTRODUCTION

There are several experimental approaches to the determination of the physical dimensions of a molecular species, each with its own set of limitations (Aiken and others, 1985). In general, obtaining clear, unambiguous values for these properties always requires that some other properties of the substance (such as molecular structure, configuration, weight, rigidity, and chemical composition) be accurately known. In the absence of such supporting data, there is always an uncertainty in the interpretation of volume and shape measurements.

In this chapter, we present the theory and results of measurements on fulvic acid (FA) using fluorescence spectrometry. The fluorescence techniques are attractive for this application because of the natural fluorescence of the sample, the high sensitivity of fluorescence detection, and the ability to directly observe the morphology of the molecule in aqueous solutions without the need for drying or applying harsh chemical conditions. Some interesting types of information obtained from fluorescence measurements are:

1. Fluorescence-lifetime measurements can increase the analytical specificity when analyzing mixtures (Love and Upton, 1980; Weiner and Goldberg, 1982; Demas, 1983; and Goldberg, 1984) and can indicate changes in chemical binding of the fluorophores under various environmental conditions (Lakowicz, 1983).
2. Depolarization measurements, coupled with fluorescence lifetimes, are correlated with rates of molecular rotation to obtain estimates of molecular conformation, volume, and shape.

Applications of fluorescence emission spectrometry to detect chemical signatures from water are well documented. M.C. Goldberg discusses in Meyers and Welch (1975, chapter 19, p. 1497–1501) the application of fluorescence spectrometry coupled with Raman spectrometry to measure water characteristics from a remote sensor. In many of these applications, the materials producing the signatures are humic substances. Goldberg and Devonald (1973) developed a fluorescence technique for quantitatively determining the amount of organic material in surface-water films. Specific applications were given for the Houston Ship Channel in Texas. The fluorescence excitation spectrum was used in specifying the types of materials present, such as bulk hydrocarbons. The fluorescence emission spectrum was used for quantification. The treatment depended on adequate standards and some prior knowledge of the film composition. An example of fluorescence spectrometry as an analytical technique is found in Goldberg and Wilson (1974), where thiamine and ferricyanide were determined in water and wastewater.

In this chapter, new information is reported concerning the physical properties of fulvic acid from the Suwannee River. Specialized techniques of fluorescence depolarization spectrometry and phase-shift fluorometry allow the nondestructive determination of molar volume and shape in aqueous solutions of varying *ionic strength* and pH. The techniques also provide sufficient data to make a reliable estimate of the number of different fluorophores in the molecule and their respective excitation and emission spectra. These measurements are possible even in instances where two fluorophores have almost the same emission spectrum. The general theoretical background of each method is presented first, followed by the specific results of measurements by that method on fulvic acid. Parts of the theoretical treatment of depolarization and phase-shift fluorometry given here are more fully expanded upon in Parker (1968), Weber (1977, 1981), and Lakowicz (1983).

## PRINCIPLES OF DEPOLARIZATION MEASUREMENTS

Any factor that affects the size or shape of a molecule or the hindered movement of a fluorophore within a molecule will affect the measured depolarization of its fluorescence emission. Therefore, the conformation of fulvic acid in solution can be studied as a function of pH, ionic strength, temperature, and other factors by depolarization measurements. The principle of the method is that excitation of fluorescent samples with polarized light stimulates emission that is also polarized. This occurs because molecules with absorption-dipole moments parallel to the polarization plane of the exciting light are selectively excited. Molecules excited by polarized light have absorption-dipole moments closely parallel to one another. Because the emission dipole moments of excited molecules have a fixed angular relation to their absorption moments, the emission moments will also tend to be parallel, resulting in polarized emission in the absence of rotation. Any rotation of a molecule that occurs after excitation by polarized light and before fluorescence emission takes place contributes to the partial depolarization of the emitted light, which is easily measured. From these data and the fluorescence lifetimes, the rotational relaxation time can be calculated.

A depolarization measurement consists of exciting a fluorescent sample with linearly polarized light and measuring the polarization of light emitted at right angles to the axis of excitation. The polarization of the emitted light is defined as:

$$P = \frac{(I_{\parallel} - I_{\perp})}{(I_{\parallel} + I_{\perp})} \qquad (1)$$

where

$I_{\parallel}$ and $I_{\perp}$ are the measured intensities when the detector polarizer is respectively oriented parallel and perpendicular to the plane of the exciting light.

There are several different factors that contribute to the depolarization of emitted fluorescence relative

to the polarization of the excitation light. Most of these can be controlled by experimental parameters, but two factors are intrinsic to the method and must be evaluated:

1. One intrinsic cause of depolarization is the nature of the photoexcitation process. When a compound is excited with polarized light, the probability of excitation is greatest for those fluorophores with absorption-dipole moments parallel to the excitation polarization plane. In a sample with a random distribution of absorption-dipole moments, as in an isotropic solution, the probability of excitation by linearly polarized light is proportional to $\cos^2\theta$ (where $\theta$ is the angle between an absorption-dipole moment and the plane of excitation polarization). This results in an angular distribution of absorption-dipole moments in the excited molecules. The light emitted from excited fluorophores is polarized parallel to their emission dipole moment. If the absorption and emission dipoles are colinear and no other causes of depolarization exist, then the angular distribution of dipoles results in a maximum emission polarization of P=0.5 (Lakowicz, 1983).
2. In general, the absorption and emission dipoles are not colinear but have an intrinsic angular difference within the fluorophore. This causes an additional degree of depolarization of the emitted light relative to the exciting light. The angular displacement of the emission and absorption dipoles can be determined by measuring the depolarization in a dilute, vitrified solution, where rotation is not possible and radiationless energy transfer has only a small probability.

In addition to the intrinsic causes of depolarization, there are several experimentally controllable factors that can contribute to a change in orientation of the emission dipole moment:

1. Radiationless transfer of energy among fluorophores results in depolarization because of a broadening in the distribution of emission dipole orientations. Because energy transfer is strongly dependent on the distance between emitter and absorber, depolarization can be used to measure the average distance between fluorophores when other contributions to depolarization are subdued. The method is most useful when the average separation of absorbing centers is about 5 nm, which corresponds to concentrations approximately 0.013 M. Therefore, radiationless energy transfer is measured in viscous, moderately concentrated solutions (to minimize rotational diffusion).
2. Rotational diffusion during the lifetime of the excited state of the fluorophores also contributes to a change in orientation of the emission dipole moment between the times of energy absorption and emission. The molecular rotation rate, and hence the degree of depolarization from this cause, is greatest in solutions of low viscosity. Rotational diffusion, therefore, is measured in solutions of low viscosity and low concentration to minimize radiationless energy transfer.

It is generally easy to experimentally separate the depolarization effects of energy transfer and rotational diffusion in fulvic acid samples. Viscosity can be controlled in aqueous solutions by adding glycerol or sucrose, and concentrations can be as small as $10^{-5}$ M or less and still provide conveniently strong fluorescence signals. By using dilute, low-viscosity solutions, the effects of intermolecular energy transfer are minimized, and the effects of rotationally caused depolarization are maximized. The resultant measured depolarization is determined by rotational motion of the fluorophore and the intrinsic offset of its absorption and emission dipoles. The following section describes how the contribution from the intrinsic dipole offset can be measured and the rate of molecular rotational diffusion determined by making measurements at a series of viscosities or a series of temperatures or both. The rate of rotational diffusion can be related to molar volume if the shape of the molecule and the microviscosity of its environment are known or assumed.

## Molecular Rotational Diffusion

Rotational diffusion is the dominant intrinsic cause of depolarization under conditions of low solution viscosity and low fluorophore concentration. Polarization measurements under these conditions are accurate indicators of molecular size. Two types of measurements are used: steady-state depolarization and time-dependent (dynamic) depolarization.

### Steady-State Fluorescence Depolarization Spectrometry

For steady-state depolarization measurements, the sample is excited with linearly polarized light of

constant intensity. Observed values of P depend on the angle between the absorption and emission dipole moment vectors. In equation 2 (from Parker, 1968, p. 57), $P_o$ is the limiting value of polarization for a dilute solution of fluorophores that are randomly oriented in a rigid medium that permits no rotation and with no energy transfer to other fluorophores:

$$P_o=(3\cos^2\beta-1)/(\cos^2\beta+3) \quad (2)$$

where

$\beta$ is the angle between the absorption and emission dipole moment vectors.

$P_o$ can range from a maximum of 1/2 to a minimum of −1/3 as $\beta$ varies from 0 to 90 degrees. To a first approximation, $P_o$ is a property of the fluorophore only, the environmental effects being small enough to neglect. For the case of a fluorophore bound to a larger molecule, $P_o$ depends on the intrinsic offset of the absorption and emission dipoles and on any restricted rotation of the fluorophore that is independent of the overall rotation of the larger molecule. Restricted rotation of the fluorophore will increase with temperature, decreasing the value of $P_o$. This effect is discussed by Lakowicz (1983, chap. 5).

When rotational diffusion occurs, additional depolarization will result. At fast rates of rotation, where the molecule turns through a large angle during the emission lifetime, complete randomization of polarization can occur. At slower rates of rotation, where the rotational period is of the same order of magnitude as the emission lifetime, there will be partial depolarization of the emitted light. Under conditions of partial depolarization, a molar volume can be calculated from equation 3, which is one form of the Perrin equation (Lakowicz, 1983, p. 136):

$$(1/P-1/3)=(1/P_o-1/3)[1+(3\tau/\rho)] \quad (3)$$

where

P is the observed depolarization of emission from all causes,

$P_o$ is the depolarization of the emission due only to intrinsic causes, without any other depolarizing factors,

$\tau$ is the lifetime of the excited fluorophore, and

$\rho$ is the rotational relaxation time of the molecule after excitation.

The rotational relaxation time, $\rho$, is related to the molecular volume by the equation:

$$\rho = \frac{3\eta V}{RT} \quad (4)$$

where

$\eta$ is the solution viscosity,

V is the equivalent volume of a spherically rotating molecule,

R is the gas constant, and

T is the temperature in degrees Kelvin.

The quantity $\eta V/RT$ is known as the rotational correlation time and is equal to six times the rotational period. The rotational relaxation time, $\rho$, should be shorter than the fluorescence lifetime, $\tau$, for these equations to apply.

It is possible to perform calculations for nonspherical molecules such as prolate and oblate ellipsoids of revolution, but, in such cases, there are different rotational rates about the different principal axes. If the measured rotation rate varies with changes in excitation wavelength, it indicates that the depolarizing rotations are not isotropic and that the molecule is nonspherical.

When equation 4 is substituted into 3, equation 5 is obtained:

$$(1/P-1/3)=(1/P_o-1/3)+(1/P_o-1/3)\,(\tau RT/\eta V) \quad (5)$$

Equation 5 has the form y=mx+b, the equation of a straight line, with y=(1/P−1/3), x=T/$\eta$, m (the slope) =$(1/P_o-1/3)(\tau R/V)$, and b (the y intercept) =$(1/P_o-1/3)$. Therefore, a graph of 1/P versus T/$\eta$, known as a Perrin plot (see fig. 1), should yield a straight line with slope m and y intercept $1/P_o$. The molecular equivalent-sphere volume can be determined from the slope by substituting $P_o$, $\tau$, R, and m into equation 6:

$$V=(1/P_o-1/3)\,(\tau R/m) \quad (6)$$

The intercept, $1/P_o$, is called the anisotropy of the molecule and is an indication of the nonrotational depolarization of the molecule. This "intrinsic" depolarization is due to the segmental motion of the fluorophores within the molecule and the depolarization due to energy transfer.

Experimentally, the quantity T/$\eta$ is varied for Perrin plots either by holding T constant and changing $\eta$, or by holding $\eta$ constant and changing T. Usually, $\eta$ is varied in aqueous solutions by adding sucrose or glycerol. When T is the constant, the viscosity of pure water and the viscosity of pure glycerol or

saturated sucrose solution determine the upper and lower limiting values of $T/\eta$. If the molecule containing the fluorophore changes its conformation at some given temperature, as might occur with high temperature denaturation, a temperature-varying Perrin plot will have an abrupt change of slope at a particular temperature (Ellerton and Isenberg, 1969). It is possible to identify a conformation change dependent on a variable such as pH, by plotting 1/P against pH (or another variable) at constant $T/\eta$. Because 1/P is related to 1/V through equations 5 or 9, its value will remain constant at constant $T/\eta$, as long as V does not change.

### Effect of Multicomponent Signals on Depolarization Measurements

If two or more components contribute to the signal, as with fulvic acid, the measured value of P will be a weighted average of the P values from each component. Therefore, weighted averages of $\tau$ and P must be used in equation 5. To our knowledge, this concern has not been discussed in the literature. We offer a method for obtaining weighted values for $\tau$ and P that requires measuring the phase-resolved lifetimes of each fluorophore. Weighted values for $\tau$ and P can be calculated from equations 7 and 8:

$$\tau' = \sum_i \tau_i f_i \qquad (7)$$

$$P' = \sum_i P_i f_i \qquad (8)$$

where

the subscript "i" indicates measured values for the pure ith component,

$f_i$ is a weighting factor proportional to the phase-resolved signal intensity of the ith component, and

$\tau'$ and $P'$ are the sample weighted average lifetime and limiting polarization, respectively.

The weighting factors are determined from the phase-resolved lifetime measurements, which are discussed later in the "Phase-Resolved Emission Spectra" section. For a sample with more than one fluorophore, equations 5 and 6 become the equivalent equations 9 and 10:

$$(1/P-1/3)=(1/P_o-1/3)+(1/P_o-1/3)\ (\tau'RT/\eta V) \qquad (9)$$

$$V=(1/P_o-1/3)\ (\tau'R/m) \qquad (10)$$

Equations 9 and 10 were used to obtain the molar volumes of fulvic acid.

## MOLAR VOLUME AND SHAPE OF FULVIC ACID

The molar volumes of Suwannee River fulvic acid are listed in table 1 at pH 6.5 and pH 4.0 in water. The molar volume of fulvic acid in water at pH 6.5 is 3,950 $cm^3$ and 4,390 $cm^3$ at pH 4.0. In dimethylsulfoxide (DMSO), the volume is 2,860 $cm^3$, which is smaller than water—a fact that allows some speculative comment in the "Discussion" section. It should be noted that the precision of measuring molar volumes is lessened by the heterogeneity of the material.

In figure 1, the Perrin plot is approximately linear to a value of 23 on the x-axis. Below 23, the depolarization of the molecule is influenced by segmental motion of the fluorophores and other "intrinsic" mechanisms of depolarization that are not based on rotational motion. The difference between the linear extrapolated part of the curve and the extrapolated value from the point nearest the ordinate (called $P_o-P_o'$) is a measure of the segmental motion of the fluorophores. These values are listed in table 1 as the

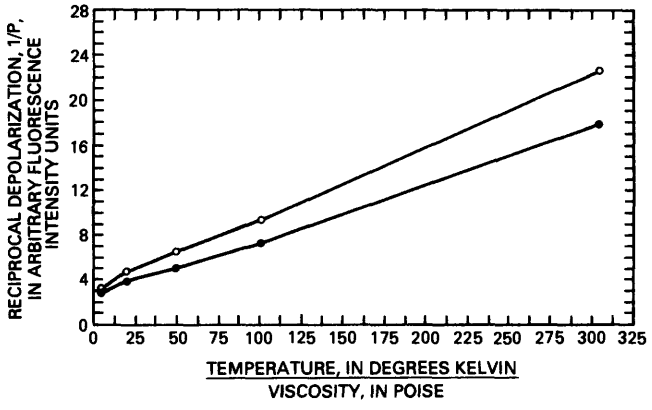


**Figure 1.** Representative Perrin plot of fulvic acid. The reciprocal polarization of each of two fluorophores is plotted against temperature divided by solution viscosity.

**Table 1.** Depolarization data for fulvic acid in water and in dimethysulfoxide

[$\tau'$, weighted fluorescence lifetimes in seconds; $P_o$, depolarization of the molecule with zero segmental motion; $P_o'$, depolarization of the molecule including the segmental motion of the fluorophores; °C, degrees Celsius; T, temperature in degrees Kelvin; $\eta$, solution viscosity (poise)]

| Material | $\tau'$[1] calculated by equation 7 | Molar volume in cubic centimeters | Radius in nanometers | Segmental anisotropy[2] $P_o - P_o'$ | Percent of depolarization due to segmental motion of the fluorophores |
|---|---|---|---|---|---|
| Suwannee River fulvic acid pH 6.5 in water, 30°C. | $4.01\times10^{-9}$ | 3,950 | 1.17 | 0.157 | 31.3 |
| Suwannee River fulvic acid pH 4.0 in water, 30°C. | $3.93\times10^{-9}$ | 4,390 | 1.16 | 0.153 | 30.6 |
| Suwannee River fulvic acid in DMSO, 30°C. | $3.99\times10^{-9}$ | 2,860 | 1.04 | 0.110 | 22.1 |

[1]Weighted sum lifetimes calculated by equation 7.

[2]Anisotropy calculated as the difference between the straight line extrapolation to the ordinate of the Perrin plot and the extrapolation to the ordinate of the lowest $T/\eta$ value measured. This is the 'hindered' or fluorophoric intramolecular internal motion that results in depolarization.

segmental anisotropy. These values are attributed to "hindered" rotation of the fluorophores within the molecule. For fulvic acid in water, this accounts for almost 40 percent of the "intrinsic" ($P_o'$) depolarization, and in the case of fulvic acid in DMSO, it accounts for about 24 percent of the "intrinsic" depolarization.

## STEADY-STATE DEPOLARIZATION MEASUREMENTS ON FULVIC ACID

In figure 2, 1/P versus pH is plotted at constant $T/\eta$ for fulvic acid. Within measurement error, 1/P is constant between pH 3 and pH 11, indicating that the shape of the fulvic acid molecule does not change over eight orders of magnitude of hydronium-ion concentration. In fact, this points to a very stable molecular conformation over a wide pH range. Outside of this range, the value of 1/P changes sharply, indicating a shape change at very high and very low pH values. This result is confirmed by the work of Lapen

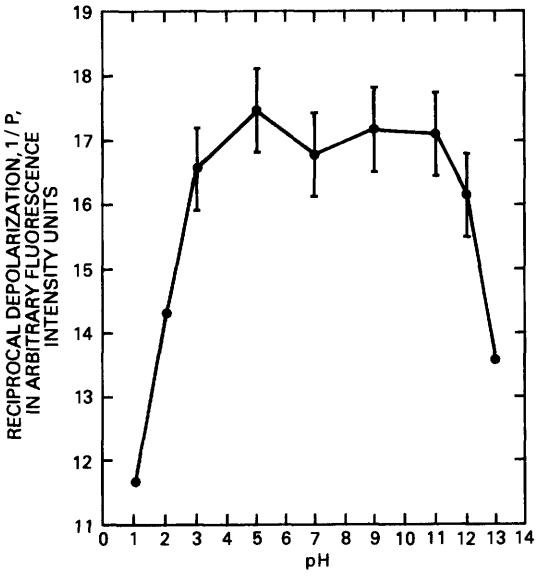


**Figure 2.** Reciprocal depolarization of fulvic acid from the Suwannee River as a function of pH in the range from 1 to 13. Vertical bars are error limits in the data.

and Seitz (1982). They reported no conformational changes between pH 5 and pH 8 for a soil-derived fulvic acid. As stated, our results confirm this for an aquatic fulvic acid and further specify the stability range below pH 5 to pH 3 and above pH 8 to pH 11. One might speculate that, at high pH values, the fulvic acid becomes completely deprotonated, losing some hydrogens that are important to intramolecular hydrogen bonding, which results in conformational changes. In the low pH range, protons could be forced onto oxygen atoms that are present in linkages of a ketonic, etheric, phenolic, or carboxylic nature, resulting in the suspected conformational changes. It is suggested that the molecular conformation changes only with severe internal changes such as forced protonation or deprotonation of a fairly stable molecular group.

## DYNAMIC DEPOLARIZATION SPECTROMETRY

Using a pulsed or modulated excitation light source instead of constant illumination allows investigation of the time dependence of emission polarization. In the case of pulsed excitation, the measured quantity is the time decay of fluorescent emission polarized parallel and perpendicular to the excitation plane of polarization. Emitted light polarized parallel to the excitation plane decays faster than the excited-state lifetime because the molecule is rotating its emission dipole away from the polarization plane of measurement. Emitted light polarized perpendicular to the excitation plane decays more slowly because the emission dipole moment is rotating toward the plane of measurement.

With a modulated light source, the phase difference between the perpendicular and parallel components of the emission is measured. The time delay of the perpendicular component of emission is longer than that of the parallel component because the molecule requires a certain period of time to rotate into the perpendicular orientation where it can be detected through the perpendicular polarizer.

Both pulse and modulation methods permit an estimate of a spherical volume for a macromolecule and are especially useful for determining deviations from molecular sphericity.

### Pulsed Method

#### Time-Resolved Fluorescence Depolarization

In equation 3, it is assumed that the polarization decays to zero as a single exponential function—this is equivalent to assuming that the molecular shape is spherical with isotropic rotational motion. Multiexponential decays arise from *anisotropic* rotational motion, which might indicate: a nonspherical molecule, a molecule rotating in a nonuniform environment, a fluorophore bound to the molecule in a manner that hinders its motion, or a mixture of fluorophores with different rotational rates.

In principle, pulsed excitation measurements can provide direct observation of time-resolved polarization decays and permit the single-exponential or multiexponential nature of the decay curves to be measured. In practice, however, accurate quantification of a multiexponential curve often requires that the emission decay be measured down to low intensity values, where obtaining a satisfactory signal-to-noise ratio can be a time-consuming process. In addition, the accuracy of rotational-rate measurements close to a nanosecond or less are severely limited by the pulse width of the flash lamps. As a result, pulsed-excitation polarization measurements are not commonly used for short rotational periods or for careful measurements of rotational anisotropy.

#### Measurements on Fulvic Acid

Lochmuller and Saavedra (1986) used the pulsed method to examine a soil-derived fulvic acid and found rotational relaxation times that ranged from 2.64 ns to 4.44 ns as pH, ionic strength, and sample concentration were varied. At low ionic strengths, they measured a "small but detectable" size increase when pH was increased from 2 to 8 and attributed this to an expansion of the fulvic acid due to accumulation of negative charge from carboxylate ionization. Because their sample was a soil-derived fulvic acid, which is likely to have structural differences from an aquatic-derived fulvic acid, and because their conclusion was based on measurements at only four different pH values with no indication of limits of error, we do not feel that their results necessarily are in conflict with our finding that the conformation of fulvic acid is essentially constant from pH 3 to pH 11.5. Over the pH range 2 to 8, our measurements also

would indicate an increase in size because of the conformational change below pH 3. At pH values less than 4.0, Lochmuller and Saavedra (1986) observed a biexponential decay of the polarization, indicating that their sample either was nonspherical or that it consisted of two differently sized fulvic acid components. This result is consistent with our conclusion that the molecular conformation changes at a pH of less than 3.

## Modulation Method

The interpretation of emission phase-angle shifts in the light-modulation method is more complicated than the analysis of decay curves from pulsed excitation, but the modulation technique is more rapid and can determine rotational rates and degree of anisotropy on a sub-nanosecond scale. Experimentally, the sample is excited with polarized, sinusoidally modulated light. The phase-angle difference, $\Delta$, between the perpendicular and parallel polarized components of the emission is measured. The relationship between tan $\Delta$ and molecular rotations has been derived by Weber (1977). The dependence of tan $\Delta$ on the limiting polarization, excited-state lifetime, rotational diffusion rate, and the light modulation frequency is shown in equation 11:

$$\tan\Delta = \frac{2R\tau^2\omega r_o}{\frac{1}{9}m_o(1+\omega^2\tau^2) + (\frac{2R\tau}{3})(2+r_o) + 2(R\tau)^2} \qquad (11)$$

where

R is the rotational rate,

$\tau$ is the fluorescence lifetime,

$\omega$ is the modulation frequency of the oscillator,

$\nu$ is equal to 2, which is the excitation modulation frequency,

$r_o$ is the anisotropy that would be observed in the absence of rotational diffusion, and

$m_o=(1+2r_o)/(1-r_o)$.

The maximum value of tan $\Delta$ is given by:

$$\tan\Delta_{max} = \frac{P_o\omega\tau}{\{1+[(1-P_o^2)(1+\omega^2\tau^2)]^{1/2}\}} \qquad (12)$$

The value of tan $\Delta$ depends upon the modulation frequency, the excited-state lifetime, and the rate of rotation. The value decreases to zero when the rotation period is either longer or shorter than the excited-state lifetime and is a maximum when the two times are comparable in magnitude. Tan $\Delta$ also increases as the modulation frequency increases. For spherical rotators, the measured value of tan $\Delta$ for a given modulation frequency and excited-state lifetime allows the rotational rate to be calculated from:

$$2R\tau^2 + 2R\tau\left[\frac{2+r_o}{3} - \left|\frac{r_o}{\tan\Delta}\right|(\omega\tau)\right] + \frac{1}{9}m_o(1+\omega^2\tau^2) = 0 \qquad (13)$$

Then, the molecular volume may be determined as for steady-state measurements using equation 6.

For spherical rotators, the measured value of tan $\Delta_{max}$ is independent of the rate of rotation. However, for nonspherical molecules, the measured value of tan $\Delta_{max}$ depends on the molecular shape and is always smaller than the measured value of tan $\Delta_{max}$ for spherical molecules. Nonsphericity can be detected by calculating the tan $\Delta_{max}$ for spherical molecules (using equation 12) and then measuring the sample throughout a temperature range that causes the sample rotation rate to vary in the region of tan $\Delta_{max}$. If the measured tan $\Delta_{max}$ is smaller than the calculated value, the molecule is nonspherical. One can distinguish between anisotropic rotation and the segmental motion of the fluorophores in the molecule (hindered rotation), both of which cause a tangent defect (the difference between the experimentally measured tangent maximum and the calculated tangent maximum). The decrease in value of tan $\Delta_{max}$ (tangent defect) caused by hindered rotation (segmental motion of the fluorophores within the molecule) can be as large as 100 percent, much larger than for anisotropic rotations, for which tangent defects seldom exceed 25 percent. By noting the magnitude of the tangent defect, the cause can be assigned to either anisotropic rotation of the molecule or hindered rotation of the fluorophores.

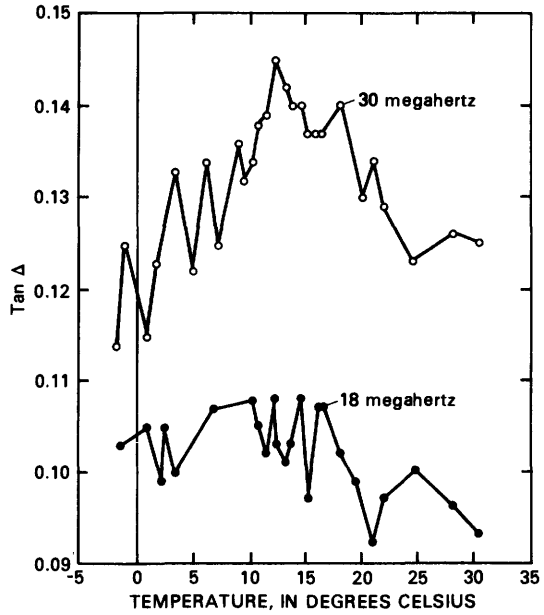


**Figure 3.** Dynamic depolarization showing the tangent defect of fulvic acid from the Suwannee River. The differential tangent determined by dynamic depolarization measurements is plotted against temperature in degrees Celsius at two frequencies (18 and 30 megahertz).

### Measurements

Our measurements of tan Δ as a function of temperature are plotted in figure 3 and have a maximum between 13°C and 15°C. These measurements were made at pH 6.5 using two frequencies, 18 and 30 megahertz. A calculation of the tan $\Delta_{max}$ according to equation 12 is in agreement with the measured tan $\Delta_{max}$. This indicates the fulvic acid molecule conforms to the mathematical description in equations 11, 12, and 13 and is rotating isotropically as a sphere. Correlating this information with the discussion in the "Steady-State Fluorescence Depolarization Spectrometry" section leads to the conclusion that fulvic acid, in water, is approximately spherical between the pH range of 3 to 11.5.

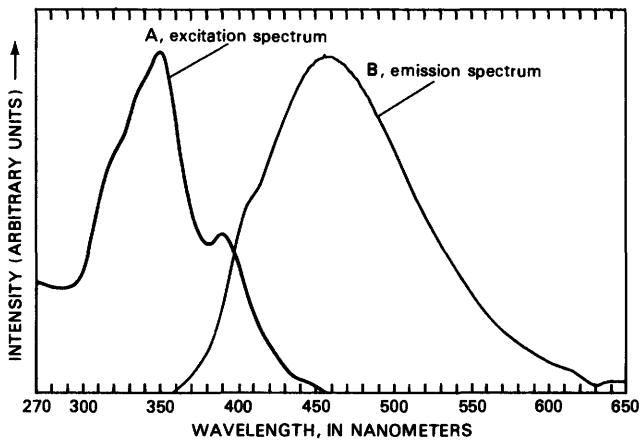


**Figure 4.** Fluorescence excitation and emission spectra of fulvic acid from the Suwannee River. Arbitrary intensity is plotted against wavelength from 270 to 650 nanometers.

## FLUOROPHORE COMPOSITION OF FULVIC ACID

If two or more fluorophores are present, they generally have different fluorescence lifetimes and excitation and emission spectra. Using these criteria, it can be shown that fulvic acid has at least two chromophores.

Figure 4 shows the excitation (curve A) and emission (curve B) spectra of aquatic Suwannee River fulvic acid at pH 6.5. The excitation spectrum was obtained by monitoring the fluorescence emission at a single wavelength while scanning the excitation wavelength and corresponds to a normal UV-visible absorption measurement. The emission spectrum was obtained by exciting the sample at a constant wavelength while scanning the fluorescence emission wavelength. The emission spectrum has a single broad peak between 450 and 470 nm with a shoulder at 405 nm, suggesting a multicomponent emission spectrum. The short-wavelength limit of the emission spectrum should correspond to the 0-0 band emission from the first excited electronic state of a fluorophore. The long-wavelength limit of the excitation spectrum should correspond to the 0-0 band excitation from the ground electronic state of a fluorophore. If only a single fluorophore is present, the excitation band should end at the wavelength where the emission band begins. A small amount of band overlap can be explained by thermal vibrational excitation that occurs after electronic excitation but before fluorescent emission (Parker, 1968). However, the large overlap of the bands in figure 4, about 90 nm, most likely results from more than one fluorophore contributing to the spectra.

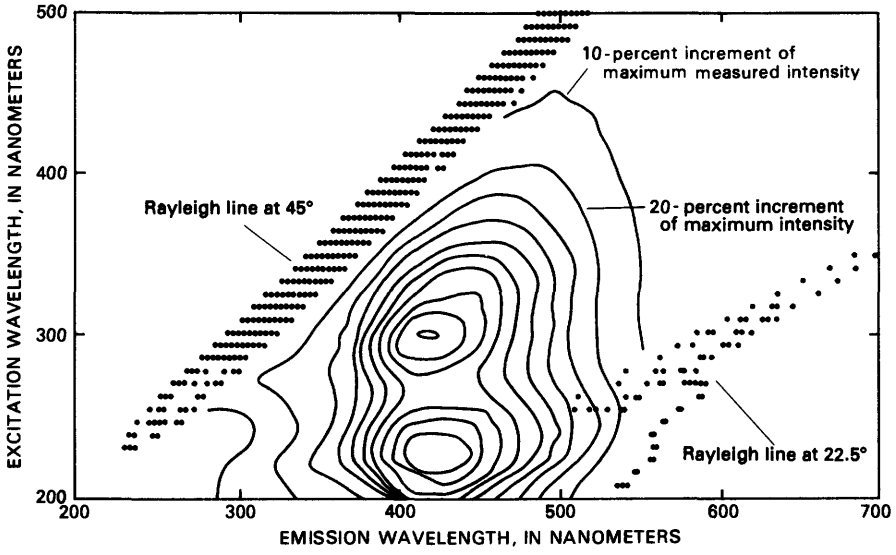


**Figure 5.** Three-dimensional fluorescence spectrum of fulvic acid from the Suwannee River at pH 4.5. Signal intensity is indicated as a 10-percent contour-line increment of the maximum concentration measured. The typical 45-degree Rayleigh line is noted at 45 degrees and the second-order line is noted at 22.5 degrees to the abscissa.

## Three-Dimensional Excitation-Emission Matrix

A more encompassing presentation of the excitation and emission characteristics of a fluorescent sample is obtained by plotting the three-dimensional excitation-emission matrix (EEM), which presents with a single display the emission spectra obtained at all possible excitation wavelengths and the excitation spectra obtained at all possible emission wavelengths (Warner, and others, 1977; Weiner, 1978; Weiner and Goldberg, 1982). An EEM for aquatic fulvic acid at pH 4.5, obtained in our laboratory, is presented in figure 5. The line at 45 degrees to the origin is caused by *Rayleigh scattering* of the incident light from the excitation grating. A second-order Rayleigh scattering is noted at 22.5 degrees to the right of the 550-nm position on the abscissa. These lines are artifacts and are not part of the fluorescence spectrum of the molecule. The EEM of fulvic acid has two distinct peaks, one at about 230-nm excitation and 420-nm emission and the other at 300-nm excitation and 415-nm emission—a pattern that is consistent with the presence of two fluorophores. (The Raman peak has been suppressed with cut-off filters in front of the detector.)

If a vertical line is drawn from 450 nm on the emission axis, a plot of the values of the incremented contour lines that intersect this vertical line is the same as an excitation spectrum monitored at 450 nm. Such a plot may be compared with the excitation spectrum, curve A, in figure 4, which was obtained with the emission monitored at 470 nm. The important difference between the two samples was pH. The excitation spectrum, monitored at an emission of 450 nm of the sample shown in figure 5, would have peaks at 240 and 330 nm, compared with the sample of figure 4, which has peaks at 350 and 390 nm. (Note that the spectrum in figure 4 does not extend to less than 270 nm because of excitation-lamp limitations.) The peak at 390 nm in figure 4 is due to Raman scattering of water. The two samples appear to be similar. The peaks at 350 nm in figure 4 and 330 nm in figure 5 could be from the same fluorophore but shifted because of the difference in pH. In figure 4, the rising signal at 270 nm suggests another possible peak at shorter wavelengths that may correspond to the 240-nm peak in figure 5.

It is of interest to compare the excitation spectra in figures 4 and 5 with excitation spectra of a soil-derived fulvic acid published by Ghosh and Schnitzer (1980, 1981). Their spectra have peaks near 360 and 465 nm, but no peaks at less than 360 nm that might correspond to the 240-nm peak in figure 5. The only common feature of the two sets of data is that both

have a peak in the 330 nm to 360 nm region. These differences are not surprising, considering the different origins of the samples: The samples of Ghosh and Schnitzer (1980, 1981) are all soil derived, whereas our samples are aquatic derived. Lochmuller and Saavedra (1986) have published an EEM of a soil-derived fulvic acid that has one main peak at 390-nm excitation, 509-nm emission; and two much smaller peaks around 350-nm excitation, 492-nm emission; and 455-nm excitation, 521-nm emission. It is possible that these spectral differences are related to structural differences between soil- and aquatic-derived fulvic acids.

## Phase-Resolved Emission Spectra

### Theory

If two or more fluorophores with different emission lifetimes contribute to the same broad, unresolved emission spectrum, their separate emission spectra sometimes can be resolved by the technique of phase-resolved fluorometry. In this method the excitation light is modulated sinusoidally, usually in the radio-frequency range, and the emission is analyzed with a phase-sensitive detector. The emission appears as a sinusoidally modulated signal, shifted in phase from the excitation modulation and partially demodulated by an amount dependent on the lifetime of the fluorophore excited state (Lakowicz, 1983, chap. 4). The detector phase can be adjusted to be exactly out-of-phase with the emission from any one fluorophore, so that the contribution to the total spectrum from that fluorophore is suppressed. For a sample with two fluorophores, suppressing the emission from one fluorophore leaves a spectrum caused only by the other component, which then can be directly recorded. With more than two fluorophores the problem is more complicated but a number of techniques for deconvoluting the complex emission curve have been developed that make use of several modulation frequencies and measurement phase angles (McGown and Bright, 1984).

For single exponential fluorescence decay, as is expected for a sample containing just one fluorophore, either the phase shift or the demodulation can be used to calculate the fluorescence lifetime, $\tau$. When the excitation light is modulated at an angular frequency, $\omega=2\pi\nu$, the phase angle, $\phi$, by which the emission modulation is shifted from the excitation modulation is related to the fluorescence lifetime by:

$$\tan \phi=\omega\tau \qquad (14)$$

The demodulation, m, is related to the fluorescence lifetime by:

$$m=(1+\omega^2\tau^2)^{-1/2} \qquad (15)$$

If the signal decay is a single-exponential curve, equations 14 and 15 result in values for $\tau$ that are in agreement with each other. Dissimilar values indicate multiexponential decay, which usually means that the sample contains more than one fluorophore.

Multiexponential decay can be resolved by using a phase fluorometer with phase-sensitive detection. A time-independent, direct-current signal is produced that is proportional to the cosine of the difference between the phase angle of the detector ($\phi_D$) and the phase angle of the fluorescence ($\phi$):

$$F(\lambda,\phi_D)=kF[\lambda \cos (\phi_D-\phi)] \qquad (16)$$

where

$F(\lambda,\phi_D)$ is the measured-direct current signal,

k is a proportionality constant that includes sample and instrumental factors as well as the modulation factor of the exciting light,

$F(\phi)$ is the direct current intensity component of the fluorescence emission,

$\phi_D$ is the detector phase angle, and

$\phi$ is the fluorescence phase angle.

For a given fluorophore at a given wavelength, the maximum phase-resolved signal intensity is obtained when $\phi_D=\phi$, and the minimum signal is obtained when the entire signal from the given component is canceled out at $\phi_D=\phi+90°$.

Consider a sample with two fluorophores A and B whose lifetimes ($\lambda_A$ and $\lambda_B$) are each independent of emission wavelength and are different from one another. By setting $\phi_D=\phi_A+90°$, the contribution from A is nulled out and the scanned emission spectrum represents only the contribution from B. Similarly, the spectrum for A is obtained by setting $\phi_D=\phi_B+90°$. In practice, finding the correct value of $\phi_D$ for this direct nulling approach can be tedious; thus, more indirect but faster methods are often used. If the spectrum is scanned with a series of different detector phase angles, spectra of the separate fluorophores can be generated by best-fit routines, or a set of simultaneous equations can be generated allowing a matrix solution of the fluorophores' contributions (McGown and Bright, 1984). For these techniques, equations 17

and 18 apply (Jameson and Weber, 1981; Weber, 1981):

$$\tan\phi = \frac{\sum_i f_i \sin\phi_i \cos\phi_i}{\sum_i f_i \cos^2\phi_i} \qquad (17)$$

$$m^2 = \left(\sum_i f_i \cos^2\phi_i\right)^2 + \left(\sum_i f_i \sin\phi_i \cos\phi_i\right) \qquad (18)$$

where

$\phi_i$ is the phase-shift due to the ith component, and

$f_i$ is the fractional contribution of the ith component to the measured quantities.

By measuring $\phi$ and m at several modulation frequencies, a set of simultaneous equations can be generated that allow a determination of the best values for fluorophore lifetime and fractional contributions. The $f_i$ values from these calculations are the quantities that are used in equations 7 and 8 to obtain weighted values of $\tau$ and $P_o$.

## Measurements on Fulvic Acid

A phase-resolved spectrum of fulvic acid is presented in figure 6. Curve C is the unresolved emission envelope that is observed with standard emission spectrometry—it is the summation spectrum of spectra A and B and is the same as curve B in figure 4. Curves A and B are the resolved curves from two fluorophores with different lifetimes and correspond to the two fluorophores seen in the excitation spectrum, curve A, of figure 4. The two curves, A and B in figure 6, have emission maxima at 417 and 455 nm respectively, and have lifetimes measured as 1.05±0.12 ns and 6.54±0.14 ns in water at 30°C and 1.31±0.14 ns and 6.78±0.36 ns in DMSO. These values agree with those of Lapen and Seitz (1982), who obtained values of 1.0 ns and 6.0 ns for a soil-derived fulvic acid in water at pH 6.0 using the method of steady-state fluorescence spectrometry. Lochmuller and Saavedra (1986), using pulsed fluorescence, concluded that their decay curves indicated the presence of three fluorophores. They measured lifetimes as a function of solution pH, ionic strength, and fulvic acid

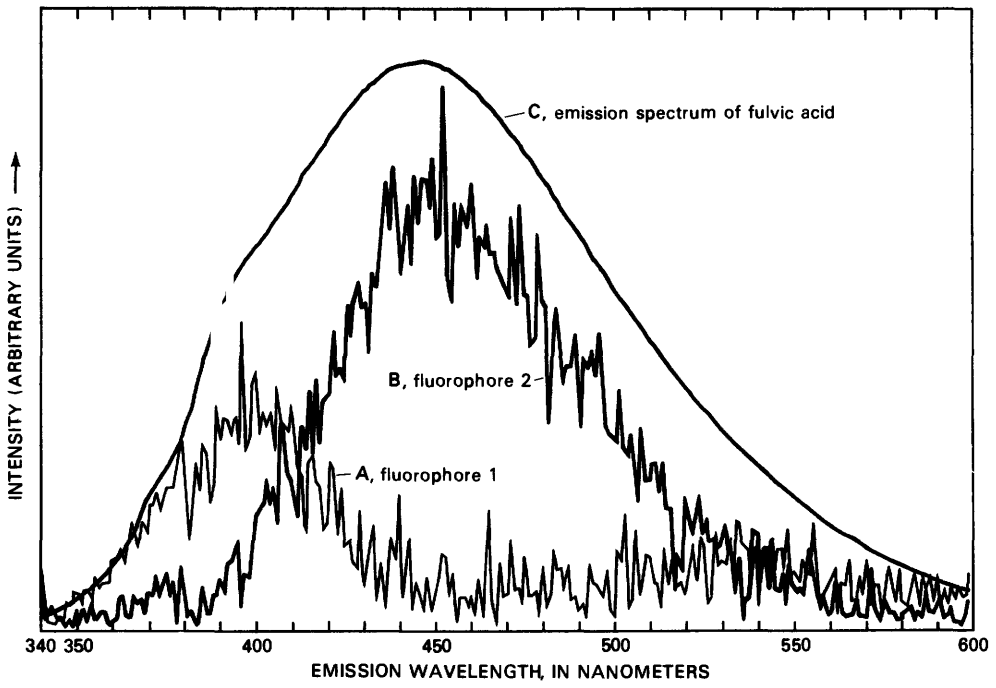


**Figure 6.** Phase-resolved spectrum of Suwannee River fulvic acid showing the complete emission spectrum of each fluorophore (curves A and B) and unresolved emission spectrum of the molecule (curve C). Arbitrary intensity is plotted as the ordinate, and the emission is scanned from 340 to 600 nanometers. Excitation was held constant at 350 nanometers. Sample concentration was 100 milligrams per liter.

concentration. No dependence on pH was observed, but lifetimes did vary with ionic strength and sample concentration. Lochmuller and Saavedra (1986) measured lifetimes that ranged from about 0.3 ns to 0.6 ns for $\tau_1$, 2.0 ns to 2.7 ns for $\tau_2$, and 6.7 ns to 8.5 ns for $\tau_3$, where $\tau_1$, $\tau_2$, and $\tau_3$ were the lifetimes of the three fluorescing components.

Lapen and Seitz (1982) and Lochmuller and Saavedra (1986) emphasize that the multiexponential decay curves they report might result from a single fluorophore measured in different environments. These different environments could affect the emission lifetime, and thus be an explanation for multiexponential decay in fulvic acid rather than multiexponential decay being due to different types of fluorophores. Our measurements, however, are evidence for two or more separate fluorophores. In figure 3, the two fluorophores having different lifetimes are seen to have emission maxima differing by about 50 nm, corresponding to an energy difference in their excited states too large to be convincingly attributed to environmental differences in a molecule as small as fulvic acid. In addition, the discussion in the text relating to figure 4 points out that the large overlap of the excitation and emission spectra for fulvic acid most likely arises from multiple fluorophores. Finally, the three-dimensional EEM spectrum shown in figure 5 shows two very distinct peaks rather than the broad featureless structure expected from a single fluorophore in different environments. The combination of these three independent measurements strongly supports the conclusion that at least two separate fluorophores are present in fulvic acid.

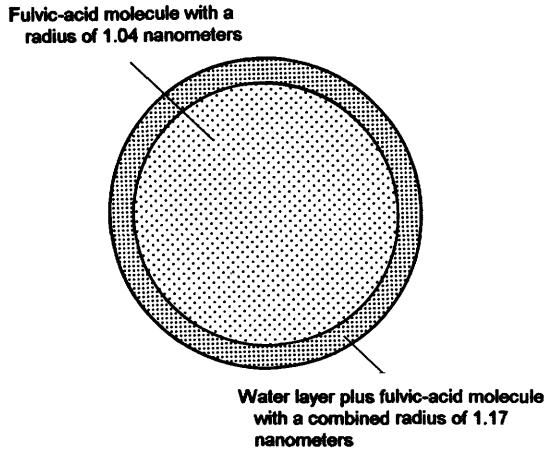


**Figure 7.** Schematic representation of the water sheath that a spherical fulvic-acid molecule would contain. The radii of equivalent spheres are given.

## DISCUSSION

Table 1 contains the data set for fulvic acid from the Suwannee River in DMSO at 30°C and in water at pH 6.5 and pH 4.0 at 30°C. Equation 2 was solved for $\beta$ and divided by the relaxation time, $\rho$. The rotation rates thus calculated are 17.17 degrees per nanosecond in water at pH 6.5 and 24.3 degrees per nanosecond in DMSO. The molar volumes are 3,950 $cm^3$ in water at pH 6.5 and 2,860 $cm^3$ in DMSO. Possible interpretations of these results are:

1. In water, the fulvic acid binds a significant number of water molecules to its side chains, increasing the apparent size of the rotating molecule. Fulvic acid rotates in the aprotic solvent, DMSO, without carrying along a water sheath. Assuming the fulvic acid molecule to be spherical in both solvents and assuming that its molecular radius of 1.043 nm in DMSO is a nonsolvent bonded radius, then a water sheath would have to increase the radius by an additional 0.124 nm to account for the apparent larger volume of fulvic acid in water (fig. 7). With this model, 72 percent by volume of the rotating body in water is fulvic acid and 28 percent is bound water. Using a similar model for globular proteins, Yguerabide and others (1970) estimated that hydrated proteins generally have about twice the volume expected for the anhydrous form, due in part to a large solvent shell.
2. In water, fulvic acid could contain weak bonds, be aggregated, or exist as a low-molecular-weight polymer. The processes of aggregation or polymerization do not occur in the highly aprotic solvent DMSO.

## CONCLUSIONS

Using several techniques based on fluorescence spectrometry, fulvic acid from the Suwannee River was found to contain at least two fluorophores with fluorescence lifetimes of 1.05±0.012 and 6.54±0.014 ns in water and 1.31±0.014 and 6.78±0.036 ns in dimethylsulfoxide. The fluorescence spectrum has an excitation maximum at 350 nm and an emission maximum at 460 nm. The emission spectrum was phase resolved into components from two fluorophores with emission maxima at 417 and 455 nm. A three-dimensional EEM more fully shows the complete fluorescence manifold. The hydrodynamic molar

volume of fulvic acid from the Suwannee River was measured to be 3,950 $cm^3$ in water at pH 6.5 and 2,860 $cm^3$ in DMSO. This corresponds to spherical radii of 1.167 and 1.043 nm, respectively. By measuring the dynamic depolarization at pH 6.5 as a function of temperature, the tangent defect was found to coincide with the peak maximum, indicating that the molecule acts as a spherical rotor in water. Measurements of the reciprocal depolarization as a function of pH showed fulvic acid to exhibit a fairly constant shape between the pH range of 3 to 11.5. Based on tangent-defect measurements, it can be concluded that the molecule is also approximately spherical in this pH range. These measurements also indicate that fulvic acid changes conformation below pH 3 and above pH 11.5. A suggested explanation for this change is the forced addition of protons to oxygen-containing groups in the molecule at pH values less than 3 and the stripping of protons from the molecule at pH values greater than 11.5. It is suggested that the different apparent volumes in water and dimethylsulfoxide could result from the fact that the molecule carries a sheath of bound water when dissolved in water, which is absent in DMSO, or because fulvic acid is loosely held together in water by bonds that are not present in DMSO.

## REFERENCES CITED


Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., 1985, Humic Substances in Soil, Sediment, and Water: New York, John Wiley, 692 p.

Demas, J.N., 1983, Excited State Lifetime Measurements [chap. 1]: New York, Academic Press, p. 1–11.

Ellerton, R.N., and Isenberg, I, 1969, Fluorescence study of DNA-proflavin complexes: Biopolymers, v. 8, p. 767–786.

Goldberg, M.C., 1984, The fluorescence lifetimes of fulvic acid chromophores: 26th Rocky Mountain Conference, Denver, Colorado, August 5–9, 1984, p. 110.

Goldberg, M.C., and Devonald, D.H., 1973, Fluorescent spectroscopy, a technique for characterizing surface films: U.S. Geological Survey, Journal of Research, v. 1, no. 6, p. 709–717.

Goldberg, M.C., and Wilson, J.K., 1974, Fluorometric determination of ferricyanide and thiamine in water [abs.]: American Chemical Society, 168th National Meeting [Atlantic City], Proceedings: Washington, D.C., American Chemical Society.

Ghosh, K., and Schnitzer, M., 1980, Fluorescence excitation spectra of humic substances: Canadian Journal of Soil Science, v. 60, p. 373–379.

———1981, Fluorescence excitation spectra and viscosity behavior of a fulvic acid and its copper and iron complexes: Soil Science Society of America Journal, v. 45, p. 25–29.

Jameson, D.M., and Weber, G., 1981, Resolution of the pH-dependent heterogeneous fluorescence decay of tryptophan by phase and modulation measurements: Journal of Physical Chemistry, v. 85, p. 953–958.

Lakowicz, J.R., 1983, Principles of fluorescence spectroscopy: New York, Plenum Press, 496 p.

Lapen, A.J., and Seitz, W.R., 1982, Fluorescence polarization studies of the conformation of soil fulvic acid: Analytica Chimica Acta, v. 134, p. 31–38.

Lochmuller, C.H., and Saavedra, S.S., 1986, Conformational changes in a soil fulvic acid measured by time-dependent fluorescence depolarization: Analytical Chemistry, v. 58, p. 1978 –1981.

Love, L.B.C., and Upton, L.M., 1980, Analysis of multicomponent mixtures through temporal resolution: Analytical Chemistry, v. 52, p. 496–499.

McGown, L.B., and Bright, F.V., 1984, Phase-resolved fluorescence spectroscopy: Analytical Chemistry, v. 56, p. 1400A–1415A.

Meyers, William, and Welch, R.I., 1975, Water resources assessment, *in* Bowden, L.W., and Pruitt E.L., eds., Manual of Remote Sensing, Volume II, Interpretation and Applications [chap. 19]: Falls Church, Virginia, American Society of Photogrammetry, p. 1478–1551.

Parker, C.A., 1968, Photoluminescence of Solutions: New York, Elsevier, 544 p.

Warner, I.M., Christian, G.D., Davidson, E.R., and Callis, J.B., 1977, Analysis of multicomponent fluorescence data: Analytical Chemistry, v. 49, p. 564–573.

Weber, G., 1977, Theory of differential phase fluorometry—Detection of anisotropic molecular motions: Journal of Physical Chemistry, v. 66, p. 4081–4091.

———1981, Resolution of the fluorescence lifetimes in a heterogeneous system by phase and modulation measurements: Journal of Physical Chemistry, v. 85, p. 949–953.

Weiner, E.R., 1978, Equivalence of simultaneous scanning and three-dimensional plotting of fluorescence spectra: Analytical Chemistry, v. 50, p. 1583–1584.

Weiner, E.R., and Goldberg, M.C., 1982, Optimizing the selectivity of standard spectrophotofluorometers with computer control: American Laboratory, v. 14, no. 9, p. 91–103.

Yguerabide, J., Epstine, H., and Stryer, L., 1970, Segmental flexibility of an antibody molecule: Journal of Molecular Biology., v. 51, p. 573–590.

Chapter L

# Acid-Base Titration and Hydrolysis of Fulvic Acid from the Suwannee River

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## CONTENTS



### FIGURES

## Abstract

The acid-base characteristics of fulvic acid from the Suwannee River were investigated by using a variety of titrimetric techniques. It is estimated that the carboxyl content of the fulvic acid is 6.1 milliequivalents per gram and the phenolic content is 1.2 milliequivalents per gram. In addition, consumption of alkali and subsequent uptake of acid were found to be time dependent, indicating that part of this consumption represents reversible hydrolysis of organic matter. At pH 10, the fulvic acid slowly consumes at least 0.9 milliequivalents of base per gram of fulvic acid after 18 hours. On the basis of theoretical analysis of the influence of hydrolytic reactions on the nature of acid-base titrations and on model experiments with synthetic esters, it is proposed that both reversible and irreversible ester hydrolysis reactions contribute to the acid-base time-dependent titrimetric characteristics of Suwannee River fulvic acid. This hypothesis is supported by infrared spectroscopic evidence.

## INTRODUCTION

One of the most fundamental characteristics of humic substances is their acidic character. In addressing the acidity of solutions of humic substances, two factors must be considered:

1. An *intensity factor*, $pK_a$. This is the pH of the solution as determined by the strength of the acid groups and the concentration of humic substances in solution.
2. A total-acid factor (capacity). This is the total equivalents of acid sites per unit mass of material.

For humic substances isolated from a given source, the total-acid factors generally decrease in the order: fulvic acid > humic acid > humin. As with the interpretation of most other humic-substance data, interpretation of acidic behavior is complicated by the multicomponent nature that is inherent to all humic substances. As a result, one cannot always unambiguously discriminate between the relative contributions of factors 1 and 2 above in controlling the pH of solutions of humic substances. Nevertheless, titration curves of humic substances do indicate that they are composed of a mixture of polyprotic acids wherein the individual $pK_a$ values encompass a relatively wide, and apparently continuous, range (Marshall and Patnaik, 1953; Pommer and Breger, 1960; Posner, 1964; Stevenson, 1982). Accordingly, titration curves of humic substances do not conform to the shapes of simple monoprotic- or polyprotic-acid titration curves; thus, these curves cannot be described in terms of a single $pK_a$ value or by a unique set of discrete $pK_a$ values.

Early work on direct potentiometric titrations of humic substances was reviewed by Marshall and Patnaik (1953) and Flaig and others (1975). Stevenson and Butler (1969) and Schnitzer and Khan (1972) summarized commonly used methods for acidic functional group determination. Perdue (1985) reviewed factors affecting acidic properties of humic substances and also updated advances in methods for acidic functional group determination in these materials.

On the basis of extensive characterization of humic substances, it is believed that their acidity is probably due primarily to the presence of carboxyl and phenolic functional groups. The $pK_a$ values of each of these classes of functional groups extend over a wide range, and there is overlap between the $pK_a$ values of both classes. For purposes of this introductory discussion, we will assume that the $pK_a$ values of the carboxyl functional groups are centered around 5.0 and that the $pK_a$ values for phenolic functional groups are centered around 10.0. For simplicity, much of this discussion also will be presented in terms of monoprotic acids; although this clearly is an oversimplification insofar as humic substances are concerned, the theoretical principles that are developed are still applicable to more complicated systems. On the basis of these assumed $pK_a$ values, the equivalence point for carboxyl functional groups in humic substances would occur at about pH 8.9 (based on 0.1 M concentration for titrand and titrant), and, at this pH, about 9 percent of the phenolic functional groups would be neutralized, showing a clear overlap between the titration of both groups.

In addition to these complexities in studying the titration curves of humic substances, there are two additional complicating factors that also need to be recognized:

1. Humic substances are oxidized in the presence of oxygen, particularly under alkaline conditions (Bremner, 1950; Swift and Posner, 1972). This oxidation is typical of many simple phenolic compounds, such as pyrogallol. To avoid this problem, humic substances generally are titrated under a nitrogen atmosphere.
2. The consumption of alkali by humic substances has been shown to be a time-dependent

phenomenon (Davis and Mott, 1981a, 1981b; Paxeus and Wedborg, 1985). In the titration of humic substances with base, the pH is observed to decrease upon standing, particularly at higher pH values. In the case of humic acids, this effect may be partly related to diffusion because of slow access of base to certain acid sites. It may also be due to hydrolysis of some components of humic acid. In the case of aquatic fulvic acids that are of lower molecular weight and are more likely to be in true solution, slow diffusion of $OH^-$ to acidic sites is improbable, and hydrolysis is likely to be the principal cause of additional alkali consumption.

This chapter presents results of a study of the acid-base properties of standard and reference aquatic fulvic acid from the Suwannee River. The purposes of this chapter are to report on how the acid-base properties of the reference aquatic fulvic acid were measured and to describe the time dependence of its acid-base titrations. The effect of ester hydrolysis and esterification on titration curves is investigated. Infrared spectroscopic evidence is presented for the presence of esters in this fulvic acid and for the occurrence of ester hydrolysis and esterification. On the basis of potentiometric titration data, estimates are given for the contents of free carboxyl and phenolic functional groups and of readily hydrolyzable esters in fulvic acid. For comparison, three synthetic esters were also investigated by titrimetry.

## THEORY OF ACID-BASE TITRIMETRY ACCOMPANIED BY HYDROLYSIS

To provide a basis for understanding the time-dependent acid-base titrations of humic substances, some theoretical considerations relating to the effect of hydrolysis on titration curves is presented here. This discussion will be presented in terms of ester hydrolysis, which has been extensively studied (Euranto, 1969; Kirby, 1972). Evidence will be presented later in this chapter for the presence of esters and the occurrence of ester hydrolysis in fulvic acid from the Suwannee River. The relevant chemical reactions are shown in equations 1, 2, and 3, where the ester, $R'CO_2R''$, hydrolyzes to the acid, $R'CO_2H$, and alcohol, $R''OH$:

$$R'CO_2R'' + H_2O \rightleftharpoons R'CO_2H + R''OH \qquad (1)$$

$$R'CO_2H + OH^- \rightleftharpoons R'CO_2^- + H_2O \qquad (2)$$

$$R''OH + OH^- \rightleftharpoons R''O^- + H_2O \qquad (3)$$

In its simplest form, hydrolysis is considered as the addition of $H_2O$ across the ester linkage, as shown in equation 1. At higher pH values, the acid, $R'CO_2H$, is neutralized as shown in equation 2; the sum of equations 1 and 2 represents saponification—that is, alkaline hydrolysis of an ester where formation of the salt drives the hydrolysis to completion and 1 mole of $OH^-$ is consumed per mole of ester. If $R'CO_2R''$ represents a phenyl ester, the resulting phenol, $R''OH$, also will consume hydroxide at high pH values, as indicated by equation 3. The sum of equations 1, 2, and 3 represents the net reaction in this case: 2 moles of $OH^-$ are consumed per mole of ester. Hydrolysis of an ester (eq. 1) can cause a decrease in pH through production of the acid, $R'CO_2H$; esterification (the reverse of eq. 1) can cause a corresponding increase in pH by consuming this acid.

The generalized experimental procedure consists of automatically titrating a solution of fulvic acid with NaOH to a specified pH value; this will be referred to as the forward titration. The forward titration usually is done quite rapidly to minimize hydrolysis. The solution then is left standing for a specified length of time, and base is automatically added to maintain the pH at a constant value (*pH-stat experiment*). Following the pH-stat experiment, the solution is rapidly back titrated with HCl and then maintained at a constant pH (using HCl to counteract the tendency for the pH to increase).

For simplicity in this theoretical analysis, we will consider the behavior of a mixture of a soluble acid, $RCO_2H$, and a soluble ester, $R'CO_2R''$. Four different cases will be addressed:

Case 1. If the ester is not susceptible to hydrolysis within the duration of the experiment, consumption of alkali will be caused by neutralization of the acid, $RCO_2H$, alone. Titration with alkali followed by back titration with acid should result in superimposable curves (as if no esters had been present), after appropriate corrections for dilution have been made.

Case 2. The ester undergoes hydrolysis prior to the back titration (that is, during the forward titration or during the pH-stat period following the forward titration), and no reesterification occurs during the experiment. Hydrolysis of the ester produces $R'CO_2H$ and $R''OH$, and a greater quantity of NaOH is required to reach or maintain a given pH value because $R'CO_2H$ consumes hydroxide in addition to $RCO_2H$. If an amount of HCl that is

equivalent to the total amount of NaOH that was added to the solution is titrated into the system, the resulting pH will be lower than the initial pH because there is now a greater concentration of acid in the system due to the presence of $RCO_2H$ plus $R'CO_2H$ (assuming correction for dilution and assuming that $R'CO_2H$ is comparable in strength to, or stronger than, $RCO_2H$). Alternatively, it would require fewer equivalents of HCl (compared to the amount of NaOH added) to restore the solution to its initial pH. The stronger the acid released by hydrolysis (that is, the smaller the $pK_a$ value) and the greater the amount of acid so released, the larger the difference is between the equivalents of NaOH added in the forward titration and the HCl added in the back titration to attain the initial pH value.

If the acid generated by hydrolysis, $R'CO_2H$, is very weak (compared to the initial acid, $RCO_2H$), the first aliquots of added HCl during the back titration protonate the conjugate base of this weak acid; furthermore, this weak acid will not significantly affect the pH of the solution under acidic conditions because it will be virtually undissociated. Under these conditions, addition of HCl in an amount equivalent to the quantity of NaOH initially added would return the solution to its original pH value (assuming correction for dilution).

Case 3. In this case, hydrolysis occurs as in case 2, but reesterification also occurs during the back titration. If reesterification were complete, then all of the acid produced ($R'CO_2H$) would be consumed in the process, and addition of a quantity of HCl equivalent to the NaOH added would return the solution to its initial pH value. If only partial reesterification occurs during the experiment, some residual acid, $R'CO_2H$, would remain, and less HCl would be required to attain the initial pH value (assuming the hydrolytically produced acid to be comparable in strength to, or stronger than, the acid originally present).

Case 4. In this case, phenyl esters are hydrolyzed to produce two potential alkali-consuming products: a carboxylic acid ($R'CO_2H$) and a phenol ($R''OH$). If the maximum pH of the solution is significantly less (that is, more than 2 pH units) than the $pK_a$ of the resulting phenol, the behavior of this system will parallel that of case 2 or case 3 above. However, at higher pH values, the phenols will be neutralized, resulting in a greater consumption of alkali. If significant amounts of phenols are generated through hydrolysis and if their reesterification rate is slow, their presence should be discernible from the shape of the back-titration curve in the high-pH region.

The behavior of some systems may not be as clear-cut as described in cases 2, 3, and 4. For example, if a molecule containing the ester linkage also contains one or more free acid groups, the $pK_a$ values of those groups may be altered by hydrolysis—an example of a discrete compound, aspirin (acetylsalicylic acid), that displays this type of behavior will be discussed later in this chapter. In addition, if reesterification does occur, it is possible that the original ester may not be formed; for example, transesterification reactions may occur. Transesterification involves the exchange of the alcohol portion of an ester with a different alcohol; for example, formation of $R^1CO_2R^3$ and $R^2OH$ from an original mixture of $R^1CO_2R^2$ and $R^3OH$ (Morrison and Boyd, 1974). The acidic and hydrolytic properties of the newly formed esters may be different from those of the original esters.

Other implications of hydrolysis/reesterification reactions during the isolation of humic substances relate to the formation of intermolecular versus intramolecular ester linkages: the former could lead to species of greater molecular weight. However, such detailed molecular information is not available at present (1987).

In the absence of additional information, it is difficult to quantitatively account for the discrepancy between the quantities of NaOH and HCl added in cases 2, 3, and 4 in order to return the solution to its initial pH. This results from the combination of intensity ($pK_a$) and total-acid factors that cannot be rigorously resolved in the case of humic substances (this is discussed in the "Introduction" section in this chapter).

By measuring forward and back titrations and by obtaining pH-stat data for humic substances, one can obtain some limited information on the hydrolysis reactions of these substances. For example, if a solution of humic substances is titrated from pH 3.0 by stepwise addition of aliquots of NaOH, onset of hydrolysis is indicated by a downward drift in pH during the intervals between alkali additions. The uptake of hydroxide through saponification, or other hydrolytic reactions, at any pH value, can then be monitored quantitatively during pH-stat experiments (assuming minimal saponification during the forward titration). Back titration with HCl then can provide information on reesterification, if any, and can also provide some information relating to the $pK_a$ values of acids ($R'CO_2H$) that are generated. For example, it can help to establish if these acids are substantially weaker or comparable in strength to the acids

($RCO_2H$) that initially were present. Reesterification under acidic conditions can be monitored by measuring the HCl that must be added to maintain a constant pH. Information relating to the hydrolysis of phenyl esters can be deduced from the shape of the back-titration curve in the alkaline pH region. It should be recognized that, regardless of the validity of the ester hydrolysis/esterification hypothesis, the theoretical arguments developed above for the consumption of alkali and acid are still valid in a more general sense.

## MATERIALS AND METHODS

### Fulvic Acid

Fulvic acid was isolated from the Suwannee River and processed as described by Malcolm and others (chap. B, this volume). IHSS reference aquatic fulvic acid was used in this research. Individual samples of the fulvic acid were dried to a constant weight over $P_2O_5$ in a vacuum desiccator (3 d at 60°C) and then were weighed accurately in glass vials. Each sample weighed about 10 mg, and its weight was reported to 0.001 mg.

### Reagents, Titrant, and Titrand Solutions

All solutions were prepared from boiled and cooled, distilled, deionized water to avoid any contribution to the acidity from dissolved carbon dioxide. The following reagents were used in this research: sodium hydroxide (V.W.R., reagent grade, A.C.S.); hydrochloric acid (Fisher Scientific Co., reagent grade, A.C.S.); potassium hydrogen phthalate (Mallinckrodt, analytical reagent, primary standard); butyl acetate (Baker, reagent grade); methyl p-hydroxybenzoate (methyl paraben, Chem Service); and acetylsalicylic acid (aspirin, Topco medicinal tablets). Primary standard potassium hydrogen phthalate was used to standardize the NaOH-titrant solution and to test the consistency of the titrant delivery and monitoring system. A batch of 0.0642 M potassium hydrogen phthalate solution was prepared for this purpose. Solutions of sodium hydroxide were prepared by diluting 5 mL of saturated NaOH solution to 1.0 L with $CO_2$-free water. This solution was standardized by titrating, in triplicate, aliquots of the primary standard potassium hydrogen phthalate solution. Derivative curves were used to establish end points in these titrations. The NaOH-titrant solution was stored in a Teflon reservoir equipped with a $CO_2$ scrubber, and its concentration was rechecked periodically. Hydrochloric-acid-titrant solutions were prepared by diluting the concentrated HCl and were standardized by titration with the standardized NaOH solution.

The various titrand (analyte) solutions were prepared as follows: The dried and weighed fulvic acid (about 10 mg) was transferred into a polypropylene titration cell and dissolved in 2 mL of $CO_2$-free water. The sample was maintained under nitrogen for the duration of the experiment. Ionic strength was not controlled in these experiments, and activity effects were not taken into consideration.

Separate 0.006 M aqueous solutions of butyl acetate and of methyl paraben were prepared. Aspirin tablets (virtually 100 percent acetylsalicylic acid) were crushed and 0.300 g of the powder was added to 100 mL of distilled water. The aspirin that remained undissolved after stirring for 2 h was removed by filtration through Whatman number 2 filter paper. The concentration of aspirin in the filtrate was determined to be 0.0168 M by rapid titration (of carboxylic functional groups) with standard NaOH.

### Equipment

A Radiometer titration system consisting of the following units was used:

1. PHM 84 research pH meter with digital readout.
2. TTT 80 titrator for automatic control of end-point titrations and end-point setting for pH-stat experiments.
3. REC 80 servograph recorder with capability for automatic chart flow corresponding to volume of titrant dispensed or a choice of different constant chart speeds. Recorder accessories included: (1) an REA derivation unit that plots the first derivative of the pH versus volume of titrant curve, and (2) an REA 270 pH-stat unit that plots the titrant consumed to maintain a constant pH as a function of time.
4. ABU 80 autoburette that has an accuracy of ±0.25 µL using a 1-mL burette for all titrations.
5. TTA 80 titration assembly with built-in stir bar providing sufficient agitation for practically instantaneous mixing and a sample chamber through which nitrogen was continuously bubbled. The nitrogen was first saturated with moisture by bubbling through distilled water to minimize

evaporative losses from the titration cell during prolonged titrations or pH-stat experiments. All experiments were done at room temperature (20°±2°C).

6. A Radiometer G2040C glass pH electrode and a Radiometer K4040 $Hg/Hg_2Cl_2$ reference electrode were used.

## Titration Procedures

### Manual Titration

Initial titrations of fulvic acid were conducted manually as follows: about 10 mg of dried, accurately weighed (as described above) fulvic acid was transferred to a titration vessel and titrated under nitrogen with standard (about 0.1 M) NaOH solution. Titrant was added manually in 20 to 60 stepwise additions of 0.010- to 0.050-mL aliquots at time intervals of 30 s to 5 min, and the titration curves were plotted manually. Initial volumes of titrand were 3 or 7 mL, depending on the choice of electrode and solution chamber used. Blanks were run by titrating corresponding volumes of distilled water.

### Automatic Continuous Titration

Titration curves of fulvic acid, potassium hydrogen phthalate, and the three synthetic esters were recorded by automatic and continuous addition of standard NaOH (about 0.1 M) with constant monitoring of the pH. Samples were titrated to pH 12.0. The fulvic-acid titration required 4 min; the potassium-hydrogen-phthalate titrations required 4 to 10 min; and the three synthetic-ester titrations required 1 to 3 min.

### Manual Discontinuous Titration

Discontinuous titration curves were recorded for fulvic acid and potassium hydrogen phthalate. In this procedure, aliquots of titrant were added manually at specified time intervals while the pH was continuously monitored on moving chart paper. Fulvic acid was discontinuously titrated with NaOH over a period of 4 h, using aliquots of 0.02 to 0.05 mL, corresponding to time intervals of 4 to 10 min; potassium hydrogen phthalate was discontinuously titrated with NaOH over a 3-h period using aliquots of 0.02 to 0.04 mL, corresponding to time intervals of 2 to 4 min. The smaller volumes and shorter intervals were used around the inflection point.

## pH-stat Experiments

A pH-stat experiment was done by titrating a sample to a specific pH value and then maintaining the pH of the solution at this value by addition of acid or base as required. The volume of titrant consumed in the initial pH adjustment was measured, and the volume of acid or base (if any) required to maintain a constant pH was recorded automatically as a function of time. For this study, two types of pH-stat experiments were done: these are referred to as discrete pH-stat experiments and a stepped pH-stat experiment. In discrete pH-stat experiments, the solution was adjusted to only a single pH value, whereas, in the stepped pH-stat experiment, the solution pH was maintained at one value for a specified time and then rapidly titrated (stepped) to another value where pH was again held constant, and so on.

Discrete pH-stat experiments using fulvic acid as titrand were conducted at pH values of 7.0, 10.0, and 11.0 for 18 h. In these experiments, the pH was initially adjusted to the desired value by rapid titration (generally within 2 min or less). Stepped pH-stat experiments also were conducted with fulvic acid. Following the pH 7.0 stat experiment described above (18 h), the solution was rapidly (30 s) titrated to pH 8.0 and maintained at that pH for 7.5 h, and then it was rapidly titrated to pH 9.0 and maintained at that pH for an additional 17 h.

The three synthetic esters were subjected to discrete pH-stat experiments. The esters were titrated separately to pH 10.0; n-butyl acetate and methyl paraben were maintained at that pH for 16 h, and aspirin was maintained at pH 10.0 for 42 h.

All pH-stat experiments were conducted in a nitrogen atmosphere. Blanks were run by titrating deionized water to the various pH values and maintaining the pH of these solutions for a specified time. Corrections were made by subtracting the quantities of alkali consumed in these experiments from the quantities consumed in the pH-stat experiments with fulvic acid and synthetic esters.

## Acid-Base Hysteresis Experiments

The reversibility of the fulvic acid titration curves was examined by first rapidly titrating a sample with NaOH solution from its initial pH to pH 11.0, maintaining the pH constant at that value for a specified duration, and then rapidly back titrating the sample with HCl to pH 3.0 and maintaining the pH constant

at that value for a given duration. The forward and back titrations were done rapidly (2 min). When changing the titrant from NaOH to HCl, the burette tip was removed from the solution, and the burette was flushed three times with HCl before replacing the tip in the solution. The solution was maintained under nitrogen at all times.

## TITRATION AND HYDROLYSIS OF FULVIC ACID

A typical forward, continuous titration curve for fulvic acid and the corresponding forward, discontinuous titration curve are shown in figure 1. The continuous titration curve (fig. 1, curve a) shows considerable consumption of base from pH 2.5 to 8.0, presumably due largely to neutralization of carboxyl functional groups—alkali consumption is also indicated from pH 8.0 to 11.5, but in a less pronounced manner, presumably due to neutralization of phenolic functional groups. No discrete end points are evident in the continuous titration curve: this is typical of titration curves of humic substances. The discontinuous titration curve (fig. 1, curve b) clearly indicates the consumption of base during the periods between addition of aliquots of titrant from about pH 7.0 to 11.5; this is indicated by the gradual downward drift of pH in those intervals. Presumably, this

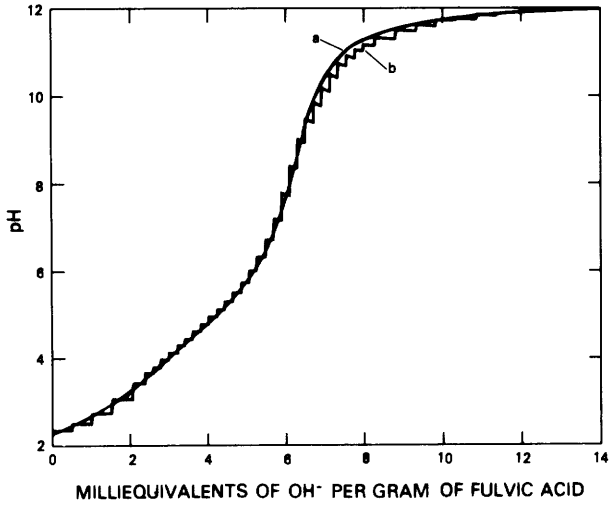



**Figure 1.** Continuous (curve a) and discontinuous (curve b) forward-titration curves for fulvic acid. Curve a: 2-milliliter solution, 4.765 milligrams fulvic acid per milliliter, 4-minute titration; curve b: 2-milliliter solution, 4.757 milligrams fulvic acid per milliliter, 4-hour titration. Titrant was 0.0989 M sodium hydroxide.

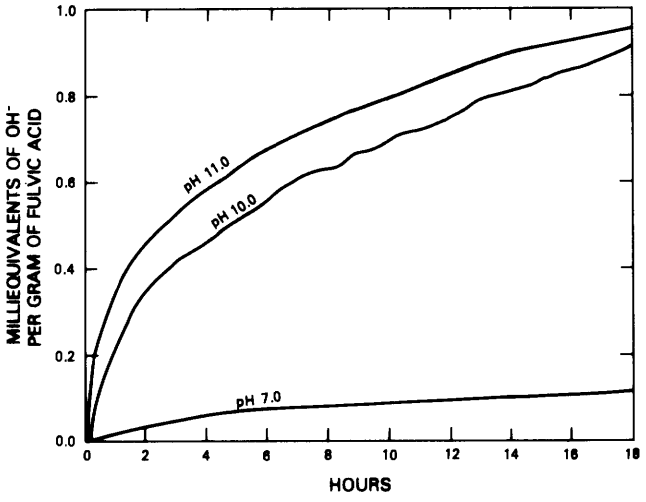



**Figure 2.** Discrete pH-stat experiments with fulvic acid at pH 7.0, 10.0, and 11.0.

consumption of base also is occurring at higher pH values but is obscured in this type of curve by the buffering of the solution at high pH values.

The fulvic acid was subjected to discrete pH-stat experiments at pH 7.0, 10.0, and 11.0 (fig. 2) and to a stepped pH-stat experiment at pH 7.0, 8.0, and 9.0 (fig. 3) in order to more quantitatively investigate the slow consumption of base. These curves exhibit two main features:

1. At all pH values, initial consumption of base is rapid, followed by a steadily decreasing rate of consumption.
2. The rate of base consumption increases with increased pH.

To investigate the reversibility of the acid-base reactions of fulvic acid, a cyclic or *hysteresis* experiment was conducted and the results (after correction for dilution) are presented in figure 4. These curves indicate that:

1. The forward- and back-titration curves are not superimposable.
2. The slope of the forward-titration curve is generally greater than that of the back-titration curve.
3. Addition of a quantity of acid during back titration equivalent to the amount of base added during forward titration plus that added during maintenance of pH at 11.0 does not restore the solution to its initial pH value; in other words, the hysteresis curve does not close.
4. A solution that is initially acidic consumes base when held constant at a high pH, such as 11.0, and consumes acid when subsequently acidified and held constant at a low pH, such as 3.0.

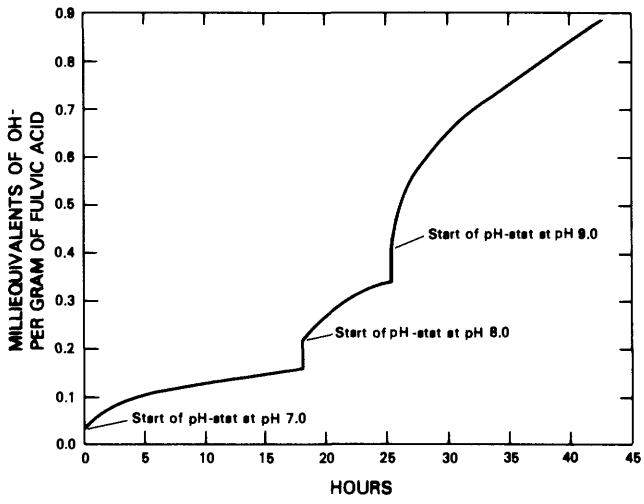



**Figure 3.** Stepped pH-stat experiment with fulvic acid at pH 7.0, 8.0, and 9.0. Titrant was 0.1022 M sodium hydroxide; 0.5322 milliliter of titrant was added to attain pH 7.0 initially.

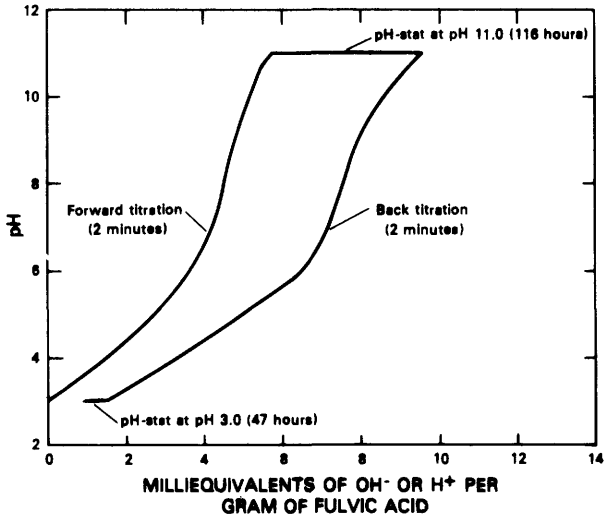
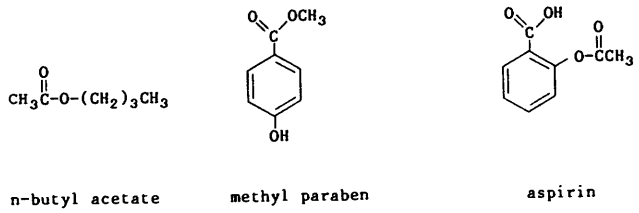



**Figure 4.** Acid-base hysteresis experiment with fulvic acid. The curves have been corrected for dilution. Experimental conditions: 9.346 milligrams fulvic acid in 2.0 milliliters distilled water; 0.1022 M sodium hydroxide; 0.1073 M hydrochloric acid. (Note: The initial part of the forward-titration curve is not shown.)

## Hydrolysis of Synthetic Esters

For comparison purposes, pH-stat experiments were run with three types of synthetic esters—an aliphatic ester (n-butyl acetate), an aromatic ester (methyl paraben), and a phenyl ester (aspirin) that have the following structures:




All experiments with the three synthetic esters were done at pH 10.0; the results are presented in figure 5. The curves for n-butyl acetate and methyl paraben showed no appreciable hydrolysis after standing for 16 h at room temperature, whereas the curve for aspirin indicated considerable hydrolysis over this same period. An hysteresis experiment was then carried out using aspirin; the results are presented in figure 6.

## Ester Hydrolysis/Esterification Hypothesis

Up to this point, this section has consisted primarily of an empirical description of the experimental results; in the following discussion, an attempt will be made to interpret these data. Based on the known properties of humic substances, one interpretation of the additional consumption of base by fulvic acid (beyond that required for neutralization of already-existing free carboxyl and phenolic functional groups) is that it is caused by ester hydrolysis. The remainder

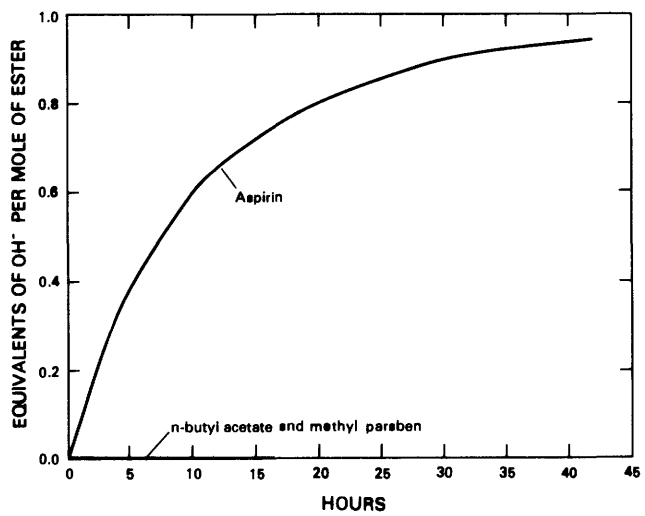



**Figure 5.** pH-stat experiments with synthetic esters at pH 10.0. Experimental conditions: initial concentrations, 0.006 M n-butyl acetate, 0.006 M methyl paraben, and 0.0125 M aspirin—each in 2 milliliters of aqueous solution; 0.0972 M sodium hydroxide.

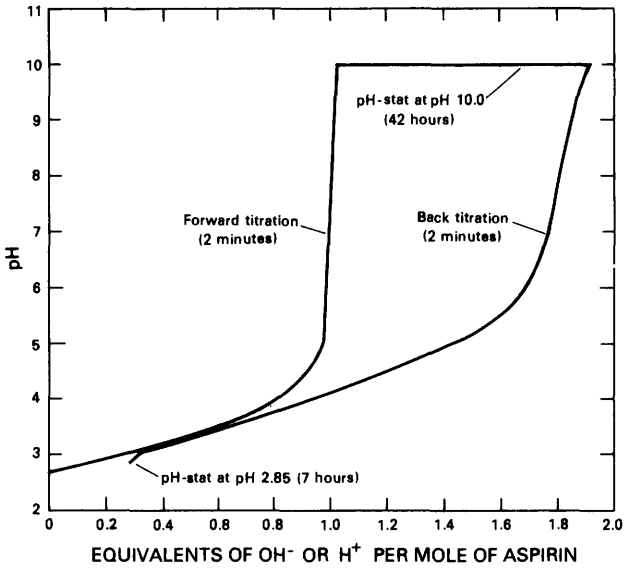



**Figure 6.** Acid-base hysteresis experiment with aspirin. The curves have been corrected for dilution. Experimental conditions: 0.0125 M aspirin in 2 milliliters of aqueous solution; 0.0972 M sodium hydroxide; 0.1073 M hydrochloric acid.

of this discussion will be presented in terms of that hypothesis. Furthermore, it is assumed that the acidity is due exclusively to carboxyl functional groups and phenols.

In this chapter, the acid-base properties of fulvic acid from the Suwannee River are discussed based on the hypothesis that the slow hydroxide consumption is caused primarily by the hydrolysis of ester moieties of fulvic acid—there are, however, other possibilities:

1. The dissociation of *enolic* hydroxyls, formed from the tautomerization of ketonic moieties—ketones comprise about 5 percent of the carbon of the fulvic acid from the Suwannee River as determined by carbon-13 nuclear-magnetic-resonance ($^{13}$C-NMR) spectroscopy (Thorn, chap. N, this volume). Some of the ketones in humic substances may be enolizable (Stevenson, 1982). If the rate of tautomerization were sufficiently slow, it could possibly manifest itself as hydroxide consumption at high pH.
2. The dissociation of carbohydrate and other alcoholic hydroxyls—Perdue (1985) presented a histogram showing the $pK_a$ distribution of 63 nonphenolic, noncarboxylic acids found in the literature (Christensen and others, 1976; Martell and Smith, 1977). Whereas most of these acids would tend to dissociate rapidly, any slow dissociation would manifest itself as slow hydroxide consumption.
3. Degradative reactions, such as the splitting of a *lignin* fragment—Breger (1951) and Pommer and Breger (1960) suggested that alkali consumption could be caused by this type of reaction.
4. Consumption of hydroxide could be due to the formation of stable free radicals, as suggested by Mikita (1980)—for example, he stated that 1,2-dioxy-substituted benzene radicals are produced from 1,2-dihydroxy-substituted benzenes by the consumption of alkali.
5. The degradation of polysaccharide moieties within the Suwannee River fulvic acid—this reaction, known as a peeling process, involves the attack of hydroxide on the end-reducing sugar of the polysaccharide, which is split off and subsequently rearranged to isomeric isosaccharinic acids. Davis and Mott (1981b) suggested that this peeling process provided a satisfactory explanation for hydroxide consumption.

The participation of one or more of these alternative mechanisms of alkali consumption in the hydrolysis of humic substances should be remembered while reading the following discussion. However, regardless of the specific mechanism(s) involved, this chapter presents quantitative data on the slow consumption of alkali (hydrolysis) and slow uptake of acid by fulvic acid from the Suwannee River.

The forward titration curve in figure 1 (curve a) indicates that, by pH 8.0, 6.1 meq of acidic functional groups per gram of fulvic acid have been neutralized, and, by pH 10.0, a total of 6.7 meq/g have been neutralized.

Because there are no discrete end points in the titration curve of the fulvic acid and because of the likely overlap of the contributions of carboxylic acids and phenols in the titration curve, there is difficulty in rigorously determining the carboxyl and phenol contents by titrimetry. As indicated in the "Introduction" to this chapter, an acid with a $pK_a$ of 5.0 (typical of monoprotic aliphatic acids) would give an equivalence point at pH 8.9, but use of this value for the carboxyl equivalence point in fulvic acid could result in an overestimate because of a contribution from phenols. For this reason, a somewhat lower pH value is sometimes chosen for the carboxyl equivalence point in humic substances; the beneficial effect of choosing this lower pH value in minimizing the contribution from phenols likely outweighs any detrimental effect of underestimating the true carboxyl content. For our purposes, a pH of 8.0 will be somewhat

arbitrarily chosen as the carboxyl equivalence point (Martin and Reeve, 1958; Schnitzer and Desjardins, 1962).

The phenol content will be estimated as twice the alkali consumption between pH 8.0 and 10.0 for the following reasons: assuming an average $pK_a$ of 10.0 for the phenols and further assuming monoprotic acid behavior, the phenols would be 50 percent titrated at pH 10.0; accordingly, the alkali consumption between pH 8.0 and 10.0 should represent one-half of the phenol content. Of course, this approach would exclude very weak phenolic groups, such as those in salicylic-acid moieties ($pK_{a_2}$ = 13.4). In addition, use of this procedure minimizes the hydrolysis problems that are more severe at higher pH values. The following estimated values of carboxyl and phenol content are based on the above suite of assumptions that are clearly simplistic; however, complete titration curves are presented for the benefit of the reader. On the basis of the above hypotheses, the estimated carboxyl content of the fulvic acid from the Suwannee River is 6.1 meq/g, and the phenolic content is 1.2 meq/g.

Curve b in figure 1 indicates that hydrolysis of fulvic acid becomes pronounced at pH greater than 7.0, and this is confirmed by the data in figures 2 and 3. The data in figure 2 show that 0.9 meq of hydroxide per gram of fulvic acid is consumed by ester hydrolysis at pH 10.0 during a period of about 18 h. There is no indication that hydrolysis was complete within the duration of these experiments (fig. 2), and, based on the significant rate of hydrolysis at the end of the experiments, it is conceivable that one or more additional milliequivalents of hydroxide could be consumed through ester hydrolysis in the fulvic acid.

The titrimetric and hydrolytic behavior of the fulvic acid and synthetic esters will now be examined in the context of the four cases that were presented in the theoretical section. Fulvic acid clearly does not conform to case 1 because it does show obvious hydrolysis. The n-butyl acetate and methyl paraben curves in figure 5 indicate that these would, however, exhibit behavior in conformance with case 1. Consumption of acid by fulvic acid during the pH 3.0 stat region of figure 4 indicates that reesterification is occurring in the freshly acidified fulvic acid, thereby eliminating it from case 2 behavior.

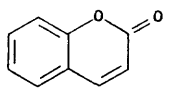
The hysteresis of a discrete compound, aspirin, will be examined as a prelude to studying the hysteresis of the more complicated fulvic acid. A solution of aspirin was rapidly titrated (2 min) with NaOH from its initial pH of 2.70 to pH 10.0 and then the pH was held constant at this value for 42 h. The solution then was rapidly back titrated (2 min) to pH 2.85 with HCl and the pH was held constant at this value for 7 h (fig. 6). The forward-titration curve shows the abrupt inflection due to neutralization of the relatively strong "weak-acid" carboxylic functional group in aspirin ($pK_a$ = 3.49). The pronounced slope in this region of the curve indicates that little hydrolysis occurred during this rapid titration. Held constant at pH 10.0, the phenyl ester in aspirin is almost completely (95 percent) hydrolyzed after 42 h as shown by the consumption of almost an equivalent amount of alkali as that required to titrate the aromatic carboxyl functional group in the forward titration. This additional alkali consumption is due to neutralization of the acetic acid produced—the phenolic hydroxyl functional group ($pK_{a_2}$ = 13.4) of salicylic acid is not neutralized at pH 10.0. The shape of the back-titration curve is quite different from that of the forward titration, particularly in terms of the smaller slope in the inflection region of the former. This is because of the presence of the weaker acetic acid ($pK_{a_1}$ = 4.75). Less acid (40.8 eq) is required to return the solution to pH 2.85 compared to the alkali added (47.2 eq) in the corresponding part of the forward titration. This results from the additional acid (acetic acid) produced by hydrolysis and the smaller $pK_{a_1}$ (2.97) of the hydrolytically produced salicylic acid compared to the $pK_a$ (3.49) of the aspirin that was present initially. When the pH of the reacidified solution was held constant at 2.85, it did not result in consumption of acid, indicating that there was no reesterification in this case. The behavior of salicylic acid conforms to that of case 2 with the added feature that the $pK_a$ of an already existing, free carboxyl functional group is decreased in one of the hydrolysis products.

Analysis of the data for fulvic acid in terms of case 3 and case 4 indicates that, because the hysteresis curve (fig. 4) does not form a closed loop, reesterification must not be complete within the time frame of the experiment. If no phenyl esters were hydrolyzed and if no other very weak acids were produced during hydrolysis, the form of the back-titration curve in the high pH region would resemble the corresponding part of the forward-titration curve. This, however, is not the case, and the increased buffer capacity in the high pH region of the back titration (as shown by the decreased slope of the back-titration curve, fig. 4) is consistent with the protonation of phenolate anions. Similarly, the enhanced buffer capacity in the lower

pH region of the back titration, compared to that in the forward titration, is consistent with the presence of hydrolytically generated carboxyl functional groups. Thus, the experimental data can be interpreted in terms of case 4 with partial reesterification occurring during the back titration. That is, phenyl esters in fulvic acid undergo hydrolysis at higher pH values, and partial reesterification occurs when the pH is decreased. It is possible that reesterification would be complete if sufficient time were allowed at the lower pH values. Given this interpretation, it is noteworthy that, of the three synthetic esters studied, only the phenyl ester (aspirin) showed noticeable hydrolysis in the same time frame as the fulvic acid (fig. 5). The aromatic and aliphatic esters were not obviously hydrolyzed under similar conditions (fig. 5).

The fact that the hysteresis curve is not closed indicates that the acids ($R'CO_2H$) that are produced during hydrolysis cannot be substantially weaker, as a whole, than the carboxylic acids that were originally present in the system. If the hydrolytically produced acids were substantially weaker, they would be undissociated at pH 3.0 and would not contribute significantly to the pH, thus enabling closure of the hysteresis loop. In fact, the hydrolytically produced acids must be comparable in strength to the acids originally present. If the former were considerably stronger than the latter, the form of the forward- and back-titration curves would be similar in the lower pH regions—this, however, is not the case. The influence of hydrolysis on the $pK_a$ values of already existing free-acid groups is difficult to assess in the case of humic substances because of the lack of discrete inflections that would allow one to differentiate between intensity ($pK_a$) and total-acid factors.

Of the synthetic esters studied, the phenyl ester (aspirin) most closely resembled fulvic acid in the forward-titration and high-pH-stat experiments. However, the aspirin hydrolysis products indicated no evidence of reesterification in the back-titration experiments or in the subsequent pH-stat experiment at pH 2.85. Likely candidate species to explain the reesterification interpretation for fulvic acid would be cyclic esters (i.e., lactones) that form in a facile manner. The presence of phenyl lactones in fulvic acid from the Suwannee River would account for the proposed facile ester hydrolysis and reesterification reactions. In future research, model experiments will be conducted with synthetic phenyl lactones in an attempt to approximate the complete hysteresis curve of fulvic acid. Coumarins, having the basic structure indicated below, are phenyl lactones that occur widely in nature, and these, or similar entities, may contribute to hydrolysis and esterification reactions in humic substances.



coumarin

Independent spectroscopic evidence for the presence of esters in fulvic acid from the Suwannee River is shown in figure 7. J.A. Leenheer (written commun., 1986) titrated fulvic acid to pH 10.0 with tetrabutylammonium hydroxide and rapidly freeze-dried the resulting salt. This salt was then dissolved in acetonitrile, and a film was cast on sodium chloride windows. The infrared spectrum (fig. 7) shows a band centered at 1,760 $cm^{-1}$ that is attributed to the carboxyl stretching frequency of esters and a band centered at 1,700 $cm^{-1}$ that is assigned to the carbonyl stretching frequency of both esters and ketones. Leenheer (written commun., 1986) found that the 1,760-$cm^{-1}$ band had disappeared and that the 1,700-$cm^{-1}$ band was diminished considerably in the spectrum of the sodium salt when the fulvic acid was refluxed at boiling point in 1 N NaOH for 6 h. This is consistent with saponification of ester groups in fulvic acid. In previous studies of soil-derived fulvic acid, an alternative interpretation of the hydrolytic reactions was given (Davis and Mott, 1981a, 1981b). In one of those studies (Davis and Mott, 1981b), the possibility of ester hydrolysis was considered but was dismissed on the basis that any esters originally present in the organic matter would have been hydrolyzed in the rather severe alkaline conditions of the extraction procedure. However, the data in the hysteresis plot of figure 4 indicate the occurrence of reversible reactions, possibly ester hydrolysis/esterification reactions. This hypothesis is further substantiated by data from J.A. Leenheer (written commun., 1986), who determined that acidification of the material that was hydrolyzed in 1 N NaOH caused reappearance of ester groups. A cast film of the tetrabutyl ammonium salt of the reacidified fulvic acid (following hydrolysis in 1 N NaOH) was prepared as described above; infrared spectroscopy showed the reappearance of absorption bands at 1,760 and 1,700 cm. Furthermore, the fulvic acid from the Suwannee River used in this study was in contact with alkali for only a very short period

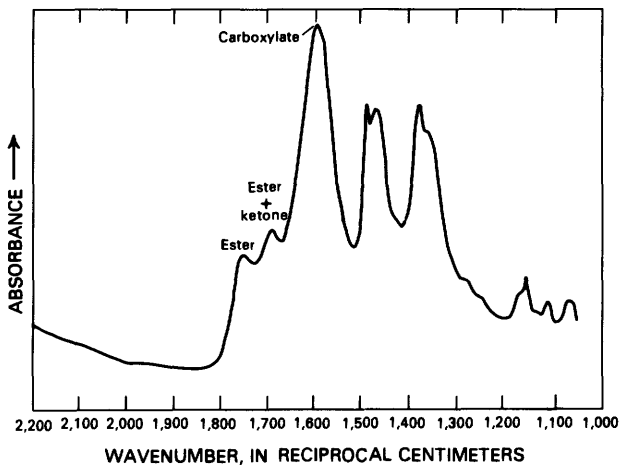



**Figure 7.** Infrared spectrum (absorbance mode) of film cast from acetonitrile of the tetrabutyl ammonium salt of fulvic acid from the Suwannee River. (Figure courtesy of J.A. Leenheer.)

during the isolation procedure (see Malcolm and others, chap. B, this volume), thereby minimizing the opportunity for hydrolysis to occur.

Thus, it appears that reversible hydrolysis/esterification reactions may be important in the chemistry of humic substances and that acidification and drying of humic substances may promote the formation of these esters. J.A. Leenheer (oral commun., 1986) also has obtained $^{13}$C-NMR spectroscopic evidence for the presence of esters and the occurrence of ester hydrolysis in fulvic acid from the Suwannee River. The role of esters and ester hydrolysis in humic chemistry needs to be investigated in a wide variety of fulvic and humic acids; further experiments need to be conducted with model compounds in an attempt to more fully simulate the titrimetric and hydrolytic behavior of humic substances.

The results of this work may have a number of important implications in humic-substances research. In the isolation of this fulvic acid from river water, the organic matter was eluted from XAD–8 resin using 0.1 N NaOH (pH ≅ 13) while exposed to the atmosphere. On the basis of the results presented here, one would expect that ester hydrolysis would occur under those conditions. Partial reesterification then may occur during subsequent acidification and freeze-drying steps. Such hydrolytic reactions could possibly transform a nonhumic hydrophobic-neutral component into a hydrophobic-acid component (a humic fraction); it might transform a humic component into a hydrophilic-acid fraction that then would be classified as a nonhumic fraction (see the papers by Leenheer and Huffman (1979) and Leenheer (1981, 1985) for a discussion of these various fractions). Subsequent reversal of these reactions following the resin separations could result in the nominally humic fractions containing some hydrophobic-neutral components and the nominally hydrophilic-acid fraction containing some humic components. These considerations have widespread implications in that virtually all extraction procedures for humic substances involve an alkali extraction followed by an acidification step.

The isolated fulvic acid may have undergone irreversible hydrolytic reactions during its extraction and, thus, may no longer be representative of the original fulvic acid in its native environment. This would have implications in attempts to interpret the geochemical and environmental behavior of humic substances such as their ability to interact with metal ions or nonionic organic compounds. Reversible and (or) irreversible hydrolysis also would have serious implications for fundamental investigations of these materials, such as molecular-weight determinations. Because of these significant implications, there is a need to study the hydrolysis and esterification reactions of humic substances in greater depth. There also may be a need to develop alternative methods of extraction that avoid contacting the sample with alkali.

## CONCLUSIONS

The titrimetric and hydrolytic data for fulvic acid from the Suwannee River can be interpreted as follows: fulvic acid possesses carboxyl and phenolic functional groups with $pK_a$ values that extend rather continuously over a wide range. This results in a somewhat indistinct titration curve where no discrete equivalence points are evident. Esters in fulvic acid undergo hydrolysis as the pH is increased, and reesterification occurs as the pH is subsequently decreased. On the basis of the shape of the back-titration curves of fulvic acid (with HCl) and on model experiments with synthetic esters, it appears that phenyl esters represent a major contributor to the above hydrolysis. The carboxylic acids that are produced by hydrolysis have comparable $pK_a$ values to the acids that are originally present in fulvic acid. Infrared spectroscopy provides independent evidence

that corroborates these interpretations. It is hypothesized that the hydrolysis and esterification reactions of fulvic acid may be due to the presence of phenyl lactones such as coumarins or coumarin-like species, but further investigation of this hypothesis is needed. In reporting titration curves of humic substances and the base-consumption capacity of these materials, the time factor in the experiments must be clearly expressed. The occurrence of hydrolysis/reesterification reactions in humic substances has a number of implications concerning our understanding the nature of the materials and their geochemical and environmental behavior.

## ACKNOWLEDGMENT

Grateful acknowledgment is made to J.A. Leenheer for initially suggesting the occurrence of ester hydrolysis in these reactions and for supplying the infrared spectrum of figure 7.

## REFERENCES CITED

Breger, I.A., 1951, Chemical and structural relationship of lignin to humic substances: Fuel, v. 30, p. 204–208.

Bremner, J.M., 1950, Some observations on the oxidation of soil organic matter in the presence of alkali: Journal of Soil Science, v. 1, p. 198–204.

Christensen, J.J., Hansen, L.D., and Izatt, R.M., 1976, Handbook of Proton Ionization Heats and Related Thermodynamic Quantities: New York, John Wiley, 269. p.

Davis, H., and Mott, C.J.B., 1981a, Titrations of fulvic acid fractions, I. Interactions influencing the dissociation/reprotonation equilibria: Journal of Soil Science, v. 32, p. 379–391.

———1981b, Titrations of fulvic acid fractions, II. Chemical changes at high pH: Journal of Soil Science, v. 32, p. 393–397.

Euranto, E.K., 1969, Esterification and ester hydrolysis, *in* Patai, S., ed., The Chemistry of Carboxylic Acids and Esters (The Chemistry of Functional Groups): New York, Wiley-Interscience, p. 505–588.

Flaig, W., Beutelspacher, H., and Rietz, E., 1975, Chemical composition and physical properties of humic substances, *in* Gieseking, J.E., ed., Soil Components—Volume 1, Organic Components: New York, Springer Verlag, p. 1–211.

Kirby, A.J., 1972, Hydrolysis and formation of esters of organic acids, *in* Bamford, C.H. and Tipper, C.P.H., eds., Comprehensive Chemical Kinetics—Volume 10, Ester Formation and Hydrolysis, Related Reactions: Amsterdam, Elsevier, p. 57–202.

Leenheer, J.A., 1981, Comprehensive approach to preparative isolation and fractionation of dissolved organic carbon from natural waters and wastewaters: Environmental Science and Technology, v. 15, p. 578–587.

———1985, Fractionation techniques for aquatic humic substances, *in* Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediments, and Water: Geochemistry, Isolation, and Characterization: New York, Wiley-Interscience, p. 409–429.

Leenheer, J.A., and Huffman, E.W.D., 1979, Analytical method for dissolved-organic carbon fractionation: U.S. Geological Survey Water-Resources Investigations 79-4, p. 1–16.

Marshall, C.E., and Patnaik, N., 1953, Ionization of soils and soil colloids, IV. Humic and hymatomelanic acids and their salts: Soil Science, v. 75, p. 153–165.

Martell, A.E., and Smith, R.M., 1977, Critical Stability Constants—Volume 3, Other Organic Ligands: New York, Plenum, 495 p.

Martin, A.E., and Reeve, R., 1958, Chemical studies of podzolic illuvial horizons, III. Titration curves of organic-matter suspensions: Journal of Soil Science, v. 9, p. 89–100.

Mikita, M.A., 1980, Studies on the structure and reactions of humic substances: Tucson, University of Arizona, unpub. Ph.D. dissertation, 91 p.

Morrison, R.T., and Boyd, R.N., 1974, Organic Chemistry (3d ed.): Boston, Allyn and Bacon, p. 682–683.

Paxeus, N., and Wedborg, M., 1985, Acid-base properties of aquatic fulvic acid: Analytica Chimica Acta, v. 169, p. 87–98.

Perdue, E.M., 1985, Acidic functional groups of humic substances, *in* Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediment, and Water—Geochemistry, Isolation, and Characterization: New York, Wiley-Interscience, p. 493–526.

Pommer, A.M., and Breger, I.A., 1960, Potentiometric titration and equivalent weight of humic acid: Geochimica et Cosmochimica Acta, v. 20, p. 30–44.

Posner, A.M., 1964, Titration curves of humic acid: Transactions of the 8th International Congress of Soil Science, v. 3, p. 161–174.

Schnitzer, M., and Desjardins, J.G., 1962, Molecular and equivalent weights of the organic matter of a podzol: Soil Science Society of America Proceedings, v. 26, p. 362–365.

Schnitzer, M., and Khan, S.U., 1972, Humic Substances in the Environment: New York, Marcel Dekker, 327 p.

Stevenson, F.J., 1982, Humus Chemistry—Genesis, Composition, Reactions: New York, John Wiley, 443 p.

Stevenson, F.J., and Butler, J.H.A., 1969, Chemistry of humic acid and related pigments, *in* Eglington, G., and Murphy, M.T.J., eds., Organic Geochemistry—Methods and Results: New York, Springer Verlag, p. 534–577.

Swift, R.F., and Posner A.M., 1972, Autoxidation of humic acid under alkaline conditions: Journal of Soil Science, v. 23, p. 381–393.

Chapter M

# Proton Nuclear-Magnetic-Resonance Studies of Fulvic Acid from the Suwannee River

*By* T.I. Noyes *and* J.A. Leenheer

# CONTENTS



## FIGURES



## TABLES

## Abstract

The nonexchangeable- and exchangeable-proton distributions of fulvic acid from the Suwannee River were investigated using proton nuclear-magnetic-resonance spectrometry. The conditions for quantitative analysis were reviewed and applied to the fulvic acid samples. Exchangeable-proton content was determined on the underivatized sample using deutero-dioxane as a solvent. Carboxyl content was determined by methylation and hydroxyl content by acetylation. The hydroxyl content was evaluated further by using two acetylation procedures: one that acetylates all hydroxyls in the sample and the other that acetylates only reactive phenolic hydroxyls. Exchangeable-proton content is approximately 10.5 millimoles per gram; this leaves 33.5 millimoles per gram of nonexchangeable (structural) protons. The carboxyl content is 6.8 millimoles per gram, and the reactive-phenolic hydroxyl content is 1.4 millimoles per gram by nuclear-magnetic resonance—an alcoholic hydroxyl content of 1.5 millimoles per gram was determined using infrared spectrometry. Determination of unreactive phenols by infrared spectrometry indicated a content of 2.7 millimoles per gram.

## INTRODUCTION

Because of the development of powerful nuclear-magnetic-resonance (NMR) spectrometers, carbon-13 ($^{13}C$) has become the predominant nucleus for characterizing humic substances by their NMR spectra. However, proton ($^{1}H$) NMR is still useful for providing complementary evidence concerning the characterization of various organic structural moieties—this is particularly true in a complex mixture of organic molecules such as fulvic acid from the Suwannee River.

Many researchers have analyzed humic substances by NMR spectrometry (Ruggiero and others, 1978; Ruggiero, Interasse, and Sciacovelli, 1979, 1980, 1981; Ruggiero and others, 1980; Hatcher and others, 1981; Harvey and Boran, 1985; Perdue, 1985). Various researchers (Ruggiero and others, 1978; Mikita and others, 1981; Wershaw and others, 1981; Thorn, 1984; Leenheer and Noyes, in press; Leenheer and others, chap. P, this volume) used chemical derivatization, such as methylation, acetylation, oximation, and reduction (as well as NMR spectrometry of the derivatives) to study the functions of oxygen in humic substances.

This chapter describes quantitative information about nonexchangeable and exchangeable protons in fulvic acid from the Suwannee River as determined by $^{1}H$-NMR spectrometry. Nonexchangeable protons were measured directly by $^{1}H$ NMR of the samples and by certain chemical derivatives dissolved in various solvents. Nonexchangeable protons were subdivided into four categories based on ranges of $^{1}H$-NMR *chemical shifts*. Exchangeable protons were measured directly by $^{1}H$ NMR and indirectly by chemical derivatives that are selective for the carboxyl and phenolic hydroxyl groups.

## METHODS AND MATERIALS USED FOR SPECTROMETRIC STUDIES

To obtain quantitative $^{1}H$-NMR spectra, one must determine proper NMR conditions. A correct flip angle (i.e., one that permits tipping of the proton nucleus onto the axis of detection) and a sufficient pulse delay (i.e., one that enables the nucleus to relax during the pulse delay) are needed. The reader is referred to Becker and others (1979), Abraham and Loftus (1980), Martin and others (1980), and Cookson and Smith (1982) for additional information about the theory and practice of $^{1}H$-NMR spectrometry.

Proton NMR spectra were obtained using a Varian FT 80–A instrument at 79.5 MHz. A pulse width of 3 μs produced a flip angle of 45 degrees; a pulse delay of 5 s was used. The sweep width was 2,000 Hz; acquisition time was 2.047 s; and sensitivity enhancement applied to the Free Induction Decay (FID) was –1.300. Fifty to 200 transients were

accumulated. A pulse delay of 5 s is adequate for complete relaxation of nuclei between pulses. Thus, the spectra are quantitatively accurate.

## Acetylations of Fulvic Acid

Acetylations of fulvic acid were made using:

1. Acetic anhydride ($Ac_2O$) and 4-dimethylaminopyridine (DMAP) (to acetylate alcoholic hydroxyls and phenols): One-hundred milligrams of sample dissolved in 5 mL pyridine, 2 mL $Ac_2O$, and a catalytic quantity of DMAP were stirred overnight. Mixed anhydrides, formed by carboxylic-acid groups, were hydrolyzed by adding 50 mL of 1 N sodium bicarbonate ($NaHCO_3$) solution and stirring for 1 h. Samples were titrated to pH 6 using hydrochloric acid (HCl) and then extracted using methylene chloride ($CH_2Cl_2$) to remove acetylated DMAP andpyridine byproducts. The solution was then titrated to pH 1 using HCl and extracted into methyl ethyl ketone (MEK). The MEK fraction was back-extracted using an equal volume of water to remove acetic acid and HCl and was vacuum-rotary evaporated to dryness. The product was methylated using diazomethane ($CH_2N_2$) according to procedure 1 in the next section.
2. Schotten-Baumann acetylation (Blau and King, 1978) (to acetylate phenols only): One-hundred milligrams of sample dissolved in 20 mL water and 1 g of $NaHCO_3$ were stirred for 30 min while maintaining pH between 7 and 8 by simultaneous additions of $Ac_2O$ and $NaHCO_3$. The pH was adjusted to 2 using HCl; acetonitrile ($CH_3CN$) was added to 50 percent by volume; and the product was isolated on a 20-mL column of XAD–8 resin using a mobile phase of pH-2 water. The product was eluted using $CH_3CN$, vacuum-rotary evaporated to dryness, and methylated using $CH_2N_2$ according to procedure 1 in the next section.

## Methylations of Fulvic Acid

Methylations were conducted using two procedures:

1. Methylation of fulvic acid using $CH_2N_2$: Nitrogen gas ($N_2$) was bubbled slowly, via Teflon tubing, through two 25×150-mm test tubes and sealed using rubber stoppers. The first tube was filled two-thirds full with diethyl ether; the second tube, kept in an ice bath, contained 8 mL diethyl ether, 8 mL 2-(2-ethoxyethoxy) ethanol, 8 mL 30-percent potassium hydroxide (KOH) solution, and 3 g of 99-percent N-methyl-N-nitroso-p-toluenesulfonamide. Diazomethane gas that was generated by the reagents was bubbled with the $N_2$ through 100 mg of sample suspended in $CH_2Cl_2$, in an ice bath, while being dispersed by ultrasonic vibration. The methylated sample was soluble in $CH_2Cl_2$. The reaction was deemed complete when small $N_2$ bubbles ceased evolving. The product was vacuum-rotary evaporated to dryness and taken up in chloroform-$d_1$ ($CDCl_3$) for NMR analysis.
2. Methylation of fulvic acid using boron trifluoride etherate ($BF_3$•$ET_2O$) and methanol-$d_4$ (MeOD): One-hundred milligrams of sample was dissolved in 5 mL 15-percent $BF_3$•$ET_2O$ and MeOD and heated at 60°C for 4 h. The reaction product was applied to a 25-mL column of XAD–8 resin; excess MeOD and other reagents were rinsed out using 50 mL of deionized water; and the sample was eluted using $CH_3CN$ and was vacuum-rotary evaporated to dryness.

## Measurement of Nonexchangeable and Exchangeable Protons by Proton Nuclear-Magnetic-Resonance Spectrometry

To measure nonexchangeable protons of underivatized samples by $^1$H-NMR spectrometry in water, the residual HDO peak due to exchangeable protons was minimized by repeated evaporation of deuterium ($D_2O$) oxide from the sample—this served to deuterium-exchange protons on carboxyl and hydroxyl functional groups. The masking effect of a large solvent peak on nonexchangeable protons was minimized by this procedure.

To measure exchangeable protons of underivatized samples by $^1$H-NMR spectrometry, the sample was evaporated repeatedly using dioxane-$d_8$, which has about the same boiling point as water. Water that was bound to fulvic acid from the Suwannee River was removed by dioxane-$d_8$ evaporation; the remaining exchangeable protons can be attributed to the sample in the $^1$H-NMR spectrum of fulvic acid dissolved in dioxane-$d_8$.

### Measurement of Alcoholic Hydroxyls and Unreactive Phenolic Hydroxyls by Infrared Spectrometry

A pyridine-solution infrared (IR) technique (Kabasakalian and others, 1959) was used to quantify alcoholic hydroxyls and unreactive phenolic hydroxyls that were not acetylated during Schotten-Baumann conditions: A 50-mg quantity of sample was acetylated using $Ac_2O$ during Schotten-Baumann conditions and methylated with $CH_2N_2$. Water that was bound to the sample was removed by three rotary-vacuum evaporations of $CH_3CN$ from the sample. The sample then was dissolved in anhydrous pyridine at 3.1 mg/mL (0.0041 N, assuming a molecular weight of 740 daltons). IR spectra were obtained using calcium fluoride ($CaF_2$) cells that had a 0.3-mm path length on a Perkin-Elmer 580 spectrophotometer. Sorbitol was used as the alcoholic-hydroxyl standard and 2-hydroxyacetophenone was used as the unreactive-phenolic-hydroxyl standard. Alcoholic hydroxyls were measured at 3,280 $cm^{-1}$; one-half of the absorbance occurring at 2,780 $cm^{-1}$ was subtracted to correct for the presence of unreactive phenolic hydroxyls that were quantitated at this wavelength. Unreactive phenolic hydroxyls were measured at 2,780 $cm^{-1}$.

## QUANTITATION OF FULVIC-ACID PROTONS

The $^1$H-NMR spectra of underivatized fulvic acid from the Suwannee River are shown in figures 1–3. The $^1$H-NMR spectrum of the sample dissolved in $D_2O$ at pH 8 is shown in figure 1. Organic solvents have different mechanisms of solvation and can affect chemical shifts dissimilarly; therefore, the same sample dissolved in dimethylformamide-$d_7$ (DMF-$d_7$) is shown in figure 2 and the same sample dissolved in dioxane-$d_8$ is shown in figure 3. The H-C-O region, which is obscured by HDO peak in figure 1 and by dioxane-$d_8$ in figure 3, is shown in figure 2.

Chemical-shift assignments for nonexchangeable protons in a variety of organic compounds are described in Simons (1978). For the $^1$H-NMR spectra shown in figures 1–3, only four assignments will be described because of the broadness of the peaks (or spectral regions). Protons attached to aliphatic carbons that are two or more carbons removed from an electronegative group occur from 0 to 1.8 ppm and

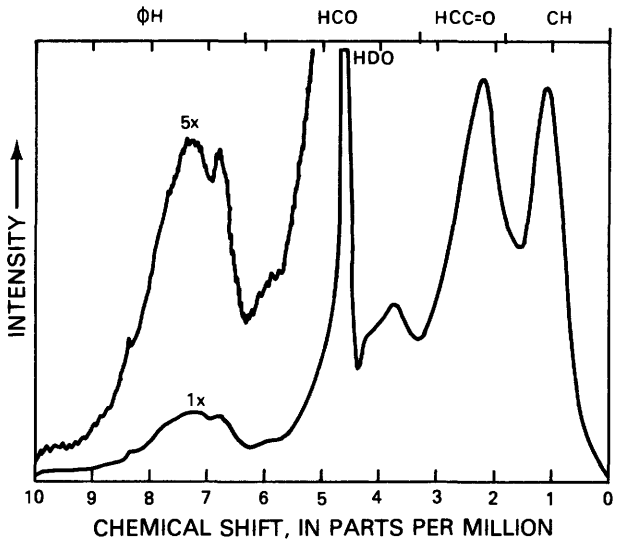



**Figure 1.** Proton nuclear-magnetic-resonance spectrum of underivatized sample of fulvic acid. Solvent is deuterium oxide at pH 8.

are designated as CH in figures 1–3. Protons attached to aliphatic carbons that are attached to electronegative groups, such as a carbonyl group or an aromatic ring, occur from 1.8 to 3.3 ppm and are designated as HCC=O in figures 1–3. Protons on carbons that are sigma-bonded to oxygen occur from 3.3 to 6.3 ppm and are designated as HCO in figures 1 and 2. Lastly, protons that occur downfield from 6.3 ppm are olefinic and aromatic protons and are designated as ØH

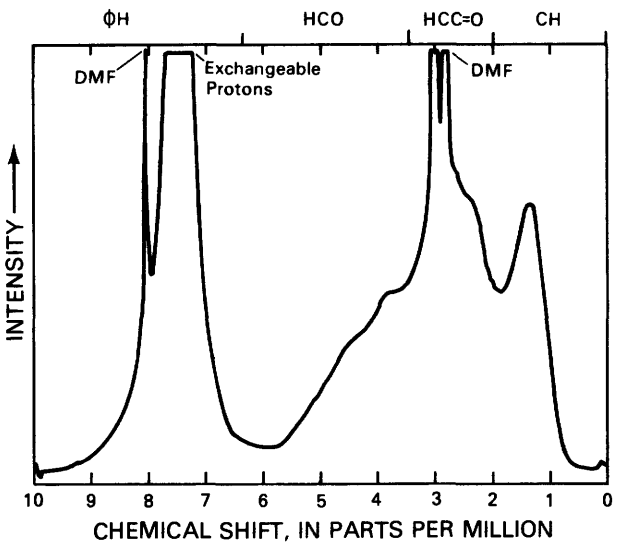



**Figure 2.** Proton nuclear-magnetic-resonance spectrum of underivatized sample of fulvic acid Solvent is dimethylformamide-$d_7$.

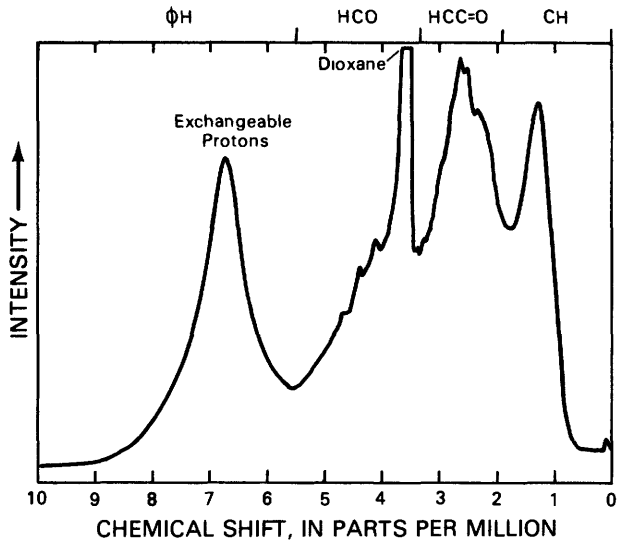



**Figure 3.** Proton nuclear-magnetic-resonance spectrum of underivatized sample of fulvic acid. Solvent is dioxane-$d_8$.

in figures 1 and 2, except for the aromatic protons that are masked by the exchangeable protons shown in figures 2 and 3.

In addition to organic-solvent effects that cause small differences in $^1$H-NMR spectra, there are charge effects that cause variations in chemical shifts. The peak of the HCC=O region occurs at 2.2 ppm in $D_2O$ (fig. 1) and at 2.5 ppm in dioxane-$d_8$ (fig. 3).

Tests on several aromatic carboxylic acids that were substituted by acetyl groups at various positions on the aromatic ring indicated that the methyl of the acetyl shifted upfield by 0.3 ppm when the aromatic carboxyl group was ionized in the salt form in water. However, $^1$H-NMR spectra did not completely account for the redistribution of protons between the four designated spectral regions of the sample in different solvents.

The approach used in this study to quantify nonexchangeable protons and exchangeable protons was threefold: First, the exchangeable-proton content was calculated using the dioxane-$d_8$ spectrum (fig. 3) and then subtracting out aromatic background protons using the spectrum of the sample dissolved in $D_2O$ (fig. 1). Second, carboxyl and total hydroxyl contents were calculated using the $^1$H-NMR spectrum of the acetylated, methylated derivative (fig. 4) and then subtracting out the $^1$H-NMR spectrum of the deutero-acetylated, deutero-methylated derivative (fig. 5). In this spectrum (fig. 4), deuterium of the derivatives is not observed: only nonexchangeable protons are seen. Third, reactive phenolic hydroxyl was calculated using the $^1$H-NMR spectrum of the derivative acetylated by the Schotten-Baumann method (fig. 6) (in which only reactive phenolic protons were acetylated) and subtracting out the background protons using the spectrum in figure 5. All spectral subtractions were normalized by matching the integrated peak areas of the CH region from 0 to 1.8 ppm. The CH peak was least affected by solvent and charge effects among the various derivatives. The various spectral subtractions are shown in figure 7 and the chemical shifts of the various derivatives are listed in table 1.

The nonexchangeable-proton backgrounds do not exactly coincide after normalization of spectra based on the CH peak as shown in figures 7*B*, 7*D*, and 7*E*. This lack of coincidence of nonexchangeable-proton backgrounds results in errors during the measurement of exchangeable protons by spectral subtraction of derivative-peak areas. The most extreme difference in nonexchangeable-proton spectral distributions is shown between figures 7*A* and 7*C*. The errors in measurement of exchangeable protons is offset somewhat by the fact that one exchangeable proton is measured by three protons of the methyl group of the derivative; an example of errors that can occur from comparison of figures 7*A* and 7*C* is shown in table 2.

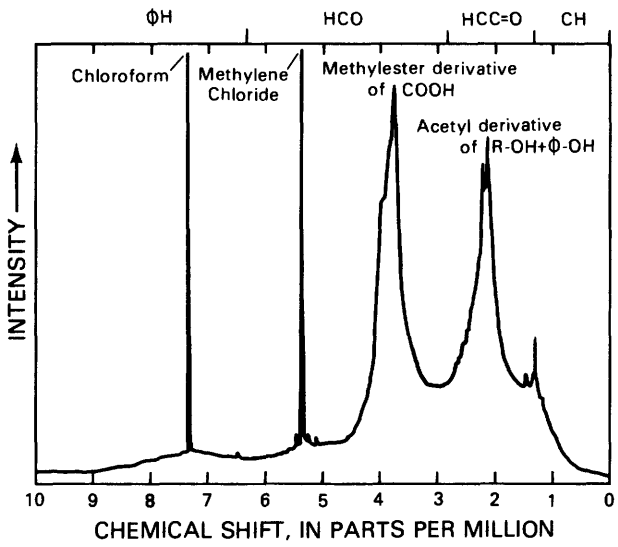



**Figure 4.** Proton nuclear-magnetic-resonance spectrum of derivatized sample of fulvic acid using acetic anhydride and 4-dimethylaminopyridine acetylation in pyridine, and diazomethane methylation. Solvent is chloroform-$d_1$.

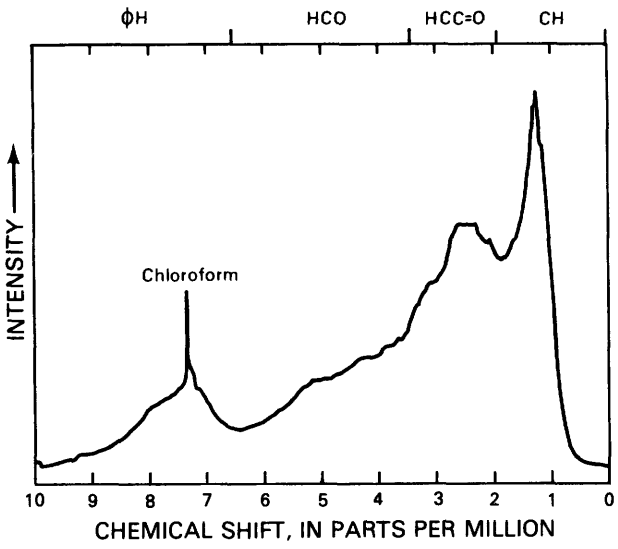



**Figure 5.** Proton nuclear-magnetic-resonance spectrum of derivatized sample of fulvic acid using methanol-$d_4$ methylation catalyzed with boron trifluoride etherate, and acetic anhydride acetylation catalyzed by 4-dimethylaminopyridine in pyridine.

The distribution of nonexchangeable versus exchangeable protons is presented in table 3; the distribution of nonexchangeable protons is presented in table 4; and the distribution of exchangeable protons is presented in table 5. These distributions were based on a total proton content of 44 mmol/g for fulvic acid from the Suwannee River (Reddy, and others, chap. I, this volume).

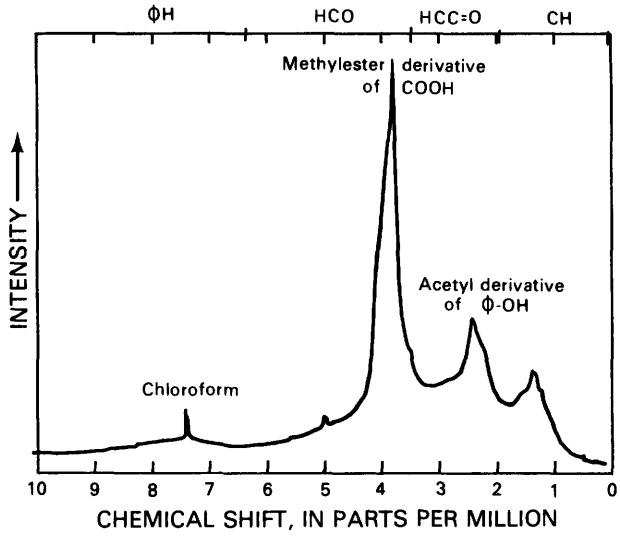



**Figure 6.** Proton nuclear-magnetic-resonance spectrum of derivatized sample of fulvic acid using Schotten-Baumann acetylation and diazomethane methylation. Solvent is chloroform-$d_1$.

The variability of the two nonexchangeable-proton distributions presented in table 4 is smaller than it appears when comparing figures 7*A* and 7*C*. This is because the differences occur within spectral regions rather than between spectral regions. For example, the 2.2-ppm peak in the $D_2O$ spectrum (fig. 7*A*) is broadened downfield to 2.2–2.6 ppm because of charge effects on HCC=O groups; the broad peak centered at 3.5 ppm in the $D_2O$ spectrum (fig. 1) is shifted downfield because of the acetylation of HCO groups in figure 7*C*.

In the exchangeable-proton distribution presented in table 5, the total of exchangeable protons measured by the sum of the derivatives (11.2 mmol/g) is slightly larger than the value measured in dioxane-$d_8$ (10.5 mmol/g). This discrepancy may be due to the hydrolysis of structural esters during the derivatization procedure that creates additional exchangeable hydrogen. The carboxyl content of 6.8 mmol/g (for fig. 7*D*, calculations in table 5) is larger than the 6.1 mmol/g reported by Bowles and others (chap. L, this volume); this supports the hydrolysis hypothesis. Some hydrolysis of previously acetylated phenols during the hydrolysis of mixed anhydrides by $NaHCO_3$ also may occur; these phenols subsequently may be methylated and counted as carboxyl because methylated carboxyl groups and methylated phenols cannot be distinguished by chemical shifts of the derivatives (as presented in table 1).

The Schotten-Baumann acetylation procedure, which was used to measure phenolic hydroxyl content reported in table 5, was tested using salicylic acid, p-hydroxy benzoic acid, gallic acid, chlorogenic acid, 2-hydroxyacetophenone, and tannic acid. Quantitative derivatives were formed for every standard (as determined by $^1$H-NMR and IR spectrometry) except for 2-hydroxyacetophenone and tannic acid; 2-hydroxyacetophenone was unreactive and tannic acid phenolic hydroxyls were only partially acetylated. This result was encouraging and somewhat surprising

**Figure 7** (facing page). Subtraction of structural background and quantitative measurements of structural moieties. *A*, deuterium-oxide structural background for *B*; *B*, dioxane-$d_8$ spectrum showing shifted exchangeable protons; *C*, nonexchangeable background for *D* and *E*; *D*, spectrum of derivatized sample showing methyl added to carboxyl and acetyl added to hydroxyl for the quantitation of both groups; *E*, spectrum of derivatized sample. Diazomethane methyl was added to carboxyl and acetyl was added to reactive phenolic hydroxyl for the quantitation of the latter group.

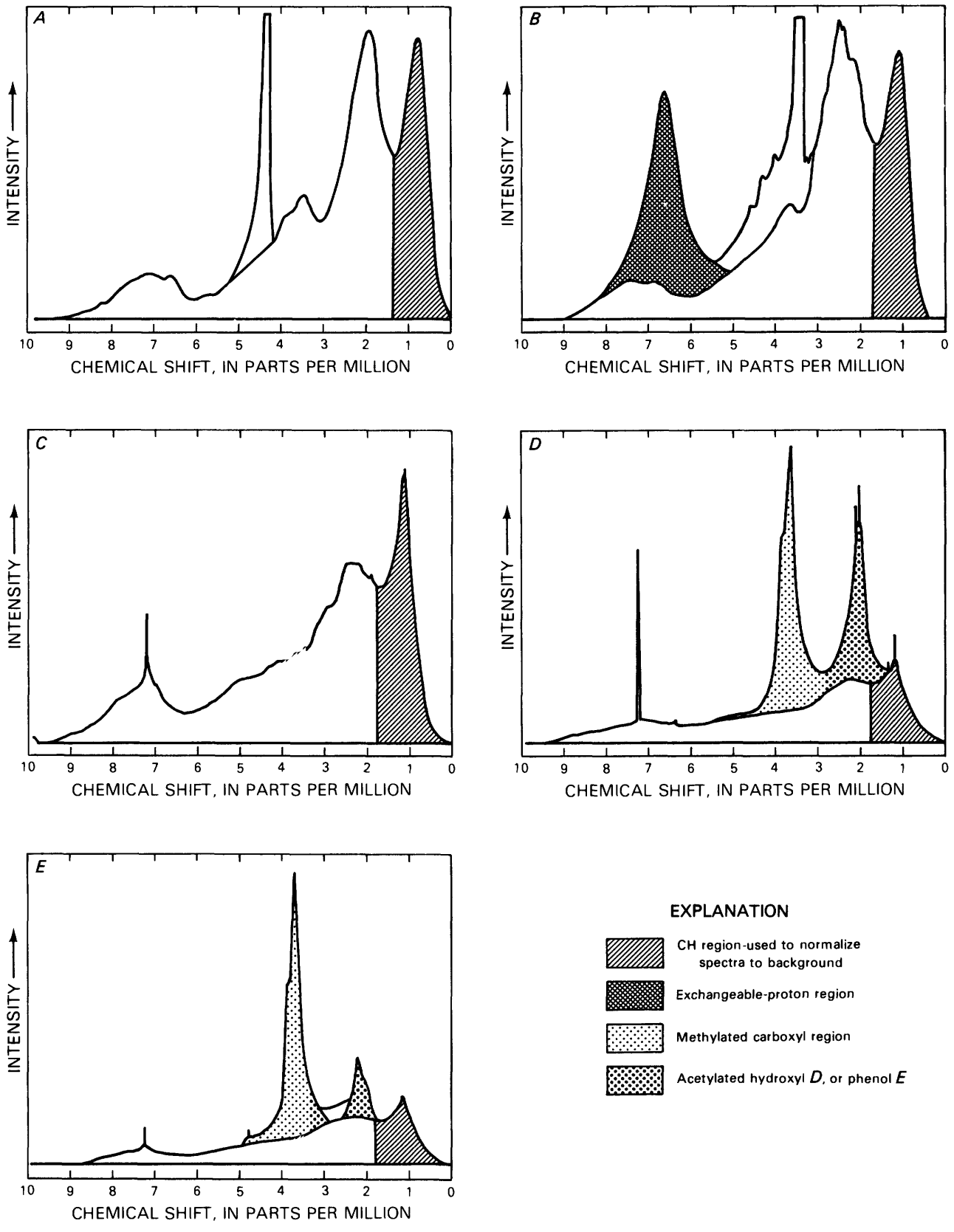

A
B
C
D
E
INTENSITY
10 9 8 7 6 5 4 3 2 1 0
CHEMICAL SHIFT, IN PARTS PER MILLION
EXPLANATION
CH region-used to normalize spectra to background
Exchangeable-proton region
Methylated carboxyl region
Acetylated hydroxyl D, or phenol E

**Table 1.** Chemical shifts, measured by proton nuclear-magnetic-resonance spectrometry, of various derivatives of fulvic acid

| Derivative | Functional group derivatized | Chemical-shift range (parts per million) |
|---|---|---|
| Acetyl ($CH_3\overset{O}{\overset{\|}{C}}$-) | | |
| Aliphatic acetoxy------- | Aliphatic alcohols--------- | 1.9-2.2 |
| Aromatic acetoxy-------- | Phenols--------------------- | 2.1-2.4 |
| Acetyl ketone----------- | Carbon alpha to ketone----- | 2.2-2.5 |
| Methyl ($CH_3$-) | | |
| Aliphatic methyl ester-- | Aliphatic carboxylic acid-- | 3.6-3.8 |
| Aromatic methyl ester--- | Aromatic carboxylic acid--- | 3.8-4.0 |
| Aromatic methoxy-------- | Phenol---------------------- | 3.6-4.0 |

**Table 2.** Percentage errors in the measurement of exchangeable protons by methyl and acetyl derivatives of fulvic acid by differences in nonexchangeable-proton distributions between figures 7*A* and 7*C*

| Spectral region (parts per million) | Nonexchangeable-proton region | Difference of total nonexchangeable protons between spectra (percent) | Variations[1] in nonexchangeable protons (millimoles of protons per gram) | Variations[1] in exchangeable protons measured by methyl group (millimoles of protons per gram) |
|---|---|---|---|---|
| 0- 1.8 | CH | 2.3 | 0.8 | 0.3 |
| 1.8- 3.3 | HCC=O | 4.5 | 1.5 | .5 |
| 3.3- 6.3 | HCO | 3.3 | 1.1 | .4 |
| 6.3-10 | ØH | 2.8 | 1.2 | .4 |

[1]Variations based on 33.5 millimoles per gram of nonexchangeable protons.

**Table 3.** Distribution of exchangeable versus nonexchangeable protons in fulvic acid

| Spectra | Nonexchangeable protons (millimoles per gram) | Exchangeable protons (millimoles per gram) |
|---|---|---|
| Figure 7*B*, which subtracts spectrum of underivatized sample in deuterium oxide (fig. 1) from spectrum of underivatized sample in dioxane-$d_8$ (fig. 3)---------- | [1]33.5±0.9 | [1]10.5±0.9 |

[1]Error based on noncoincidence of two spectra from 5 to 9 parts per million.

**Table 4.** Distribution of nonexchangeable protons in fulvic acid

| Spectra | Spectral region (parts per million) | Structural group | Nonexchangeable protons (millimoles per gram) |
|---|---|---|---|
| Figure 7*A*, underivatized sample in deuterium oxide---------- | 0- 1.8 | CH | 9.9 |
| | 1.8- 3.3 | HCC=O | 12.3 |
| | 3.3- 6.3 | HCO | 7.6 |
| | 6.3-10 | ØH | 3.7 |
| Figure 7*C*, Sample derivatized with methanol-$d_4$ catalyzed with boron trifluoride etherate, and acetic anhydride catalyzed with 4-dimethylaminopyridine---------- | 0- 1.8 | CH | 9.1 |
| | 1.8- 3.3 | HCC=O | 10.8 |
| | 3.3- 6.3 | HCO | 8.7 |
| | 6.3-10 | ØH | 4.9 |

because of the weak acid and sterically hindered nature of certain phenolic hydroxyl groups in salicylic, gallic, and chlorogenic acids. The procedure was also tested using coumarin; hydrolysis of coumarin did not occur during Schotten-Baumann conditions.

The carboxyl-group content (table 5, from spectrum in fig. 7*E*) is much too large (7.6 mmol/g), but the large value is readily explained by underivatized alcoholic hydroxyl groups occurring in the spectral region of measurement (3.0–5.5 ppm) that were not subtracted when the spectrum in figure 7*C* was used for background correction.

The magnitude of the exchangeable protons listed in table 5 is substantial when compared to the magnitude of the worst-case error listed in table 2.

**Table 5.** Distribution of exchangeable protons in fulvic acid

| Spectra | Spectral region (parts per million) | Derivatives (functional groups) | Derivative protons (millimoles per gram) | Exchangeable protons (millimoles per gram) |
|---|---|---|---|---|
| Figure 7*D*, which subtracts spectrum of d-methylated, d-acetylated sample (fig. 7*C*) from spectrum of methylated and acetylated sample (fig. 5). | 1.8-3.0 | Acetyl (total hydroxyl) | 13.2 | 4.4 |
| | 3.0-5.5 | Methyl (carboxyl) | 20.4 | 6.8 |
| | | | | 11.2 total exchangeable protons |
| Figure 7*E*, which subtracts spectrum of d-methylated, d-acetylated sample (fig 7*C*) from spectrum of Schotten-Baumann acetylated diazomethane methylated sample (fig. 6). | 1.8-2.6 | Acetyl (reactive phenol) | 4.2 | 1.4 |
| | 3.0-5.5 | Methyl (carboxyl) | 22.9 | 7.6 |

Therefore, the derivative method of measuring exchangeable protons by $^1$H-NMR spectrometry is reasonably quantitative if the various errors and side reactions are accounted for.

Derivatization conditions that were sufficiently stringent to quantitatively acetylate or methylate alcoholic hydroxyl groups also caused carbon acetylation or methylation as a side reaction. The acetylation procedure that was catalyzed using DMAP caused the least amount of side reactions (compared with $BF_3$-catalyzed acetylation or sodium-hydride methyliodide methylation), but IR spectrometry indicated that certain ketone structures were carbon-acetylated by $Ac_2O$ and DMAP. Therefore, a pyridine-solution IR technique was used with the derivative acetylated by the Schotten-Baumann procedure (fig. 7*E*) and methylated using $CH_2N_2$ to quantitate alcoholic hydroxyls and nonreactive phenolic hydroxyls (fig. 8). Kabasakalian and others (1959) reported that various alcoholic hydroxyls in steroids produced molar absorptivities that varied by only about 10 percent when these hydroxyls were hydrogen bonded to the pyridine solvent. The combined acetylation and methylation procedure eliminated reactive phenolic and carboxylic OH that interfered with alcoholic-hydroxyl measurement by IR spectrometry. The IR spectra that were used to quantitate alcoholic hydroxyls and nonreactive phenolic hydroxyls in fulvic acid from the Suwannee River are shown in figure 8. An alcoholic-hydroxyl content of 1.5 mmol/g and a nonreactive-phenolic-hydroxyl content of 2.7 mmol/g was obtained. When the content of 1.5 mmol/g for alcoholic hydroxyl is subtracted from the content of 4.4 mmol/g for total hydroxyl determined using acetylation (table 5), a difference of 2.9 mmol/g is obtained for total phenolic hydroxyl. This difference is smaller than the content of 4.1 mmol/g for reactive plus nonreactive phenolic hydroxyls and may be due to greater molar absorptivities of nonreactive phenolic hydroxyl groups in the sample than of the 2-hydroxyacetophenone standard used for quantitation. The IR spectrometric method confirms, however, the quantitative significance of nonreactive phenols. The total phenolic-hydroxyl content is probably similar to the difference of 2.9 mmol/g because of greater confidence placed in the total hydroxyl, carboxyl, and alcoholic hydroxyl contents than in the direct nonreactive phenolic hydroxyl determination.

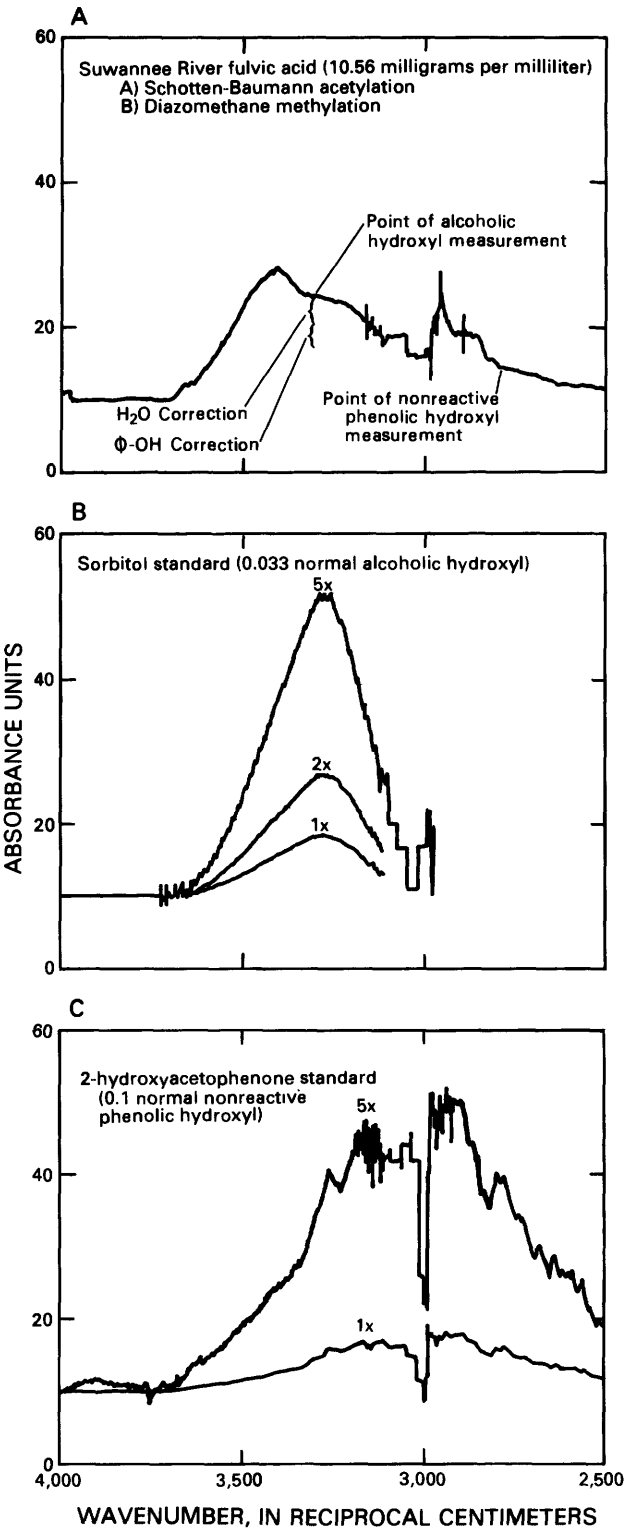



**Figure 8.** Quantitation of alcoholic hydroxyls and nonreactive phenolic hydroxyls by infrared spectrometry in pyridine. *A*, fulvic acid from Suwannee River; *B*, sorbitol standard; *C*, 2-hydroxyacetophenone standard.

## CONCLUSIONS

Proton NMR produced a quantitative measure of proton distribution in fulvic acid from the Suwannee River and compared well with other techniques for quantitation of nonexchangeable and exchangeable protons and associated structural moieties in this fulvic acid. Carboxyl-group content, determined by methylation, was clearly distinguished from total hydroxyl-group content, determined by acetylation, because of the distinct resolution of the methyl-ester peak from the acetyl peak in $^1$H-NMR spectra. Subdivision of the hydroxyl groups into alcoholic hydroxyl and phenolic hydroxyl was complicated by the incomplete reactivity of phenolic hydroxyl to Schotten-Baumann acetylation. The derivatizations used are as gentle as feasible, and complications due to various side reactions were accounted for. However, some functional groups are quite labile and cause some discrepancies in quantitation of exchangeable-proton distribution. A number of oxygen functional groups are difficult to quantitate by derivatization, including ethers, esters (particularly phenolic esters), and ketones. Better methods are needed to quantitate phenolic-hydroxyl content without interference from side reactions (such as hydrolysis) or incomplete derivatization (as in the current problems with Schotten-Baumann acetylation). Much interesting research remains in the investigation of the subtleties of oxygen functional groups in fulvic acid from the Suwannee River.

## REFERENCES CITED


Abraham, R.J., and Loftus, P., 1980, Proton and carbon-13 NMR Spectrometry—An Integrated Approach: London, Heyden and Son, 230 p.

Becker, E.D., Ferretti, J.A., and Gambhir, P.N., 1979, Selection of optimum parameters for pulse fourier transform nuclear magnetic resonance: Analytical Chemistry, v. 51, no. 9, p. 1413–1420.

Blau, K., and King, G.S., 1978, Acylation, *in* Blau, K., and King, G.S., eds., Handbook of Derivatives for Chromatography: London, Heyden and Son, p. 104–151.

Cookson, D.J., and Smith, B.E., 1982, Optimal experimental parameters for quantitative pulse fourier transform proton nuclear magnetic resonance spectrometry: Analytical Chemistry, v. 54, no. 14, p. 2591–2593.

Harvey, G.R., and Boran, D.A., 1985, Geochemistry of humic substances in seawater, *in* Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediment, and Water: New York, John Wiley, p. 233–247.

Hatcher, P.G., Maciel, G.E., and Dennis, L.W., 1981, Aliphatic structure of humic acids—A clue to their origin: Organic Geochemistry, v. 3, p. 43–48.

Kabasakalian, P., Townley, E.R., and Yudis, M.D., 1959, Infrared determination of hydroxyl equivalents in steroids: Analytical Chemistry, v. 31, p. 375–376.

Leenheer, J.A., and Noyes, T.I., in press, Derivatization of humic substances for structural studies, *in* Hayes, M.H.B., and others, eds., Humic Substances—Reactions and Interactions: Chichester, England, John Wiley.

Martin, M.L., Martin, G.J., and Delpuech, J.J., 1980, Practical NMR Spectroscopy: London, Heyden and Son, 460 p.

Mikita, M.A., Steelink, C., and Wershaw, R.L., 1981, carbon-13 enriched nuclear magnetic resonance method for the determination of hydroxyl functionality in humic substances: Analytical Chemistry, v. 53, no. 11, p. 1715–1717.

Perdue, E.M., 1985, Acidic functional groups of humic substances, *in* Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediment, and Water: New York, John Wiley, p. 493–526.

Ruggiero, P., Interesse, F.S., Cassidei, L., and Sciacovelli, O., 1980, $^1$H-NMR spectra of humic and fulvic acids and their peracetic oxidation products: Geochimica et Cosmochimica Acta, v. 44, no. 4, p. 603–609.

Ruggiero, P., Interesse, F.S., and Sciacovelli, O., 1979, $^1$H and $^{13}$C NMR studies on the importance of aromatic structures in fulvic and humic acids: Geochimica et Cosmochimica Acta, v. 43, no. 9, p. 1771–1775.

———1980, Short communication—$^1$H NMR evidence of exchangeable aromatic protons in fulvic and humic acids: Soil Biology and Biochemistry, v. 12, p. 297–299.

———1981, $^1$H-NMR and I.R. spectroscopic investigations on soil organic fractions obtained by gel chromatography: Soil Biology and Biochemistry, v. 13, p. 361–366.

Ruggiero, P., Sciacovelli, O., Testini, C., and Interesse, F.S., 1978, Spectroscopic studies on soil organic fractions. II. I.R. and $^1$H-NMR spectra of methylated and unmethylated fulvic acids: Geochimica et Cosmochimica Acta, v. 42, no. 4, p. 411–416.

Simons, W.W., ed., 1978, The Sadtler Handbook of Proton NMR Spectra: Philadelphia, Sadtler Research Laboratories, 1254 p.

Thorn, K.A., 1984, NMR structural investigations of aquatic humic substances: Tucson, University of Arizona, unpub. Ph.D. dissertation, 193 p.

Wershaw, R.L., Mikita, M.A., and Steelink, C., 1981, Direct $^{13}$C NMR evidence for carbohydrate moieties in fulvic acids: Environmental Science and Technology, v. 15, no. 12, p. 1461–1463.

Chapter N

# Nuclear-Magnetic-Resonance Spectrometry Investigations of Fulvic and Humic Acids from the Suwannee River

*By* K.A. Thorn

## CONTENTS



### FIGURES

TABLES



## Abstract

Fulvic and humic acids from the Suwannee River have been examined using solution-state carbon-13, proton, and nitrogen-15 nuclear-magnetic-resonance spectrometry. The carbon-13 nuclear-magnetic-resonance experiments included the measurement of spin-lattice relaxation times, measurement of Nuclear-Overhauser-enhancement factors, recording of quantitative spectra employing long pulse delays and inverse-gated decoupling, recording of attached-proton-test spectra, recording of spectra of samples methylated with carbon-13-enriched reagents, and the examination of solvent and pH effects. Proton nuclear-magnetic-resonance spectra were recorded in both aqueous solution and dimethylsulfoxide. The fulvic acid was derivatized with nitrogen-15-enriched hydroxylamine hydrochloride and its nitrogen-15 nuclear-magnetic-resonance spectrum was recorded.

The longest spin-lattice relaxation times measured in the fulvic and humic acids were less than or equal to 1.8 seconds; these were for the carboxyl and carbonyl carbons. Nuclear-Overhauser-enhancement factors ranged from 0.11 to 0.90 (all well below the theoretical maximum of 2.0) and were greatest for the aliphatic carbons (0 to 90 ppm). Quantitative carbon-13 nuclear-magnetic-resonance spectra indicated carbon distributions of 58 percent $sp^2$-hybridized versus 42 percent $sp^3$-hybridized carbons for the fulvic acid and 71 percent $sp^2$-hybridized versus 29 percent $sp^3$-hybridized carbons for the humic acid. Carbon aromaticities, $f_a$'s, were calculated to be 0.28 and 0.42 for the fulvic and humic acids, respectively. Attached-proton-test experiments clearly resolved the carbon-13 nuclear-magnetic-resonance spectra into methyl (0 to 26 ppm), methylene (26 to 43 ppm), methine (43 to 62 and 62 to 92 ppm), protonated aromatic and (or) acetal (90–105 ppm), protonated aromatic (105 to 135 ppm), and nonprotonated aromatic (135 to 162 ppm) carbons. The attached-proton-test spectra indicated that the aliphatic moieties of fulvic and humic acids are predominated by branched-chain, short-chain, and (or) cyclic structures. Spectra of samples methylated using carbon-13-enriched diazomethane indicated two distinct substitution

patterns of phenolic hydroxyls. Carbon-13 nuclear-magnetic-resonance spectra of the fulvic acid dissolved in dimethylsulfoxide showed superior resolution and signal-to-noise ratio compared to the corresponding spectra of the fulvic acid in aqueous solution. No major effects of pH on aqueous solution-state carbon-13 nuclear-magnetic-resonance spectra of the fulvic acid were observed. Proton nuclear-magnetic-resonance spectra indicated proton aromaticities of 0.15 and 0.22 for the fulvic and humic acids, respectively.

The nitrogen-15 nuclear-magnetic-resonance spectrum indicated that oxime formation is the major reaction of the fulvic acid with hydroxylamine. Nitrogen-15 resonances attributable to hydroxamic acids (the reaction products of hydroxylamine with esters) also were observed.

## INTRODUCTION

Since the first Fourier transform carbon-13 nuclear-magnetic-resonance ($^{13}$C-NMR) spectrum of a fulvic acid was reported over 10 years ago (Stuermer and Payne, 1976), $^{13}$C-NMR spectrometry has significantly advanced our understanding of the chemical structure of humic substances. Numerous studies on the application of solution-state NMR spectrometry to humic substances have been published recently, including the papers by Verheyen and others (1982), Gonzalez-Vila and others (1983), Skjemstad and others (1983), Newman and Tate (1984), Schnitzer and Preston (1986), and the reviews by Wershaw (1985) and Steelink and others (1989). Wilson and others (1981) have provided one of the few analyses of dissolved aquatic materials. Despite the large number of publications, the analysis of humic substances by solution-state $^{13}$C-NMR spectrometry still needs to be considered in the developmental stage because several aspects of NMR spectrometry of humic substances have not been investigated in great detail. Systematic studies of spin-lattice relaxation times ($T_1$'s) in humic substances have not been carried out, and, therefore, it is not possible to know a priori what pulse delay to choose to obtain quantitative spectra. Questions of solvent and pH effects in $^{13}$C-NMR spectra of humic substances have not been adequately addressed. The various solution-state carbon multiplicity sorting experiments introduced in the early 1980's (for example, attached proton test (APT), distortionless enhancement by polarization transfer (DEPT)) have not been evaluated against each other in their applicability to humic substances.

The purpose of this study is twofold: to provide as much structural information as possible on Suwannee River fulvic and humic acids using NMR spectrometry and to provide some further insight into these unresolved questions on the application of solution-state $^{13}$C-NMR spectrometry to humic substances. Proton ($^{1}$H-NMR) and nitrogen-15 nuclear-magnetic resonance ($^{15}$N-NMR) also were used to provide additional information. To this end, the following NMR analyses were carried out.

Carbon-13 spin-lattice relaxation times were measured to determine the pulse delays necessary to allow for complete relaxation of nuclei between pulses. Quantitative carbon distributions were then obtained for the fulvic and humic acid samples from $^{13}$C-NMR spectra acquired with inverse-gated decoupling and pulse delays determined from $T_1$ measurements; Nuclear-Overhauser-enhancement (NOE) factors were simultaneously determined. Next, $^{13}$C-NMR spectra were acquired under conditions that maximize signal-to-noise—short pulse delays and continuous decoupling—to obtain qualitative information that is suppressed in the quantitative spectra. Attached-proton-test spectra then were recorded to distinguish among methyl, methylene, methine, quaternary, and protonated and nonprotonated aromatic carbons in the fulvic and humic acids. Next, spectra of the samples methylated with $^{13}$C-enriched reagents were presented to provide information on the hydroxyl-group functionality. Proton NMR spectra were recorded to measure the proton aromaticities. A $^{15}$N-NMR spectrum of the fulvic acid derivatized with $^{15}$N-enriched hydroxylamine was obtained to provide more detailed information on the nature of the carbonyl groups. Finally, the effects of pH on aqueous-solution-state $^{13}$C-NMR spectra of the fulvic acid were examined, and spectra of the fulvic acid recorded in aqueous solution and in dimethylsulfoxide (DMSO) were compared.

The International Humic Substance Society Suwannee River fulvic and humic acid standard samples are expected to be the subject of many investigations among humic substances researchers. Hopefully, the details of chemical structure presented here can serve as a foundation upon which other findings can be related, whether they be metal-binding properties, physiological properties, or reaction

products of chemical degradations, etc. An abbreviated discussion of the NMR analyses of the Suwannee River samples has been published (Thorn, 1987).

As a final introductory note, in any discussion of the structural characteristics of the Suwannee River samples, the molecular-weight properties as defined by Aiken and others (chap. J, this volume) and the solubility properties defined by the isolation of these materials on XAD–8 resins (Malcolm and others, chap. B, this volume) are implicitly assumed. The number-average molecular weight of the fulvic acid was estimated to be 800 daltons using vapor-pressure osmometry; the molecular weight of the humic acid was estimated at 1,100 daltons using low-angle X-ray scattering.

## EXPERIMENTAL CONDITIONS

### Methylation of Fulvic and Humic Acids

The methylation of the fulvic and humic acids using $^{13}C$-enriched reagents has been described previously by Thorn (1984) and Thorn and others (1987). In brief, 50 mg of sample dissolved in 50 mL of dimethylformamide (DMF) was methylated with 2.33 mmol of ethereal diazomethane generated from 0.5 g of N-methyl-($^{13}C$)-N nitroso-p-toluenesulphonamide, 92.1 atom percent $^{13}C$. After NMR analysis, the diazomethylated sample was redissolved in 50 mL of freshly dried DMF and permethylated with 0.2 g of sodium hydride and 0.2 mL (3.21 mmol) of methyl iodide, 99 atom percent $^{13}C$, under a nitrogen atmosphere.

### Derivatization of Fulvic Acid with Nitrogen-15-Enriched Hydroxylamine Hydrochloride

The derivatization was performed by dissolving 0.19 g of hydroxylamine hydrochloride (99 atom percent $^{15}N$) and 0.81 g of the fulvic acid (H-saturated form) in 100 mL of deionized and distilled water, titrating to pH 5 with 1 N NaOH, and refluxing for 4 h at 88°C. The solution of the derivatized product was passed through an H-saturated MSC–1 cation-exchange column and freeze-dried. The amount of product recovered was 682 mg. This was dissolved in 2 g of DMSO-$d_6$ for NMR analysis.

### Nuclear-Magnetic-Resonance Spectrometry

The $^{13}C$-NMR spectra of the fulvic and humic acids methylated with $^{13}C$-enriched reagents were recorded on a Varian FT80A NMR spectrometer at 20.0 MHz. All other $^{13}C$-NMR spectra were recorded on a Varian XL–300 NMR spectrometer at 75.4 MHz. The $^{1}H$- and $^{15}N$-NMR spectra also were recorded on the Varian XL–300, at 299.9 and 30.4 MHz, respectively. The probe temperature for all spectra was at ambient room temperature, 20°±1°C. Sample concentrations and acquisition parameters for each spectrum are listed with the appropriate figure. Acquisition parameters are abbreviated as follows: spectral window, SW; decoupling mode, DM; pulse width, PW; line broadening, LB; acquisition time, AT; sensitivity enhancement, SE; pulse delay, PD; number of transients, NT. All $^{13}C$-NMR spectra were referenced on the solvent peaks. Peak areas of spectra were measured using both electronic integration by the spectrometer and cut-and-weigh methods; results from both methods were in good agreement. No artificial manipulations or flattening procedures were applied to the spectral baselines.

## CARBON-13 NUCLEAR-MAGNETIC-RESONANCE SPECTRA OF FULVIC AND HUMIC ACIDS

### Quantitation in Carbon-13 Nuclear-Magnetic-Resonance Spectrometry

To generate solution-state $^{13}C$-NMR spectra that are quantitative (i.e., where there is an accurate proportionality between signal intensities and the number of resonating carbon nuclei), two conditions must be met. First, differential saturation effects must be eliminated. This is accomplished by employing a pulse delay on the order of 3 to 5 times the longest $T_1$ present in the sample. The spin-lattice relaxation time, $T_1$, is a time constant that is inversely proportional to the rate at which energy is transferred from the spins to the lattice, allowing the magnetization to return to its equilibrium value after a radiofrequency pulse (Wehrli and Wirthlin, 1980). If too short a pulse delay is used, carbon nuclei with shorter $T_1$'s will be exaggerated in intensity relative to carbon nuclei with longer $T_1$'s. Secondly, differential NOE

effects must be eliminated. This is accomplished by employing inverse-gated decoupling: the decoupler is on during the acquisition but off during the pulse delay. The NOE effect occurs because proton decoupling causes changes in the population distribution of the $^{13}C$ spins, resulting in an enhancement of the $^{13}C$ signals:

$$\eta = \frac{S - S_o}{S_o}$$

where

$S_o$ is the $^{13}C$-signal intensity without NOE,

$\eta$ is the NOE factor, and

S is the resulting enhanced $^{13}C$-signal intensity.

For $^{13}C$ with $^{1}H$ decoupling, where $^{13}C$ spin-lattice relaxation occurs solely by $^{1}H$-$^{13}C$ dipole-dipole interactions, and, within the extreme narrowing limit of fast molecular motion, $\eta$ has its maximum value of 1.988, resulting in $S = 3S_o$. Therefore, at its maximum, the NOE can result in an approximately threefold increase in signal intensity.

The problems in obtaining quantitatively accurate $^{13}C$-NMR spectra of humic substances in the solid state using cross polarization with magic-angle spinning (CP/MAS) are more formidable than in the solution state. Some of the problems include unfavorable cross relaxation rates, $T_{CH}$'s, and proton rotating frame spin-lattice relaxation times, $T_{1H}$'s, in the humic materials; presence of paramagnetic materials; and problems of molecular motion. If each major band in the CP/MAS spectrum of the humic material has a different optimum contact time, it may not be possible to obtain a single spectrum where the peak areas accurately reflect the number of nuclei present. Although the commonly used acquisition parameters of 1-ms contact time and 1-s repetition rate have been shown to yield quantitative CP/MAS spectra for many simple organic molecules (Alemany and others, 1983a, 1983b), these parameters do not yield quantitatively accurate spectra for humic substances. Schnitzer and Preston (1986) showed that CP/MAS spectra of humic substances acquired with 1-ms contact times and 1-s repetition rates underestimated the aromatic carbons compared to solution-state spectra. In our own laboratory, we found that the solid-state CP/MAS spectrum of the Williams Fork Reservoir (Colorado) fulvic acid, acquired at room temperature using a 1-ms contact time and 1-s repetition rate, underestimated the aromaticity by 50 percent compared to the quantitative solution-state spectrum.

Earl and others (1987) showed that problems of molecular motion at room temperature prevented efficient cross polarization in the Armadale soil fulvic acid so that CP/MAS spectra of the Armadale did not agree with solution-state spectra acquired with long pulse delays and inverse-gated decoupling. For more comprehensive discussions of problems in quantitation in solid-state CP/MAS spectra of humic substances, the reader is referred to papers by Earl (1987), Vassalo (1987), and Frye and Maciel (1987) in the volume by Wershaw and Mikita (1987). Many researchers continue to publish aromaticities of humic substances from CP/MAS spectra without providing any quantitative analysis of the cross-polarization dynamics. Data that has been published without any analysis of the cross polarization time constants or, even worse, without a listing of the acquisition parameters, should be viewed with caution. Such omissions in the application of solid-state CP/MAS $^{13}C$-NMR to humic substances have, unfortunately, added much confusion to the ongoing debate on the true aromaticity of fulvic and humic acids.

## Measurements of Carbon-13 Nuclear-Magnetic-Resonance Spin-Lattice Relaxation Times

The $T_1$'s of the fulvic and humic acids were measured using the progressive saturation method (Freeman and Hill, 1971) to determine the pulse delays necessary for generating quantitative $^{13}C$-NMR spectra. These experiments are shown in figures 1 and 2. In the progressive-saturation method, the $^{13}C$ spins are subjected to repetitive 90° pulses separated by pulse delays, $\tau$, of increasing value. For each value of $\tau$, a dynamic equilibrium is established in which the saturation effect of the radiofrequency pulses and that of the relaxation balance each other. Peak intensities then will increase with an increase in the pulse delay until the pulse delay is on the order of approximately five times the $T_1$ of the particular peak. The $T_1$'s are calculated from a plot of $\ln(M_z - M_z^0)$ against $\tau$, which produces a straight line with a gradient of $-1/T_1$. $M_z^0$ is the peak intensity at equilibrium and $M_z$ the peak intensity for each value of $\tau$.

The $T_1$ values calculated from these progressive-saturation experiments are listed in table 1. As one would expect, the aliphatic I peaks (0 to 60 ppm;

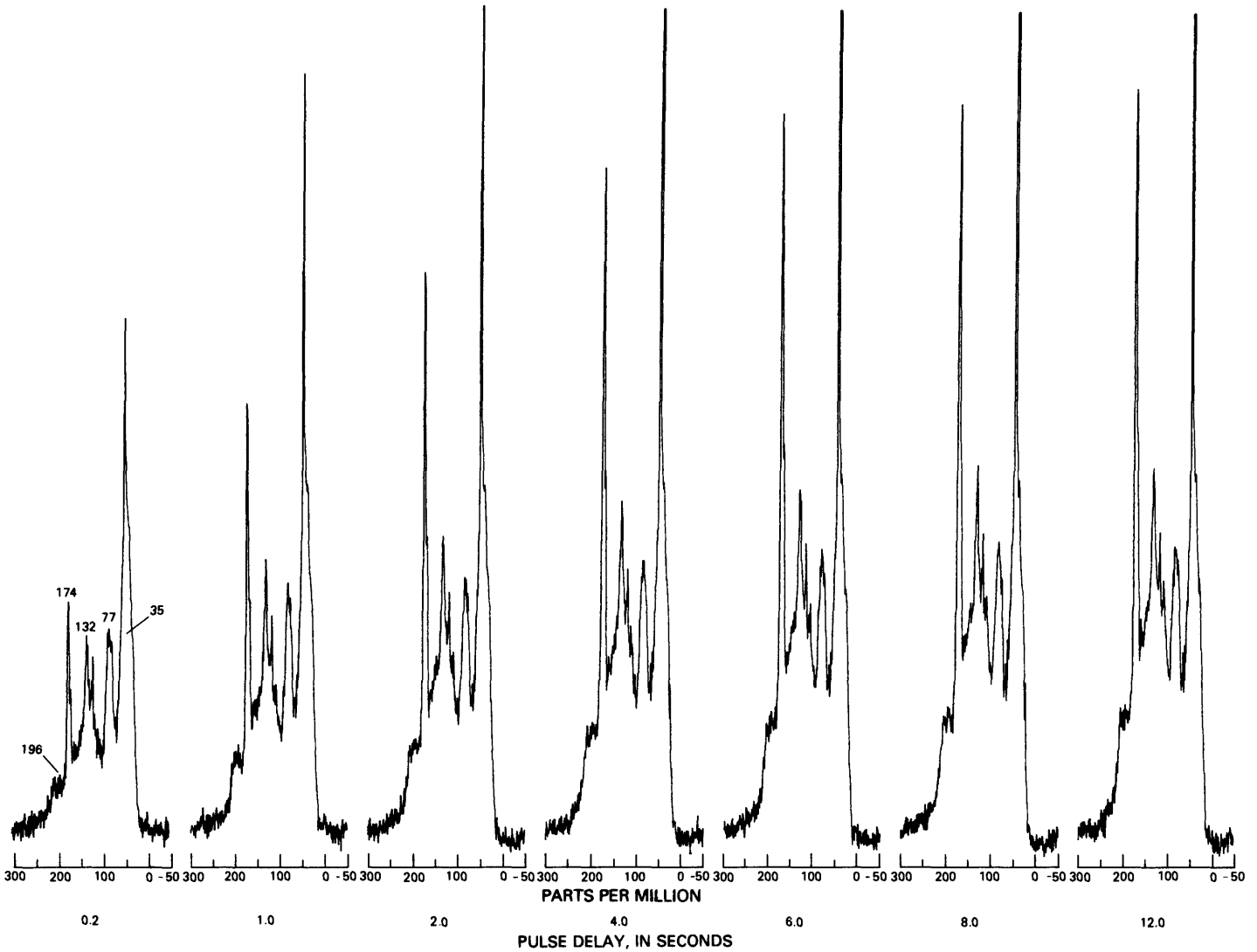



**Figure 1.** Carbon-13 nuclear-magnetic-resonance spectra for progressive-saturation experiment of fulvic acid. "Parts per million" label on x-axis refers to chemical shift in parts per million. Concentration = 200 milligrams in 2.0 grams of dimethylsulfoxide-$d_6$, carbon-13 depleted. Spectral window = 30,000 hertz; pulse width = 90°; acquisition time = 0.2 second; decoupling mode = continuous WALTZ decoupling; number of transients for each value of pulse delay = 4,000; line broadening = 20.0 hertz.

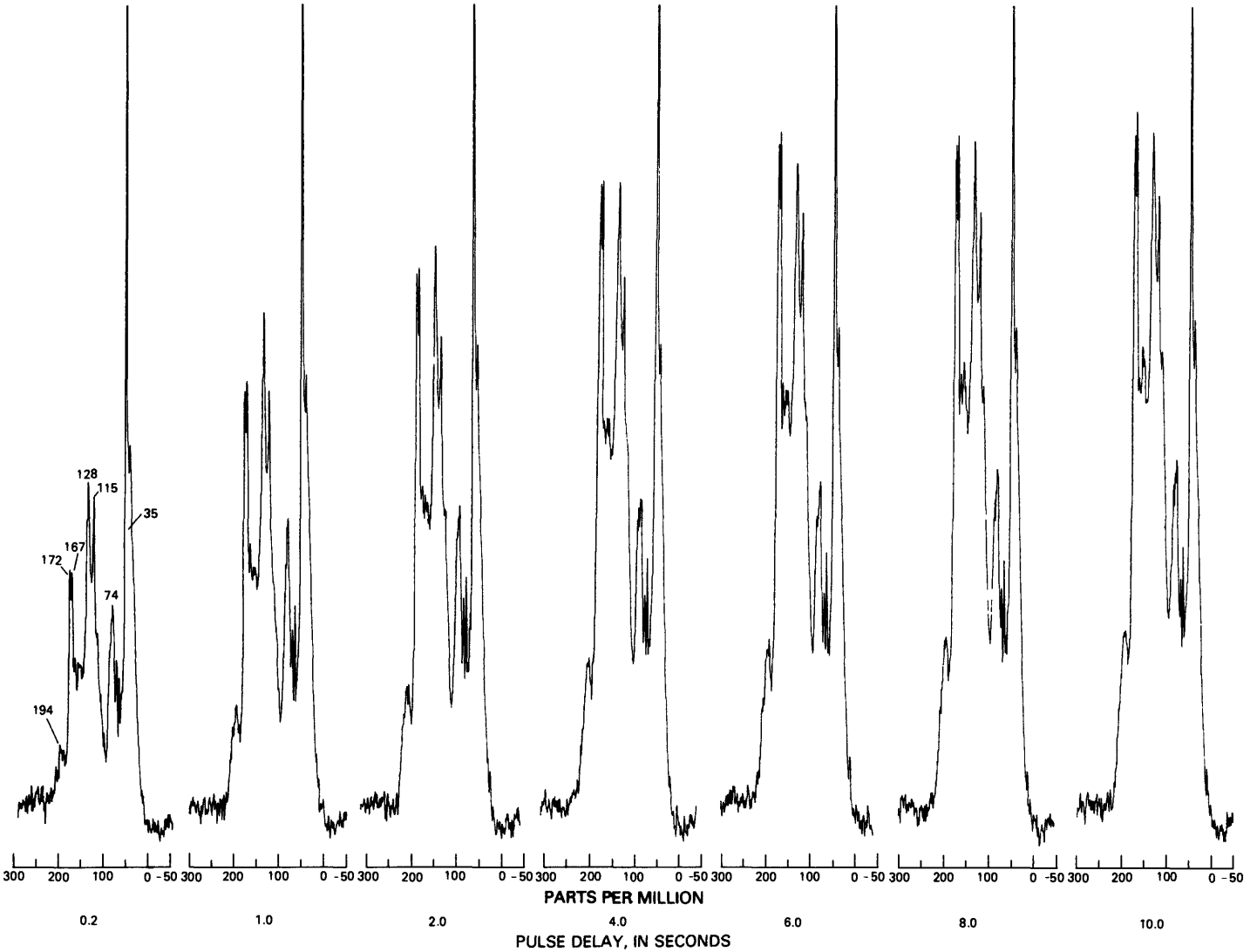



**Figure 2.** Carbon-13 nuclear-magnetic-resonance spectra for progressive-saturation experiment of humic acid. "Parts per million" label on x-axis refers to chemical shift in parts per million. Concentration = 300 milligrams in 2.0 grams of dimethylsulfoxide-$d_6$, carbon-13 depleted. Spectral window = 30,000 hertz; pulse width = 90°; acquisition time = 0.2 second; decoupling mode = continuous WALTZ decoupling; number of transients for each value of pulse delay = 2,500; line broadening = 100.0 hertz.

**Table 1.** Carbon-13 spin-lattice relaxation times ($T_1$'s), in seconds, of fulvic and humic acids

[ppm, chemical shift in parts per million; $T_1$, carbon-13 spin-lattice relaxation time; see figs. 1 and 2 for experimental details]

| | Ketone (220 to 180 ppm) | Carboxyl (180 to 160 ppm) | Aromatic (160 to 90 ppm) | Aliphatic II (90 to 60 ppm) | Aliphatic I (60 to 0 ppm) |
|---|---|---|---|---|---|
| Fulvic acid-- | $1.4 \leqq T_1 \leqq 1.8$ | $1.4 \leqq T_1 \leqq 1.8$ | $0.7 \leqq T_1 \leqq 1.4$ | $0.2 \leqq T_1 \leqq 0.4$ | $0.1 \leqq T_1 \leqq 0.2$ |
| Humic acid--- | $T_1 \cong 1.4$ | $0.7 \leqq T_1 \leqq 1.8$ | $0.7 \leqq T_1 \leqq 1.4$ | $T_1 \cong 0.4$ | $T_1 \cong 0.2$ |

carbons bonded to other carbons) and aliphatic II peaks (60 to 90 ppm; primarily carbons bonded to oxygens, such as carbohydrate, alcohol, and ether carbons) have the shortest $T_1$'s, and the aromatic-carbon peaks (90 to 160 ppm) and carbonyl-carbon peaks (160 to 220 ppm) have the longest $T_1$'s. For both the fulvic and humic acids, the longest $T_1$'s are less than or equal to 1.8 s; the range of $T_1$'s from 0.2 to 1.8 s is similar to what other researchers have found for soil humic substances in aqueous solution (Newman and others, 1980; Preston and Blackwell, 1985). The progressive-saturation results presented in table 1 also were confirmed using the spin-inversion recovery method.

## Quantitative Carbon-13 Nuclear-Magnetic-Resonance Spectra and Measurement of Nuclear-Overhauser-Enhancement Factors

Because the longest $T_1$'s for both the fulvic and humic acids are less than or equal to 1.8 s, $^{13}$C-NMR spectra acquired with a pulse delay of 8.0 s in conjunction with a 45° pulse should allow for complete relaxation of nuclei in between pulses. These spectra are shown in figures 3 and 4 for the fulvic and humic acids, respectively. The spectra in figures 3*A* and 4*A* were acquired with continuous decoupling, and the spectra in figures 3*B* and 4*B* were acquired with inverse-gated decoupling. The spectra in figures 3*B* and 4*B* are quantitative; increases in peak areas from the inverse-gated-decoupled spectra (figs. 3*B* and 4*B*) to the continuous-decoupled spectra (figs. 3*A* and 4*A*) are due to NOE. The peak areas for the quantitative spectra and the NOE factors for these spectra determined from the difference between the *A* and *B* spectra in figures 3 and 4 are listed in table 2.

The description of the spectra will follow the subdivisions of the spectra delineated above: aliphatic I carbons (0 to 60 ppm); aliphatic II carbons (60 to 90 ppm); aromatic carbons (90 to 160 ppm); carbonyl carbons (160 to 180 ppm and 180 to 220 ppm).

The aliphatic I (0 to 60 ppm) peak consists primarily of carbons bonded to other carbons, and, as the APT spectra presented later will demonstrate, it is comprised of methyl, methylene, and methine carbons. Some $sp^3$ carbons that are bonded to sulfur and nitrogen also occur in this region.

The aliphatic II peak (60 to 90 ppm) consists primarily of carbons bonded to oxygen and may be comprised of carbohydrates, ethers, and alcohols.

The broad resonance from 90 to 160 ppm consists mainly of aromatic carbons. Acetal and ketal carbons, and the *anomeric* carbons of carbohydrates, may overlap in the region from 90 to 105 ppm. As an example of an aromatic carbon shifted upfield, Wagner and others (1976) have reported the chemical shift of the C-8 carbon of quercetin to be 93.5 ppm. The range of chemical shifts of phenolic carbons in DMSO-$d_6$ is 135 to 165 ppm. The chemical shifts of phenolic carbons of model compounds are listed in table 3.

In order to describe the nature of the carbonyl carbons, 160 to 180 ppm and 180 to 220 ppm, it is useful to define the chemical-shift ranges of these carbons in DMSO-$d_6$: ketones, 220 to 190 ppm; aldehydes, 220 to 190 ppm; quinones, 190 to 178 ppm; carboxylic acids, 175 to 160 ppm; esters, 172 to 165 ppm; amides, 173 to 165 ppm; and lactones, 178 to 160 ppm.

The chemical shifts of selected model carbonyl compounds in DMSO-$d_6$ are listed in table 3. The carbonyl peak from 160 to 180 ppm represents primarily carboxylic-acid carbons; esters, amides, and lactones may overlap with the carboxylic acids. In both the fulvic- and humic-acid spectra, there is a splitting of the carboxyl peak, with maxima at 167 and 172 ppm. These maxima represent two broad categories of carboxylic-acid groups. The peak centered at 167 ppm encompasses benzenecarboxylic and

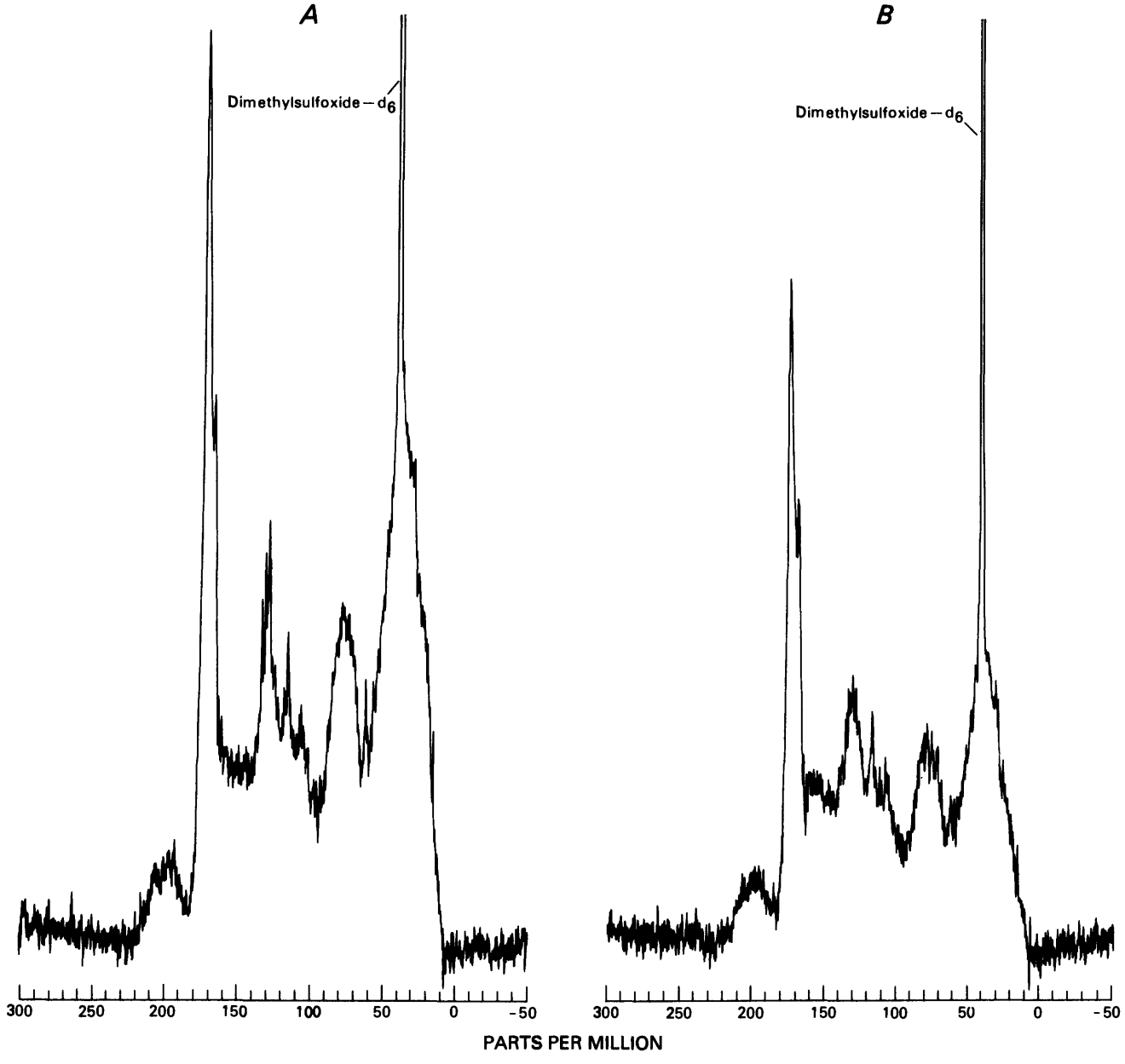



**Figure 3.** Nuclear-Overhauser-enhancement measurement and quantitative carbon-13 nuclear-magnetic-resonance spectrum of fulvic acid. "Parts per million" label on x-axis refers to chemical shift in parts per million. Concentration = 75 milligrams in 0.5 gram of dimethylsulfoxide-$d_6$, carbon-13 depleted; spectral window = 50,000 hertz; pulse width = 45°; acquisition time = 0.2 second; pulse delay = 8.0 seconds; number of transients = 15,000; line broadening = 20.0 hertz. *A*, Continuous WALTZ decoupling, Nuclear-Overhauser enhancement retained; *B*, Inverse-gated decoupling, Nuclear-Overhauser enhancement eliminated.

α, β-unsaturated carboxylic acids (such as benzoic and crotonic acids, respectively; table 3). The peak centered at 172 ppm encompasses aliphatic, benzylic, and ortho-hydroxybenzenecarboxylic acids (for example, n-heptanoic, phenylacetic, and 2,3-dihydroxybenzoic acids, respectively). It is interesting to note that the ratio of the intensity of the 167 ppm peak with respect to the 172 ppm peak is greater in the humic acid compared to the fulvic acid; this correlates with the greater aromaticity of the humic compared to the fulvic acid. The peaks centered at 167 ppm constitute direct evidence for the presence of aromatic carboxylic-acid groups in the fulvic and humic acids. The carbonyl peaks from

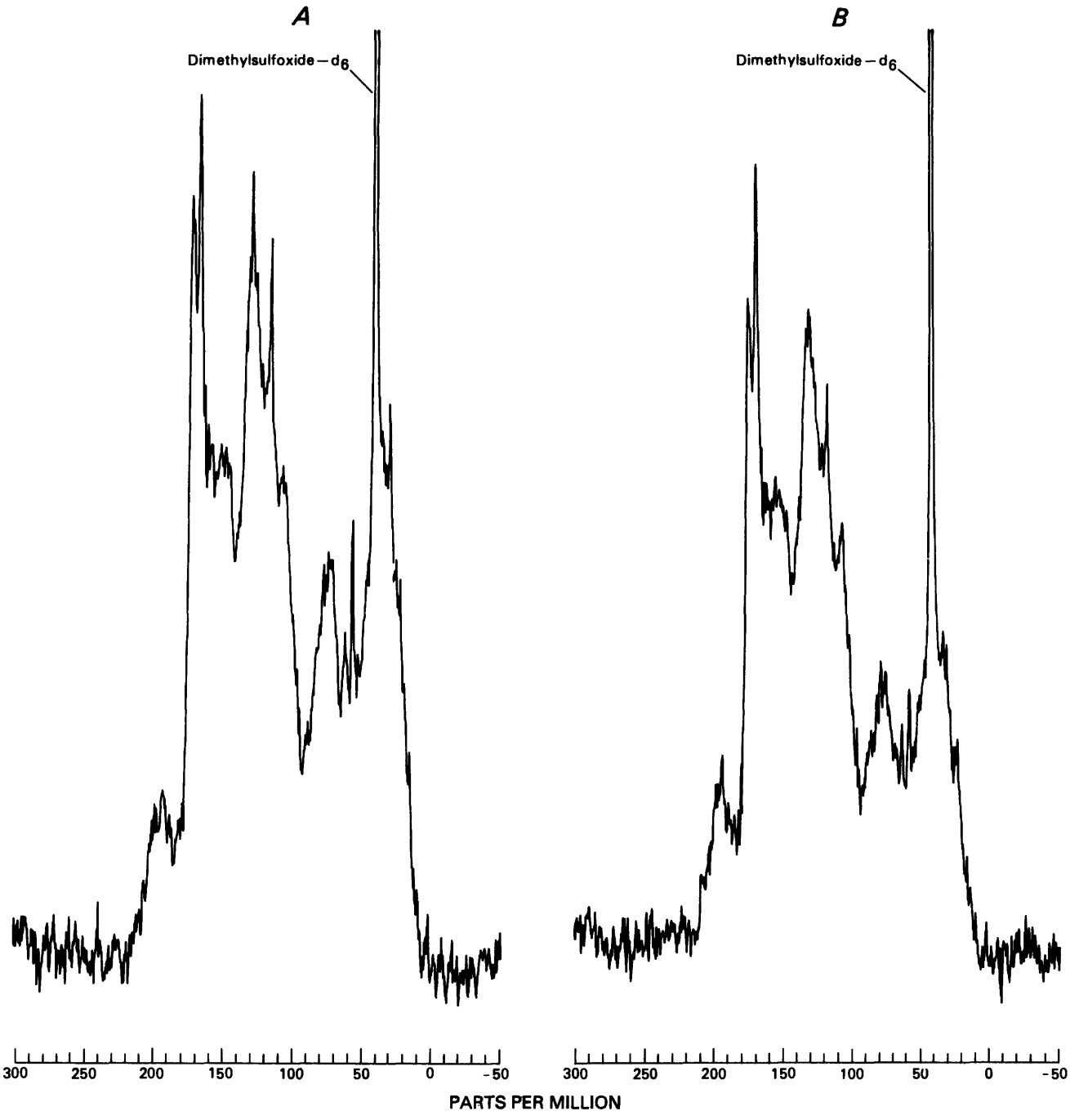



**Figure 4.** Nuclear-Overhauser-enhancement measurement and quantitative carbon-13 nuclear-magnetic-resonance spectrum of humic acid. "Parts per million" label on x-axis refers to chemical shift in parts per million. Concentration = 75 milligrams in 0.5 gram of dimethylsulfoxide-$d_6$, carbon-13 depleted; spectral window = 50,000 hertz; pulse width = 45°; acquisition time = 0.2 second; pulse delay = 8.0 seconds; number of transients = 9,300; line broadening = 50.0 hertz. *A*, Continuous WALTZ decoupling, Nuclear-Overhauser enhancement retained; *B*, inverse-gated decoupling, Nuclear-Overhauser enhancement eliminated.

180 to 220 ppm, centered at 195 ppm in the fulvic acid and 192 ppm in the humic acid, are interpreted as representing primarily ketone carbons with some overlap of quinone carbons. Although aldehyde carbons would appear in this region, the APT spectra presented later show that these carbons are nonprotonated. Furthermore, aldehyde protons are not evident in the $^1$H-NMR spectra of the fulvic and humic acids.

**Table 2.** Peak areas in quantitative carbon-13 nuclear-magnetic-resonance spectra, Nuclear-Overhauser-enhancement factors, and aromaticities for fulvic and humic acids[a]

[ppm, chemical shift in parts per million; Nuclear-Overhauser-enhancement factor, η; aromaticity, $f_a$]

| | Ketone (220 to 180 ppm) | Carboxyl/ carbonyl (180 to 160 ppm) | Aromatic (160 to 105 ppm) | Aromatic/ acetal (105 to 90 ppm) | Aliphatic II (90 to 60 ppm) | Aliphatic I (60 to to 0 ppm) | $sp^2$ (220 to 90 ppm) | $sp^3$ (90 to 0 ppm) | Aromaticity[b], $f_a$ |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Fulvic acid | | | | | |
| Peak area, percent of total carbon. | 6 | 19 | 28 | 5 | 15 | 27 | 58 | 42 | 0.28 |
| Nuclear-Overhauser-enhancement factor, η. | 0.20 | 0.35 | -- | 0.31[c] | -- | 0.67 | 0.90 | | |
| | | | | Humic acid | | | | | |
| Peak area, percent of total carbon. | 7 | 16 | 42 | 6 | 12 | 17 | 71 | 29 | 0.42 |
| Nuclear-Overhauser-enhancement factor, η. | 0.11 | 0.14 | -- | 0.19[c] | -- | 0.48 | 0.66 | | |

[a] In the previous report of these samples (Thorn, 1987), the spectra were integrated from 220-185 ppm, 185-165 ppm, 165-90 ppm, 90-60 ppm, and 60-0 ppm. The different subdivisions of the spectra account for the slight discrepancies in peak areas between these two reports.

[b] $f_a$ = spectrum area from 160-105 ppm divided by total spectrum area.

[c] Values represent η for peaks from 160-90 ppm.

Aldehydes, then, do not appear to be significant functional groups in these fulvic and humic acids. The general trend in $^{13}$C-NMR chemical shifts of ketones is that diaryl ketones are upfield of alkyl-aryl ketones that, in turn, are upfield of dialkyl ketones. The fact that the ketone peaks in both the fulvic and humic acids encompass the range from approximately 180 to 220 ppm suggests that diaryl, alkyl-aryl, and dialkyl ketones all are present in these samples.

A point worth mentioning here is that the $^{13}$C-NMR chemical shifts of carboxylic-acid carbonyls are solvent dependent and are a function of the polarity and H-bonding capacity of the solvent. As an example, consider the chemical shifts of these carboxylic acids in DMSO-$d_6$ and $CDCl_3$:

| | Chemical shift, in ppm | |
|---|---|---|
| | DMSO-$d_6$ | $CDCl_3$ |
| Phenylacetic acid---------- | 172.6 | 178.4 |
| Benzoic acid---------- | 167.4 | 172.7 |
| Valeric acid---------- | 174.8 | 180.5 |

The carbonyls are shifted downfield approximately 5 ppm in $CDCl_3$ compared with DMSO-$d_6$. In aqueous solution, the chemical shift of carboxylate carbons would depend on the pH of the sample (see discussion of pH effects that follows). When making assignments in the carboxyl region of $^{13}$C-NMR spectra of humic materials, the solvent system is an important consideration.

Referring back to table 2, if the spectral region from 90 to 160 ppm is considered to be aromatic carbon, the proportion of aromatic carbons in both the fulvic and humic acids is high—33 percent in fulvic and 48 percent in humic acid. In the fulvic acid, there is approximately one aromatic carbon for each aliphatic carbon. Another way to look at this is to consider that the spectral region from 0 to 90 ppm represents $sp^3$-hybridized carbons, and the region from 90 to 220 ppm represents $sp^2$-hybridized carbons. Thus, $sp^2$-hybridized carbons constitute 58 percent and 71 percent of the carbons in the fulvic and humic acids, respectively; $sp^3$-hybridized carbons constitute 42 percent and 29 percent of the fulvic and humic acids, respectively. To convey the quantitative data in the more conventional manner, the aromaticities, $f_a$'s, can be calculated for the samples. The aromaticity is defined here as the spectrum area from 105 to 160 ppm divided by the total spectrum area. The $f_a$ is 0.28 for the fulvic acid and 0.42 for the humic acid. The aromaticity takes into consideration the possibility of overlap among aromatic carbons, acetal carbons, and the anomeric carbons of carbohydrates in the region from 90 to 105 ppm.

The carbon distributions presented in table 2 must be considered with two related caveats in mind. First, in any spectrum of a complex mixture, resonances of

**Table 3.** Carbon-13 nuclear-magnetic-resonance chemical shifts in dimethylsulfoxide-$d_6$ of carbonyl and phenolic carbons of model compounds

| Ketone | Chemical shift (ppm) |
|---|---|
| 4-Heptanone | 209.6 |
| Methyl ethyl ketone | 208.0 |
| Levulinic acid | 207.3 |
| Butyl levulinate | 205.5 |
| Phenyl-2-propanone | 205.4 |
| 2, 2', 4, 4'-tetrahydroxybenzophenone | 199.3 |
| 3-penten-2-one | 197.3 |
| Acetophenone | 197.2 |
| 2, 4, 4'-trihydroxy benzophenone | 197.2 |
| Benzophenone | 195.6 |
| Benzophenone tetracarboxylic acid anhydride | 192.6 |
| Chalcone | 189.2 |
| Quercetin | 176.0 |

| Aldehyde | Chemical shift (ppm) |
|---|---|
| Butyraldehyde | 202.4 |
| Salicylaldehyde | 193.5 |
| Benzaldehyde | 192.8 |

| Quinones | Chemical shift (ppm) |
|---|---|
| Benzoquinone | 187.6 |
| Naphthoquinone | 184.6 |
| 1-hydroxyanthraquinone | 181.8, 188.1 |
| 2-OH-1, 4-naphthoquinone | 181.2, 184.5 |
| 2-amino-anthraquinone | 180.1, 183.4 |
| 1,2-naphthoquinone | 178.8, 180.0 |

| Carboxylic acid | Chemical shift (ppm) |
|---|---|
| Heptanoic | 174.5 |
| Levulinic | 174.1 |
| Trans-3-hexenoic | 173.4 |
| 1-hydroxy-2-naphthoic | 173.4 |
| 2,3-dihydroxybenzoic | 172.7 |
| Phenylacetic | 172.6 |
| 2,6-dihydroxybenzoic | 172.4 |
| Salicylic | 172.2 |
| 2,4-dihydroxybenzoic | 172.1 |
| 2,5-dihydroxybenzoic | 172.0 |
| Galacturonic | 170.8 |
| Phthalic | 169.0 |
| Gallic | 168.0 |

different types of carbons overlap, and the chemical-shift position where spectra are subdivided is, by necessity, somewhat subjective. Second, because the spectra are comprised of broad, overlapping resonances, it is impossible to know what the true line shapes of the major resonances are, and so the integrations are performed by simply dropping a line from the spectrum to the baseline.

Another note of caution in the interpretation of the peak areas of the spectra listed in table 2 is that

**Table 3.** Carbon-13 nuclear-magnetic-resonance chemical shifts in dimethylsulfoxide-$d_6$ of carbonyl and phenolic carbons of model compounds—Continued

| Carboxylic acid--Continued | Chemical shift (ppm) |
|---|---|
| 3,4-dihydroxybenzoic | 167.7 |
| 4-hydroxybenzoic | 167.7 |
| Crotonic | 167.5 |
| Benzoic | 167.4 |
| 3-furoic | 164.0 |
| 2-furanoic | 159.6 |

| Ester | Chemical shift (ppm) |
|---|---|
| Allyl butyrate | 171.9 |
| Ethyl butyrate | 171.8 |
| Butyl levulinate | 171.7 |
| Ethyl phenylacetate | 170.8 |
| Benzyl acetate | 170.0 |
| Ethyl salicylate | 169.3 |
| Resorcinol monoacetate | 169.2 |
| Phenyl acetate | 168.5 |
| Tannic acid | 168.0 |
| Phenyl salicylate | 167.1 |
| Ethyl crotonate | 165.3 |
| Ethyl p-hydroxybenzoate | 165.6 |
| Benzyl benzoate | 165.3 |
| Phenyl benzoate | 164.5 |

| Amides | Chemical shift (ppm) |
|---|---|
| Acetamide | 173.2 |
| Benzamide | 168.5 |
| Benzanilide | 165.6 |

| Lactones | Chemical shift (ppm) |
|---|---|
| γ - Butyrolactone | 177.9 |
| ε - Caprolactone | 175.6 |
| Coumarin | 159.9 |

| Phenolic carbons | Ring position | Chemical shift (ppm) |
|---|---|---|
| Gallic acid | $C_3$, $C_5$ | 145.8 |
| | $C_4$ | 138.4 |
| 4-hydroxybenzoic acid | $C_4$ | 162.0 |
| 2,4-dihydroxybenzoic acid | $C_2$,$C_4$ | 163.6, 164.2 |

resonances with very short spin-spin relaxation times, $T_2$'s, may not be observed in the spectra. Carbon nuclei in close proximity to paramagnetic metal ions (for example, $Fe^{3+}$) or stable organic free radicals can have short $T_2$'s and, therefore, may have line widths that are too broad to be observed.

In principle, spin counting experiments could be performed to see if this is a problem in Suwannee River samples. Taylor and Garbarino (chap. E, this volume) have measured the concentration of trace metals in the fulvic and humic acids. The concentrations of paramagnetic metals in the fulvic acid are

**Table 3.** Carbon-13 nuclear-magnetic-resonance chemical shifts in dimethylsulfoxide-$d_6$ of carbonyl and phenolic carbons of model compounds—Continued

| Phenolic carbons--Continued | Ring position | Chemical shift (ppm) |
|---|---|---|
| 3,4-dihydroxybenzoic acid--------- | $C_3$ | 145.1 |
| | $C_4$ | 150.3 |
| Quercetin------------------------- | $C_3$ | 135.9 |
| | $C_5$ | 156.3 |
| | $C_7$ | 164.1 |
| | $C_3'$ | 145.2 |
| | $C_4'$ | 147.9 |
| 1-hydroxy-2-naphthoic acid-------- | $C_1$ | 160.8 |
| 2,3-dihydroxybenzoic------------- | $C_2$ | 150.7 |
| | $C_3$ | 146.1 |
| 3,5-dihydroxybenzoic acid--------- | $C_3, C_6$ | 160.8 |
| 2,5-dihydroxybenzoic acid--------- | $C_2$ | 154.5 |
| | $C_5$ | 149.7 |
| 1-hydroxyanthraquinone----------- | $C_1$ | 161.5 |

low and are most likely not a problem. The concentration of iron in the humic acid, however, (estimated at 440 μg/g) might warrant concern. Saleh and others (chap. G, this volume) determined that Suwannee River fulvic and humic acids are comparable to most other humic samples reported in the literature with respect to concentrations of organic-free radicals in spins per gram.

The NOE factors for the major spectral regions of the $^{13}$C-NMR spectra of the fulvic and humic acids are listed in table 2. They are all less than the theoretical maximum of 2.0. The maximum NOE factors in the humic samples ($\eta$ = 0.90, 0.66) are for the aliphatic I carbons (0 to 90 ppm)—these values decrease downfield with the aromatic carbons (90 to 160 ppm), carboxyl carbons (160 to 180 ppm), and ketone carbons (180 to 220 ppm). This trend is to be expected, given that the aliphatic I peak consists of methyl, methylene, and methine carbons (APT spectra); the peak is, therefore, comprised of the most highly protonated carbons, whereas the aliphatic II peak consists mainly of methine carbons; the degree of protonation subsequently decreases with aromatic and carbonyl carbons. The NOE factors presented here match fairly closely the range of values reported in previous studies with soil humic samples by Newman and others (1980), Wilson and others (1981), Newman and Tate (1984), and Preston and Blackwell (1985). However, the NOE factor of the carboxyl peak in the fulvic acid, $\eta$ = 0.35, is greater than most $\eta$'s reported for carboxyl groups in humic substances.

The reduction of the NOE values below the theoretical maximum of 2.0 can be effected both by slow molecular motion in solution (outside the extreme narrowing limit) and by the presence of relaxation mechanisms other than dipolar relaxation. The extreme narrowing condition normally is met for samples of molecular weight under 1,000 in nonviscous solutions (Abraham and Loftus, 1980). Interestingly, the number-average molecular weight of the fulvic acid reported by Aiken and others (chap. J, this volume), 800 daltons, would imply that the fulvic-acid molecules meet the extreme narrowing condition. Measurements of the NOE factors at different field strengths could provide further insight into mechanisms leading to the reduction of the NOE below the theoretical maxima in these humic samples.

## Nonquantitative Carbon-13 Nuclear-Magnetic-Resonance Spectra

Spectra of humic substances acquired with continuous decoupling and short pulse delays will exhibit superior resolution and signal-to-noise compared to spectra acquired with inverse-gated decoupling and longer pulse delays. Also, discrete, sharp-line resonances, with short $T_1$'s or large NOE's, may be suppressed in spectra acquired under quantitative conditions. It is useful, therefore, to record $^{13}$C-NMR spectra under conditions that maximize resolution and signal-to-noise to obtain qualitative information not readily discernible in the quantitative spectra. Carbon-13 NMR spectra of the fulvic and humic acids acquired with 45° pulse angles, 1.0-s pulse

delays (total of 1.2 s between pulses), and continuous decoupling are shown in figures 5 and 6.

Several discreet resonances are present in the fulvic- and humic-acid spectra. In the fulvic acid, these occur at 133.2, 130.9, 128.8, and 127.9 ppm in the aromatic region and at 61.4, 28.9, and 14.2 ppm in the aliphatic region. These same seven resonances also are present in the humic acid. These peaks correlate with the chemical shifts of dialkyl phthalates (assuming the ester carbonyl of the dialkyl phthalates is overlapped by the carboxyl carbons of the humic samples) and indicate that trace amounts of dialkyl phthalates may be present in the fulvic- and humic-acid samples. Schnitzer and Kahn (1972) reported the isolation of dialkyl phthalates from soil fulvic and humic acids. The origin of these materials in the Suwannee River samples is uncertain.

Additional discreet peaks in the humic acid also occur at 164.6, 163.2, and 161.0 ppm (one of which probably represents carbonate) and at 79.5, 79.0, 78.6, 76.9, 70.3, and 67.8 ppm. These latter peaks remain unidentified. The humic acid has a prominent signal at 56 ppm that the APT experiment will demonstrate to be *methoxyl* carbon. The fulvic acid has a less pronounced methoxyl signal at 55.9 ppm.

## Attached-Proton-Test Carbon-13 Nuclear-Magnetic-Resonance Spectra

Several multiplicity sorting experiments for solution-state NMR have been introduced in recent years. These include: (1) the attached proton test (APT; Patt and Shoolery, 1982) that is alternatively referred to as J-modulated spin echo Fourier transform, J-SEFT, or gated spin echo, GASPE, and (2) distortionless enhancement by polarization transfer (DEPT; Doddrell and others, 1982). Preston and Blackwell (1985) have reported J-SEFT spectra for soil humic materials. The APT experiment is applied here to Suwannee River fulvic and humic acids.

The APT sequence is a spin echo, with the decoupler gated off during one of the tau delays ($\tau$, which are multiples of 1/J milliseconds, where J is the carbon-proton one-bond coupling constant in hertz):

$$(\text{PD–PW–}\tau\text{ (decoupler off)–}180^\circ\text{–}(\tau + \Delta)\text{–}180^\circ\text{–}\Delta\text{–acquire})_n$$

where

PD is the pulse delay between the spin-echo sequences, in seconds,

PW is the normal observe pulse, and

$\Delta$ is the short delay, in milliseconds.

In the experiments performed on the humic samples, PD, PW, and $\Delta$ were 0.0 s, 45°, and 1.0 ms, respectively. The total time between collection of free induction decays (FID's) was less than 0.25 s, so that intensities of nuclei with shorter $T_1$'s are enhanced relative to those with longer $T_1$'s. The NOE also is retained in this pulse sequence.

The signal intensities of the carbon nuclei as a function of the delay are governed by the following equations (Patt and Shoolery, 1982):

$$I_{CH} : \cos(J\tau\pi)$$

$$I_{CH_2} : 0.5 + 0.5\cos(2J\tau\pi)$$

$$I_{CH_3} : 0.25\cos(3J\tau\pi) + 0.75\cos(J\tau\pi)$$

When $\tau = 1/J$, quaternary and methylene carbons are positive, and methyl and methine carbons are negative. At $\tau = 1/(2J)$, all carbons except quaternaries are canceled. At $\tau = 2/(3J)$, methyl carbons are reduced to approximately one-fourth the intensity of methine carbons. The average coupling constants for aromatic C-H and aliphatic C-H are 160 Hz and 125 Hz, respectively.

To distinguish among quaternary carbons, protonated and nonprotonated aromatic carbons, and methine, methylene, and methyl carbons, several spectra were run at various values for $\tau$ and J for both the fulvic and humic acid (figs. 7 and 8):

In the spectra in figures 7*A* and 8*A*, $\tau$ equals 1/J, but continuous decoupling is employed so that all carbon peaks are refocused with the same phase. The spectra in figures 7*A* and 8*A* are commonly referred to as the double-spin-echo spectra and demonstrate the limits of quantitation under the acquisition parameters used with these samples. Additionally, the spin-echo-spectra sequence discriminates against broad resonance components with short $T_2$'s. It is useful to compare the double-spin-echo spectra with normal broadband-decoupled spectra to check if any sharp-line resonances are concealed beneath broad components.

The spectra in figures 7*B* and 8*B* should negatively invert protonated aromatic, methine, and methyl carbons while quaternary, nonprotonated aromatic, and methylene carbons remain positive. Because J equals 160 Hz, the protonated aromatic

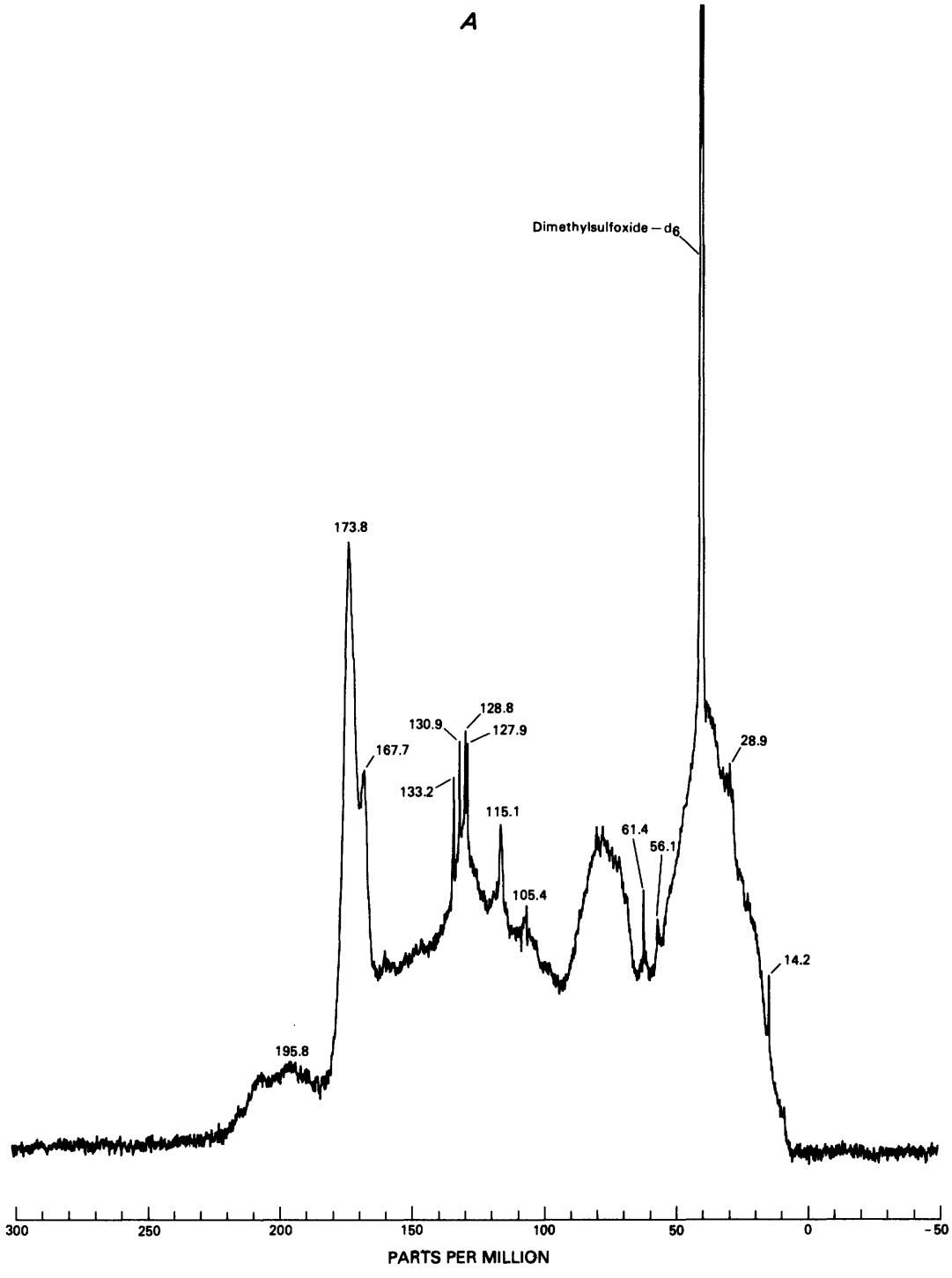
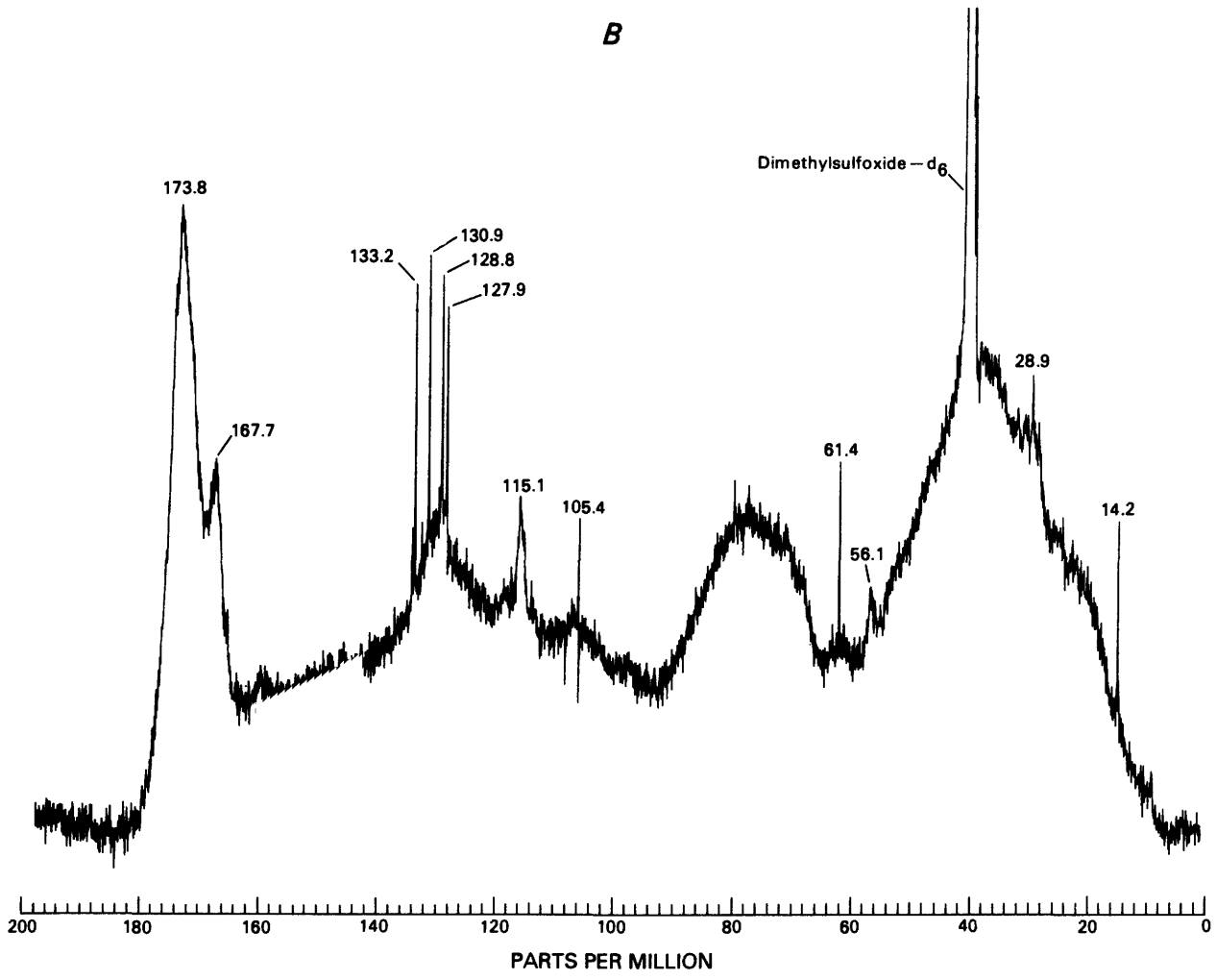



**Figure 5** (above and facing page). Nonquantitative carbon-13 nuclear-magnetic-resonance spectrum of fulvic acid. "Parts per million" label on x-axis refers to chemical shift in parts per million. Concentration = 200 milligrams in 2.0 grams of dimethylsulfoxide-$d_6$, carbon-13 depleted; spectral window = 30,000 hertz; pulse width = 45°; acquisition time = 0.2 second; pulse delay = 1.0 second; decoupling mode = continuous WALTZ decoupling; number of transients = 66,000. *A*, Full-scale plot, line broadening = 10.0 hertz; *B*, Horizontal expansion, line broadening = 1.0 hertz.




carbons should have full intensity in the negative direction while the aliphatic methine and methyl carbons have less than full intensity. The spectra in figures 7*C* and 8*C* also should negatively invert the protonated aromatic, methine, and methyl carbons while quaternary, nonprotonated aromatic, and methylene carbons remain positive. However, since J now equals 125 Hz, complete inversion occurs for the aliphatic methyl and methine carbons, while the protonated aromatic carbons are less than fully inverted. The spectra in figures 7*D* and 8*D* show only quaternary carbons and therefore allow one to distinguish between methylene and quaternary carbons among the positive peaks in the 1/J spectra (in figs. 7*B*, 7*C* and 8*B*, 8*C*). The spectra in figures 7*E* and 8*E* allow one to distinguish between methyl and methine carbons because the methyl carbons are reduced to approximately one-fourth the intensity of the methine carbons.

There are several important observations to be made in the APT spectra of the fulvic acid (fig. 7). Comparison of the double-spin-echo spectrum (fig. 7*A*) with the inverse-gated decoupled spectrum in figure 3 indicates, as expected, that the spectrum in figure 7*A* does not represent a quantitative distribution of carbons because of differential saturation and differential NOE effects. Also, no sharp resonances are apparent in figure 7*A* that are not already discernible in the normal broadband-decoupled spectrum in figure 5.

The spectrum in figure 7*B* clearly shows that the aromatic carbons from approximately 105 to 135 ppm are protonated and the aromatic carbons from approximately 135 to 162 ppm are nonprotonated. The carboxyl peak remains positive, and there is a negatively inverted peak at 106 ppm that could correspond to both protonated aromatic carbons or the anomeric carbons of carbohydrates. Discreet protonated

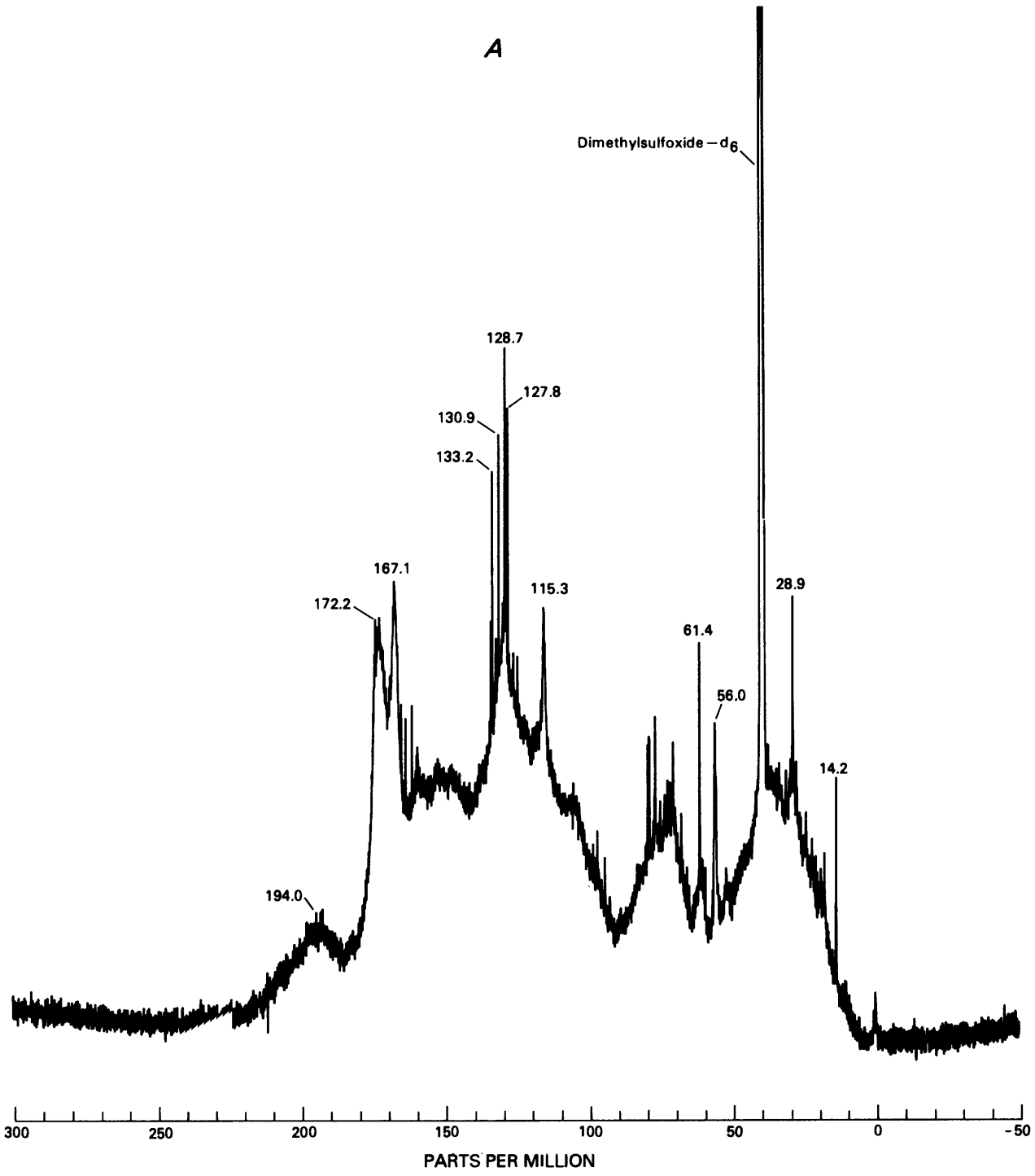
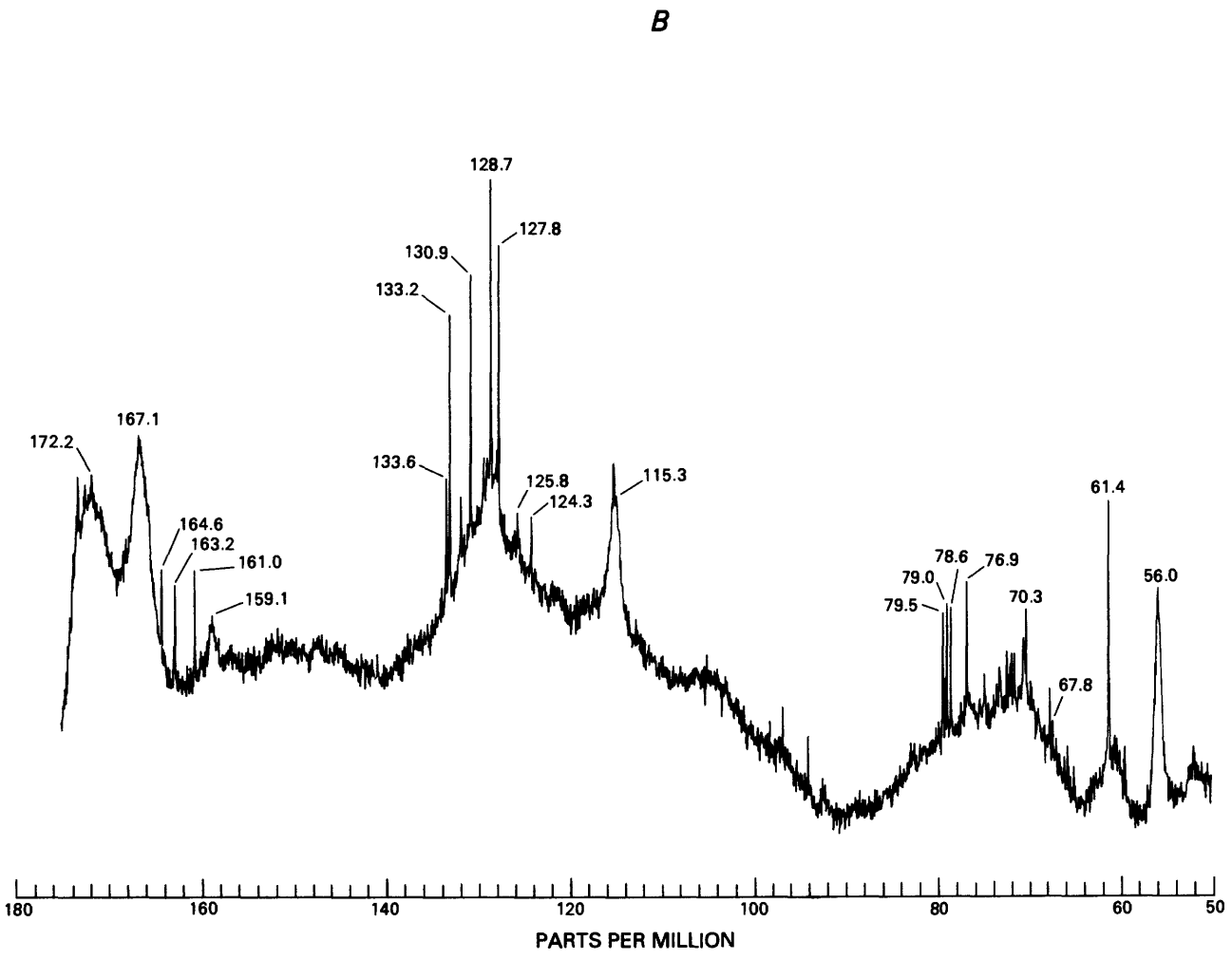



**Figure 6** (above and facing page). Nonquantitative carbon-13 nuclear-magnetic-resonance spectrum of humic acid. "Parts per million" label on x-axis refers to chemical shift in parts per million. Concentration = 300 milligrams in 2.0 grams of dimethylsulfoxide-$d_6$, carbon-13 depleted; spectral window = 30,000 hertz; pulse width = 45°; acquisition time = 0.2 second; pulse delay = 1.0 second; decoupling mode = continuous WALTZ decoupling; number of transients = 260,000. *A*, Full-scale plot, line broadening = 1.0 hertz; *B*, Horizontal expansion, line broadening = 1.0 hertz.

aromatic resonances occur at 115 and 129 ppm. The nonprotonated aromatic carbons from 135 to 162 ppm should be comprised in part of phenolic carbons. Aromatic carbons substituted with other substituents and aromatic carbons in ring junctures also may contribute to the peak from 135 to 162 ppm.




In figure 7*C*, the aliphatic I peak is resolved into three separate resonances (centered at 20, 35, and 50 ppm) corresponding to methyl carbons, methylene and (or) quaternary carbons, and methine and (or) methoxyl carbons, respectively. The aliphatic II peak is completely inverted downwards, indicating methine carbons. This is in agreement with the common interpretation of the aliphatic II peak as being comprised, in part, of carbohydrate carbons. Methine carbons of secondary alcohols and secondary ethers also would contribute to this peak. In fact, these latter types of methine carbons probably are the major contributors to the aliphatic II peak—carbohydrate analyses of aquatic fulvic acids, including the Suwannee River fulvic acid, have shown that carbohydrates comprise less than 2 to 4 percent of the carbon of fulvic acids (Thurman, 1985). The data in table 2 indicate that the aliphatic II peak represents approximately 15 percent of the fulvic-acid carbon. If the carbohydrate analyses are accurate, the carbohydrate content of Suwannee River fulvic acid is too low to account for the total area of the aliphatic II peak. Interestingly, the complete negative inversion of the aliphatic II peak excludes methylene carbons involved in ether linkages and primary alcohols as significant moieties in the fulvic acid.

In practice, instead of recording the 1/J spectra at two different values of J, a single 1/J spectrum would be acquired using a value of J that is the average between the aromatic and aliphatic C-H coupling constants, $J_{CH}$ = 142 Hz, or $\tau$ = 7.0 ms. However, figures 7*B* and 7*C* nicely illustrate the effect of the tau delay ($\tau$) on signal intensities in the aromatic and aliphatic regions.

Figure 7*D* should exhibit only quaternary carbons. The nonprotonated aromatic carbons and the carbonyl carbons are present; there is incomplete cancellation of the protonated aromatic carbons and the aliphatic II peak. The most interesting feature of this spectrum is that there is no indication of quaternary

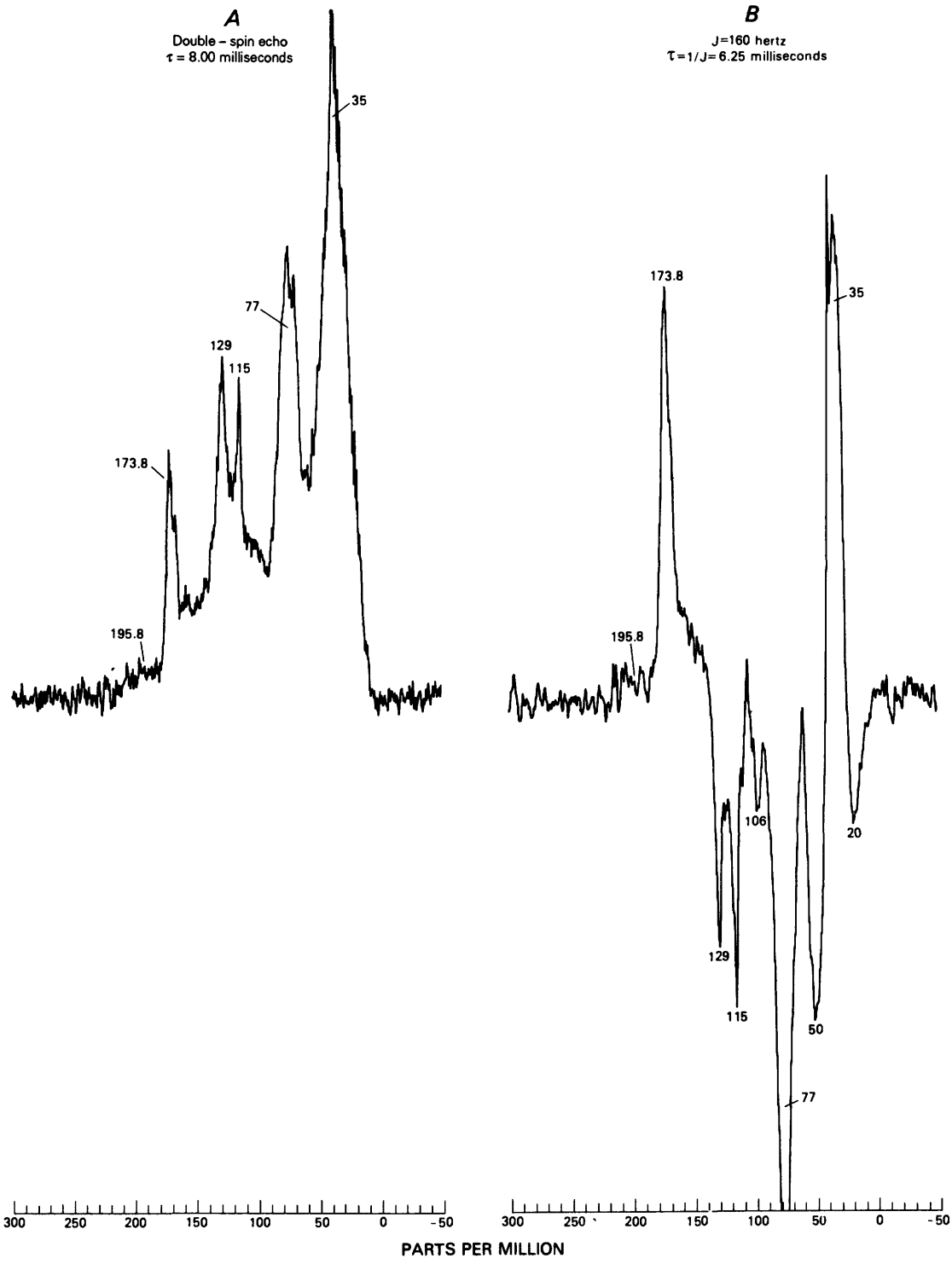
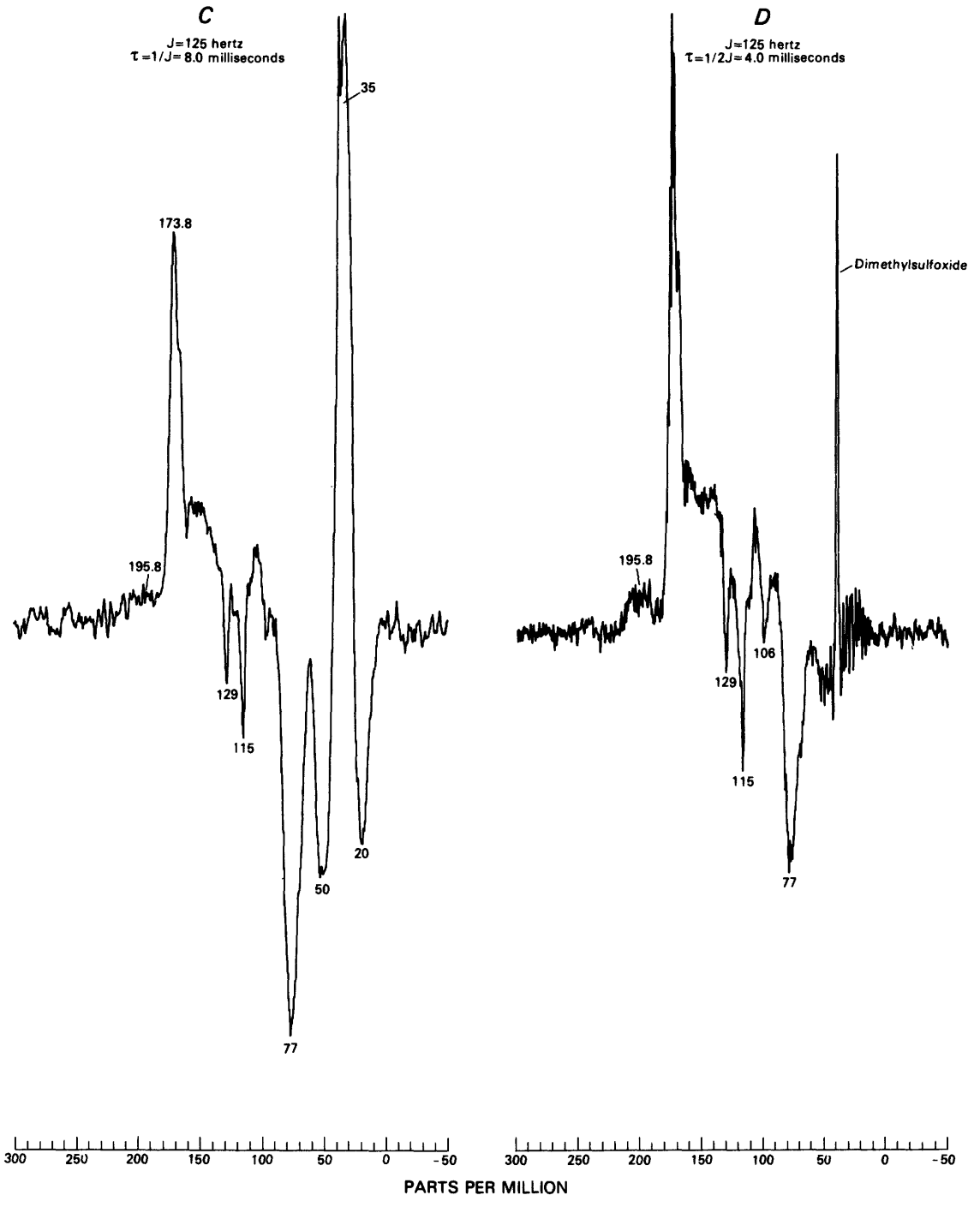
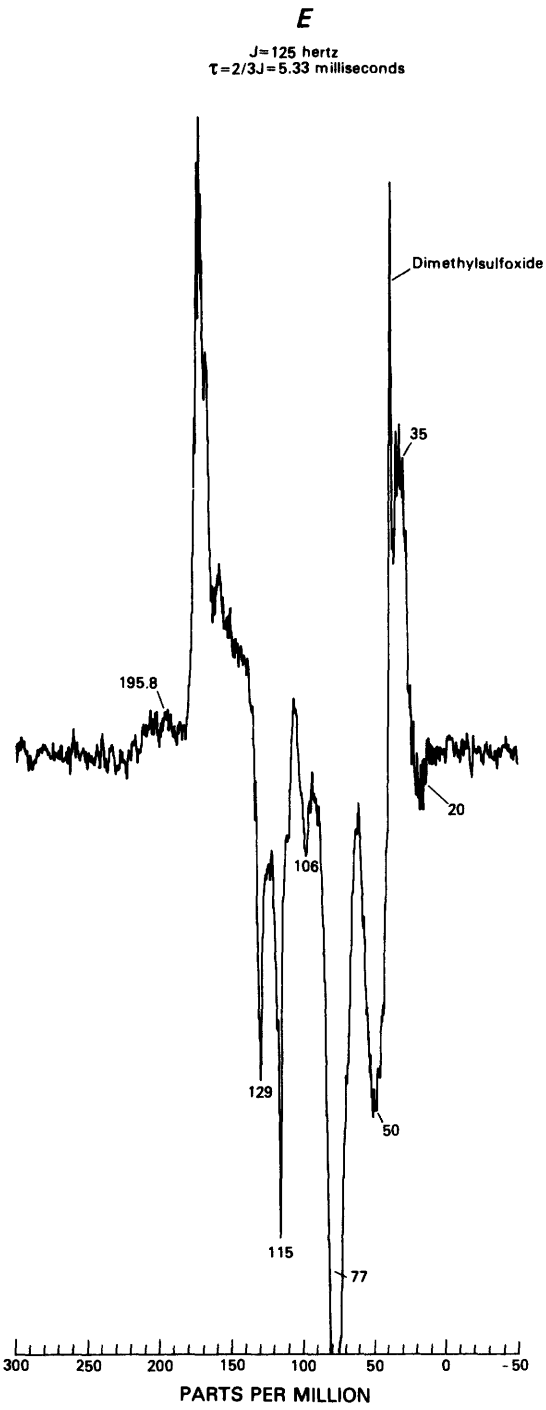



**Figure 7** (above and on following pages). Attached-proton-test carbon-13 nuclear-magnetic-resonance spectra of fulvic acid. "Parts per million" label on x-axis refers to chemical shift in parts per million. Concentration = 200 milligrams in 2.0 grams of dimethylsulfoxide-$d_6$, carbon-13 depleted. Spectral window = 30,000 hertz; pulse width = 45°; acquisition time = 0.2 second; pulse delay = 0.0 second; line broadening = 20.0 hertz. *A*, $\tau$=1/J, J=125 Hz (double-spin echo); *B*, $\tau$=1/J, J=160 Hz; *C*, $\tau$=1/J, J=125 Hz; *D*, $\tau$=1/(2J), J=125 Hz; *E*, $\tau$=2/(3J), J=125 Hz.




$sp^3$-hybridized aliphatic carbons in the region from 0 to 90 ppm. The $-CD_3$ peak of the DMSO-$d_6$ behaves as a quaternary carbon. The positive peak from 26 to 43 ppm (centered at 35 ppm in figs. 7*B* and 7*C*) therefore can be assigned as methylene carbon. In figure 7*E*, methyl carbons should be reduced to approximately one-fourth the intensity of methines.

The intensity of the peak that is centered at 20 ppm is reduced, confirming the assignment of this peak as methyl carbon. Significantly, the inverted resonance from 42 to 62 ppm (centered at 50 ppm) is not reduced in intensity, indicating that this peak is comprised predominantly of methine and not methoxyl carbons. Supporting this conclusion, the methoxyl




content of the fulvic acid was determined by the Zeisel method to be 0.4 percent (R.L. Malcolm, written commun., 1986)—this value is far too low to account for the total area of this peak.

The APT spectra of the humic acid exhibit the same details as the spectra of the fulvic acid. There are methyl carbons centered at 19 ppm, methylene carbons centered at 35 ppm, methine carbons centered at 51 and 74 ppm, protonated-aromatic carbons from 105 to 135 ppm, and nonprotonated aromatic carbons from 135 to 160 ppm. There is a negatively inverted peak at 96 ppm in figure 8*B* that is most likely attributable to carbohydrate anomeric carbon. The sharp peak at 56 ppm is reduced in intensity from figure 8*C* to figure 8*E*, confirming the earlier assignment of this peak as methoxyl. The methoxyl content of the humic acid was determined to be 1.02 percent (R.L. Malcolm, written commun., 1986). The area of nonprotonated aromatic carbon in figure 8*B* is substantial. The high phenolic hydroxyl content of Suwannee River humic acid indicated by the $^{13}$C-NMR spectrum of the methylated sample (fig. 10*A*, next section) correlates well with the large peak area of nonprotonated aromatic carbon in figure 8*B*.

In summary, the APT experiments have resolved $^{13}$C-NMR spectra of the fulvic and humic acids into several carbon types: methyl, 0 to 26 ppm; methylene, 26 to 43 ppm; methine, 43 to 62 and 62 to 92 ppm; protonated aromatic/acetal, 92 to 105 ppm; protonated aromatic, 105 to 135 ppm; nonprotonated aromatic, 135 to 162 ppm; and carboxyl/carbonyl/ketone, 162 to 220 ppm. The large proportion of aliphatic methine and methyl carbons with respect to methylene carbons indicates that the aliphatic moieties are predominated by branched-chain, short-chained, and (or) cyclic structures. In the total mixture of molecules constituting the fulvic and humic acids, long, linear alkyl chains are not significant entities. This is reasonable because fulvic and humic acids are refractory in the aquatic environment and are resistant to biodegradation. Long, linear alkyl-chain moieties would be susceptible to attack by microorganisms.

The branched, short-chained, or cyclic nature of the aliphatic components in humic and fulvic acids has been correctly inferred simply from chemical-shift considerations in normal broadband-decoupled $^{13}$C-NMR spectra—they have been correctly inferred in proton spectra by other researchers in previous studies. The APT spectra clearly confirm these observations. However, they also provide information not at all apparent in proton or normal broadband-decoupled $^{13}$C-NMR spectra: namely the relative absence of quaternary sp$^3$-hybridized carbons in the 0 to 90 ppm region and the relative absence of methylene carbons bonded to oxygens in primary alcohol and primary ether structures.

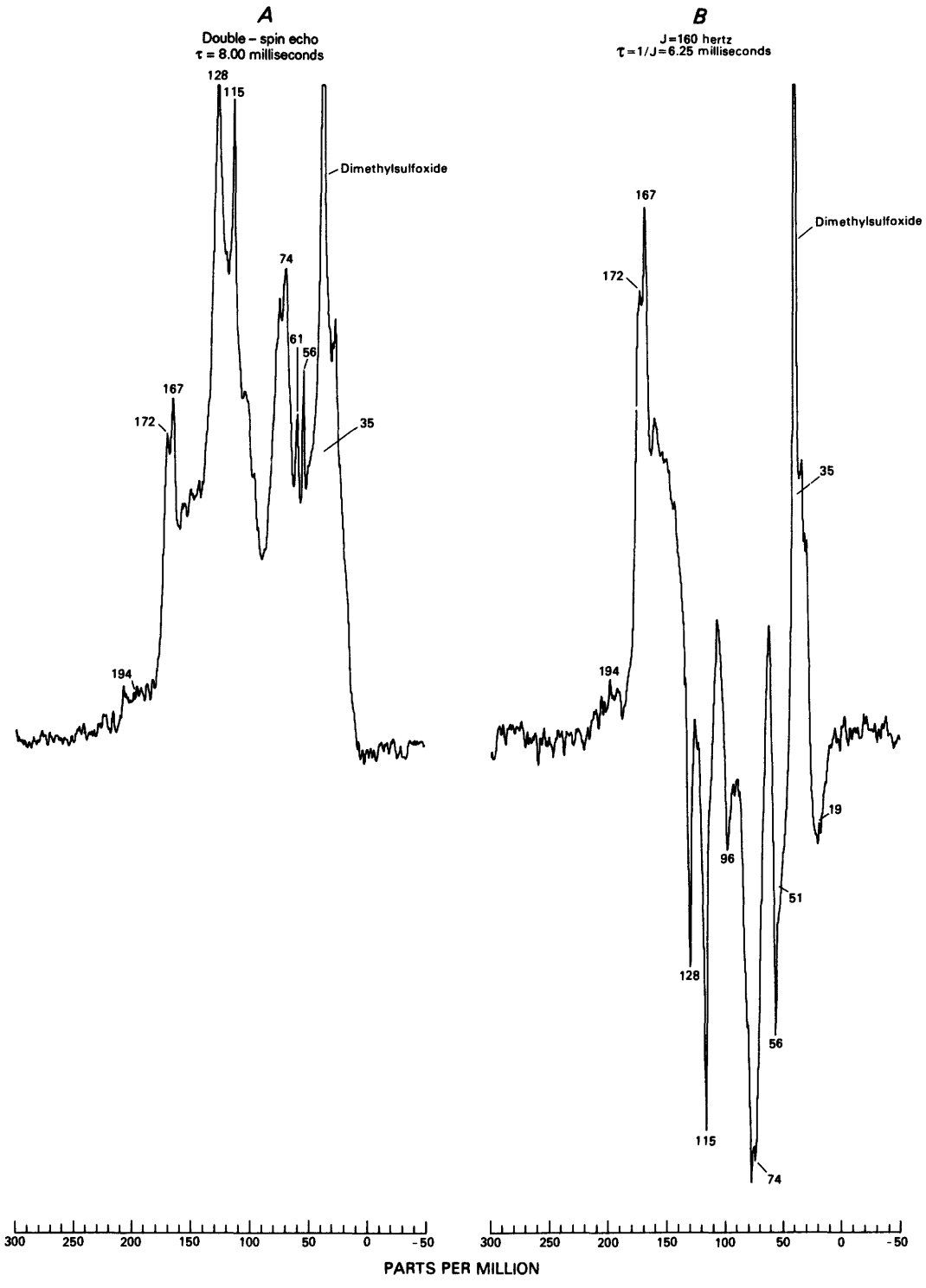
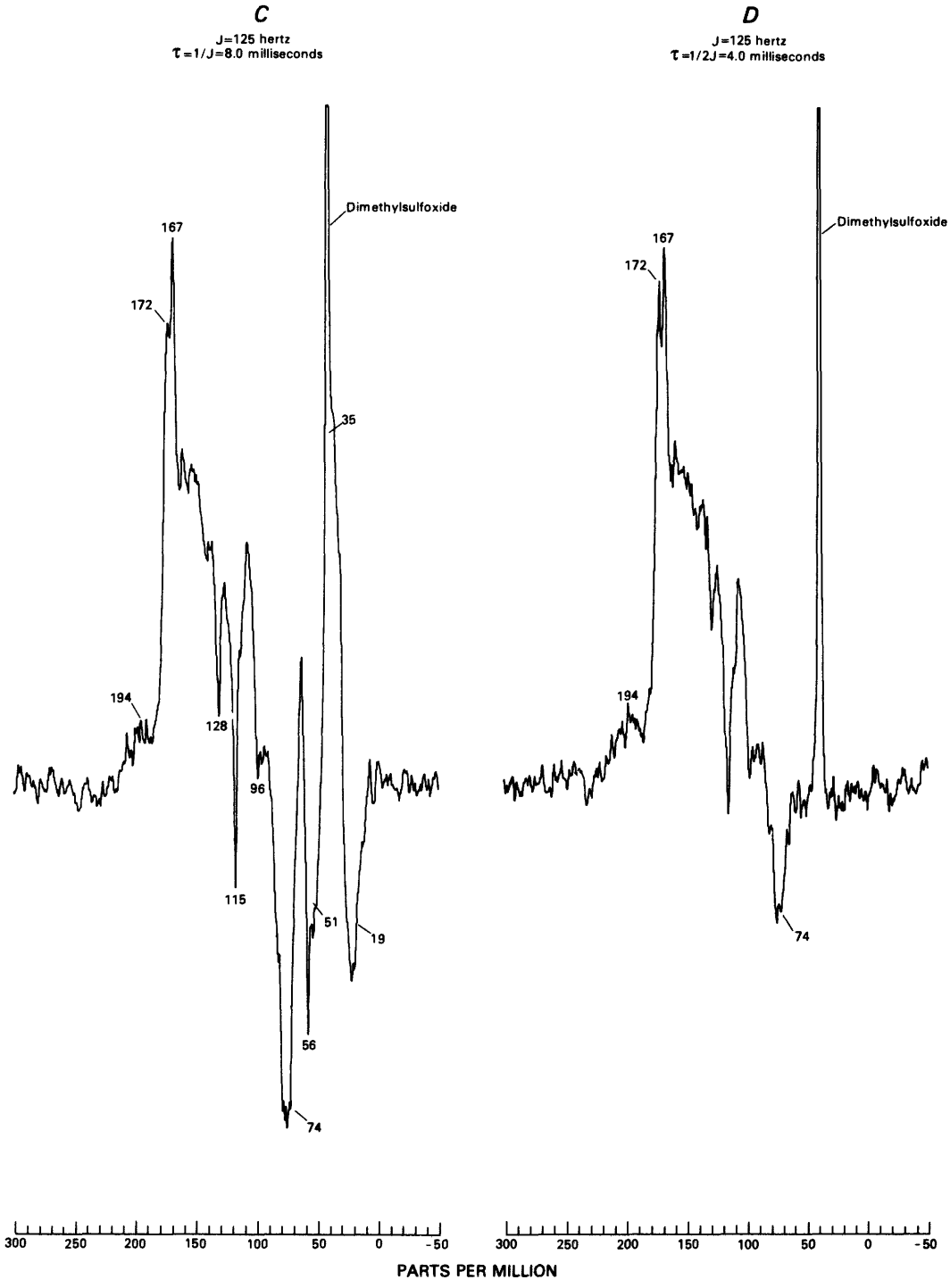
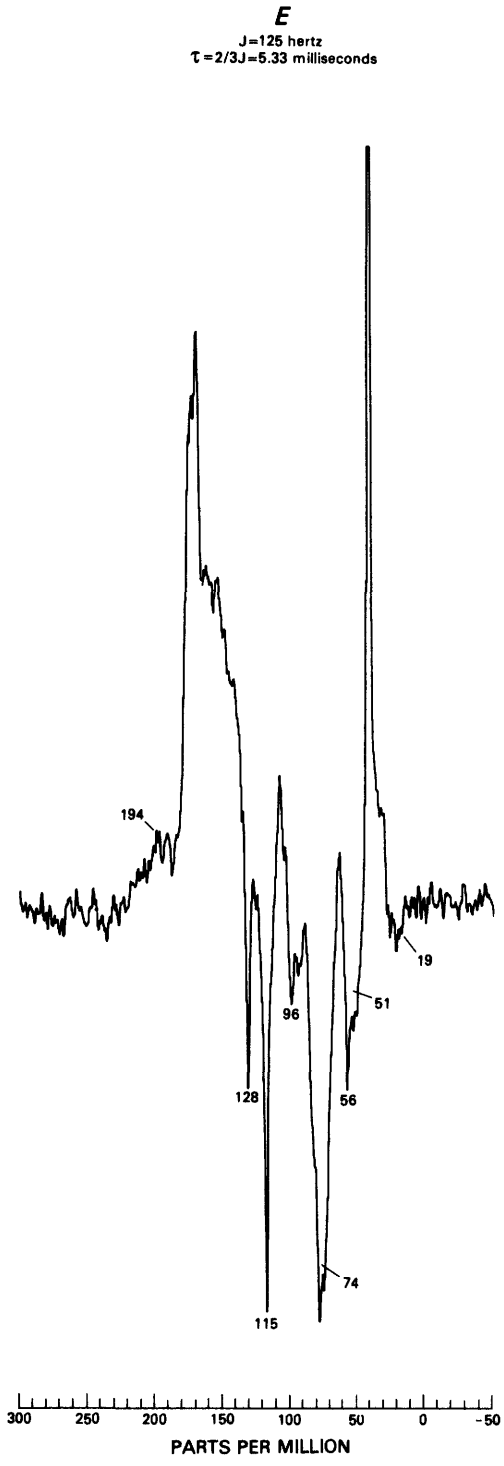



**Figure 8** (above and on following pages). Attached-proton-test carbon-13 nuclear-magnetic-resonance spectra of humic acid. "Parts per million" label on x-axis refers to chemical shift in parts per million. Concentration = 200 milligrams in 2.0 grams of dimethylsulfoxide-$d_6$, carbon-13 depleted. Spectral window = 30,000 hertz; pulse width = 45°; acquisition time = 0.2 second; pulse delay = 0.0 second; line broadening = 100.0 hertz. *A*, $\tau$=1/J, J=125 Hz (double-spin echo); *B*, $\tau$=1/J, J=160 Hz; *C*, $\tau$=1/J, J=125 Hz; *D*, $\tau$=1/(2J), J=125 Hz; *E*, $\tau$=2/(3J), J=125 Hz.




A shortcoming of the APT experiment is its dependence on the magnitude of the one-bond $^{13}C$-$^{1}H$ coupling constant. As a tau delay is employed that is based on an assumed average value for the coupling constant, errors can arise when there are protonated carbon nuclei present with $^{1}J_{CH}$ values significantly different from the average. The DEPT sequence is much less sensitive to a spread in $^{1}J_{CH}$, but it is a more difficult experiment to execute because it requires carefully calibrated 90° proton pulses along the decoupler channel. Preliminary results of the DEPT experiment on Suwannee River fulvic acid




have been obtained, and no discrepancies with the assignments provided by the APT spectra have been found. However, more work needs to be done. A thorough comparison of the analyses of humic substances by various carbon multiplicity sorting experiments, both one and two dimensional, is an important area of research.

## CARBON-13 NUCLEAR-MAGNETIC-RESONANCE SPECTRA OF FULVIC AND HUMIC ACIDS METHYLATED USING CARBON-13-ENRICHED REAGENTS

Concentrations of various hydroxyl groups present in humic substances cannot be directly determined from natural abundance $^{13}$C-NMR spectra of underivatized humic substances because of the problem of overlapping resonances. In natural abundance $^{13}$C-NMR spectra of underivatized humic substances, carboxylic-acid carbons overlap with ester, amide, lactone, and some phenolic carbons (160 to 180 ppm); phenolic carbons overlap with other nonprotonated aromatic carbons (135 to 165 ppm); and carbohydrate and alcohol carbons may overlap with ether carbons (60 to 77 ppm). Information on the hydroxyl group functionality can be obtained by methylating the hydroxyl groups and then directly observing the methyl esters and methyl ethers in the $^{13}$C-NMR spectra.

Spectra of the fulvic and humic acids methylated with $^{13}$C-enriched reagents are presented in figures 9 and 10. The spectra in figures 9*A* and 10*A* represent samples methylated with $^{13}$C-enriched diazomethane only. The spectra in figures 9*B* and 10*B* represent permethylated or "doubly methylated" samples—methylation with $^{13}$C-enriched diazomethane followed by methylation with $^{13}$C-enriched methyl iodide and sodium hydride (NaH).

The analysis of these spectra has been discussed previously by Thorn (1984) and Thorn and others (1987). This approach to examining the hydroxyl-group functionality of humic substances first was described by Mikita and others (1981).

In the spectra of samples methylated with diazomethane alone, the peaks at about 52 ppm represent the methyl esters of carboxylic acids; the peaks at about 56 ppm, the methyl ethers of phenolic and (or) enolic hydroxyls; the peaks at about 61 ppm, the methyl ethers of phenolic hydroxyls adjacent to two substituents, where the ring juncture on a condensed aromatic-ring structure counts as a substituent. Diazomethane, without the use of a catalyst, does not methylate aliphatic or carbohydrate hydroxyls (Black, 1983); the peaks at 56 ppm and 61 ppm cannot be assigned as the methyl ethers of carbohydrate or aliphatic hydroxyls. Examples of phenolic hydroxyls that yield methyl ethers in the 60 to 62 ppm chemical-shift range when methylated are the 4-hydroxyl of gallic acid, the 3-hydroxyl of quercetin, and the hydroxyls of 1-hydroxy-2-naphthoic acid and 2-methyl-1-naphthol:

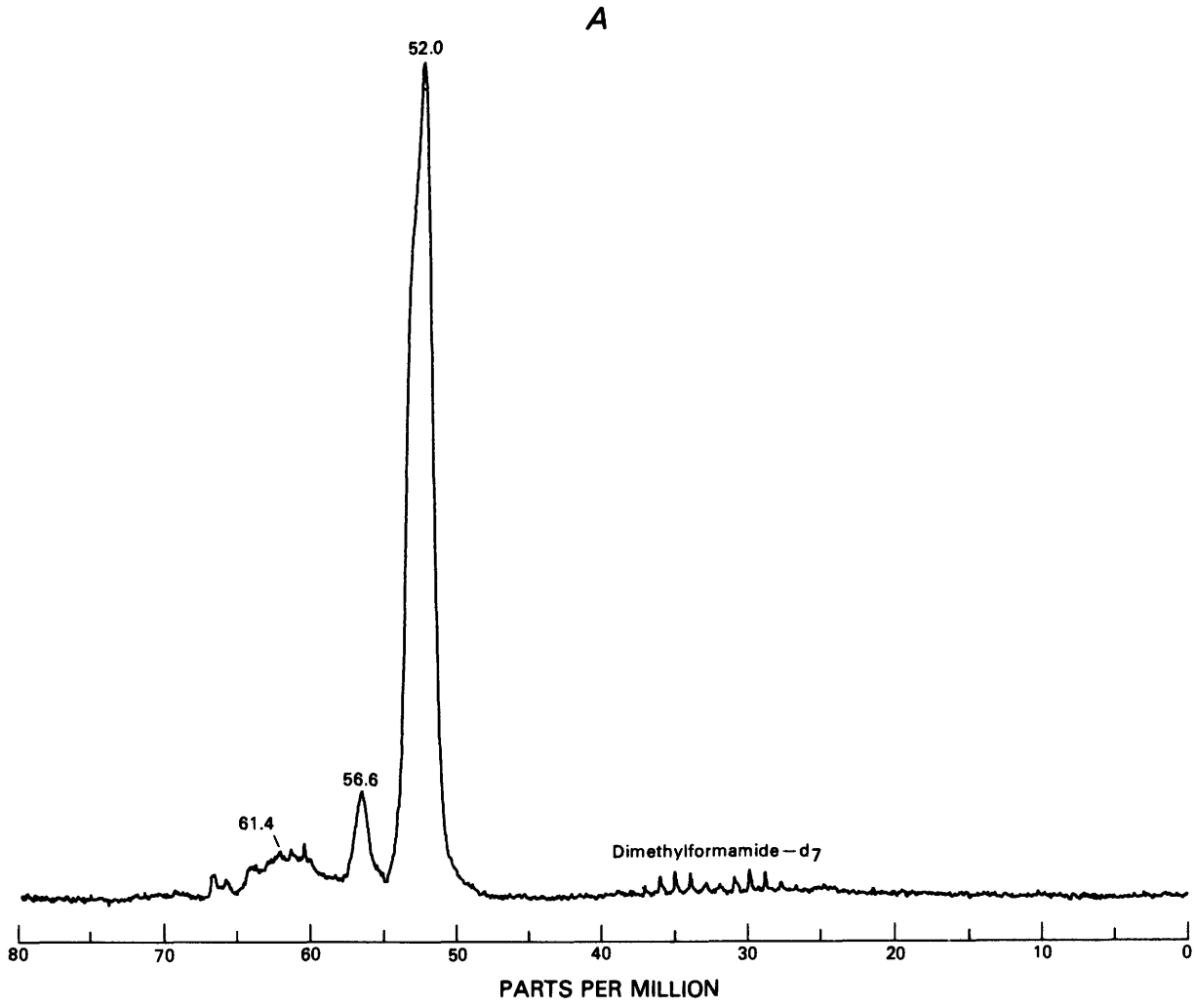
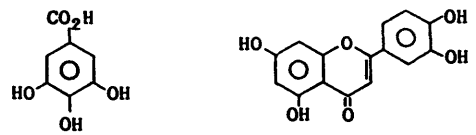


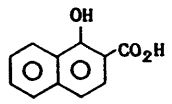

**Figure 9** (above and facing page). Carbon-13 nuclear-magnetic-resonance spectra of fulvic acid methylated using carbon-13-enriched reagents. "Parts per million" label on x-axis refers to chemical shift in parts per million. Spectral window = 5,000 hertz; pulse width = 45°; acquisition time = 1.023 seconds; pulse delay = 0.0 second; decoupling mode = continuous broadband decoupling; sensitivity enhancement = −1.00. *A*, Methylated with carbon-13-enriched diazomethane, concentration ≅ 50 milligrams in 0.5 milliliter of dimethylformamide-$d_7$; *B*, Permethylated with carbon-13-enriched diazomethane followed by carbon-13-enriched methyl iodide and sodium hydride, concentration ≅ 50 milligrams in 0.5 milliliter of deuterated chloroform.



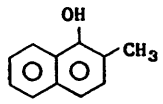

Gallic Acid




Quercetin

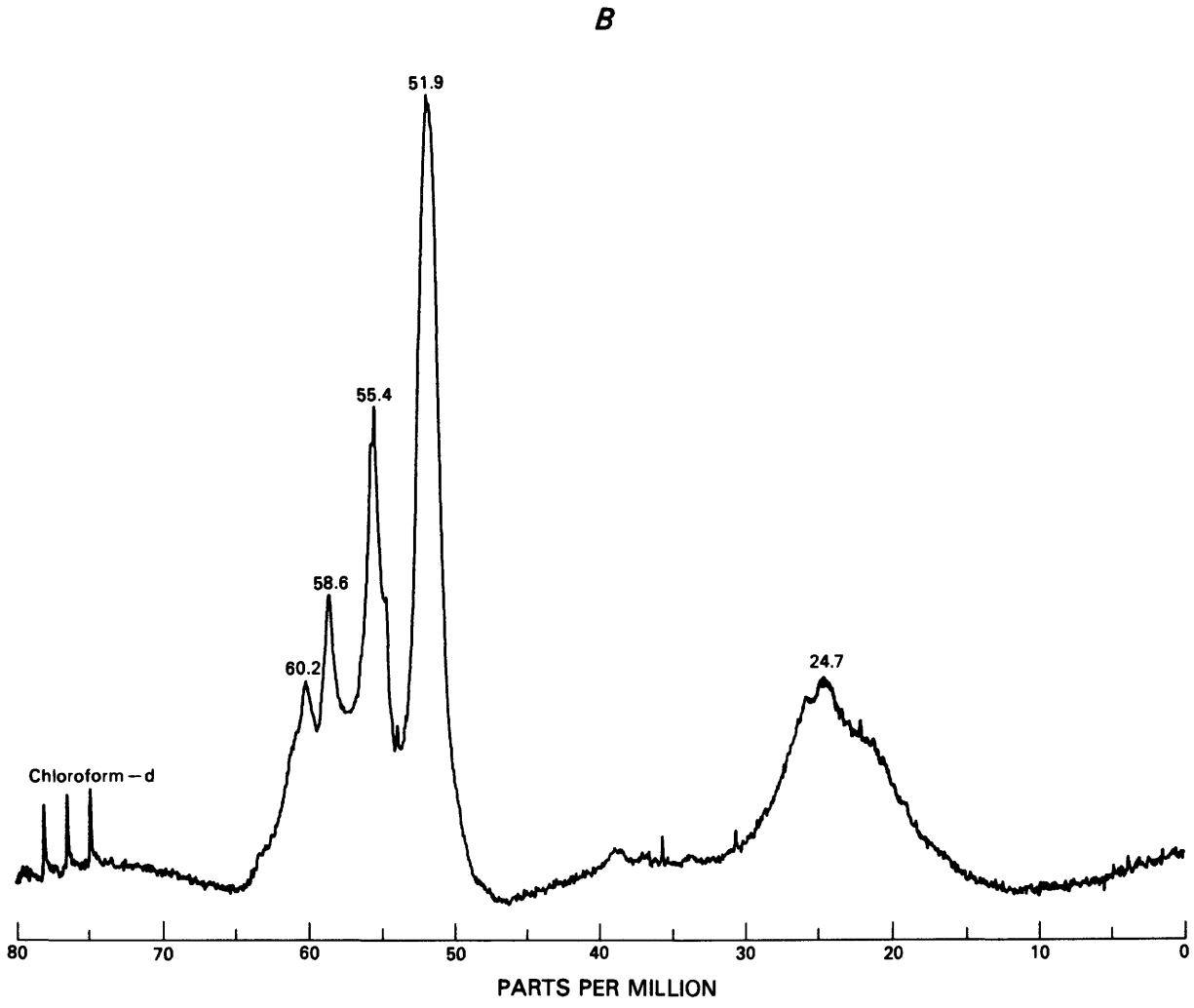



1-hydroxy-2-naphthoic acid




2-methyl-1-napththol

It is interesting to consider the methyl ether peaks centered at about 61 ppm in the spectra of figures 9*A* and 10*A* in relation to the peaks representing nonprotonated aromatic carbons from about 135 to 165 ppm in the APT spectra (figs. 7 and 8). The chemical shifts of the C-4 carbon of gallic acid and the C-3 carbon of quercetin are 139 and 136 ppm, respectively. The fact that the diazomethylated fulvic and humic acids exhibit broad methyl-ether peaks centered at about 61 ppm supports the contention that the nonprotonated aromatic carbons from about 135 to 165 ppm in the APT spectra may be comprised, in part, of phenolic carbons throughout the entire




chemical-shift range of the nonprotonated aromatic-carbon peak.

The presence of phenolic hydroxyls adjacent to two substituents, indicated by the spectra of the diazomethylated samples, suggests that condensed aromatic structures, lignin-derived fragments (originating from sinapyl-alcohol-derived units in lignin), tannin-like, or flavanol-like moieties may occur in the fulvic- and humic-acid samples.

The spectra of the permethylated samples in figures 9*B* and 10*B* exhibit methyl-ester peaks at about 52 ppm and methyl-ether peaks at about 56, 59, and 61 ppm. Since methyl iodide/NaH does derivatize carbohydrate, aliphatic, and weakly acidic phenolic hydroxyls, the interpretation of the peaks from 56 to 61 ppm is now more complicated. The methyl ethers of phenolic, enolic, carbohydrate, and aliphatic hydroxyls overlap with one another in this region. The methyl ethers of aliphatic and carbohydrate hydroxyls range from about 58 to 62 ppm; the methyl ethers of the hydroxyls attached to the anomeric carbons of carbohydrates range from about 54 to 57 ppm. It is quite apparent that a significant fraction of the hydroxyl groups of the fulvic and humic acids are not methylated with diazomethane alone.

The spectra of the methylated samples in figures 9 and 10 were recorded using a 45° pulse angle, 1.023-s acquisition time, no pulse delay, and continuous broadband decoupling. Although these acquisition parameters would not generate quantitative spectra of the naturally abundant $^{13}C$ nuclei in humic substances, they are adequate for quantitative analysis of the methyl esters and ethers in the spectra of the methylated samples because these methoxyl groups have very similar $T_1$'s and NOE's. The integrated-peak areas for the spectra of the diazomethylated and permethylated fulvic and humic acids are presented in table 4. In a previous study (Thorn, 1984),

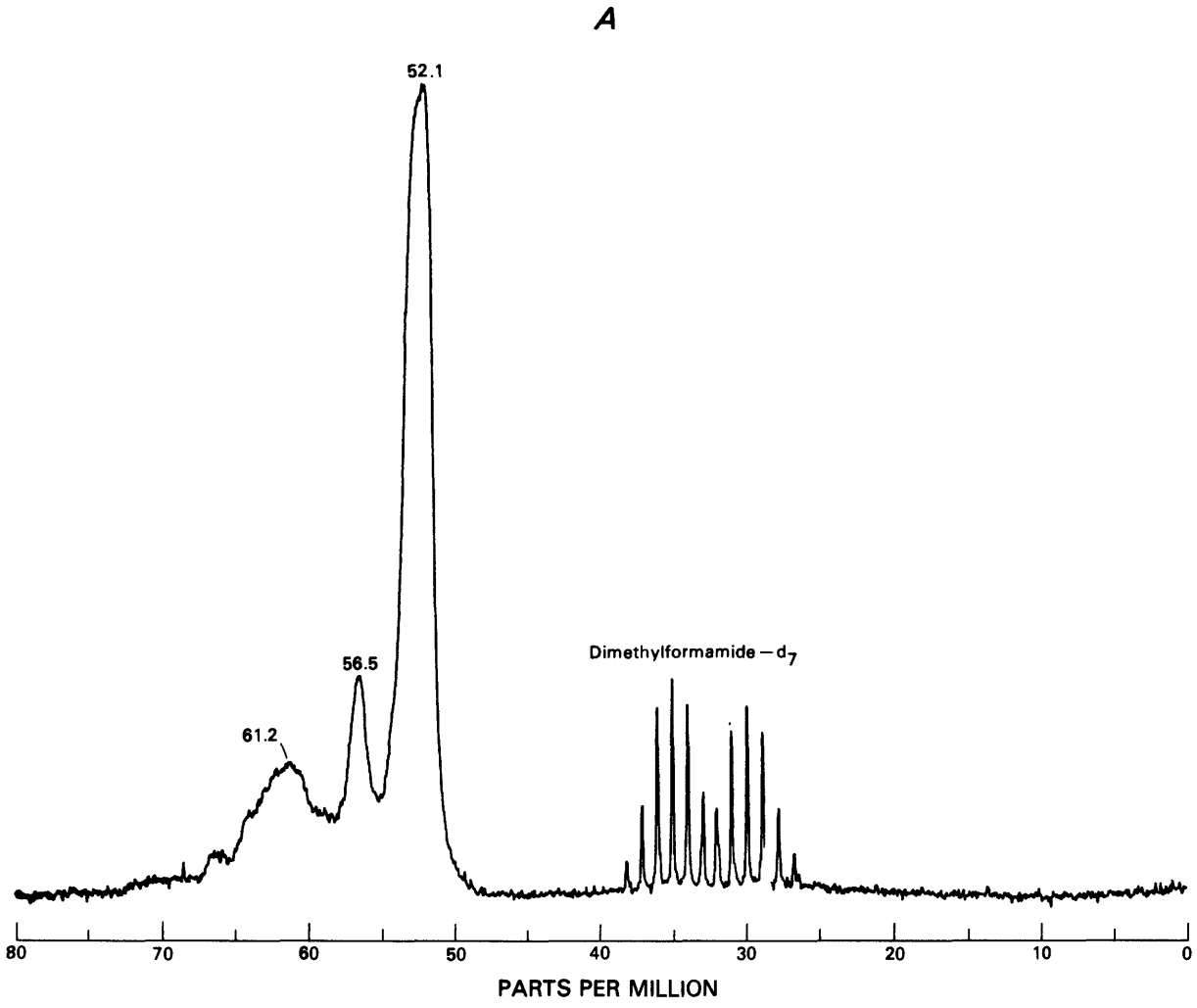



**Figure 10** (above and facing page). Carbon-13 nuclear-magnetic-resonance spectra of humic acid methylated using carbon-13-enriched reagents. "Parts per million" label on x-axis refers to chemical shift in parts per million. Spectral window = 5,000 hertz; pulse width = 45°; acquisition time = 1.023 seconds; pulse delay = 0.0 second; decoupling mode = continuous broadband decoupling; sensitivity enhancement = −1.00. *A*, Methylated with carbon-13-enriched diazomethane, concentration ≅ 50 milligrams in 0.5 milliliter of dimethylformamide-$d_7$; *B*, Permethylated with carbon-13-enriched diazomethane followed by carbon-13-enriched methyl iodide and sodium hydride, concentration ≅ 50 milligrams in 0.5 milliliter of deuterated chloroform.

comparison of these areas to peak areas obtained from spectra acquired with long pulse delays and inverse-gated decoupling showed no differences. The relative peak areas in these spectra were found to be quantitatively accurate.

The data in table 4 indicate that, in the spectra of both the diazomethylated fulvic and humic acids, the area of the phenolic-hydroxyl peak that is centered at 60 ppm is about twice the area of the phenolic-hydroxyl peak centered at 56 ppm. If the area of the methyl-ester peak in the diazomethylated samples is set equal to the carboxylic-acid content determined by potentiometric titration (fulvic acid = 6.1 meq/g and humic acid = 4.9 meq/g; Bowles and others, chap. L, this volume), then values for phenolic-hydroxyl content are calculated to be 1.5 meq/g and 2.9 meq/g for the fulvic acid and humic acid, respectively. From the permethylated spectra, if again the methyl-ester peak is set equal to the carboxylic-acid content from potentiometric titration, the total noncarboxylic-acid hydroxyl contents (phenolic, carbohydrate, and aliphatic hydroxyl) are calculated as 7.0 meq/g and 9.0 meq/g for the fulvic and humic acid, respectively. If the carboxylic acids are not completely methylated with diazomethane, then the area of the methyl-ester peak does not represent a

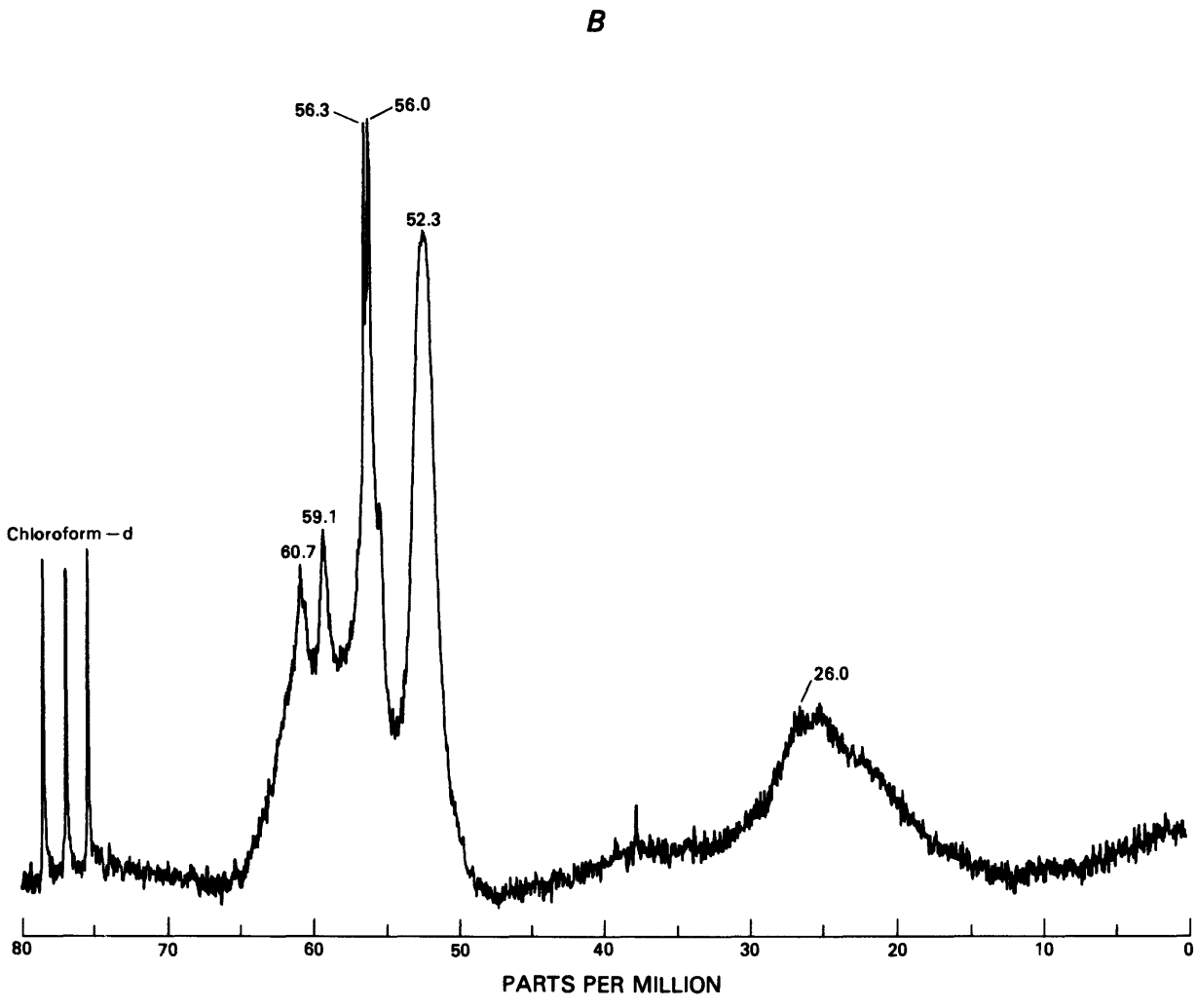



**Table 4.** Peak areas in carbon-13 nuclear-magnetic-resonance spectra of methylated samples and hydroxyl-group contents calculated from spectra of methylated samples

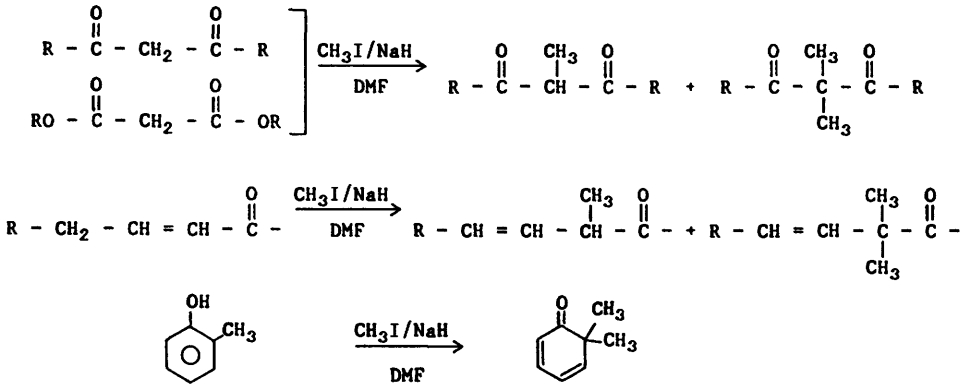
[ppm, chemical shift in parts per million; mmol/g, millimoles per gram]

| Sample | Peak areas in carbon-13 nuclear-magnetic-resonance spectra of methylated samples[1] | | | | | Phenolic hydroxyl and total hydroxyl content calculated from methylation spectra | | |
|---|---|---|---|---|---|---|---|---|
| | Diazomethylated sample | | | Permethylated sample | | $-CO_2H$ (mmol/g)[2] | Phenolic -OH (mmol/g)[3] | Total non $-CO_2H$ hydroxyl (mmol/g)[4] |
| | (60 ppm) | (56 ppm) | (52 ppm) | (60-55 ppm) | (52 ppm) | | | |
| Fulvic acid | 12.6 | 7.5 | 79.9 | 53.5 | 46.5 | 6.1 | 1.5 | 7.0 |
| Humic acid | 24.0 | 13.4 | 62.6 | 64.7 | 35.3 | 4.9 | 2.9 | 9.0 |

[1]Percentage of total area.
[2]From potentiometric titration.
[3]From spectra of diazomethylated sample; area of methyl-ester peak of carboxylic acid set equal to titration value for carboxylic-acid content.
[4]From spectra of permethylated sample; area of methyl-ester peak of carboxylic acid set equal to titration value for carboxylic-acid content.

constant value in going from the diazomethylated to the permethylated spectrum—this would introduce error into the calculations.

The broad resonances that are centered at approximately 23 ppm in the spectra of the permethylated samples are interpreted as the C-$CH_3$ groups arising from carbon methylation. Structures that can carbon alkylate with the sodium hydride and methyl iodide ($CH_3I$/NaH) methylating reagent include activated methylene and methine carbons, α, β-unsaturated ketones, and phenols:




The $^{13}$C-NMR chemical shifts of the C-$CH_3$ groups of the C-methylated derivatives of such model compounds as benzoylacetone, methyl acetoacetate, dimethyl malonate, methyl phenylacetate, and ortho-cresol fall within the range of 20 to 26 ppm and correlate with the broad resonance in the spectra of the permethylated fulvic and humic acids.

Carbons that can undergo carbon alkylation are significant structural units in these samples. The implication of these results is that a significant fraction of the methine and methylene carbons in the fulvic- and humic-acid molecules are in activated positions—that is, alpha to aromatic rings or carbonyl groups. Further studies of carbon alkylation in conjunction with the APT experiments could confirm this hypothesis.

The use of $^{13}$C-enriched methylating reagents has a distinct advantage: only the carbons added onto the humic molecules are observed in the spectra, and, therefore, overlap with the naturally occurring $^{13}$C nuclei in the humic samples is avoided. The reason for this is that the concentration of $^{13}$C in the methylating reagents is 100 times the concentration of the naturally abundant $^{13}$C in the humic molecules. For the sake of quantifying hydroxyl groups, therefore, more accurate peak areas can be generated from spectra methylated with enriched reagents.

## HYDROGEN-1 NUCLEAR-MAGNETIC-RESONANCE SPECTRA OF FULVIC AND HUMIC ACIDS

Proton NMR spectra of the fulvic and humic acids are presented in figures 11 and 12. Both spectra of the sodium salts of the samples in $D_2O$, without homonuclear decoupling of the residual HOD peak, and spectra of the $^1$H-saturated forms of the samples in DMSO-$d_6$, with homonuclear decoupling of the residual HOD peaks, are shown. These $^1$H-NMR spectra exhibit the four major broad resonances characteristic of humic substances: (1) 0 to 1.6 ppm, protons on methyl and methylene carbons directly bonded to other carbons; (2) 1.6 to 3.0 ppm, protons on methylene and methine carbons alpha to aromatic rings, carboxyl, and carbonyl groups; (3) 3.3 to 5.5 ppm, protons on methyl, methylene, or methine carbons directly bonded to oxygen or nitrogen, including carbohydrate and amino acid protons; and (4) 5.5 to 9.0 ppm, protons attached to unsaturated carbons and aromatic protons. These are general assignments—protons in a multitude of different structures overlap within these spectral regions. Noteworthy is the fact that, in the region from approximately 5.5 to 9.0 ppm in both the fulvic and humic acids, the peak maxima occur at approximately 7.0 ppm. This spectral region, therefore, is predominated by aromatic and not olefinic protons. Most olefinic protons occur in the range 5 to 7 ppm

and most aromatic protons in the range 6.5 to 8.5 ppm.

The humic acid differs strikingly from the fulvic acid in that it has a higher proportion of aromatic protons (peak centered at 7.6 ppm) and protons on carbons bonded to hetero-atoms (peak centered at 3.7 ppm). Also noteworthy is the absence of aldehyde protons in the 9 to 11 ppm region in the fulvic- and humic-acid spectra. These observations agree with the results obtained from the $^{13}$C-NMR analyses.

Proton spin-lattice relaxation times for both the fulvic and humic acids were determined to be less than or equal to 0.4 s by the progressive-saturation method. Since the spectra were acquired with a 45° pulse width and 5.0-s pulse delays, the spectra are quantitative. Peak areas of the aqueous-solution proton NMR spectra are given in table 5. The fulvic-acid and humic-acid proton aromaticities determined from the aqueous-solution spectra (and calculated by dividing the peak area of the aromatic protons by the total spectrum area) are 0.15 and 0.22, respectively. These numbers represent only the nonexchangeable protons of the fulvic and humic acids. Additionally, interpretation of the peak areas must be tempered with the possibility of deuterium exchange into both aromatic and aliphatic structures.

## NITROGEN-15 NUCLEAR-MAGNETIC-RESONANCE SPECTRUM OF HYDROXYLAMINE-DERIVATIZED FULVIC ACID

The assignment of the broad carbonyl peak, from 180 to 220 ppm in the $^{13}$C-NMR spectrum of the fulvic acid, as ketone carbon can be confirmed by converting the ketones into their oxime derivatives with hydroxylamine and directly observing the oxime nitrogens by $^{15}$N-NMR. In a study of the reaction of hydroxylamine with humic substances, Thorn and others (1986) derivatized Suwannee River fulvic acid with $^{15}$N-enriched hydroxylamine and recorded the $^{15}$N-NMR spectrum. This spectrum is shown in figure 13. The broad resonance from 340 to 380 ppm, the major resonance in the spectrum, represents the oxime nitrogens. The $^{15}$N-NMR chemical shifts, in DMSO-$d_6$, for a series of oximes of model ketones, ketoses, aldehydes, aldoses, and para-quinones were determined; the chemical shifts all were within the range from 340 to 390 ppm. Unlike the $^{13}$C-NMR chemical shifts of ketones, the oxime $^{15}$N-NMR chemical shifts do not exhibit any general trend, such as oximes of dialkyl ketones downfield of oximes of diaryl ketones. Nevertheless, the broad nature of the oxime resonance in the $^{15}$N-NMR spectrum would appear to support the conclusion drawn from the $^{13}$C-NMR spectrum: dialkyl, alkyl-aryl, and diaryl ketones all are present in the fulvic acid.

Another broad resonance in the $^{15}$N-NMR spectrum occurs from about 160 to 185 ppm. The upfield portion of this peak, from approximately 164 to 168 ppm, is assigned as hydroxamic-acid nitrogen. Hydroxamic acids would arise from the reaction of hydroxylamine with naturally occurring esters in the fulvic acid. The $^{15}$N-NMR chemical shifts of model hydroxamic acids in DMSO-$d_6$ were determined to range from 164 to 168 ppm.

The band from approximately 240 to 255 ppm, centered at 247 ppm, is tentatively assigned as nitrile nitrogen. Nitriles could arise from the dehydration of aldoximes or from the fragmentation of certain types of ketoximes (March, 1985)—they would thus represent secondary reaction products. In the former case, nitrile resonances would constitute evidence for the presence in the fulvic acid of minor amounts of

**Table 5.** Peak areas of aqueous-solution hydrogen-1 nuclear-magnetic-resonance spectra of fulvic and humic acids[1]

[ppm, chemical shift in parts per million]

| Sample | 9.0-5.5 ppm | 5.5-3.0 ppm | 3.0-1.6 ppm | 1.6-0.0 ppm |
|---|---|---|---|---|
| Fulvic acid----- | 15 | 23 | 30 | 32 |
| Humic acid------ | 22 | 34 | 26 | 17 |

[1]Percent of total area.

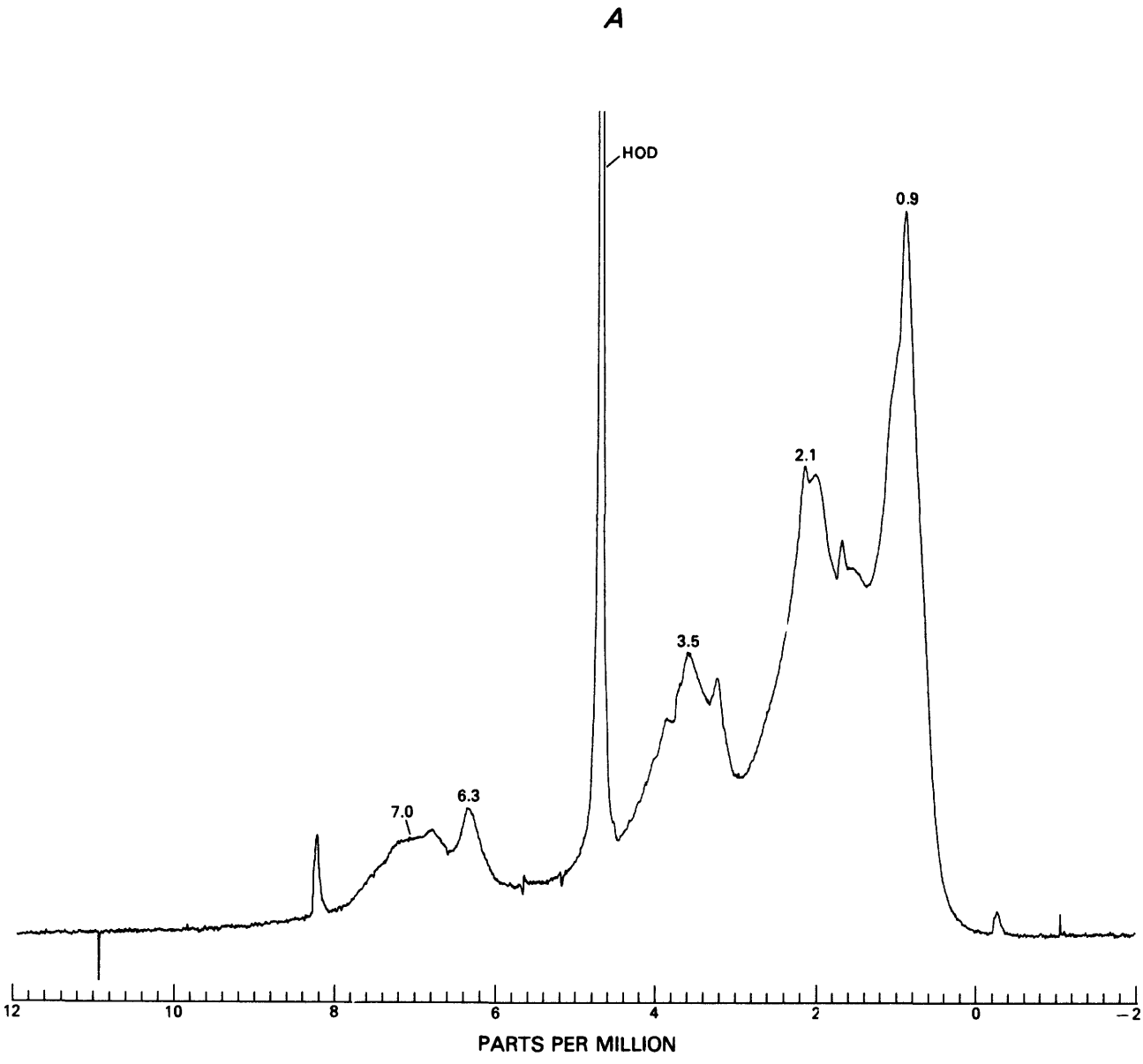
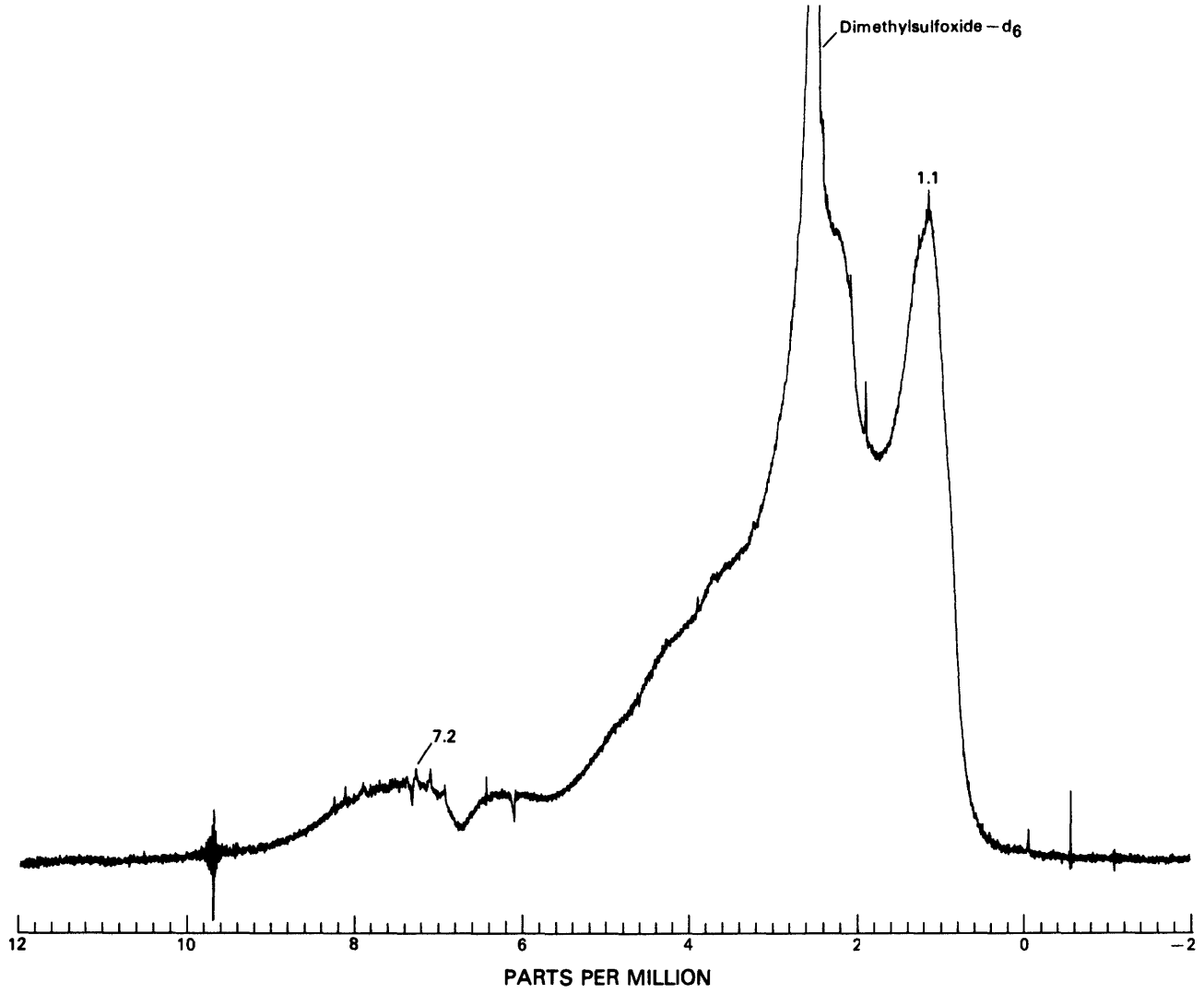



**Figure 11** (above and facing page). Hydrogen-1 nuclear-magnetic-resonance spectra of fulvic acid. "Parts per million" label on x-axis refers to chemical shift in parts per million. Spectral window = 8,000 hertz; pulse width = 25°; acquisition time = 1.9 seconds; pulse delay = 5.0 seconds; line broadening = 1.0 hertz. *A*, Sodium salt in deuterium oxide; *B*, Hydrogen-saturated form in dimethylsulfoxide-$d_6$, with homonuclear decoupling of residual water peak.

aldehydes not discernible from the $^{13}C$- and $^{1}H$-NMR spectra.

The other resonances in the $^{15}N$-NMR spectrum have not been identified as of this writing. Obviously, reactions other than the formation of oximes and hydroxamic acids are occurring between the fulvic acid and the hydroxylamine. There is some interesting organic chemistry to be unraveled here. An approximate $^{15}N$-$T_1$ measurement of the derivatized fulvic acid indicated that the spectrum presented in figure 13 is essentially quantitative. We can conclude that oxime formation is the major reaction of the fulvic acid with hydroxylamine. The baseline roll in the spectrum is a distortion arising from acoustic ringing, a problem often encountered in $^{15}N$-NMR (Fukushima and Roeder, 1981; Patt, 1982).

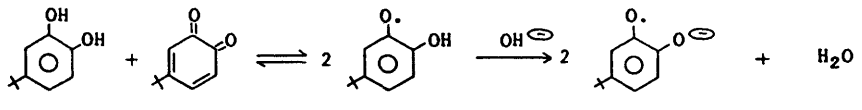
*B*




## EFFECTS OF pH ON AQUEOUS-SOLUTION CARBON-13 NUCLEAR-MAGNETIC-RESONANCE SPECTRA OF FULVIC ACID

Steelink and others (1983) noted that fulvic and humic acids form stable free radicals, attributable to quinone-hydroquinone complexes, when titrated to basic pH. The reaction proceeds as follows:

Stable organic-free radicals may broaden NMR signals via paramagnetic relaxation (Abraham and Loftus, 1980). In fact, line broadening and losses of signal intensity in the aromatic regions of $^{13}$C-NMR spectra of model flavonoids and polyphenols were observed when these samples were brought from neutral to basic pH (Steelink and others, 1983)—they suggested that the intensities of the aromatic regions in the $^{13}$C-NMR spectra of humic substances acquired under basic pH may be distorted.



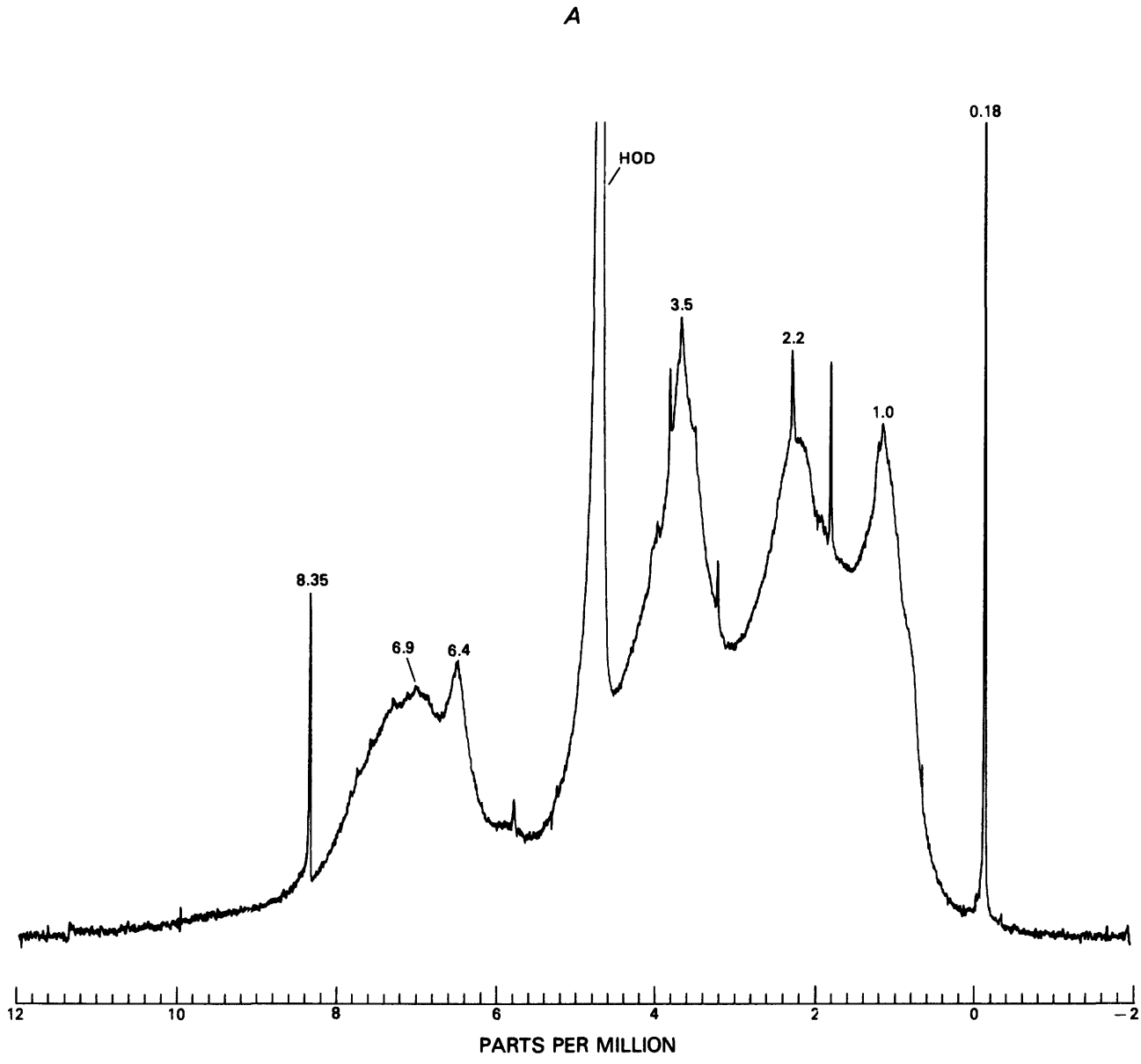



**Figure 12** (above and facing page). Hydrogen-1 nuclear-magnetic-resonance spectra of humic acid. "Parts per million" label on x-axis refers to chemical shift in parts per million. Spectral window = 8,000 hertz; pulse width = 25°; acquisition time = 1.9 seconds; pulse delay = 5.0 seconds; and line broadening = 1.0 hertz. *A*, Sodium salt in deuterium oxide; *B*, Hydrogen-saturated form in dimethylsulfoxide-$d_6$, with homonuclear decoupling of residual water peak.

To investigate the possibility that $^{13}$C-NMR spectra of humic substances that are acquired under basic pH may be distorted, spectra of Suwannee River fulvic acid were acquired in aqueous solution at pH values of 3, 5, 7, 9, 11, and 13 (fig. 14). The acquisition parameters were identical for each spectrum: 45° pulse angle, 1.0-s pulse delay, and continuous decoupling. By visual inspection, there is a definite change in the intensities of some of the peaks across the pH range. The carboxyl peak decreases in intensity from pH 3 through pH 9, where it reaches a minimum, and then increases again to pH 13. The changes in intensities of the carboxyl peak result from the chemical shifts of the individual carboxyl carbons moving downfield as the individual carboxyl groups are converted from the H-saturated to the dissociated form. For example, the chemical shift of the carboxyl carbon of butyric acid in aqueous solution

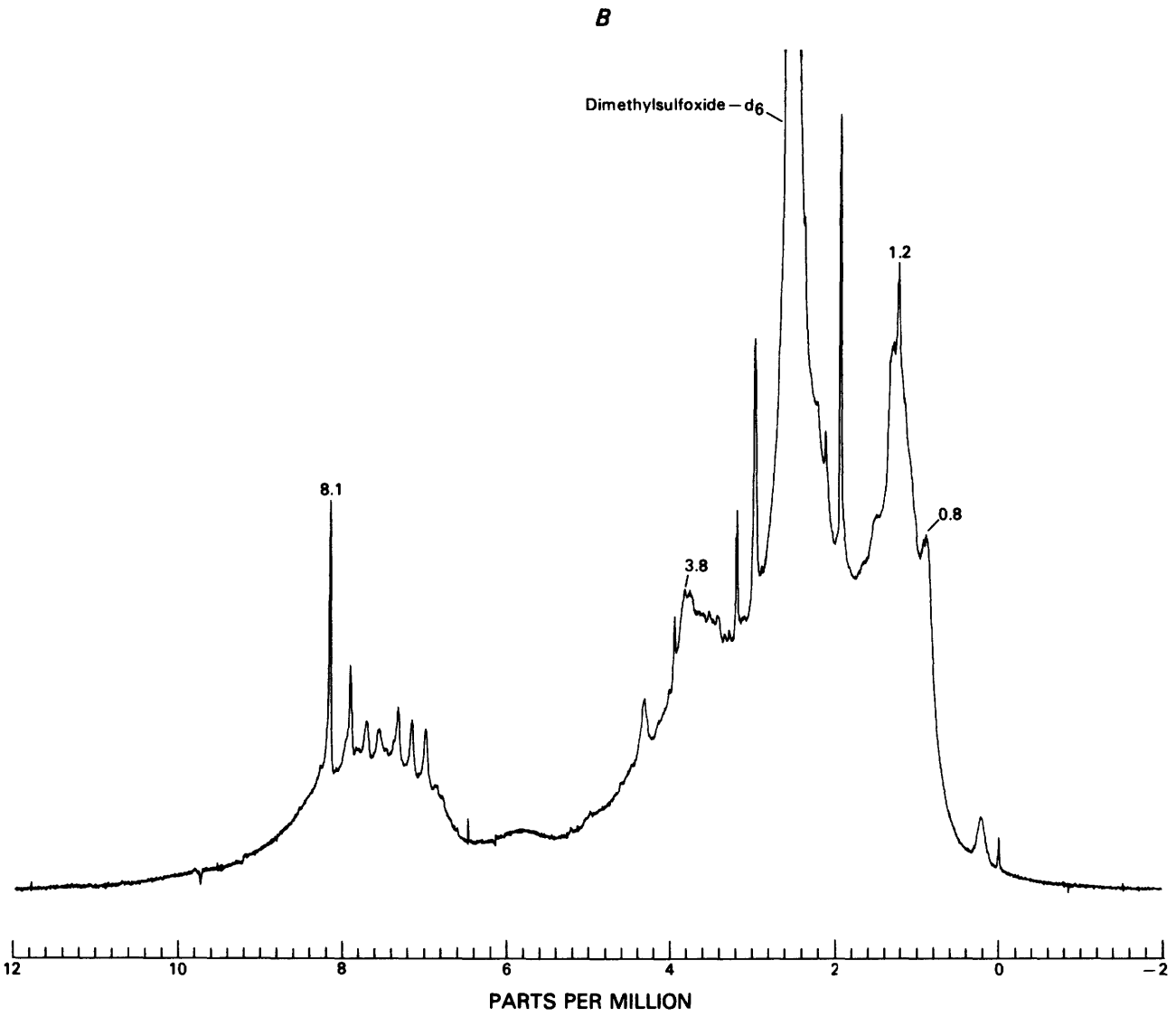



**Table 6.** Peak areas of aqueous-solution carbon-13 nuclear-magnetic-resonance spectra of fulvic acid as a function of pH

[Integration areas are in arbitrary units; ppm, chemical shift in parts per million]

| pH | Ketone (220 to 185 ppm) | Carboxyl (185 to 165 ppm) | Aromatic (165 to 90 ppm) | Aliphatic II (90 to 60 ppm) | Aliphatic I (60 to 0 ppm) |
|---|---|---|---|---|---|
| 3 | 10.2 | 26.0 | 60.8 | 32.0 | 63.6 |
| 5 | 8.3 | 23.4 | 59.4 | 33.8 | 72.1 |
| 7 | 7.1 | 22.7 | 59.7 | 34.0 | 67.2 |
| 9 | 8.6 | 21.7 | 58.1 | 34.3 | 71.1 |
| 11 | 10.0 | 23.4 | 58.3 | 33.8 | 64.0 |
| 13 | 7.9 | 23.9 | 54.8 | 32.9 | 67.4 |

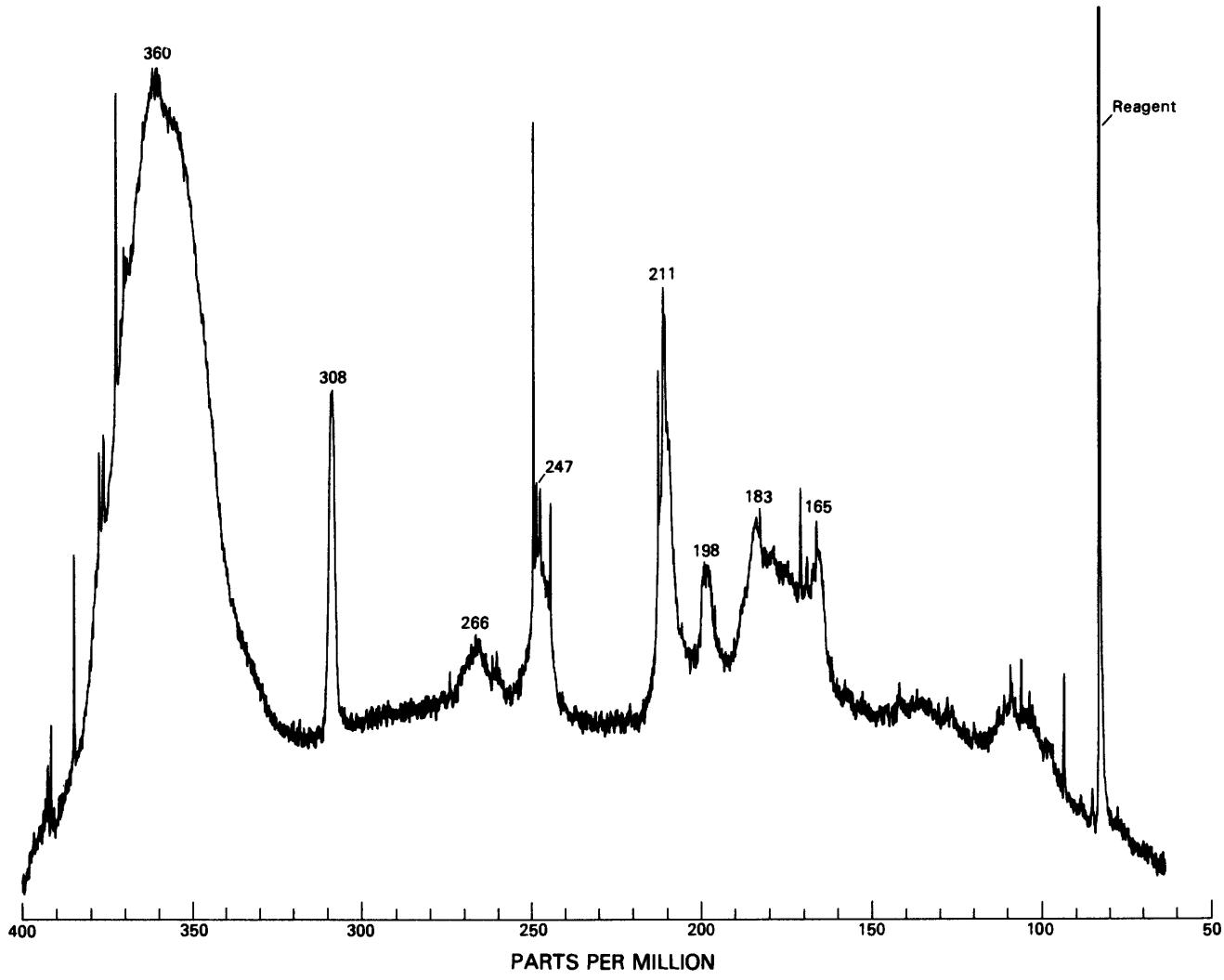



**Figure 13.** Nitrogen-15 nuclear-magnetic-resonance spectrum of fulvic acid derivatized using nitrogen-15-enriched hydroxylamine hydrochloride. "Parts per million" label on x-axis refers to chemical shift in parts per million. Concentration = 682 milligrams of hydrogen-saturated derivative in 2.0 grams of dimethylsulfoxide-$d_6$. Spectral window = 21,276.6 hertz; acquisition time = 0.5 second; pulse width = 45°; pulse delay = 2.0 seconds; decoupling mode = inverse-gated decoupling; line broadening = 1.0 hertz; number of transients = 118,300.

moves downfield from 178.4 to 183.7 ppm as the pH is increased from 2 to 13. Phenolic carbons also will move downfield as phenolic hydroxyls are converted to phenolate anions with increasing pH. The intensities of the aromatic, aliphatic II, and aliphatic I peaks increase slightly from pH 3 to pH 5 and then remain constant through pH 13. The changes in peak intensities are not reflected in any significant changes in peak areas, which are listed in table 6. The changes in peak areas are within the range of error of the spectral integrations. No effect, with respect to loss of aromatic carbon signals as might be expected because of paramagnetic relaxation due to stable free-radical formation in base, can be observed in Suwannee River fulvic acid. Schnitzer and Preston (1986) also have reported that the $^{13}C$-NMR spectrum of a soil fulvic acid dissolved in NaOD (pH 12) showed no major differences compared to the spectrum of the same sample in $D_2O$ (pH 2.5). However, effects of pH need to be examined in a broader range of samples, including humic acids.

## COMPARISON OF CARBON-13 NUCLEAR-MAGNETIC-RESONANCE SPECTRA OF FULVIC ACID IN AQUEOUS SOLUTION AND IN DIMETHYLSULFOXIDE

Carbon-13 NMR spectra of the fulvic acid dissolved in aqueous solution at pH 6 and in DMSO-$d_6$ are shown in figure 15. Both spectra were acquired using the same acquisition parameters—45° pulse angle, 1.0-s pulse delay, continuous decoupling—and represent the same number of transients, sample concentrations, and line broadening. The DMSO spectrum shows better signal-to-noise and resolution than does the aqueous-solution spectrum. The splitting of the carboxyl peak in the DMSO spectrum cannot be observed in the aqueous-solution spectrum. Additionally, the sharp resonances in the DMSO spectrum, possibly representing pthalate ester contaminants, are not observed in the aqueous-solution spectrum.

## CONCENTRATIONS OF OXYGEN-CONTAINING FUNCTIONAL GROUPS

Quantitative estimates of the oxygen-containing functional groups of the fulvic and humic acids can be made from a combination of the $^{13}$C-NMR data, elemental analysis, and potentiometric titration data. Elemental analysis indicates the percent carbon of the fulvic acid to be 51.3 percent (Reddy, and others, chap. I, this volume), and potentiometric titration indicates the carboxylic-acid content to be 6.1 mmol/g (Bowles and others, chap. L, this volume). The carbonyl band from 160 to 180 ppm in the quantitative $^{13}$C-NMR spectrum of the fulvic acid represents 19 percent of the total carbon (table 2). A carbon content of 51.3 percent translates into 42.8 mmol/g of carbon; 19 percent of this is 8.1 mmol/g of carbon. Therefore, the carbonyl band from 160 to 180 ppm represents 8.1 mmol/g of carbon in the fulvic acid. If 6.1 mmol/g of this is comprised of carboxylic acid, the remaining 2.0 mmol/g would be comprised of a combination of ester, amide, and (or) lactone groups. Similarly, the ketone band from 180 to 220 ppm in the quantitative $^{13}$C-NMR spectrum of the fulvic acid represents 6 percent of the total carbon; 6 percent of 42.8 mmol/g of carbon translates into 2.6 mmol ketone per gram of fulvic acid. Estimates of a phenolic hydroxyl content of 1.5 mmol/g and a total noncarboxylic-acid hydroxyl content of 7.0 mmol/g were made from the methylation results discussed previously. These results are summarized in table 7. The same calculations were made for the humic acid, assuming a carboxylic-acid content of 4.9 mmol/g from potentiometric titration and a carbon content of 54.2 percent by elemental analysis.

## SUMMARY AND CONCLUSIONS

Each set of NMR analyses performed on Suwannee River fulvic and humic acids provided complementary information on the structural characteristics of these samples. Clearly, a comprehensive approach to the NMR analyses of materials as complex as humic substances is needed in order to obtain all the information potentially available from NMR. The general structural characteristics of the Suwannee River samples are summarized here. Quantitative $^{13}$C-NMR spectra show that both the fulvic and humic acids have significant aromatic carbon contents: the fulvic acid has an $f_a$ of 0.28, and the humic acid has an $f_a$ of 0.42. A combination of the quantitative $^{13}$C-NMR spectra and methylation analysis indicates that the concentrations of oxygen-containing

**Table 7.** Concentration of oxygen-containing functional groups in fulvic and humic acids estimated from a combination of potentiometric titration, elemental analysis, and carbon-13 nuclear-magnetic-resonance data

[mmol/g, millimoles per gram]

| Functional group | Fulvic acid, in mmol/g | Humic acid, in mmol/g |
|---|---|---|
| Carboxylic acid | 6.1 | 4.9 |
| Ketone | 2.6 | 3.2 |
| Ester, amide, lactone | 2.0 | 2.3 |
| Phenolic hydroxyl | 1.5 | 2.9 |
| Total noncarboxylic-acid hydroxyl | 7.0 | 9.0 |

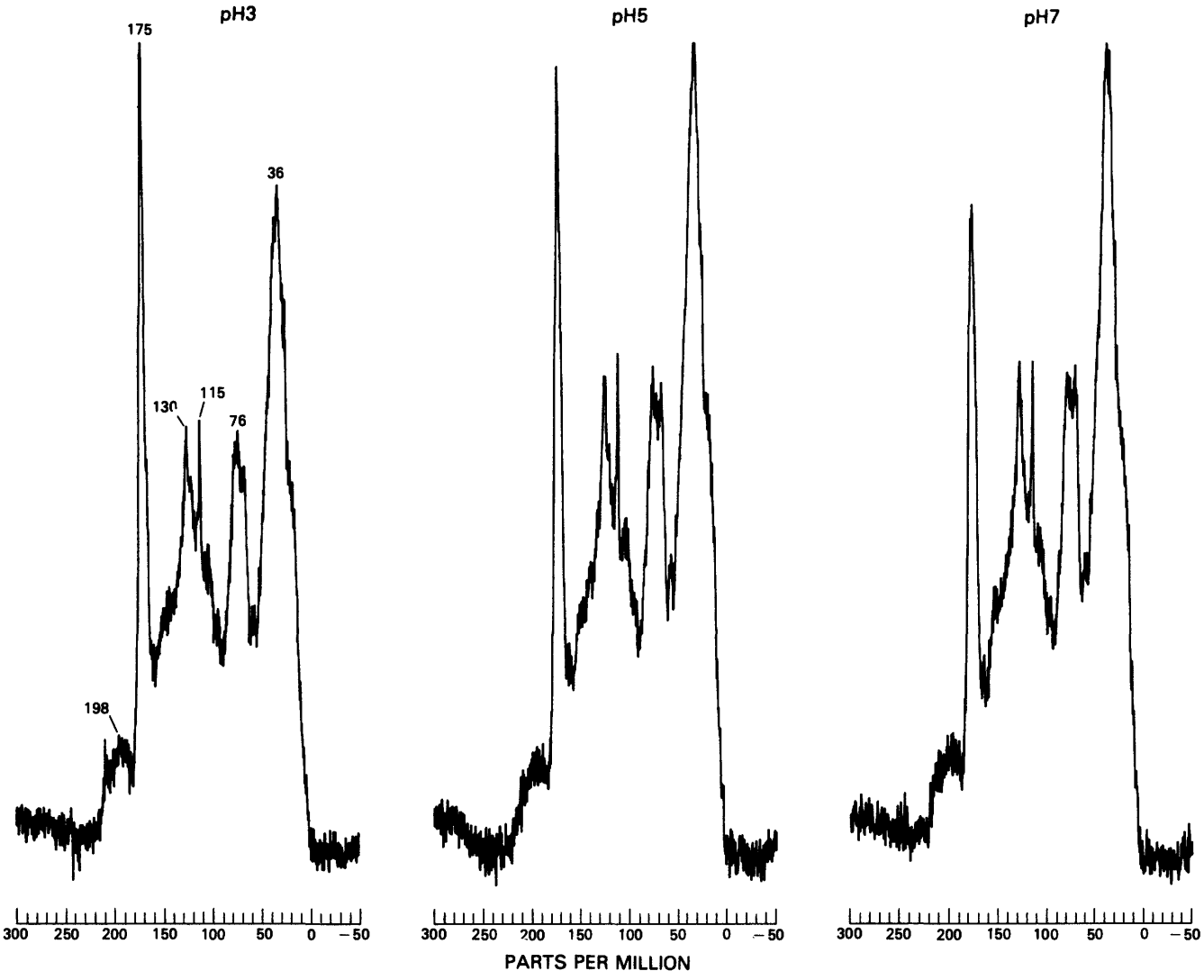
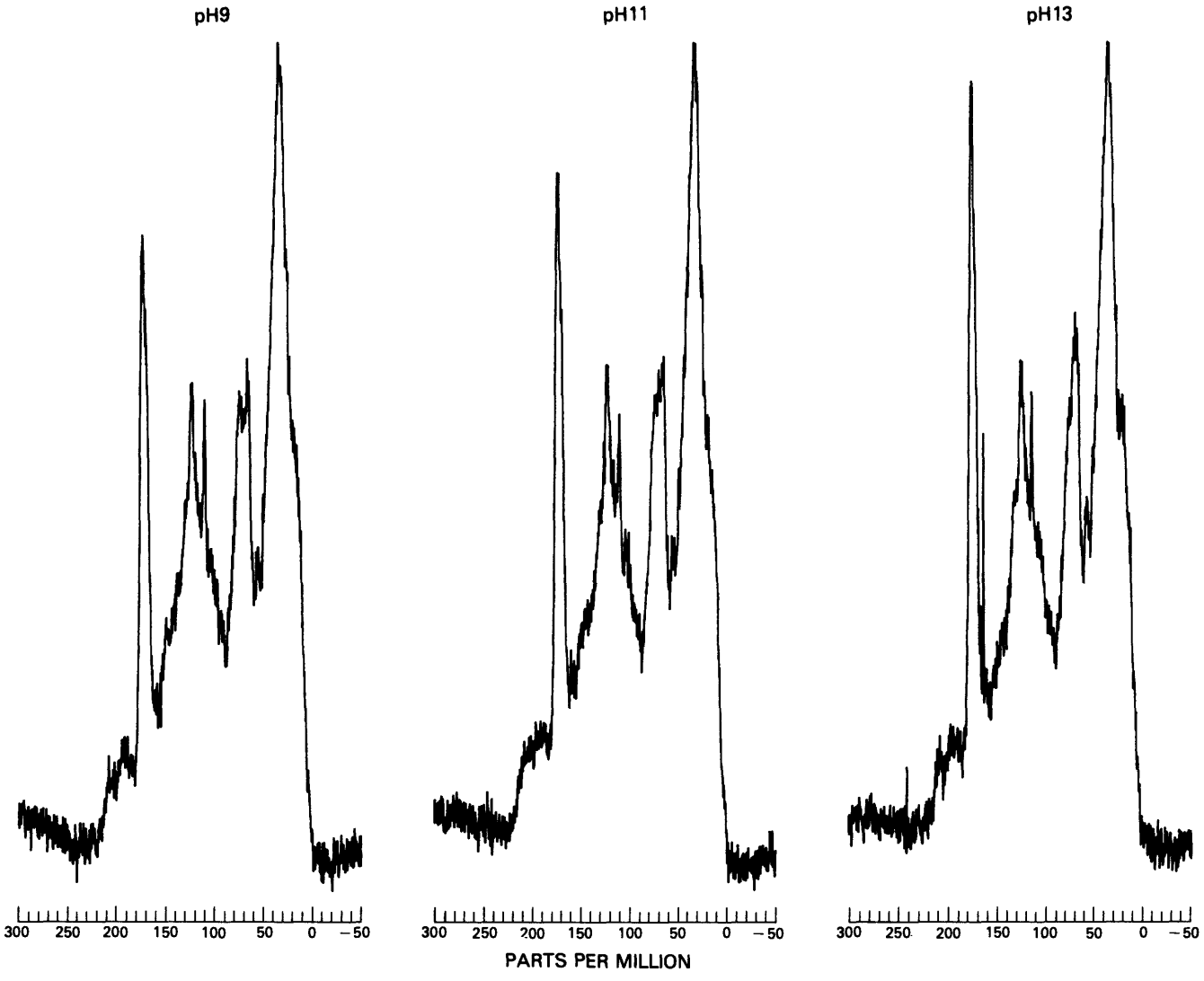



**Figure 14** (above and facing page). Carbon-13 nuclear-magnetic-resonance spectra of fulvic acids at different pH values. "Parts per million" label on x-axis refers to chemical shift in parts per million. Concentration = 200 milligrams in 0.5 milliliter deuterium oxide and 1.5 milliliters deionized and distilled water; pH adjusted using 20 percent sodium deuteroxide. Spectral window = 30,000 hertz; acquisition time = 0.2 second; pulse width = 45°; pulse delay = 1.0 second; decoupling mode = continuous WALTZ decoupling; line broadening = 20.0 hertz; number of transients = 25,000.

functional groups in both the fulvic and humic acids follow the order: carboxylic acid > hydroxyl – OH (combined phenolic OH, carbohydrate OH, and aliphatic OH) > ketone > other carbonyl groups (including esters, amides, and lactones). No direct evidence for ether oxygen is available in any of the NMR spectra—concentrations of ether oxygens must be inferred from difference calculations. Some uncertainty remains as to the precise nature of the hydroxyl groups in the fulvic and humic acids that are not derivatized with diazomethane but that are derivatized with the stronger methyl iodide and sodium hydride methylating reagent. These can be any combination of weakly acidic phenolic hydroxyls, carbohydrate hydroxyls, or noncarbohydrate alcoholic hydroxyls. The APT spectra indicate that, in the case of non-carbohydrate alcoholic hydroxyls, these would be predominated by secondary hydroxyls. A combination of the APT $^{13}C$- and $^{1}H$-NMR spectra indicate the presence of both protonated and nonprotonated aromatic carbons in the fulvic and humic acids. The $^{13}C$-NMR spectra show that the carboxylic-acid groups may occur in several configurations, including aliphatic, benzylic, orthohydroxybenzenecarboxylic, benzenecarboxylic, and possibly α, β-unsaturated carboxylic acids. The presence of benzenecarboxylic




acids and phenolic hydroxyls in the fulvic and humic acids is consistent with the production of benzene carboxylic acids and hydroxybenzene carboxylic acids upon oxidative degradations of aquatic humic materials—this as has been reported by several researchers (Ogner and Gjessing, 1975; Liao and others, 1982). Chemical-shift considerations in the $^{13}$C-NMR spectra indicate that ketones occur as dialkyl, alkyl-aryl, and diaryl ketones in the fulvic and humic acids. The APT spectra clearly indicate the presence of aliphatic methine, methylene, and methyl carbons and indicate an absence of quaternary aliphatic carbons in the two samples; these spectra also indicate that the aliphatic structures in the fulvic and humic acids are primarily branched, short-chained, and (or) cyclic.

A review of the literature suggests that the quantitative $^{13}$C-NMR spectra of Suwannee River fulvic and humic acids presented here are the first natural abundance $^{13}$C-NMR spectra of dissolved aquatic humic materials that have been acquired under convincingly quantitative conditions. Quantitative $^{13}$C-NMR analyses of fulvic and humic acids from other aquatic environments is an area of future research. Such studies may finally resolve the long-standing uncertainties over the true distribution of carbons in aquatic humic substances.

## ACKNOWLEDGMENTS

The author thanks M.A. Mikita and E.M. Thurman for their helpful reviews of the manuscript.

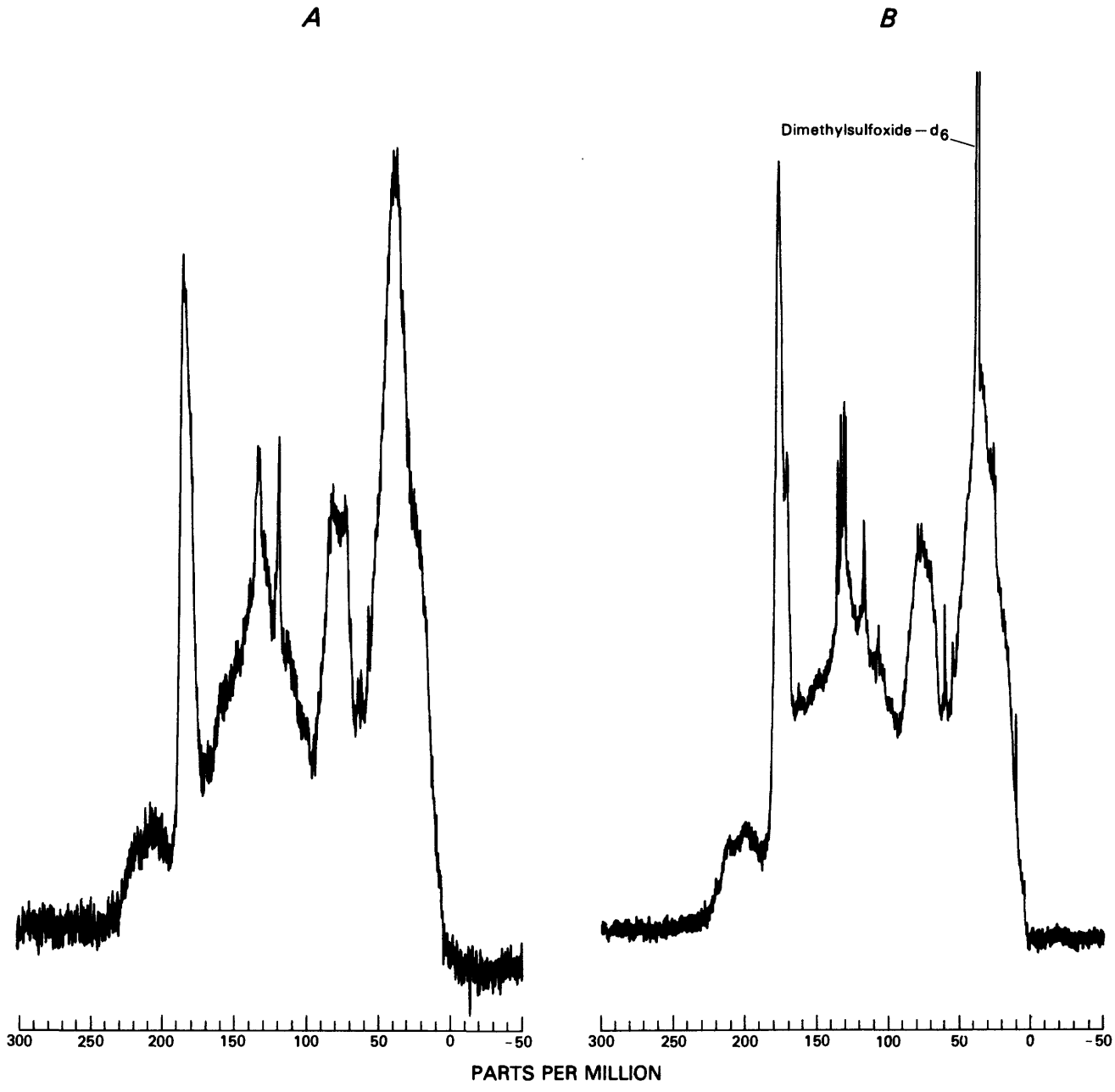



**Figure 15.** Comparison of carbon-13 nuclear-magnetic-resonance spectra of fulvic acid in aqueous solution and in dimethylsulfoxide. "Parts per million" label on x-axis refers to chemical shift in parts per million. Spectral window = 30,000 hertz; pulse width = 45°; acquisition time = 0.2 second; pulse delay = 1.0 second; decoupling mode = continuous WALTZ decoupling; line broadening = 20.0 hertz; number of transients = 66,000. *A*, Concentration = 200 milligrams in 0.5 milliliter deuterium oxide and 1.5 milliliters deionized and distilled water, pH = 6; *B*, Concentration = 200 milligrams in 2 grams dimethylsulfoxide-$d_6$, carbon-13 depleted.

## REFERENCES CITED

Abraham, R.J., and Loftus, P., 1980, Proton and carbon-13 NMR Spectroscopy—An Integrated Approach: London, Heyden, 230 p.

Alemany, L.B., Grant, D.M., Pugmire, R.J., Alger, T.D., and Zilm, K.W., 1983a, Cross polarization and magic angle sample spinning NMR spectra of model organic compounds. 1. Highly protonated molecules: Journal of the American Chemical Society, v. 105, p. 2133–2141.

———1983b, Cross polarization and magic angle sample spinning NMR spectra of model organic compounds. 2. Molecules of low or remote protonation: Journal of the American Chemical Society, v. 105, p. 2142–2147.

Black, T.H., 1983, The preparation and reactions of diazomethane: Aldrichimica Acta, v. 16, no. 1, p. 3–10.

Doddrell, D.M., Pegg, D.T., and Bendall, M.R., 1982, Distortionless enhancement of NMR signals by polarization transfer: Journal of Magnetic Resonance, v. 48, p. 323–327.

Earl, W.L., 1987, Effects of molecular mobility on high resolution solid state NMR Spectra—Model systems, *in* Wershaw, R.L., and Mikita, M.A., eds., NMR of Humic Substances and Coal—Techniques, Problems and Solutions: Chelsea, Michigan, Lewis Publishers, p. 167–188.

Earl, W.L., Wershaw, R.L., and Thorn, K.A., 1987, The use of variable temperature and magic-angle spinning in studies of fulvic acids: Journal of Magnetic Resonance, v. 74, p. 264–274.

Freeman, R., and Hill, H.E.W., 1971, Journal of Chemical Physics: v. 54, 3,367 p.

Frye, J., and Maciel, G.E., 1987, Solid state NMR of humic materials, *in* Wershaw, R.L. and Mikita, M.A., eds., NMR of Humic Substances and Coal: Chelsea, Michigan, Lewis Publishers, p. 47–72.

Fukushima, E., and Roeder, S.B.W., 1981, Experimental Pulse NMR—A Nuts and Bolts Approach: Reading, Massachusetts, Addison-Wesley, 539 p.

Gonzalez-Vila, F.J., Ludemann, H.D., and Martin, F.D., 1983, $^{13}C$ NMR structural features of soil humic acids and their methylated, hydrolyzed and extracted derivatives: Geoderma, v. 31, p. 3-15.

Liao, W., Christman, R.F., Johnson, J.D., Millington, D.S., 1982, Structural characterization of aquatic humic material: Environmental Science and Technology, v. 16, p. 403–410.

March, J., 1985, Advanced Organic Chemistry, 3d ed.: New York, John Wiley, p. 930–932.

Mikita, M.A., Steelink, C., and Wershaw, R.L., 1981, Carbon-13 enriched nuclear magnetic resonance method for determination of hydroxyl functionality in humic substances: Analytical Chemistry, v. 53, p. 1715–1717.

Newman, R.H., Tate, K.R., Barron, P.F., and Wilson, M.A., 1980, Towards a direct, non-destructive method of characterizing soil humic substances using $^{13}C$ NMR: Journal of Soil Science, v. 31, p. 623–631.

Newman, R.H., and Tate, K.R., 1984, Use of alkaline soil extractants for $^{13}C$ NMR characterization of humic substances: Journal of Soil Science, v. 35, p. 47–54.

Ogner, G., and Gjessing, E.T., 1975, Permanganate oxidation of methylated and nonmethylated aquatic humus in Norway: Geoderma, v. 14, p. 139–145.

Patt, S.L., 1982, Pulse strategies for the suppression of acoustic ringing: Journal of Magnetic Resonance, v. 49, p. 161–163.

Patt, S.L., and Shoolery, J.N., 1982, Attached proton test for carbon-13 NMR: Journal of Magnetic Resonance, v. 46, p. 535–539.

Preston, C.M., and Blackwell, B.A., 1985, Carbon-13 nuclear magnetic resonance for a humic and fulvic acid—Signal-to-noise optimization, quantitation, and spin echo techniques: Soil Science, v. 139, p. 88–96.

Schnitzer, M., and Kahn, S.U., 1972, Humic Substances in the Environment: New York, Marcel Dekker, 327 p.

Schnitzer, M., and Preston, C.M., 1986, Analysis of humic acids by solution and solid state carbon-13 nuclear magnetic resonance: Soil Science Society of America Journal, v. 50, p. 326–331.

Skjemstad, J.O., Frost, R.L., and Barron, P.F., 1983, Structural units in humic acids from southeastern Queensland soils as determined by $^{13}C$ NMR spectroscopy: Australian Journal of Soil Research, v. 21, p. 539–547.

Steelink, C., Mikita, M.A., and Thorn, K.A., 1983, Magnetic resonance studies of humates and related model compounds, *in* Christman, R.F., and Gjessing, E.T., eds., Aquatic and Terrestrial Humic Materials: Ann Arbor, Michigan, Ann Arbor Science, p. 83–105.

Steelink, C., Wershaw, R.L., Thorn, K.A., and Wilson, M.A., 1989, Application of liquid-state NMR spectroscopy to humic substances, *in* Swift, R.S., Hayes, M.H.B., MacCarthy, P., and Malcolm, R.L., eds., Humic Substances in Soil, Sediment, and Water: Structure: New York, John Wiley.

Stuermer, D.H., and Payne, J.R., 1976, Investigations of seawater and terrestrial humic substances with carbon-13 and proton nuclear magnetic resonance: Geochimica et Cosmochimica Acta, v. 40, p. 1109–1114.

Thorn, K.A., 1984, NMR structural investigations of aquatic humic substances: Tucson, University of Arizona, Ph.D. dissertation, 193 p.

———1987, Structural characteristics of the IHSS Suwannee River fulvic and humic acids determined by solution-state $^{13}C$ NMR spectroscopy: Science of the Total Environment, v. 62, p. 175–183.

Thorn, K.A., Arterburn, J.B., and Mikita, M.A., 1986, Nitrogen-15 NMR investigation of hydroxylamine derivatized humic substances, *in* Book of Abstracts, Rocky Mountain Regional Meeting of the American Chemical Society, 8th, Denver, 1986: Denver, Colorado Section of the American Chemical Society, p. A–140.

Thorn, K.A., Steelink, C., and Wershaw, R.L., 1987, Methylation patterns of aquatic humic substances determined by $^{13}C$ NMR spectroscopy: Organic Geochemistry, v. 11, no. 3, p. 123–137.

Thurman, E.M., 1985, Organic Geochemistry of Natural Waters: Boston, Massachusetts, Junk Publishers, 497 p.

Vassalo, A.M., 1987, Solid state $^{13}C$ measurements of humic substances: an examination of factors affecting their reliable interpretation, *in* Wershaw, R.L. and Mikita, M.A., eds., NMR of Humic Substances and Coal: Chelsea, Michigan, Lewis Publishers, p. 211–224.

Verheyen, T.V., Johns, R.B., and Blackburn, D.T., 1982, Structural investigations of Australian coals: 2. A $^{13}C$ NMR study of the humic acids from Victorian

brown coal lithotypes: Geochimica et Cosmochimica Acta, v. 42, p. 269–277.

Wagner, H., Chari, V.M., and Sonnenbichler, J., 1976, Tetrahedron Letters No. 21, p. 1799.

Wehrli, F.W., and Wirthlin, T., 1980, Interpretation of carbon-13 NMR Spectra: London, Heyden, 310 p.

Wershaw, R.L., 1985, Application of nuclear magnetic resonance spectroscopy for determining functionality in humic substances, *in* Aiken, G.R., and McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediment, and Water: New York, John Wiley, p. 561–582.

Wershaw, R.L., and Mikita, M.A., eds., 1987, NMR of Humic Substances and Coal—Techniques, Problems and Solutions: Chelsea, Michigan, Lewis Publishers, 236 p.

Wilson, M.A., Barron, P.F., and Gillam, A.H., 1981, The structure of freshwater humic substances as revealed by $^{13}C$ NMR spectroscopy: Geochimica et Cosmochimica Acta, v. 45, p. 1743–1750.

Chapter O

# Significance of Density Determination in Molecular Structures Comprising Fulvic Acid from the Suwannee River

*By* P.A. Brown *and* J.A. Leenheer

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## Abstract

Density values of various organic structural units were calculated by using published and experimental data. Because of the additivity of atomic volumes and densities, structural-unit density values were used to calculate the density of fulvic acid from the Suwannee River from liquid-state, carbon-13 nuclear-magnetic-resonance data; solid-state, carbon-13 nuclear-magnetic-resonance data; proton nuclear-magnetic-resonance data; and oxygen-functional-group distribution. The density of the fulvic acid was then determined in various solvents, and the determined density values were compared to the calculated density values. The degree of aromaticity could not be determined accurately from the density because the elemental ratios included in the empirical formula had the most influence on density. However, the high density value (1.679 grams per milliliter) for the fulvic acid in water at 2 percent concentration (compared to its calculated density value (1.454 grams per milliliter) and experimental density value in acidified dimethylsulfoxide (1.465 grams per milliliter)) indicated that extensive intramolecular and intermolecular hydrogen bonding occurs in aqueous solutions of fulvic acid and that the solute mixture is likely to be aggregated extensively.

## INTRODUCTION

Material density first was determined by Boyle in 1665 (Partington, 1960) and is significant in the determinations of chemical structures because of the additivity of atomic volumes and densities incorporated into molecular density (Traube, 1895). Determination of the molecular weight of fulvic acid from the Suwannee River by equilibrium ultracentrifugation requires measurement of the density of the fulvic acid in various solvent systems (Chervenka, 1973).

Once the density values were known, there was a need to determine what additional characteristics (other than molecular weight) could be deduced about the fulvic acid from the density. The primary questions were:

1. Can an accurate prediction of density be made when only the molecular structure is known?
2. Can an accurate prediction of the structure be made when density, molecular formula, proton distribution, carbon distribution, and oxygen-functional-group distributions of the fulvic acid are known?

Published literature provided various methods in which atomic volumes for each molecular group were used to determine various organic structural moieties (Van Krevelen and Chermin, 1954; Bondi, 1964; and Traube, 1895). Traube (1895) reported that "...the molecular volume of an organic liquid may be calculated approximately as the sum of the 'atom volume' ($V_i$) of the number ($n_i$) of i atoms, if necessary, with a correction ($W_j$) for a number ($Z_j$) of structural effects (j):"

$$\frac{m}{d} = \sum_i n_i V_i + \sum_j Z_j W_j + \phi \qquad (1)$$

where

m is the molecular weight,
d is the specific gravity at 20°C, and
$\phi$ represents the "residual volume" of end groups (Van Krevelen and Chermin, 1954, p. 82).

The structural effects that are corrected in the equation by the term $Z_jW_j$ are the olefinic double bonds, triple bonds, and rings, including aromatic double bonds. The olefinic double and triple bonds have small correction factors (–1.7 $cm^3$ for double bonds and –3.4 $cm^3$ for triple bonds) compared to that for the rings (–13.2 $cm^3$). The large correction factor for aromaticity was used by Van Krevelen and Chermin (1954) to determine aromaticity of various polymeric resins and coals. The same approach can be applied to determine the density and the aromaticity of fulvic acid from the Suwannee River; the limitations are:

1. The term was disregarded by Van Krevelen and Chermin (1954) because high polymeric resins and condensed coal structures contain few end groups, whereas the fulvic acid from the Suwannee River contains many end groups, especially the carboxylic-acid functional group.
2. The high oxygen content of the fulvic acid results in reactive functional groups that hydrogen bond to each other. This causes large increases in molecular density that are independent of the summation of atomic volumes in a monodisperse structure.

To overcome the above limitations, an experimental design was devised that provided density data on low-molecular-weight, carboxylic-acid compounds that had many end groups; this design more

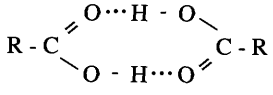
accurately modeled fulvic-acid characteristics. Hydrogen-bonding interactions, "which make the structure more flexible, permit the 'building blocks' to aggregrate and disperse reversibly depending on pH and ionic strength..." (Schnitzer, 1978, p. 47)—these interactions were minimized by determining solute density in polar organic-solvent systems that disrupt solute-solute hydrogen bonding.

The objectives for the study described in this chapter were: (1) Correlate data from the various carbon-13 nuclear-magnetic-resonance ($^{13}$C-NMR) spectra, proton-NMR spectra, and oxygen-functional-group distributions (Leenheer and others, chap. P, this volume) with the data presented in this chapter to determine if primary structural data fit the density determined, and (2) determine the density of fulvic acid from the Suwannee River and its derivatives in various solvents and explain why the density values differ in different solvents. This information might indicate secondary and tertiary structural configurations of fulvic acid in various solvents.

## METHODS USED FOR DETERMINING DENSITY VALUES IN MOLECULAR STRUCTURES COMPRISING FULVIC ACID

### Calculations Using Published Data for Functional Groups

Density values were calculated from handbook data (Weast, 1973) for all functional groups except the carboxyl (COOH) functional group because the published density values for this group were inconsistent; an explanation for this is the dimeric association of carboxylic acids through hydrogen bonding as shown below (Ferguson, 1963):




The calculations for each functional group required the use of published density values (Weast, 1973) at 20°C that were based on each carbon functional group in the compound. To determine the density of the methyl ($CH_3$) functional group, a series of hydrocarbons was chosen and their densities were plotted against the ratio of methylene ($CH_2$) carbons to total carbons (expressed as a percentage) in each hydrocarbon. The line connecting the data then was extrapolated until there was zero percent $CH_2$. This point was identified as the density contribution of the $CH_3$ functional group (fig. 1).

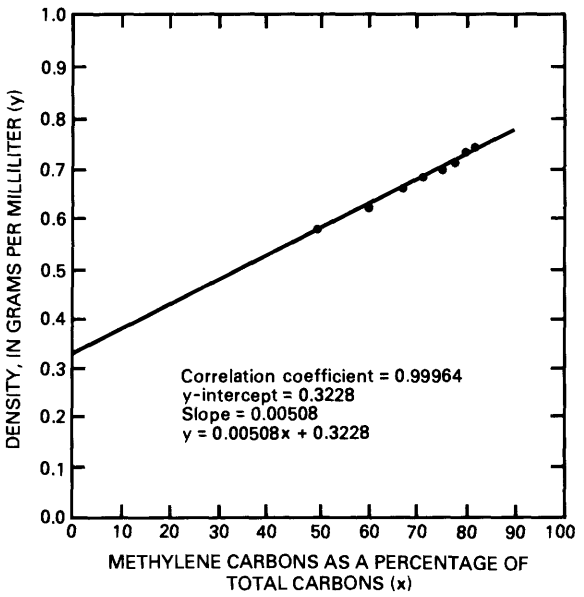



**Figure 1.** Density of linear hydrocarbons as a function of percentage of methylene carbons.

To calculate the density of the methylene ($CH_2$) functional group, the same series of aliphatic hydrocarbons as above was used. An equation was derived in which the sum of the percentage of each functional group in the chemical formula multiplied by the density of the appropriate functional group was set equal to the known density of the compound. The unknown density to be determined was set equal to X and the equation was solved for X. Using hexane as an example, the equation is:

$$\text{Hexane} = CH_3\text{–}CH_2\text{–}CH_2\text{–}CH_2\text{–}CH_2\text{–}CH_3$$

$$0.333(A)+0.666(X) = 0.660 \qquad (2)$$

where

A is the known density of the $CH_3$ functional group, in grams per milliliter,

X is the unknown density of the $CH_2$ functional group, in grams per milliliter, and

0.660 is the known density of hexane at 20°C compared to water at 4°C, in grams per milliliter.

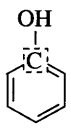
The density for the $CH_2$ functional group was determined to be 0.83 g/mL. After solving equations similar to equation 2 to determine the density of the $CH_2$ functional group in the series of hydrocarbons, the density values were averaged and the average value (0.83 g/mL) was used for that functional group.

The density of the methine (CH) functional group was calculated in the same manner. Similarly, a series of alcohols was used to calculate the density of the $CH_2OH$, CHOH, and COH functional groups. Ethers were used to calculate the density of the $CH_2$-O-, $CH_3$-O-, and CH-O- functional groups, and ketones were used to calculate the density of the C=O functional groups. The density of the benzene functional group was used to estimate the aromatic C-H density.

For aromatic compounds, the substituted carbon functional group had a greater density than that of the benzene functional group and was calculated in a manner similar to that shown in the following examples:

Example 1:

phenol: 0.833(A) + 0.167(X) = 1.057



where

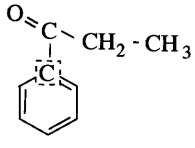
A is the known density of benzene functional group, in grams per milliliter,

X is the unknown density of C-OH functional group, in grams per milliliter, and

1.057 is the known density of phenol at 20°C compared to water at 4°C, in grams per milliliter.

Example 2:

propiophenone: 0.556(A) + 0.111(B) + 0.111(C) +0.111(D) + 0.111(X) = 1.009



where

A is the known density of benzene functional group, in grams per milliliter,

B is the known density of ketone functional group, in grams per milliliter,

C is the known density of $CH_2$ functional group, in grams per milliliter,

D is the known density of $CH_3$ functional group, in grams per milliliter,

X is the unknown density of aromatic carbon functional group, in grams per milliliter, and

1.009 is the known density of propiophenone at 20°C compared to water at 4°C, in grams per milliliter.

Because the density of the COOH functional group for carboxylic acids could not be determined in the same way as that for the other functional groups, solution density values were determined by *pycnometry* at a concentration of 5 percent by weight/volume at 20°C (American Society for Testing and Materials, 1955). Once the solution density, the solvent density, and the exact concentration are known, the following equation (Chervenka, 1973) can be solved for $\bar{v}$ and for the density of the solute, $1/\bar{v}$:

$$\rho_{sol} = \rho_{solv} + C[1 - \rho_{solv}(\bar{v})] \quad (3)$$

where

$\rho_{sol}$ is the density of the solution, in grams per milliliter,

$\rho_{solv}$ is the density of the solvent, in grams per milliliter,

C is the concentration, in grams per milliliter,

v is the *apparent specific volume* of the solute, in milliliters per gram, and

1/v is the density of the solute, in grams per milliliter.

The v is apparent specific volume and not partial specific volume because the sample was not analyzed at various concentrations and the resulting data were not extrapolated to zero. Once the density is determined, the calculation for the COOH functional group in the compound can be made (this was done for the compounds mentioned above).

## Dissolution of Fulvic Acid from the Suwannee River in Various Solvents

The density of the fulvic acid from the Suwannee River in various solvents was determined by

pycnometry, as previously described, with the exception of correcting for the water content in the dried sample. The following solvents were used: tetrahydrofuran (THF); dimethylformamide (DMF); dimethylsulfoxide (DMSO); 0.1 N trifluoroacetic acid/DMF; 0.1 N trifluoroacetic acid/DMSO; 0.1 N hydrochloric acid; a solution consisting of 1 percent hydrochloric acid, 5 percent distilled water, and 94 percent DMSO (Hayes, 1985); and distilled water.

A density versus concentration curve was determined for fulvic acid from the Suwannee River in distilled water at concentrations of 2.0, 1.0, 0.6, 0.4, and 0.2 percent. A similar curve also was determined for a methylated sample of the fulvic acid in anhydrous DMF at concentrations of 1.09, 0.61, 0.40, and 0.20 percent.

The density of the fulvic acid from the Suwannee River was calculated using the actual values of the proton distribution, carbon distribution, and oxygen-functional-group distribution (Leenheer and others, chap. P, this volume). The calculations were performed for the aliphatic region of the liquid-state $^{13}$C-NMR data as follows:

$$\frac{\text{Number of aliphatic hydrogens}^{1}}{\text{Number of aliphaic carbons}^{2}} = \frac{16H}{7.2C} = 2.22$$

2.22–2 = 0.22 (amount in excess of $CH_2$)

Density of $CH_2$ functional group = 0.83 g/mL
Density of $CH_3$ functional group = 0.32 g/mL
Δ density = 0.51 g/mL

0.51 g/mL
× 0.22 (amount in excess of $CH_2$)
0.11 g/mL

0.83 g/mL (density of $CH_2$ functional group)
– 0.11 g/mL
0.72 g/mL (density for aliphatic carbons)

0.72 g/mL (density for aliphatic carbons)
× 0.218 (percentage of carbons for the aliphatic region)
0.154 g/mL (density contribution for the aliphatic region)

[1]From proton-NMR data.
[2]From $^{13}$C-NMR data.

This calculation was repeated for each spectral region using the appropriate density values; then, the values for each region were added to give the density of the total sample. The process was repeated for the solid-state $^{13}$C-NMR data.

### Derivatives of Fulvic Acid from the Suwannee River

Lastly, two derivatives of the fulvic acid from the Suwannee River were made. In the first derivative, 1 g of the sample was dissolved in distilled water, titrated to pH 8 with potassium hydroxide, freeze-dried, and then dissolved in a 0.2 N potassium chloride solution with a pH of 8. This was done to determine the density of the sample as a potassium salt. The second derivative was the methylation of an aliquot of the sample in methylene chloride using diazomethane (Noyes and Leenheer, chap. M, this volume), and then the density of the derivative was determined in various solvents by pycnometry.

## EFFECTS OF SOLVENTS ON HYDROGEN BONDING AND RELATED DENSITY VALUES OF THE STANDARDS

The density of most of the functional groups was calculated using published density data of related compounds (table 1). Discrepancies between published density values and calculated density values were noted for the homologous series of monocarboxylic and dicarboxylic acids. Unlike other functional groups, there was no consistency in the calculated density values of the COOH functional group because of hydrogen-bonded aggregates. The first solvent chosen for the study of hydrogen-bonding aggregation of the carboxylic acids was THF. Using this solvent, carboxylic acids tended to remain as aggregates (fig. 2), as evidenced by the increasing density of the COOH functional group with increasing chain length. The shorter chained mono-carboxylic acids have density values of about 1.90 g/mL for the COOH functional group (equal to the density of oxalic acid, the simplest dicarboxylic acid), but, as the chain length increases, the density also increases. This probably is a result of the inability of THF to break up the hydrogen bonding. With the dicarboxylic acids, the chance of intramolecular hydrogen bonding has increased

greatly. In fact, Hendrickson, and others (1964, p. 355) reported that "intramolecular hydrogen bonding is favored over intermolecular hydrogen bonding" when they stated

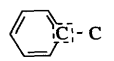
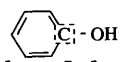
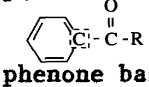
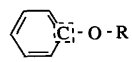
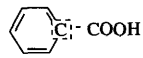
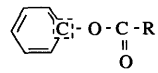
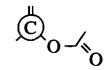
> ...A common example of entropy effects is found in cyclizations, reactions which create rings by internal or intramolecular reaction between two functional groups on the same molecule. The cyclization can be compared to the identical reaction between two separate molecules, which is called intermolecular. Both reactions have the same ΔH (assuming the cyclization does not form a strained ring) but differ in entropy. In the intermolecular case, the two molecules need to be brought together before they can react, and this constraint lowers the entropy and costs energy, whereas, in the cyclic molecule, the reactants are already together in the same molecule, and the entropy (ΔS) cost is small in their reacting (ΔS≅0)....

The effect is dramatic in most cyclizations, which are much more favored reactions than their intermolecular counterparts. Therefore, cyclization of the dicarboxylic acids is occurring and cannot be broken up with THF.

The density of the carboxylic acids was then determined in DMF; density values for the COOH functional group that were measured in THF are generally

**Table 1.** Density of functional groups

[g/mL, gram per milliliter; ND, not determined]

| Functional group | Number of related compounds used to determine density | Density (g/mL) | Standard deviation about the mean (g/mL) | Functional group | Number of related compounds used to determine density | Density (g/mL) | Standard deviation about the mean (g/mL) |
|---|---|---|---|---|---|---|---|
| $CH_3$ | 8 | 0.32 | ND | R-COOH<br>HOOC-R-COOH | 11 | 1.90 | ±0.11 |
| $CH_2$ | 7 | 0.83 | ±0.02 |  | 6 | 1.41 | ±.06 |
| CH | 4 | 1.33 | ±.04 | | | | |
| | | | | toluene base | | | |
| $CH_2OH$ | 6 | 1.27 | ±.01 |  | 6 | 2.16 | ±.18 |
| | | | | phenol base | | | |
| CHOH | 10 | 1.76 | ±.02 |  | 4 | 1.81 | ±.08 |
| | | | | phenone base | | | |
| COH | 8 | 2.31 | ±.10 |  | 6 | 1.93 | ±.06 |
| C=O | 6 | 1.74 | ±.02 |  | 5 | 2.04 | ±.08 |
| $CH_2$-O- | 6 | 1.10 | ±.02 |  | 1 | 2.46 | ND |
| $CH_3$-O- | 4 | .62 | ±.05 |  | 1 | 2.21 | ND |
| CH-O- | 1 | 1.56 | ND |  | 2 | 1.57 | ±.08 |
|  | 7 | 1.89 | ±.05 |  | 5 | .88 | ±.03 |

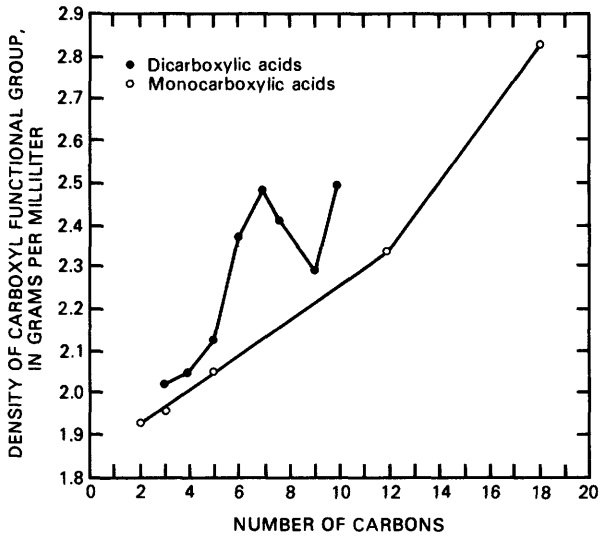
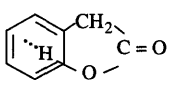



**Figure 2.** Density of the carboxyl functional group in various carboxylic acids dissolved in tetrahydrofuran.

greater than the density values for the COOH functional group that were measured in the other solvents shown in table 2. Various combinations of organic solvents (THF, DMF, or DMSO), distilled water, and hydrochloric acid were tested (based on the previous work and recommendations of Porter (1967) and Hayes (1985)), and the density of the COOH functional group was reduced to a minimum value of about 1.9 g/mL for the most polar solvent combinations (in 0.1 N trifluoroacetic acid/DMSO, and in the solution of DMSO, distilled water, and hydrochloric acid). This reduction in density is consistent with disruption of both intramolecular and intermolecular hydrogen bonding as solvent polarity is increased. The density data for the COOH functional group of acetic and succinic acid in distilled water (table 2) indicate that distilled water is not sufficiently polar to disrupt hydrogen bonding of the COOH functional groups. The acids that had the COOH functional group on an aliphatic side chain attached to an aromatic ring (benzilic, phenylacetic, hydrocinnamic, mandelic acids) consistently had larger density values for the COOH functional group than did the other carboxylic acids tested. Strong intramolecular bonding between the carboxylic acid and the $\pi$ electrons in the aromatic ring is postulated to occur as shown below (Ferguson, 1963):

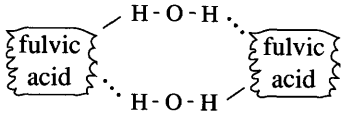



Benzilic and phenylacetic acid had some decrease in density in the solvents containing DMSO and acids, but these acids never decreased in density to the predicted value of 1.9 g/mL for the free COOH functional group. This intramolecular hydrogen bonding between the COOH functional group and the rings may be so great that even DMSO-acid water is not polar enough to break the intramolecular bond.

The solvent consisting of DMSO, distilled water, and hydrochloric acid (Hayes, 1985) was strong enough to disrupt the hydrogen bonding in the succinic, adipic, and benzoic acids. This produced a density for the COOH functional group of about 1.90 g/mL in a totally disaggregated system.

## EFFECTS OF SOLVENTS ON FULVIC ACID FROM THE SUWANNEE RIVER

After calculating the density of the various functional groups, the next step was to determine the density of fulvic acid from the Suwannee River in the various solvents (table 3). The THF (either by itself or in conjunction with distilled water or hydrochloric acid or both) did not disaggregate the sample. This is because the carboxylic acids (of which there are four in the proposed average molecule (Leenheer and others, chap. P, this volume)) seem prone to associate with water molecules in aprotic solvents, such as THF, at the partial expense of dimerization (Davis, 1968). The increase in the density of the sample in THF and distilled water compared to that in THF alone can be explained by the fact that the water can be used as a bridge between polar groups in fulvic acid structures and can form a mixed water/fulvic-acid aggregate as shown below:



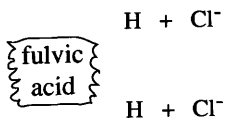

When the density of the sample was determined in THF, distilled water, and hydrochloric acid, the density increased only slightly in comparison to the density in THF alone. This is because the hydrochloric acid is more powerful than water and will form an attachment to polar functional groups. At this point,

**Table 2.** Density of carboxylic acid and associated carboxyl functional groups measured in various solvents

[COOH, carboxyl functional group; g/mL, grams per milliliter; --, not determined]

| Carboxylic acid | Tetrahydrofuran | | Dimethyl-formamide | | 0.1 N trifluoro-acetic acid/ dimethylformamide | | 0.1 N trifluoro-acetic acid/ dimethylsulfoxide | | 94 percent dimethylsulfoxide 5 percent distilled water 1 percent hydrochloric acid | | Distilled water | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Density (g/mL) | COOH density (g/mL) | Density (g/mL) | COOH density (g/mL) | Density (g/mL) | COOH density (g/mL) | Density (g/mL) | COOH density (g/mL) | Density (g/mL) | COOH density (g/mL) | Density (g/mL) | COOH density (g/mL) |
| Acetic-------- | 1.123 | 1.93 | 1.120 | 1.92 | -- | -- | -- | -- | -- | -- | 1.196 | 2.07 |
| Propionic----- | 1.026 | 1.96 | 1.037 | 1.99 | -- | -- | -- | -- | -- | -- | -- | -- |
| Valeric------- | 0.9719 | 2.05 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| Heptanoic----- | -- | -- | 0.9366 | 2.70 | -- | -- | -- | -- | -- | -- | -- | -- |
| Octanoic------ | -- | -- | 0.9211 | 2.06 | -- | -- | -- | -- | -- | -- | -- | -- |
| Nonanoic------ | -- | -- | 0.9215 | 2.18 | -- | -- | -- | -- | -- | -- | -- | -- |
| Lauric-------- | 0.9103 | 2.33 | 0.8929 | 2.22 | -- | -- | -- | -- | -- | -- | -- | -- |
| Stearic------- | 0.9271 | 2.82 | 0.8782 | 2.00 | -- | -- | -- | -- | -- | -- | -- | -- |
| Malonic------- | 1.625 | 2.02 | 1.589 | 1.96 | -- | -- | -- | -- | 1.468 | 1.81 | -- | -- |
| Succinic------ | 1.438 | 2.05 | 1.434 | 2.04 | 1.458 | 2.09 | 1.362 | 1.89 | 1.347 | 1.87 | 1.498 | 2.17 |
| Glutaric------ | 1.348 | 2.13 | 1.291 | 1.98 | -- | -- | -- | -- | 1.262 | 1.91 | -- | -- |
| Adipic-------- | 1.337 | 2.37 | 1.285 | 2.21 | 1.261 | 2.14 | 1.225 | 2.03 | 1.220 | 2.01 | -- | -- |
| Pimelic------- | 1.308 | 2.48 | 1.219 | 2.17 | -- | -- | -- | -- | 1.169 | 2.00 | -- | -- |
| Suberic------- | 1.225 | 2.41 | 1.138 | 2.06 | -- | -- | -- | -- | 1.111 | 1.95 | -- | -- |
| Azelaic------- | 1.150 | 2.29 | 1.138 | 2.23 | -- | -- | -- | -- | 1.098 | 2.05 | -- | -- |
| Sebacic------- | 1.163 | 2.49 | 1.114 | 2.25 | -- | -- | -- | -- | 1.065 | 2.01 | -- | -- |
| Citric-------- | -- | -- | 1.848 | 2.37 | -- | -- | -- | -- | 1.605 | 1.88 | -- | -- |
| Tartaric------ | -- | -- | 1.845 | 1.93 | -- | -- | -- | -- | 1.704 | 1.65 | -- | -- |
| Malic--------- | -- | -- | 1.619 | 1.94 | -- | -- | -- | -- | -- | -- | -- | -- |
| Benzoic------- | -- | -- | 1.1985 | 2.08 | -- | -- | -- | -- | 1.178 | 1.93 | -- | -- |
| Phthalic------ | -- | -- | 1.471 | 2.10 | -- | -- | -- | -- | -- | -- | -- | -- |
| Terephthalic-- | -- | -- | 1.429 | 1.94 | -- | -- | -- | -- | -- | -- | -- | -- |
| Paratoluic---- | -- | -- | 1.170 | 2.09 | -- | -- | -- | -- | -- | -- | -- | -- |
| Hydroxybenzoic | -- | -- | 1.390 | 2.16 | -- | -- | -- | -- | -- | -- | -- | -- |
| Benzilic------ | -- | -- | 1.286 | 4.31 | 1.306 | 4.60 | 1.267 | 4.04 | 1.293 | 4.41 | -- | -- |
| Phenylacetic-- | -- | -- | 1.186 | 2.85 | 1.186 | 2.85 | -- | -- | 1.1385 | 2.47 | -- | -- |
| Hydrocinnamic- | -- | -- | 1.1345 | 2.76 | 1.139 | 2.80 | -- | -- | -- | -- | -- | -- |
| Mandelic------ | -- | -- | 1.352 | 3.25 | 1.331 | 3.08 | -- | -- | -- | -- | -- | -- |

no further attachment can be made in this solvent system as shown below:




DMF and DMSO are considered by Hayes (1985, p. 362) to be the best organic solvents. He states that, "Their solvation action can be partially explained by the abilities of these solvents to provide polar and nonpolar moieties for solvation and to act as acceptors in hydrogen-bonding systems." DMSO is the best organic solvent tested for humic acids, and its effectiveness is improved by the presence of small amounts of concentrated acids (Hayes, 1985). This is shown by the large decrease in the density resulting from the use of DMSO, distilled water, and hydrochloric acid rather than only DMSO as the solvent. Hayes also explains that "DMSO-water interactions are stronger than the associations between the water molecules, and the organic molecules associate preferentially with two molecules of water. It also can be expected to associate with the phenolic and carboxyl group and to break the interstrand and intrastrand hydrogen bonds" (Hayes, 1985, p. 361); this produces a smaller density value.

## DENSITY VERSUS CONCENTRATION CURVES

The density versus concentration curve for fulvic acid from the Suwannee River in distilled water (fig. 3) indicates that aggregation is taking place at higher concentrations. Chebaevskii and others (1971) reported that, at higher concentrations, the partial specific volume, of which density is the inverse, is decreasing. This is due to the ability of the smaller particles, which unite together through hydrogen bonds, to form aggregates. Wershaw and Pinckney (1973) have determined that the degree of aggregation is a function of both pH and concentration. As shown in figure 3, density decreases as concentration decreases. According to Pimentel and McClellan (1960), intramolecular hydrogen bonds are retained, even at the lowest concentrations; whereas, intermolecular hydrogen bonds are broken at small concentrations as the association is disrupted. Therefore, one can determine from this curve that intermolecular hydrogen bonding is occurring, and one also can determine a valid density by extrapolating to a zero concentration. This density value would be approximately 1.45 g/mL.

The density of methylated fulvic acid from the Suwannee River was determined in DMSO, DMF,

**Table 3.** Density of fulvic acid from the Suwannee River in various solvents

| Solvent | Density (grams per milliliter) |
|---|---|
| Potassium salt in 0.2 N potassium chloride at pH 8 | 2.198 |
| 95 percent tetrahydrofuran and 5 percent distilled water | 1.827 |
| 94 percent tetrahydrofuran, 5 percent distilled water, and 1 percent hydrochloric acid | 1.757 |
| Tetrahydrofuran | 1.755 |
| 0.1 N trifluoroacetic acid/dimethylformamide | 1.731 |
| Distilled water | 1.679 |
| 0.1 N hydrochloric acid | 1.670 |
| 0.1 N trifluoroacetic acid/dimethylsulfoxide | 1.658 |
| Dimethylsulfoxide | 1.646 |
| 94 percent dimethylformamide, 5 percent distilled water, and 1 percent hydrochloric acid | 1.628 |
| Dimethylformamide | 1.616 |
| Methylated; dimethylformamide | 1.533 |
| 94 percent dimethylsulfoxide, 5 percent distilled water, and 1 percent hydrochloric acid | 1.465 |
| Methylated; dimethylsulfoxide | 1.379 |
| Methylated; anhydrous dimethylformamide | 1.379 |

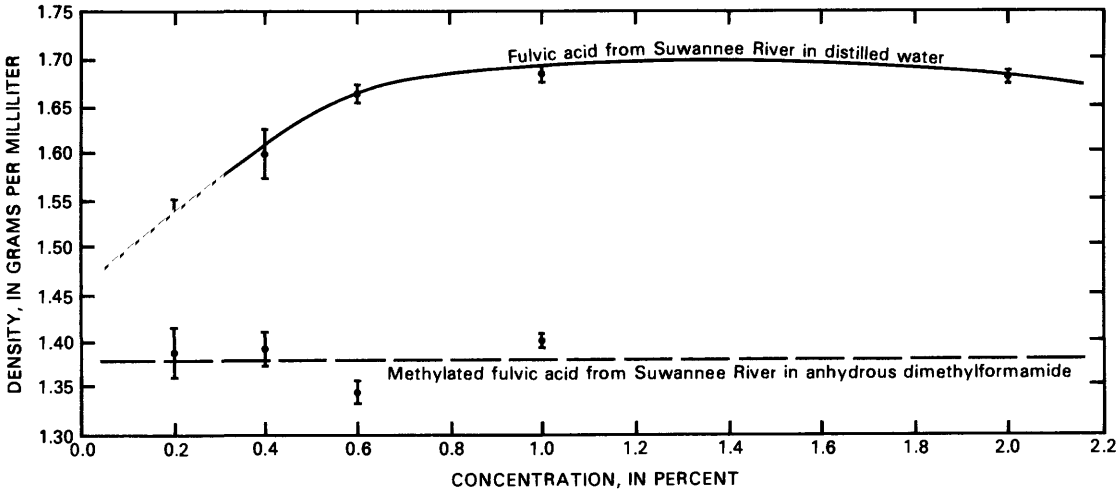



**Figure 3.** Concentration dependence of density of fulvic acid from the Suwannee River. Vertical bars indicate analytical error.

and anhydrous DMF. By methylating the COOH functional groups, there were no COOH functional groups available to participate in hydrogen bonding. Although the DMSO eliminates any intramolecular bonding, it is not anhydrous. At lower sample concentrations, it forms a complex of water, solvent, and sample in which the water lowers the density excessively. However, in DMF, although the sample was methylated, a small quantity of water that hydrogen bonds with the sample to lower the density (as was the case in DMSO). It was not until the use of anhydrous DMF that a valid density in DMF was determined. The density was independent of concentration—this indicates the methylated sample was completely disaggregated by anhydrous DMF. The average density value of 1.38 g/mL in DMF was lower than the extrapolated density value of 1.45 g/mL for the underivatized sample at infinite dilution (fig. 3); this density decrease between the methylated and nonmethylated samples corresponds to the calculated density decrease due to methylation.

## CALCULATED DENSITY OF FULVIC ACID FROM THE SUWANNEE RIVER

The calculated density of the proposed structural model of fulvic acid from the Suwannee River was 1.454 g/mL based on the liquid-state $^{13}$C-NMR data (table 4) and was 1.485 g/mL based on the solid-state $^{13}$C-NMR (table 5). There was insufficient difference between the two calculated density values to determine aromaticity. It appears that the empirical formula is the main constraint on the density because, as the density contribution from aromaticity decreases when going from the liquid-state $^{13}$C-NMR data to the solid-state $^{13}$C-NMR data, the aliphatic H/C ratio also decreases—this results in a compensatory increase in density. For example, the percentage carbon distribution in the aromatic region decreases from 35.0 to 24.9 percent from the liquid-state $^{13}$C-NMR data to the solid-state $^{13}$C-NMR data (tables 4 and 5), but the percentage carbon distribution in the methine region must increase from 0 to 21 percent to stay within the constraints of the empirical formula. The decrease in aromaticity between the two data sets decreases density contribution by 0.163 g/mL (tables 4 and 5), but the increase in methine carbon (density = 1.33 g/mL) results in a concomitant increase in density distribution by 0.234 g/mL (tables 4 and 5). However, the calculated density values of fulvic acid from the Suwannee River are similar to the measured the density value in the DMSO, distilled water, and hydrochloric acid mixture (table 3); the density value is similar to that in distilled water when concentrations are extrapolated to infinite dilution (fig. 3).

The liquid-state $^{13}$C-NMR data are believed to be quantitative based on the study of Thorn (chap. N, this volume); however, no quantitative studies were performed on the fulvic acid in the solid state. The initial objective of assessing the quantitative nature of both liquid-state and solid-state $^{13}$C-NMR data by measurement of density was not met because of the great constraint of the empirical formula on density.

**Table 4.** Calculated density of fulvic acid from the Suwannee River, by spectral regions, for liquid-state, carbon-13 nuclear-magnetic-resonance data

| Nuclear-magnetic-resonance region | Percentage carbon distribution | Density (grams per milliliter) | Density contribution = percentage carbon × density (grams per milliliter) |
|---|---|---|---|
| Aliphatic---------- | 21.8 | 0.72 | 0.157 |
| H-C-O[2]---------- | 20.7 | 1.620 | .335 |
| Aromatic---------- | 35.0 | 1.601 | .560 |
| COOH + ester------ | 16.9 | 1.878 | .317 |
| Ketone------------ | 4.9 | 1.74 | .085 |
| Calculated density--- | | | 1.454 |

[1]Actual values were used (not rounded values) to determine average structural models.
[2]Hemiacetal included with ether.

**Table 5.** Calculated density of fulvic acid from the Suwannee River, by spectral regions, for solid-state, carbon-13 nuclear-magnetic-resonance data

| Nuclear-magnetic-resonance region | Percentage carbon distribution | Density (grams per milliliter) | Density contribution= percentage carbon × density (grams per milliliter) |
|---|---|---|---|
| Aliphatic---------- | 34.6 | 1.13 | 0.391 |
| H-C-O[2]---------- | 14.8 | 1.537 | .227 |
| Aromatic---------- | 24.9 | 1.596 | .397 |
| COOH + ester------ | 18.0 | 1.984 | .338 |
| Ketone------------ | 7.6 | 1.74 | .132 |
| Calculated density--- | | | 1.485 |

[1]Actual values were used (not rounded values) to determine average structural models.
[2]Hemiacetal included with ether.

## CONCLUSIONS

From the data presented herein, it was determined that one cannot predict the degree of aromaticity on the basis of density of the sample. This was shown by comparing the liquid-state $^{13}$C-NMR data (which has 35.0 percent aromatic carbons) to the solid-state $^{13}$C-NMR data (which has 24.9 percent aromatic carbons). Instead, it was found that, by determining the density of fulvic acid from the Suwannee River in various solvents, one could determine the type and degree of aggregation occurring in the system.

The increase in density of fulvic acid from the Suwannee River in distilled water exceeded both the calculated density and the measured density in acidified DMSO (which disrupts solute-solute hydrogen bonds). The density increase provides substantial evidence that this fulvic acid is extensively intermolecularly hydrogen bonded in distilled water at moderate to large concentrations. Intermolecular hydrogen bonding in the fulvic acid causes aggregation that may result in molecular-weight and molecular-size measurements being too large if the measurements are made in an aqueous solution at concentrations exceeding 0.1 percent.

## REFERENCES CITED

American Society for Testing and Materials, 1955, Standard test method for density and relative density (specific gravity) of liquids by Lipkin bicapillary pycnometer: Philadelphia, Standard D941, 6 p.

Bondi, A., 1964, van der Waals volumes and radii: Journal of Physical Chemistry, v. 68, no. 3, p. 441–451.

Chebaevskii, A.L., Tuer, N.A., and Stepanova, N.P., 1971, Densities and partial specific volumes of fulvic acids in aqueous solutions: Pochvovedeniye v. 7, p. 31–37.

Chervenka, C.H., 1973, A Manual of Methods for the Analytical Ultracentrifuge: Palo Alto, California, Spinco Division of Beckman Instruments, Inc., 100 p.

Davis, M.M., 1968, Acid-base behavior in aprotic organic solvents: National Bureau of Standards Monograph 105, 151 p.

Ferguson, L.N., 1963, The Modern Structure Theory of Organic Chemistry: Englewood Cliffs, N.J., Prentice-Hall, 600 p.

Hayes, M.H.B., 1985, Extraction of humic substances from soil, *in* Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediment, and Water: New York, John Wiley, p. 329–362.

Hendrickson, J.B., Cram, D.J., and Hammond, G.S., 1964, Organic Chemistry: New York, MacGraw-Hill, 1279 p.

Partington, J.R., 1960, A Short History of Chemistry: New York, Harper and Brothers, 416 p.

Pimentel, G.C., and McClellan, A.L., 1960, The Hydrogen Bond: San Francisco, W.H. Freeman and Company, 475 p.

Porter, L.K., 1967, Factors affecting the solubility and possible fractionation of organic colloids extracted from soil and leonardite with an acetone-$H_2O$-HCl solvent: Journal of Agriculture and Food Chemistry, v. 15, p. 807–811.

Schnitzer, M., 1978, Humic substances, chemistry and reactions *in* Schnitzer, M., and Khan, S.U., eds., Soil Organic Matter, Developments in Soil Science 8: Amsterdam, The Netherlands, Elsevier, 319 p.

Traube, J., 1895, Über das Molekularvolumen: Berichte der Deutschen chemischen Gesellschaft, v. 28, p. 2722–2728.

Van Krevelen, D.W., and Chermin, H.A.G., 1954, Chemical structures and properties of coal. I. Elementary composition and density: Fuel, v. 33, p. 79–87.

Weast, R.C., ed., 1973, Handbook of Chemistry and Physics (53d ed.): Cleveland, The Chemical Rubber Co., 2287 p.

Wershaw, R.L., and Pinckney, D.J., 1973, The fractionation of humic acids from natural water systems: U.S. Geological Survey Journal of Research, v. 1, p. 361–366.

Chapter P

# Structural Components and Proposed Structural Models of Fulvic Acid from the Suwannee River

*Compiled by* J.A. Leenheer, D.M. McKnight, E.M. Thurman, *and* Patrick MacCarthy

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### FIGURES

TABLES



## Abstract

The data for the various structural moieties of fulvic acid from the Suwannee River were synthesized into a number of chemical structural models. Major elements of carbon, hydrogen, and oxygen, which occur in every molecule of the fulvic acid, were assembled into three models that fit the number-averaged data. The process of model synthesis revealed errors in analytical data that were corrected by development of improved methods. The models aided in development of hypotheses of various biogeochemical processes that transform plant precursors into the complex mixture of organic solutes that comprise the fulvic acid. The models demonstrate the importance of spatial arrangements between functional groups (such as lactone formation, intramolecular hydrogen bonding, and negative-charge potential on the exterior of the molecules).

Minor characteristics of fulvic acid from the Suwannee River, defined as occurring less than once in a number-average model, are quantified in terms of their frequency of occurrence and are discussed in terms of their significance to environmental processes. Minor characteristics that are quantified include nitrogen, sulfur, phosphorus, amino acids, numbers of different metal binding sites, and organic free-radical contents. Fluorophores and molecular aggregate structures are discussed in a qualitative sense. Speculative molecular models are presented for each of the minor characteristics of the fulvic acid.

## INTRODUCTION

The concept of structural models for humic substances will continue to be a controversial topic until there are structural determinations of a number of individual components that comprise humic-substance mixtures. Some researchers maintain that structural models have little meaning because the diversity of chemical structures within humic-substance fractions and subfractions is so great that an "average" or "representative" structural model has little meaning given the heterogeneity of the mixture. Those who maintain an alternative viewpoint claim that structural models are figurative representations of data that are useful for predicting gross chemical and physical properties of humic substances and discovering possible spatial arrangements of various structural moieties. The structural-model approach is adopted in this chapter. Proponents of models state that models are not intended to represent accurate structures, but they do represent approximations that can be modified when new data become available.

There are two differing objectives into which the various contributions to this volume are grouped; the nature of the objective determines the relevance of structural models to each study. Those contributions in the first part of this volume (which concern biogeochemical interactions of humic substances with each other and with various introduced contaminants) invoke minor structural components, such as strong, metal-binding sites, organic free radicals, and fluorophore content to explain environmentally significant phenomena. Those contributions in the second part of this volume concern major properties of humic substances that pertain to structural studies and exclude minor components in explaining the gross chemical and physical properties of humic substances from the Suwannee River.

The objective of this summary report is to propose, from the data presented in this volume, various structural models for fulvic acid from the Suwannee River that account for both major and minor characteristics of interest. The major and minor characteristics will be presented in a number of separate models to emphasize the diversity of the mixture that is fulvic acid. Hopefully, the data synthesis presented in this summary chapter will not only summarize the overall study but also will indicate interrelations, such as spatial relations between functional groups, that lead to new hypotheses for future studies.

## MAJOR CHARACTERISTICS OF THE FULVIC ACID

Major elements that occur in every molecule of fulvic acid from the Suwannee River are carbon, hydrogen, and oxygen. The distribution of carbon in molecular frameworks that comprise the fulvic acid are described by carbon-13 nuclear-magnetic-resonance ($^{13}$C-NMR) data (Thorn, chap. N, this volume); the functional groups that contain hydrogen and oxygen are described by proton nuclear-magnetic-resonance data ($^{1}$H-NMR) (Noyes and Leenheer, chap. M, this volume), $^{13}$C-NMR data (Thorn, chap. N, this volume), and titrimetric studies (Bowles and others, chap. L, this volume). Nitrogen, sulfur, and phosphorous were not considered as major elements that occur in every molecule because of the analytical constraint imposed by molecular weight. Susceptibility of fulvic acid to hydrolysis and oxidation introduces additional uncertainties in molecular structure and functional-group composition.

### Significance of Molecular Weights on Structure and Properties

*By* G.R. Aiken

No structural model of fulvic acid from the Suwannee River would be complete without consideration of molecular-weight data (Aiken and others, chap. J, this volume). Of particular importance are weight-average to number-average molecular-weight ratios ($\overline{M}_w/\overline{M}_n$) obtained by equilibrium ultracentrifugation measurements that provide an estimate of the degree of polydispersity of the sample. For instance, the $\overline{M}_w/\overline{M}_n$ ratio of 2.0 ($\overline{M}_w$ = 1,335, $\overline{M}_n$ = 655) obtained for the fulvic acid clearly indicates this sample to be a polydisperse mixture of molecules of various molecular weights. Because humic substances are not ordered polymers, it is not possible for any model to adequately describe all the components of this mixture. However, models can be generated based on average properties of the sample, including molecular weight.

The moderate molecular weights of fulvic-acid samples from the Suwannee River also have important geochemical and biogeochemical implications. Stewart and Wetzel (1981), for instance, have suggested that smaller molecular weight fulvic acids are more inhibitory to calcite precipitation than are larger molecular weight humic acids. In other work, Chiou and others (1986) have reported that significant solubility enhancements of hydrophobic organic pollutants and pesticides by humic substances can be described in terms of a partition-like interaction. The effectiveness of humic substances in enhancing solute solubility is controlled both by the molecular size and polarity of the humic substances. Larger, soil-derived humic substances are more effective than smaller molecular weight fulvic acids.

A relation between the molecular weights of humic substances and their biological activity also has been noted (Steinberg and Muenster, 1985). Stewart and Wetzel (1982) have reported that humic substances of small apparent molecular weight are more stimulatory to both $^{14}$C assimilation and alkaline phosphatase activity by algal-bacterial assemblages than are humic substances of large apparent molecular weight. Visser (1985) also has noted the increased effect of smaller molecular weight humic substances on the physiological activity of microbial cells. Others have noted that the presence of humic substances enhanced the uptake of iron by diatoms in laboratory studies (Prakash and others, 1973). This effect may be due to changes in iron speciation in the aquatic medium external to the cell, or it may be a more indirect biochemical effect (McKnight and Feder, in press).

### Structural Instabilities in the Fulvic Acid

*By* Patrick MacCarthy *and* R.C. Antweiler

The fundamental experimental results of the acid-base titrimetric studies of fulvic acid from the Suwannee River (Bowles and others, chap. L, this volume) are:

1. Fulvic acid consumes hydroxide slowly, particularly at pH values greater than 7 (that is, alkali needs to be added to maintain a fixed pH): (1) The rate of hydroxide consumption increases as a function of pH; and (2) at a fixed pH, the rate of hydroxide consumption decreases as a function of time.

2. When a sample that has been standing at high pH for several hours is rapidly titrated to a low pH value, it then consumes acid slowly (that is, acid needs to be added to maintain a fixed pH), but total acid consumption is not equivalent to previous hydroxide consumption.

These experimental results indicate that the fulvic acid undergoes some type of hydrolysis during alkaline conditions and that the hydrolysis is partially reversible during reacidification. The hypothesis that the slow consumption of base and acid is because of slow diffusion of $OH^-$ and $H_3O^+$ ions, respectively, to the reacting sites can be reasonably dismissed on the basis that fulvic acid from the Suwannee River consists of relatively small molecules (average molecular weight about 800 daltons) and that this aquatic material occurs in the dissolved state.

The above statements largely describe fundamental experimental results—the major difficulty occurs when attempts are made to impose a molecular interpretation on the phenomena. As discussed by Bowles and others (chap. L, this volume), there are a variety of possible hydrolytic mechanisms that can explain the experimental results. On the basis of supporting infrared spectroscopic data, it is suggested that the slow consumption of alkali and subsequent slow uptake of acid by fulvic acid from the Suwannee River is due, at least in part, to ester hydrolysis/ reesterfication reactions. Considering the extreme complexity of humic substances, it is likely that other mechanisms for the uptake of alkali and acid by the fulvic acid also are involved.

However, regardless of the actual specific mechanisms involved, the fundamental experimental results have implications for the study of humic substances. The primary reasons for studying humic substances are to better understand their fundamental chemical nature as they occur in the environment and to learn more about their geochemical reactions and fate in the environment. Most studies of humic substances are done on isolated samples in the laboratory. Thus, it is critical that the isolated material be identical, or as similar as possible, to that which occurs in the environment. The introduction of artifacts during the extraction, isolation, and purification procedures need to be minimized.

Virtually all extraction procedures for humic substances from water and soil involve contacting the sample with a high pH solution (pH = 13), sometimes at high temperatures (for example, 60°C) for prolonged periods (frequently 12 h), followed by acidification of the sample to pH 1 to 2. In the specific instance of fulvic acid from the Suwannee River, the original water sample first was acidified to pH 2.0 prior to sorption onto an XAD–8 resin column; the humic substances subsequently were eluted from the column using 0.1 N NaOH. The sample then was reacidified to obtain the humic/fulvic separation. Each sample was contacted with alkali again and then passed through a strong, acid, cation exchanger (Malcolm and others, chap. B, this volume). Even though the fulvic acid was not in contact with alkali for prolonged periods and the isolation was carried out at room temperature, it seems that there was opportunity for hydrolysis of the fulvic acid to occur, based on the data presented by Bowles and others (chap. L, this volume). The extent of hydrolysis that occurred during the isolation of this specific sample and the extent of reesterification are not known. Therefore, the isolated substances were not exactly identical to those that existed in the original water sample collected from the Suwannee River.

The titrimetric and pH-stat experiments were conducted in an oxygen-free atmosphere, whereas no such precautions were taken in the actual isolation of the fulvic acid. Many phenolic compounds participate in oxidative reactions at alkaline pH values; it is well documented that humic substances consume oxygen during alkaline conditions. Consequently, it is likely that the isolated fulvic acid has undergone both hydrolytic and oxidative reactions to some degree. Even though such alterations need to be considered when extrapolating results of laboratory experiments to environmental conditions, hopefully, the extent of artifact formation is sufficiently small so as not to nullify any major conclusions based on these experiments.

Focusing more specifically on the ester-hydrolysis hypothesis, the hydrolyzed material would be expected to be more hydrophilic than the original material. Conclusions that concern the interaction of this material with nonpolar organic solutes may not be entirely relevant to the association of "true" fulvic acid with nonpolar organic compounds in the environment. Likewise, the increased content of carboxyl and hydroxyl functional groups in the hydrolyzed product could alter its metal-complexing properties compared to those of the original source material. Other properties of the material, such as its propensity for sorbing to mineral and oxide surfaces, also would be affected by these chemical reactions. Hydrolysis could result in the formation of smaller molecules—properties that are based on molecular weight, size, or shape could be substantially affected by hydrolysis reactions.

Aiken and Malcolm (1987) have reviewed the literature on alkaline hydrolysis of humic substances during extraction. They conclude that alkaline hydrolysis has been reported to decrease molecular weights of soil humic substances, but no significant effects on molecular weight were reported for aquatic fulvic acids.

The effects of the hydrolysis reactions during alkaline conditions could be partly offset by the reesterification reactions that occur at acidic pH values. However, there is no guarantee that the reesterification reactions eliminate the effects of the initial hydrolysis reactions. For example, in a mixture as complex as fulvic acid, there would be opportunity for transesterification reactions to occur. The products of reesterification reactions might be different from the components in the original humic substances. These considerations require more detailed investigation in the future.

The isolated humic substances probably have much in common with the humic substances that occur in nature. Differences may be more in degree than in kind; nevertheless, such differences are important. The implications of the hydrolytic and oxidative reactions in the extraction and isolation of humic substances are likely to be much more serious when isolation conditions are more severe, as is commonly the situation for soil humic substances.

The existence of ester groups in fulvic acid from the Suwannee River (the presence of which is consistent with the hydrolytic reactions as supported by infrared spectrometry) is of interest because it provides additional information about structural entities within the fulvic acid. Further experiments need to be conducted to learn more about the specific types of esters that are naturally present in the fulvic acid. Most esters hydrolyze readily under alkaline conditions, but the reverse reaction does not usually occur under acidic conditions. Five- and six-membered ring lactones (cyclic esters) are exceptions, however, in that they readily undergo hydrolysis and reesterification reactions; the reesterification reaction is caused by the proximity of the carboxyl and hydroxyl functional groups in the hydrolyzed product. The fulvic acid from the Suwannee River may contain a mixture of lactones and noncyclic esters—this would account for the fact that the hydrolysis reaction was not completely reversible.

## Average Structural Models for the Fulvic Acid

*By* J.A. Leenheer

The data and methods used for formulating average structural models of fulvic acid from the Suwannee River are given in table 1. The molecular weight of the structural models (732 daltons) was adjusted slightly downward from the measured number-average molecular weight (800 daltons) for convenient formulation of the models based on a core of two aromatic rings. Data for the various atomic units in the average structural models have been rounded to integer values because fractional atoms cannot be incorporated into the models. Errors due to integer rounding are a significant fraction of the measured and estimated error for most parameters in table 1.

Three structural models that were formulated based on the number-averaged data of table 1 are shown in figures 1*A*, 1*B*, and 1*C*. Evaluation of both data and methods by formulation of average structural models for the complex mixture that comprises fulvic acid from the Suwannee River has greatly expanded our knowledge of the chemistry of this fulvic acid. For example, the percentage of aromaticity (Thorn, chap. N, this volume), the significant ester content, (Noyes and Leenheer, chap. M, this volume) and the nature of ketones (Leenheer and others, 1987) in the sample were unknown prior to this study. Formulation of three average structural models (based on data in table 1 and depicted in figures 1*A*, 1*B*, and 1*C*) revealed and verified conformational constraints on the structure due to intramolecular hydrogen bonding between phenol and carbonyl functional groups. This intramolecular hydrogen bonding also promotes a more hydrophobic interior to the fulvic-acid models. The diaryl-ketone structure (figs. 1*A*, 1*B*) provides a $\pi$-electron system with extended conjugation that results in molar absorptivity in an extended region of the ultraviolet and visible spectrum. The two esters illustrate functional groups that can hydrolyze in a reversible (phenolic lactone) and nonreversible (aliphatic ester) manner. The carbohydrate moiety is mostly substituted so that only one alcoholic hydroxyl group remains. The aliphatic hydrocarbon moieties generally do not have extended chains; the chains generally are branched and frequently are terminated by carboxyl groups.

The average structural models are also based on hypothetical processes that may transform plant tannins, lignins, carbohydrates, and lipids into fulvic acid. The lactone structures (figs. 1*A*, 1*B*, and 1*C*) might result from esterification of carboxyl groups with an adjacent phenol or alcohol, photocyclization of a phenyl propane acid, or reduction of a coumarin ring. The diaryl ketone and aryl-aliphatic ketone structures (figs. 1*A*, 1*B*, and 1*C*) might result from photo-Fries rearrangements of phenolic-ester structures in hydrolyzable tannins (Leenheer and others, 1987). The uronic-acid structure (figs. 1*A*, 1*C*) could result from hydrolysis of plant pectins and hemicelluloses; the aldaric-acid structure (fig. 1*B*) could result from oxidation of a hexose sugar. The redox conditions in the Okefenokee Swamp, the origin of the fulvic acid, water, and peat vary greatly (Bosserman, 1984), so that both oxidation and reduction processes likely affect fulvic-acid structures.

**Table 1.** Molecular data used for formulating average structural models of the fulvic acid

[NMR, nuclear-magnetic resonance; *, measured; **, estimated; ppm, parts per million]

| Parameter | Method | Value | Error (average deviation) |
|---|---|---|---|
| Number-average molecular weight. | Vapor-pressure osmometry in water[1] | 823 daltons | ±50 daltons* |
| | Vapor-pressure osmometry in tetrahydrofuran[1] | 781 daltons | ±30 daltons* |
| | Average of above | 800 daltons | |
| Elemental contents corrected for moisture and ash contents: | | | |
| Carbon (C) | Combustion[2]; measurement of $CO_2$ | 53.8 percent | ±0.1 percent* |
| Hydrogen (H) | Combustion[2]; measurement of $H_2O$ | 4.3 percent | ±0.1 percent* |
| Oxygen (O) | Reductive pyrolysis[2]; measurement of CO | 40.9 percent | ±0.8 percent* |
| Nitrogen (N) | Dumas method[2] | 0.7 percent | ±0.05 percent* |
| Sulfur (S) | Combustion[2]; measurement of $SO_4$ | 0.6 percent | ±0.05 percent* |
| Phosphorus (P) | Acid digestion[2] | <0.1 percent | ±0.1 percent |
| Average molecular formula. | Synthesis of number-average molecular weight and elemental analysis. | $C_{33}H_{32}O_{19}$; molecular weight = 732 daltons | Rounded by ±0.5 percent; N and S <0.5 percent |
| Average moles of unsaturation (Φ). | $\Phi = [(2C + 2) - H]/2$ | 18 rings or pi bonds or both | -- |
| Carbon distribution by type of carbon: | Quantitative $^{13}C$-NMR spectrometry in $^{12}C$ enriched dimethylsulfoxide[3] for indicated integration range: | 33-C | -- |
| Aliphatic | 0-50 ppm | 7-C | ±1-C** |
| H-C-O (alcohol, ether, ester, acetal, ketal) | 50-95 ppm | 5-C | ±1-C** |
| O-C-O (acetal, ketal) plus aromatic | 95-110 ppm | 2-C | ±1-C** |
| Aromatic | 110-145 ppm | 8-C | ±1-C** |
| Phenols, phenolic esters, aromatic ethers | 145-165 ppm | 3-C | ±1-C** |
| Carboxyl plus ester | 165-185 ppm | 6-C | ±1-C** |
| Ketone | 185-210 ppm | 2-C | ±1-C** |

The three models in figure 1 indicate that a large diversity of structures are allowed by the constraints of the analytical data in table 1. The aromatic structure in figure 1*A* is a substituted benzophenone that might be related to tannin and lignin precursors. In figure 1*B*, the aromatic structure is a substituted anthrone that might result from the reduction of plant anthraquinones. The aromatic structure in figure 1*C*

**Table 1.** Molecular data used for formulating average structural models of the fulvic acid—Continued

[NMR, nuclear-magnetic resonance; *, measured; **, estimated; ppm, parts per million]

| Parameter | Method | Value | Error (average deviation) |
|---|---|---|---|
| Hydrogen distribution: | | 32-H | |
| Exchangeable-hydrogen distribution by type of hydrogen: | | 7-H | |
| Carboxyl | Titrimetry[4] | 4-H | ±0.2H* |
| | Methylation and proton NMR[5] | 4-H | ±0.5H** |
| Phenol | Acetylation and proton NMR[5] | 2-H | ±0.5H** |
| Alcohol | Infrared spectrometry[5] | 1-H | ±0.5H** |
| Nonexchangeable-hydrogen distribution by type of hydrogen: | Quantitative proton NMR spectrometry as sodium salt, in $D_2O$ at pH 8[6] for indicated integration range: | 25-H | |
| Isolated aliphatic---- $H_3C$-C=O, $H_2C$-C=O, H-C-C=O, | 0-1.9 ppm | 7-H | ±0.5H** |
| $H_3$-C-Φ, $H_2$-C-Φ, H-C-Φ | 1.9-3.2 ppm | 9-H | ±0.5H** |
| H-C-O | 3.2-6.2 ppm | 6-H | ±0.5H** |
| H-Φ | 6.2-8.6 ppm | 3-H | ±0.5H** |
| Oxygen distribution by type of oxygen: | | 19-O | |
| Carboxyl | Titrimetry[4] | 8-O | ±0.4-O* |
| | Methylation and proton NMR[5] | 8-O | ±1.0-O** |
| Ester | Infrared spectronetry[6] | 4-O | ±0.5-O* |
| Carboxyl+ester | $^{13}$C-NMR spectrometry[3] | 12-O | ±2.0-O** |
| Ketone | $^{13}$C-NMR spectrometry[3] | 2-O | ±0.5-O** |
| Phenol | Combination of acetylation and proton NMR[5], and infrared spectrometry. | 2-O | ±0.5-O** |
| Alcohol | Infrared spectrometry[5] | 1-O | ±0.5-O** |
| Alcohol | Acetylation-proton NMR[5] | 2-O | ±1.0-O** |
| Acetal and ketal | $^{13}$C-NMR spectrometry[3] | 1-O | ±1.0-O** |
| Ether | Deficit from total oxygen | 1-O | ±2-O |
| Ether | Excess by carbon accounting | 1-O | ±1-O |

References:


[1]Aiken and others, chap. J, this volume.
[2]Huffman and Stuber, 1985.
[3]Thorn, chap. N, this volume.
[4]Bowles and others, chap. L, this volume.
[5]Noyes and Leenheer, chap. M, this volume.
[6]Bloom and Leenheer, 1989.


is based on ellagic acid, which is a common component of condensed tannins. Many more structures are undoubtedly possible.

Three spatial relations are indicated by the models that should affect the chemistry of the fulvic acid from the Suwannee River. First, lactone formation should be statistically favored, given the abundance of carboxyl and hydroxyl groups that might exist in positions to form five- and six-membered ring lactones. Similarly, the approximately equal abundance of aromatic carbonyl groups (from ketones, acids, and esters) to phenol groups results in a high probability

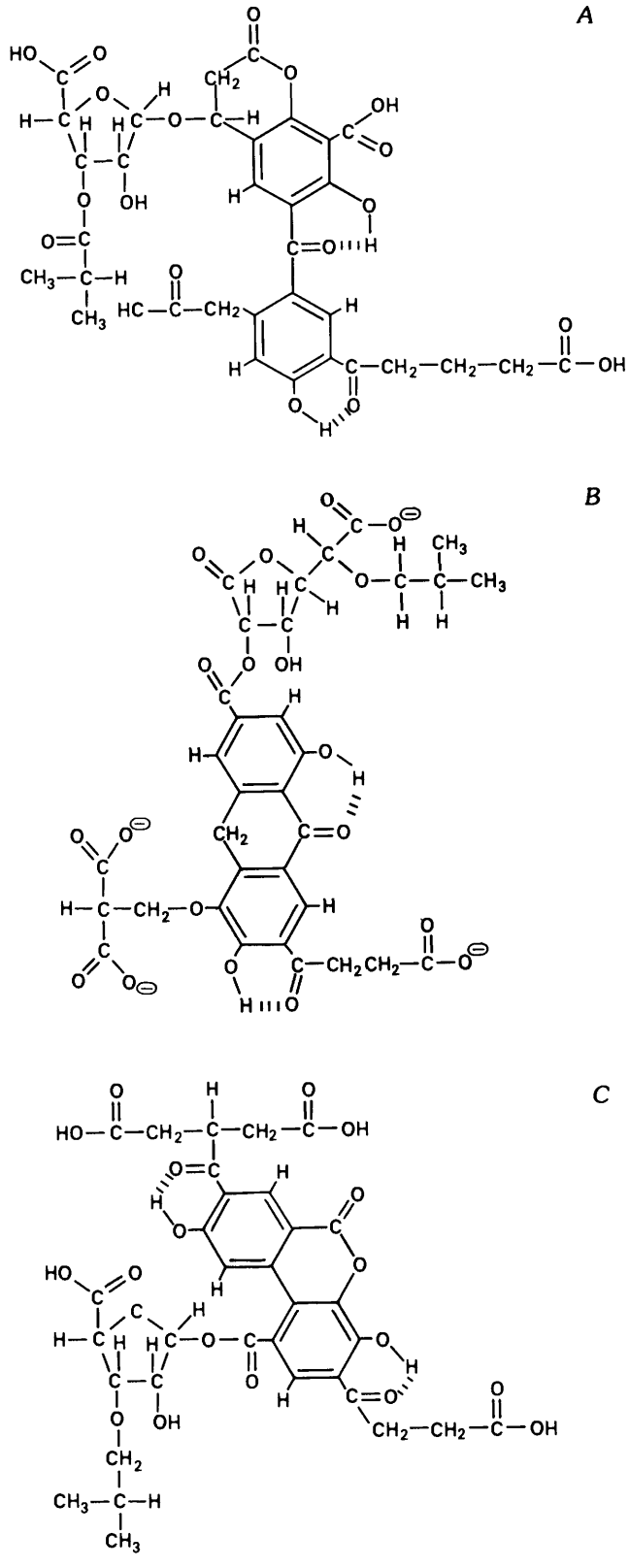



**Figure 1.** Three proposed average structural models (*A*, *B*, and *C*) of the fulvic acid.

that phenols may be adjacent to a carbonyl group where it will interact by hydrogen bonding. This may explain why many phenols in humic substances are much weaker acids than are normal phenols without adjacent substituents. Last, the predominance of aliphatic carboxyl groups will result in a negatively charged electrical double layer on the exterior of the molecule (fig. 1*B*) that will exclude hydroxide-ion attack on ester structures. This feature may explain stability of base-labile linkages of fulvic acid in certain aquatic environments.

## MINOR CHARACTERISTICS OF THE FULVIC ACID

In the first section of this report, the structural relations of the three major elements—carbon, hydrogen, and oxygen—that compose fulvic acid from the Suwannee River were considered. In presenting average structural models for the fulvic acid, it was emphasized that the sample is heterogeneous: it is a mixture composed of many nonidentical molecules that have some common properties. In this section, we further emphasize this heterogeneity by discussing minor characteristics of the fulvic acid. A minor characteristic is defined as one that occurs at an average frequency of less than one per fulvic-acid molecule.

Again, molecular weight is a critical constraint in this context. The molecular-weight of 800 daltons, which is used for the following discussion, has some uncertainty because of a possible range of 300 to 3,000 daltons. This uncertainty is a source of error in calculations of the frequency of occurrence of a given moiety. However, a molecular weight at least an order of magnitude greater than 800 daltons is required to meet the constraints for the average carbon, hydrogen, and oxygen distribution if the molecule is to contain one of every minor characteristic found in the fulvic acid.

Although these characteristics are "minor" compared to the fulvic-acid sample as a whole, these moieties are "major" when considered in the context of the subset of fulvic-acid molecules in which these moieties occur. To emphasize this point, complete structures are used to illustrate these minor characteristics. Because much less is known about the chemical nature of these minor characteristics compared to the chemical nature of carbon, hydrogen, and oxygen in fulvic acid from the Suwannee River, these structures are speculative and are presented as examples from a host of different possible structures.

Beyond the heterogeneity aspect, the minor characteristics of humic substances are quite significant for environmental chemistry and biogeochemical cycles. For example, minor characteristics include strong metal-binding sites and groups that contain nitrogen and sulfur. The contents of minor characteristics in the fulvic acid are listed in table 2.

## Minor Elements and Amino Acids

*By* E.M. Thurman *and* D.M. McKnight

Nitrogen, sulfur, and phosphorous commonly occur in trace concentrations in aquatic and terrestrial humic substances. For each of these three elements, their frequency of occurrence is too limited to affect the average structural models (fig. 1). However, the biogeochemical cycling of these elements in the environment substantially affects biological productivity. For example, in many lakes, phytoplankton abundance and species distribution is dependent on the bioavailability of nitrogen and phosphorus. The production of humic substances containing trace amounts of structural nitrogen, sulfur, and phosphorus may correspond to production of biologically unavailable or refractory nitrogen, sulfur, and phosphorus and, therefore, a loss in terms of their biogeochemical cycle. In Thoreau's Bog, an ombrotrophic bog in New England, production of aquatic humic substances is more important for the nitrogen and sulfur budgets than for the carbon budget (McKnight and others, 1985). Although the Okefenokee Swamp is a more complex ecosystem than Thoreau's Bog (Malcolm and others, chap. A, this volume), it is possible that nitrogen and sulfur cycling in the Okefenokee Swamp are similarly affected by production of aquatic humic substances. The nature of the functional groups that contain nitrogen, sulfur, and phosphorus will determine the biological availability as well as the geochemical reactivity of these groups.

The nature of sulfur and phosphorus in humic substances has received little study. The two structural models (fig. 2*A* and 2*B*) that show a sulfate ester and a phosphate ester are speculative. However, the nature of nitrogen in soil humic substances has been studied in some detail (for example, Schnitzer, 1985). Amino-acid groups are a significant form of nitrogen in humic substances; amino-acid composition of fulvic acid from the Suwannee River is discussed by Thurman and Malcolm, chapter F, this volume.

**Table 2.** Minor characteristics of the fulvic acid

[nmol/mg, nanomoles per milligram; mol/mg C, moles per milligram of carbon]

| Characteristic | Content[1] | Frequency of occurrence (one per stated number of molecules with a molecular weight of 800 daltons) |
|---|---|---|
| Nitrogen (N) | 0.7 percent | 2.5 |
| Sulfur (S) | 0.6 percent | 6.9 |
| Phosphorus | <0.1 percent | 42 |
| Amino acids | 34 nmol/mg | 10.0 |
| Metal-binding sites, nitrogen-containing functional groups[2]. | (½ of N) | 5.0 |
| Metal-binding sites, sulfur-containing functional groups[2]. | (½ of S) | 13.8 |
| Organic free radicals | 1 or 2 | 500 to 1,000 |
| Metal-binding sites | | |
| Type I: log k = 6 | $1.2 \times 10^{-6}$ mol/mgC | 2 |
| Type II: log k = 8 | $2.7 \times 10^{-7}$ mol/mgC | 9 |

[1]Analytical error in these values is about 15 percent.
[2]Hypothetical sites.

Concentrations of amino-acid residues in the fulvic (34 nmol/mg) and humic (110 nmol/mg) acids from the Suwannee River were substantially less than those reported for soil fulvic and humic acids, which range from 150 to 775 nmol/mg for fulvic acid and from 700 to 1,800 nmol/mg for humic acid (Schnitzer, 1985; Thurman, 1985). One simple explanation for this difference may be because of different isolation methods. The extraction procedure for soil-derived humic substances has not used XAD–8 resin isolation followed by cation exchange, as was used for fulvic acid from the Suwannee River. The cation-exchange step removes amino acids that are associated with humic substances and also removes humic fractions that are enriched in amino acids, such as cationic humic substances (MacCarthy and O'Cinneide, 1974). Thus, data for soil humic substances may correspond to both associated and structural amino acids.

Past studies of amino acids in aquatic humic substances (Lytle and Perdue, 1981; DeHaan and DeBoer, 1978, 1979) also did not use cation exchange. Because cation exchange was used in the isolation of fulvic and humic acids from the Suwannee River, the amino acids that remain in the fulvic and humic acids are solely structural.

Fulvic acid from the Suwannee River contains 34 nmol/mg amino acids—this is approximately 1 amino-acid residue per 10 molecules. Thus, amino acids represent a minor characteristic in the fulvic acid. A possible structural model for fulvic acid from the Suwannee River that contains the amino acid alanine is shown in figure 3. In contrast to the fulvic acid, humic acid from the Suwannee River contains three times more amino acids and is heavier in molecular weight. Therefore, the humic acid contains one amino-acid residue per molecule, on the average, and amino acids are a more significant factor in the humic-acid structure.

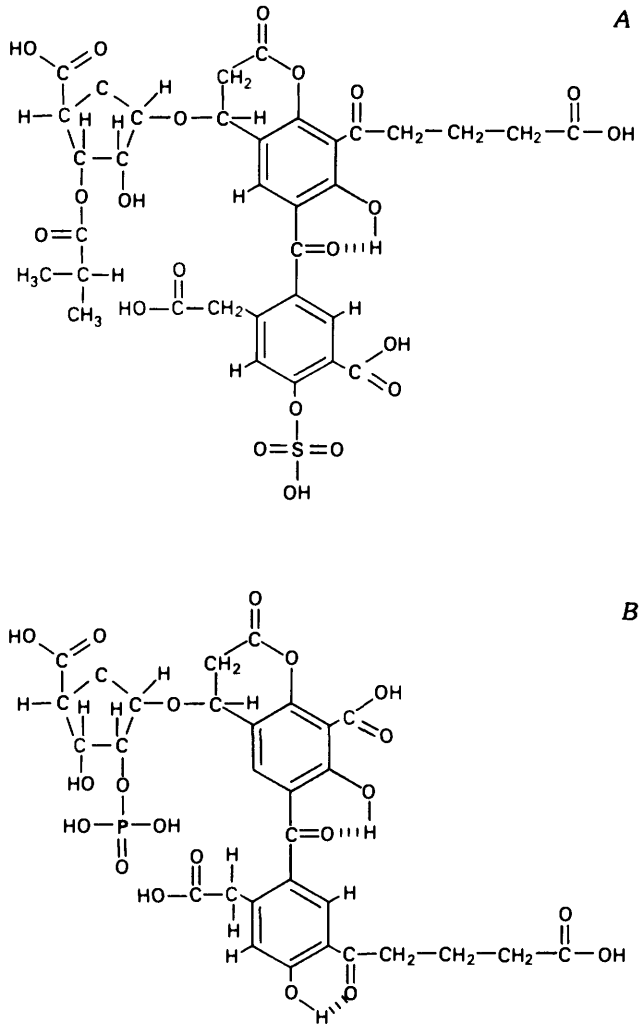



**Figure 2.** Structural models of the fulvic-acid molecule containing: *A*, sulfate ester; and *B*, phosphate ester.

## Metal-Binding Structures

*By* D.M. McKnight

Conceptually, two types of metal binding can be identified in humic substances: (1) strong, site-specific binding that involves electron-transfer interactions and (2) weak binding that may be thought of as either "territorial binding" of counter ions in the vicinity of the humic polyion or simple electrostatic attraction. These types of binding are not expected to be entirely independent of each other. Theoretically, the degree of weak binding of counter ions may affect the effective formation constants for the site-specific metal binding. Weak binding properties probably are more common in fulvic acid from the Suwannee River because all fulvic-acid molecules contain some carboxylic-acid groups; whereas, strong metal-binding sites are definitely a minor characteristic, because they are not present for every fulvic-acid molecule.

The dominant acidic functional groups that cause territorial binding almost certainly are ubiquitous carboxylic-acid groups. However, the charge distribution around a fulvic-acid molecule will depend on the structural arrangement of these carboxylic-acid groups, which is likely to be extremely variable. For example, the $^{13}C$-NMR spectra of the fulvic acid indicate that a majority of carboxylic acids (about 75 percent) are attached to aliphatic moieties, but some are attached to aromatic moieties. For both aliphatic and aromatic carboxylic acids, the major factor affecting their contribution to territorial binding is probably

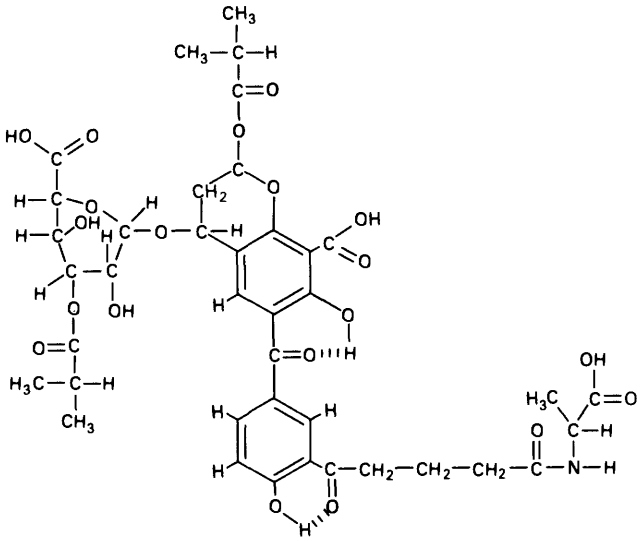


**Figure 3.** Structural model of the fulvic-acid molecule containing an amino-acid (alanine) moiety.

their proximity to other functional groups, especially other carboxyl groups and hydroxyl groups (Perdue, 1985). Therefore, weak binding that is sufficient to cause a measurable decrease in metal-ion activity (McKnight and Wershaw, chap. D, this volume) may be somewhat restricted in occurrence and may vary for different metal ions (for example, calcium and copper).

The heterogeneity within a humic substance becomes even more prominent when the myriad possibilities for the structural nature of the strong (site-specific) metal-binding sites are considered. From the molecular-weight data (number-average molecular weight equal to 800 daltons) (Aiken and others, chap. J, this volume) and the abundance of strong copper-binding sites (McKnight and Wershaw, chap. D, this volume), it can be shown that the incidence of these sites is just less than one for every fulvic-acid molecule for the sites with apparent conditional formation constants of about $1.0\times10^6$ and one for every nine fulvic-acid molecules for the sites with apparent conditional formation constants of about $1.0\times10^8$. Although it generally has been hypothesized, based on the abundance of carboxylic-acid groups, that these strong metal-binding sites are mainly phthalic-acid or salicylic-acid sites (Gamble and others, 1980), the only direct evidence for the involvement of carboxylates in copper complexation by humic substances in aqueous systems has come from spectrometric data. Such spectrometric data are: (1) data indicating absorbance at 240 nm, which is characteristic of copper carboxylate charge transfer complexes (McKnight and Wershaw, chap. D, this volume), (2) infrared spectra of copper/humic-acid complexes dissolved in deuterium oxide (MacCarthy and Mark, 1975), and (3) Fourier-transform infrared spectra of humic acid in water (MacCarthy and others, 1975). A model of a fulvic-acid molecule containing a phthalic-acid copper-binding site is shown in figure 4. To an even greater degree than for territorial binding, the metal-binding properties of carboxylic-acid sites will depend greatly on the structural configuration of these sites; this partially explains the lesser incidence of strong metal-binding sites relative to carboxyl and hydroxyl groups.

An additional reason for the lesser incidence of strong metal-binding sites in the humic-substance molecular mix is the presence of trace, non-oxygen-containing functional groups, such as groups that contain nitrogen or sulfur. As shown in table 2, the incidence of metal-binding sites for functional groups containing nitrogen or sulfur is comparable to that for strong metal-binding sites even if only one-half of the functional groups that contain nitrogen or sulfur are considered.

Several biologically significant metals that are present in trace concentrations in the environment are capable of forming strong complexes with functional groups that contain nitrogen or sulfur as well as with functional groups that contain oxygen. These metals include cadmium, cobalt, copper, iron, lead, manganese, and nickel and are classified as "borderline" metals in the classification proposed by Nieboer and Richardson (1980). Comparison of typical concentrations of these metals in aquatic humic substances in natural water provides circumstantial evidence that these strong complexes may be some of the major chemical species for these metals.

The possible involvement of functional groups containing nitrogen or sulfur in site-specific metal binding opens up many possibilities for the chemical structure of these sites, especially if the possibilities of "mixed-ligand" sites (nitrogen and oxygen, and sulfur and oxygen) and bridging between molecules also are considered. Several examples of possible structural models of the fulvic-acid molecule are shown in figure 5. Knowledge of the chemical nature of nitrogen and sulfur in aquatic humic substances would be valuable for constraining the possibilities for different structural models and for developing more sensitive spectrometric techniques for studying metal complexation. Potentiometric techniques and other techniques that indirectly measure the metal-humic complex are of limited utility in this context, although through metal-competition experiments, they may provide some important information.

Questions about the chemical structure of strong metal-binding sites and about the configuration of

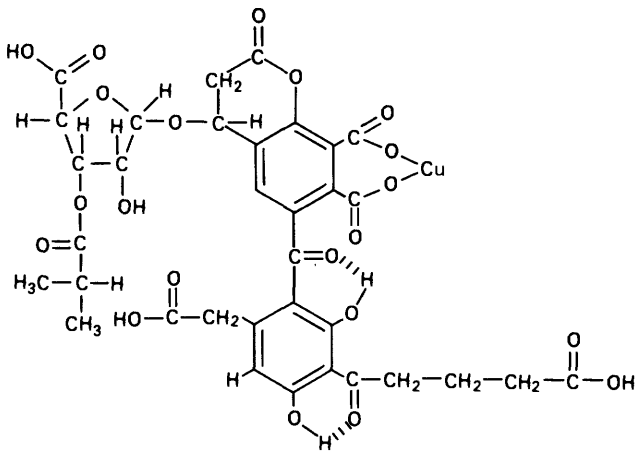


**Figure 4.** Structural model of the fulvic-acid molecule containing a metal-binding site of phthalic acid.

sites that cause territorial binding are ones that, if the answers were known, would have direct environmental and ecological significance. If more were known about charge distribution on the humic-substance molecule, such processes as adsorption of humic-substance molecules to particulate surfaces, such as metal oxides, could be understood more thoroughly. If the chemical structures of strong metal-binding sites were known, the dependence of such binding on pH and competitive interactions with different metals could be predicted. Such knowledge also would increase understanding of the relation between complexation of metals by humic substances and their biological availability.

## Organic Free Radicals

*By* F.Y. Saleh

An electron-spin resonance (ESR) study of fulvic acid from the Suwannee River indicated that organic free radicals are integral components of the fulvic-acid molecules in which they occur (Saleh and others, chap. G, this volume). The estimated frequency of occurrence is 1 or 2 organic free radicals per 1,000 fulvic-acid molecules (table 2); it is, therefore, probably the most "trace" of all the trace constituents considered in this chapter. An example of a possible fulvic-acid molecule containing a semiquinone free radical is presented in figure 6.

Many other aquatic and soil-humic substances also have been found to contain trace quantities of organic free radicals. Despite their infrequent occurrence relating to the total sample, the presence of organic free radicals in humic substances may be involved in several different chemical processes, such as oxidative polymerization and photochemical degradation of natural organic material and organic contaminants. The organic free-radical content of humic substances also may affect the response of different aquatic organisms to increasing concentrations of humic substances.

## Fluorophores

*By* M.C. Goldberg

A comprehensive fluorescence analysis of fulvic acid from the Suwannee River has been made using standard excitation-emission spectra, phase-resolved-emission spectra, three-dimensional excitation-emission spectra, and differential-phase depolarization measurements (Goldberg and Weiner, chap. K this volume). A broad maximum in the excitation spectrum at 350 nm indicates a strongly absorbing $\pi$-electron system that has an extended conjugation, as in polycyclic aromatic-ring or conjugated ketone structures. Two distinct fluorescence lifetimes of 1.05 and 6.54 ns were measured by phase-resolved fluorimetry and indicate the presence of two different fluorophores. The fluorophore with a lifetime of 1.05 ns accounts for 46 percent of the total emission and has its emission maximum at 472 nm. The shorter lifetime generally is a characteristic of the greater energy component. The 55-nm separation of the emission maxima substantiates the assignment of the two different lifetimes to two different fluorophores rather than to one fluorophore in two different environments, because the emission maxima separation corresponds to an energy difference too large to be readily attributed to environmental differences in a fulvic-acid molecule.

The presence of two fluorophores also is indicated by the three-dimensional excitation-emission spectra that have two prominent, well-separated peaks and by the large overlap of the excitation and emission bands in the standard fluorescence spectrum. If only one fluorophore is present, the short wavelength limit of the emission band and the long wavelength limit of the excitation band should correspond closely to the 0-0 band transitions and should have only a small overlap region. However, the 90-nm overlap is evidence for at least two separate fluorophores in the fulvic acid. These measurements indicate the possibility that the different fluorophores are in the same

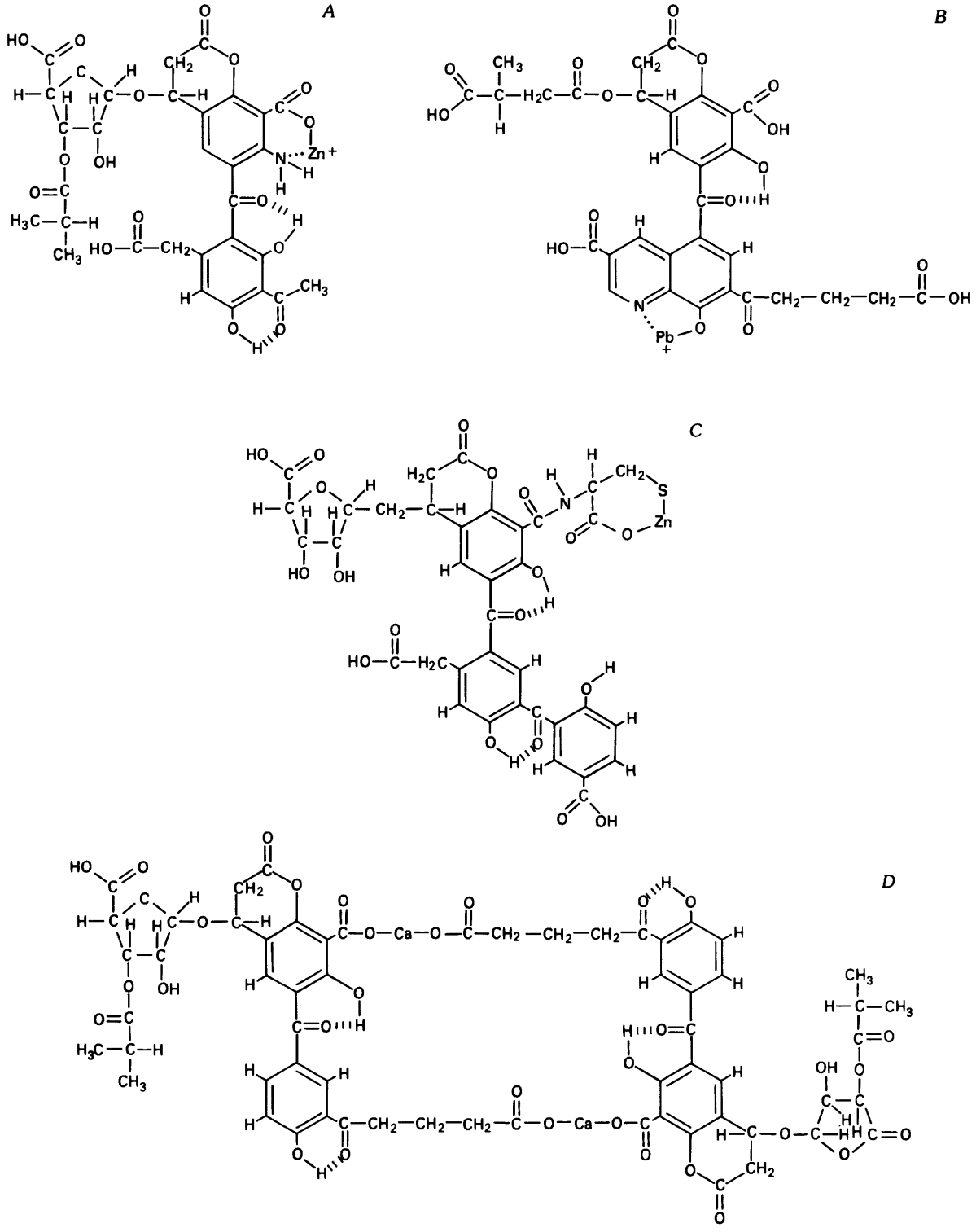



**Figure 5.** Structural models showing: *A*, a fulvic-acid molecule containing a nitrogen (anthranilic-acid type) metal-binding site; *B*, a fulvic-acid molecule containing a nitrogen (8-hydroxyquinoline type) metal-binding site; *C*, a fulvic-acid molecule containing an amino acid (cysteine) complexed with a metal; and *D*, a bridging of two fulvic-acid molecules by calcium complexation.

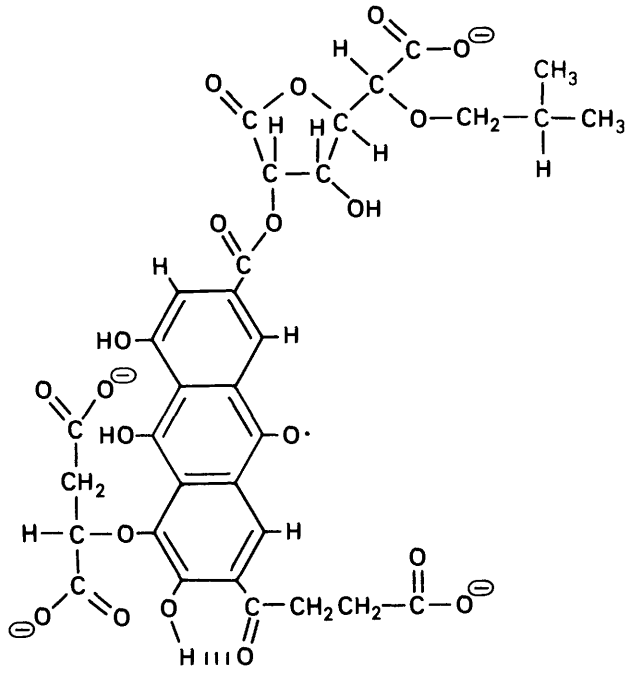


**Figure 6.** Structural model of the fulvic-acid molecule containing a semiquinone free radical.

fulvic-acid molecule, in separate fulvic-acid molecules, or both; however, fluorescence measurements cannot distinguish whether or not each fluorophore is in separate fulvic-acid molecules.

Fluorescence depolarization measurements were made as a function of temperature and of pH to obtain shape and volume information about the fulvic acid (Goldberg and Weiner, chap. K, this volume). The fulvic acid has an equivalent spherical molar volume of 3,950 $cm^3$ in water at pH 6.5 and 2,860 $cm^3$ in dimethylsulfoxide. Nonspherical molecules will function as spherical rotors in water if they can include at least two bound water molecules; thus, the fulvic acid may not be a spherical molecule, but it functions as a spherical rotor in water. The shape seems to be stable between pH 3 and 11, and conformational changes occur outside this range. At pH 3 and less, the protons in solution may be forced onto oxygens, causing intramolecular hydrogen bonding with hydroxyls that would tend to change the molecular conformation. Similarly, at pH values more than 11, protons may be stripped from phenolic-type linkages, again resulting in a conformational change. All of these measurements are consistent with a structural model for the fulvic acid that contains two or more fluorophores in conjugated structures such as are shown in figure 7.

## Molecular Aggregate Structures

*By* R.L. Wershaw

Measurements of apparent molar volume (Goldberg and Weiner, chap. K, this volume) and colligative properties by vapor-pressure osmometry (Aiken and others, chap. J, this volume) as functions of concentration in water indicate that fulvic acid from the Suwannee River is largely dissaggregated at concentrations as large as 1 percent (weight/volume) in water. Decreases in apparent specific volume in concentrations of about 1 percent and greater qualitatively indicate the presence of molecular aggregates. Although molecular aggregates of fulvic acid commonly exist only in trace concentrations at ambient concentration in water, they still may be significant in causing the partitioning of trace organic contaminants into the unsolvated organic phase of the aggregate. Molecular-aggregate formation in water may affect the interpretation of molecular size from X-ray scattering data and may affect the resolution of solution-state NMR data because sample concentrations in X-ray scattering measurements and solution-state NMR measurements are sufficiently high where molecular-aggregate formation is expected (Wershaw and Pinckney, 1987).

In previous studies, Wershaw (1986) and Wershaw and others (1986) proposed that soil humic substances are composed of partially degraded molecular components from living organisms (mainly plants) that are bound together in ordered, membrane-like, aggregated structures by various interactions such as hydrogen bonding, π-bonding, van der Waals interactions, and hydrophobic forces. These aggregated structures may constitute, to some extent, a separate phase in soil-water, sediment-water, and peat-water systems. The physical-chemical properties of the humic membrane-like or micelle-like phases are more a function of the nature and structure of the phases than of the properties of the individual molecular species of the aggregates.

The aggregation model for humic substances proposed by Wershaw (1986) originally was developed for soil humic substances. However, aquatic fulvic acids have surface-active properties that are similar to those for soil humic and fulvic acids that indicate that they are composed of amphiphilic molecules (that is, molecules that have separate hydrophobic and hydrophilic groups). In addition, solid-state NMR studies by Earl and others (1987) indicate that, in solid fulvic acid from the Suwannee River, there are domains of greater and lesser molecular mobility. One possible explanation for this difference in mobility may be the

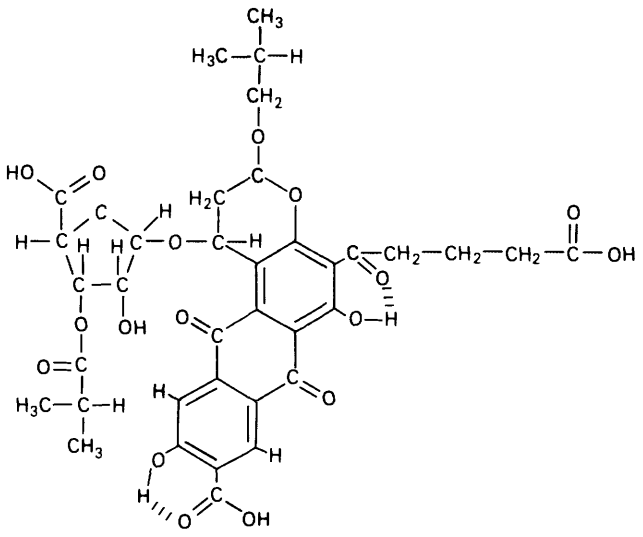


**Figure 7.** Structural model of a Suwannee River fulvic-acid molecule with multiple fluorophores.

aggregation of amphiphilic molecules into membranes or micelles. The interiors of these ordered structures are composed of the hydrophobic ends of the amphiphilic molecules that have much more segmental motion than the hydrophilic groups that comprise the exterior surfaces of the aggregates.

The results outlined in the preceding paragraph were obtained from only one fraction of the organic material in a river. In nature, one would expect that this fraction will interact with the other organic and inorganic components in the water; Hunter and Lee (1986) have reported that aquatic fulvic acids form mixed aggregates with humic acids in aquatic environments. They detected this aggregation in unextracted humic material in the water of a stream in New Zealand. Thus, substantially larger humic aggregates may exist in aquatic environments than can be detected in aquatic fulvic-acid isolates in which particle weights in the range of 600 to 1,500 daltons have been detected.

## RESEARCH NEEDS

Research on chemical structure and composition of humic substances is one of the most challenging problems in the field of analytical chemistry. The problem is difficult because of the extreme heterogeneity of humic substances: Certain researchers have stated that perhaps no two molecules of humic substances are identical. Therefore, the primary research need is to perform additional chromatography and fractionation to reduce the heterogeneity factor. Chromatography is a useful technique even if no two molecules of humic substances are identical because structural model analysis can be performed on fractions having component structural characteristics that are only slightly different.

Many promising avenues of research could be explored in chromatography of humic substances. New types of chromatography, such as field-flow fractionation and supercritical-fluid chromatography, are just beginning to be applied to studies of humic substances. Conventional normal-phase and reverse-phase chromatography is improving because of new column technology. The practice of forming chemical derivatives of humic substances to improve chromatography has great promise when using conventional chromatography. Mass spectrometry is gaining in potential for structural analysis of humic substances as new techniques of molecular ionization are being developed that do not fragment labile molecules. The coupling of mass spectrometers in tandem mass spectrometry may enable the bypassing of high-resolution chromatographic separation of humic substances because of the great resolution of mass spectrometers compared to liquid chromatography. The utility of chemical and thermal degradative studies of humic substances for structural analysis increases in proportion to the degree of fractionation and homogeneity of the fraction subjected to degradative analysis.

A second research need is to apply the findings of structural studies of humic substances to contaminant interactions. Mechanisms of trace-metal binding and organic-contaminant association are not specifically known because of the lack of structural information. Of particular importance are the development of easily determined surrogate measurements of the humic structural components that cause contaminant binding.

Lastly, it is important to do more research on humic substances that are derived from end-member environments that have a simple source term. Selection of humic substances for study from end-member environments is needed to distinguish whether the heterogeneity factor is due to multiple source terms or whether biogeochemical processes that degrade humic substances cause humic-substance heterogeneity. Also, minimizing the diversity of the source term should enable much better definition of biogeochemical-decay processes.

Structural studies of humic substances at the molecular level are not hopeless analytical problems despite the heterogeneity of the mixture. More analytical tools exist to study the problem than there are fiscal resources and personnel devoted to the problem.

Given the limited size of the field of analytical chemistry of humic substances compared to the field of analytical biochemistry of living organisms, scientific breakthroughs still can be made by innovative application of techniques developed by others.

## SUMMARY

Synthesis of major element (carbon, hydrogen, and oxygen) data for fulvic acid from the Suwannee River into diverse structural models has demonstrated the importance of common spatial relations among functional groups on molecules within the heterogeneous mixture. Both the spatial proximity and relative abundances of carboxyl and hydroxyl groups favor lactone formation; the same factors for aromatic carbonyl groups and phenol groups favor intramolecular hydrogen bonding that affects solute conformation and polarity, and the predominance of aliphatic carboxyl groups render the exterior of the molecule negatively charged and polar. The process of model systhesis has lead to improved methods that converged divergent data, such as different carbon distributions, depending on NMR conditions and whether the sample was in the liquid or solid state. Significant ester content was revealed by divergence of titrimetry data, $^{1}$H-NMR data after methylation of carboxyl groups, and $^{13}$C-NMR data of carboxyl plus ester carbons. The aromatic and aliphatic structures of the models indicate residues of parent structures, such as plant lignins, tannins, carbohydrates, and lipids, but the significant carboxylic-acid content indicates extensive oxidation of parent compounds—the aromatic ketone content may be an indicator of photolytic rearrangements of phenolic esters or photolytic oxidation of aliphatic side chains or aromatic rings.

Minor characteristics of the fulvic acid, defined as occurring less than once in a number-average model, still may be important with regard to chemical properties and to biogeochemical processes. The incidence of nitrogen and sulfur functional groups are comparable to the number of strong metal-binding sites. Because nitrogen and sulfur groups form metal complexes with greater stability constants than oxygen groups, trace quantities of nitrogen and sulfur in fulvic acid may be the cause of metal binding of certain trace elements. Trace quantities of free radicals found in the fulvic acid may cause oxidative polymerization and photochemical degradation of natural organic material and organic contaminants. Fluorescence depolarization measurements were able to determine conformational changes in fulvic-acid molecules that fluoresce. Molecular aggregates of fulvic acid from the Suwannee River may only exist in trace quantities as mixed aggregates with humic acid, but these aggregates may still be important as an organic phase into which organic contaminants may partition.

## REFERENCES CITED

Aiken, G.R., and Malcolm, R.L., 1987, Molecular weights of aquatic fulvic acids by vapor pressure osmometry: Geochimica et Cosmochimica Acta, v. 51, no. 8, p. 2177–2184.

Bloom, P.R., and Leenheer, J.A., 1989, Vibrational, electronic, and high energy spectroscopic methods for characterizing humic substances, *in* Hayes, M.H.B., MacCarthy, P., Malcolm, R.L., and Swift, R.S., eds., Humic Substances II: In Search of Structure: Chichester, England, John Wiley, p. 404–448.

Bosserman, R.W., 1984, Diurnal variations of water chemistry parameters in the Okefenokee Swamp, *in* Cohen, A.D., Casagrande, D.J., Andrejko, M.J., and Best, G.R., eds., The Okefenokee Swamp: Los Alamos, New Mexico, Wetland Surveys, p. 296–319.

Chiou, C.T., Malcolm, R.L., Brinton, T.I., and Kile, D.E., 1986, Water solubility enhancement of some organic pollutants and pesticides by dissolved humic and fulvic acids: Environmental Science and Technology, v. 20, no. 5, p. 502–508.

DeHaan, H., and DeBoer, T., 1978, A study of the possible interactions between fulvic acid, amino acids, and carbohydrates from Tjeukemeer, based on gel filtration: Water Research, v. 12, no. 12, p. 1035–1040.

———1979, Seasonal variations of fulvic acids, amino acids, and carbohydrates in Tjeukemeer, The Netherlands: Archiv für Hydrobiologie, v. 85, no. 1, p. 30–40.

Earl, W.L., Wershaw, R.L., and Thorn, K.A., 1987, The use of variable temperature and magic angle spinning in studies of fulvic acids: Journal of Magnetic Resonance, v. 74, p. 264–274.

Gamble, D.S., Underdown, A.W., and Langford, C.H., 1980, Copper (II) titration of fulvic acid ligand sites with theoretical potentiometric and spectrophotometric analysis: Analytical Chemistry, v. 52, no. 12, p. 1901–1908.

Huffman, E.W.D., Jr., and Stuber, H.A., 1985, Analytical methodology for elemental analysis of humic substances *in* Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediment, and Water: New York, John Wiley, p. 433–456.

Hunter, K.A., and Lee, K.C., 1986, Polarographic study of the interaction between humics and other surface-active organics in river waters: Water Research, v. 20, no. 12, p. 1489–1491.

Leenheer, J.A., Wilson, M.A., and Malcolm, R.L., 1987, Presence and potential significance of aromatic ketone groups in aquatic humic substances: Organic Geochemistry, v. 11, no. 4, p. 273–280.

Lytle, C.R., and Perdue, E.M., 1981, Free, proteinaceous, and humic-bound amino acids in river water containing high concentrations of aquatic humus: Environmental Science and Technology, v. 15, no. 2,. p. 224–228.

MacCarthy, Patrick, and Mark, H.B., Jr., 1975, Infrared studies of humic acid in deuterium oxide. Part I. Evaluation and possibilities of the technique: Soil Science Society of America Proceedings, v. 39, no. 4, p. 663–669.

MacCarthy, Patrick, Mark, H.B., Jr., and Griffiths, P.R., 1975, Direct measurement of the infrared spectra of humic substances in water by Fourier transform infrared spectroscopy: Journal of Agricultural and Food Chemistry, v. 23, no. 3, p. 600–602.

MacCarthy, Patrick, and O'Cinneide, S., 1974, Fulvic acid. Part I. Partial fractionation: Journal of Soil Science, v. 25, no. 4, p. 420–428.

McKnight, D.M., and Feder, G.L., in press, Ecological aspects of humic substances in the environment, *in* MacCarthy, Patrick, and Hayes, M.H.B., eds., Humic Substances—Reactions and Interactions: Chichester, England, John Wiley.

McKnight, D.M., Thurman, E.M., Wershaw, R.L., and Hemond, Harold, 1985, Biogeochemistry of aquatic humic substances in Thoreau's Bog, Concord, Massachusetts: Ecology, v. 66, no. 4, p. 1339–1352.

Nieboer, E., and Richardson, D.H., 1980, The replacement of the nondescript term "heavy metals" by a biologically and chemically significant classification of chemical ions: Environmental Pollution Series B, v. 1, no. 1, p. 3–26.

Perdue, E.M., 1985, Acidic functional groups of humic substances, *in* Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediment, and Water: New York, John Wiley, p. 493–526.

Prakash, A., Rashid, M.A., and Rao, D.V.S., 1973, Influence of humic substances on the growth of marine phytoplankton-diatoms: Limnology and Oceanography, v. 18, no. 3, p. 516–524.

Schnitzer, Morris, 1985, Nature of nitrogen in humic substances, *in* Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediment, and Water: New York, John Wiley, p. 303–325.

Steinberg, Christian, and Muenster, Uwe, 1985, Geochemistry and ecological role of humic substances in lake water, *in* Aiken, G.R., McKnight, D.M., Wershaw, R.L., and MacCarthy, Patrick, eds., Humic Substances in Soil, Sediment, and Water: New York, John Wiley, p. 105–145.

Stewart, A.J., and Wetzel, R.G., 1981, Dissolved humic materials—Photodegradation, sedimentation effects, and reactivity with phosphate and calcium precipitation: Archiv für Hydrobiologie, v. 92, no. 3, p. 265–286.

———1982, Influence of dissolved humic materials on carbon assimilation and alkaline phosphatase activity in natural algal bacterial assemblages: Freshwater Biology, v. 12, no. 4, p. 369–380.

Thurman, E.M., 1985, Organic Geochemistry of Natural Waters: Dordrecht, The Netherlands, Martinus Nijhoff–Dr. Junk Publishers, 497 p.

Wershaw, R.L., 1986, A new model for humic materials and their interactions with hydrophobic organic chemicals in soil-water or sediment-water systems: Journal of Contaminant Hydrology, v. 1, no. 1, p. 29–45.

Wershaw, R.L., and Pinckney, D.J., 1987, NMR evidence for a membrane model of soil humic acid, *in* Wershaw, R.L., and Mikita, M.A., eds., NMR of Humic Substances and Coal—Techniques, Problems, and Solutions: Chelsea, Michigan, Lewis Publishers, p. 189–210.

Wershaw, R.L., Thorn, K.A., and Pinckney, D.J., 1986, Application of a membrane model to the secondary structure of humic materials in peat, *in* Fuchsman, C.H., ed., Peat and Water: New York, Elsevier Applied Science, p. 133–157.

Visser, S.A., 1985, Physiological action of humic substances on microbial cells: Soil Biology and Biochemistry, v. 17, no. 4, p. 457–462.

# GLOSSARY

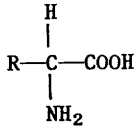
[Glossary terms are italicized where first used in the report]

*acetylation.*—Insertion of an acetyl radical, $\overset{O}{\overset{\|}{\cdot C-CH_3}}$, into an organic molecule containing OH or $NH_2$ groups.

*aliphatic.*—Of or pertaining to a broad category of carbon compounds distinguished only by a straight or branched, open-chain arrangement of the constituent carbon atoms. The carbon-carbon bonds may be saturated or unsaturated.

*alum.*—An aluminum sulfate of some type, commonly used as a flocculent in water treatment.

*amino acid.*—One of a group of related biochemicals that form the basic subunits of proteins; they have the general chemical formula:



*anisotropic.*—Possessing properties with different values when measured along axes in different directions.

*anodic-stripping voltammetry.*—The initial reduction and collection of metals at the surface of an anode and the subsequent stripping of the metals by oxidation, resulting in a voltammogram that identifies the metals and their respective concentrations.

*anomeric.*—Referring to a cyclic stereoisomer of the carbohydrate series with isomerism involving only the arrangement of atoms or groups at the aldehyde or ketone position.

*apparent formation constant.*—The term apparent formation constant is normally applied to the formation of a metal complex of an organic ligand in a homogeneous thermodynamic system. In such a system, a thermodynamic equilibrium will be established between the unbound metal ions and the metal ions bound to the organic ligand. Strictly speaking, this equilibrium can be characterized by an equilibrium constant that will be a function of the thermodynamic activities of the reacting species. These activities, in turn, will be functions of the ionic strength and the pH of the solution. If the activities of the reacting species are not known, then the equilibrium constant is replaced by the apparent formation constant where the activities of the reacting species have been replaced by concentrations.

*apparent specific volume.*—Reciprocal of density not corrected for intermolecular and intramolecular interactions.

*aromatic.*—Of or pertaining to organic compounds that resemble benzene in chemical characteristics.

*autochthonous.*—Produced within the system, for example, within a lake (opposite of *allochthonous*).

*boiling-point elevation.*—A method for determining molecular weights of soluble materials. A weighed quantity of material of unknown molecular weight is dissolved in a weighed quantity of a solvent whose boiling-point-elevation constant is known. The measured increase in the boiling point permits calculation of the molality of the solution, and, from this, the molecular weight of the solute can be determined.

*calorimetry.*—The measurement of heat of combustion, determined in a bomb calorimeter, that provides information about molecular bonding.

*chemical shift.*—The resonance position, usually expressed in parts per million (ppm), of a nucleus in a nuclear-magnetic-resonance spectrum. The chemical shift of a particular nucleus is dependent on the distribution of its surrounding electrons.

*colligative property.*—A thermodynamic property that depends on the number of particles in solution and not on the nature of these particles.

*colloidal.*—The colloidal state represents a condition intermediate between true solutions, where the particles are of ionic or molecular dimensions, and particulate suspensions, where the particles are sufficiently large to settle under the influence of gravity. The range is generally regarded as extending from 10 to 2,000 angstroms.

*column distribution coefficient.*—The column distribution coefficient, k′, in the context of frontal adsorption chromatography using XAD resins, is defined as the mass of solute sorbed on XAD resin divided by mass of solute dissolved in water.

*dalton.*—An atomic mass unit, equivalent to one-twelfth the mass of the carbon atom (mass number 12).

*decoupling.*—A technique in nuclear-magnetic-resonance spectrometry, sometimes referred to as double resonance, used to remove the effects of spin-spin coupling in nuclear-magnetic-resonance spectra. In heteronuclear decoupling in $^{13}C$ or $^{15}N$ nuclear-magnetic resonance for example, protons are irradiated with a radio-frequency field to remove splitting due to $^{13}C$-$^{1}H$ or $^{15}N$-$^{1}H$ spin-spin coupling.

*density.*—The mass of a substance per unit volume, usually expressed in grams per milliliter.

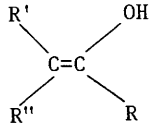
*dielectric constant.*—The dielectric constant, ε, measures the relative effect of the medium on the force with which two opposite charges attract each other. The dielectric constant of a liquid is determined readily by measuring the electrical capacitance of a condenser when empty and when filled with the liquid.

*dissolved organic carbon.*—Organic carbon in natural waters can be divided into dissolved and particulate

organic carbon. The dissolved organic carbon is operationally defined as that fraction which passes through a 0.45-micrometer filter.

*$E_4/E_6$ ratio.*—The $E_4/E_6$ ratio is defined as the absorbance of alkaline humate solution at 465 nanometers divided by the absorbance of alkaline humate solution at 665 nanometers.

*enolic.*—Pertaining to the enol group (also called alkenol),




, where a carbon atom is double bonded to another carbon atom and is singly bonded to both a hydroxyl group and to another atom.

*esterification.*—The formation of an ester, $R-\overset{\overset{O}{\|}}{C}-OR$.

A common type of esterification is the reaction of an alcohol with a carboxylic acid.

*electron-spin-resonance spectrometry.*—When molecules containing unpaired electrons are placed in a magnetic field, the energy level of each electron is split into two discrete states through interaction of the magneticmoment of the electrons with the applied field. These molecules can be excited from the lower to the higher energy level by absorption of electromagnetic radiation in the microwave region (about 6 to 9 hertz). This isreferred to as ESR spectrometry (alternatively, electron paramagnetic resonance (EPR) spectrometry) and is used specifically for the study of molecules with unpaired electrons or free radicals.

*FITEQL.*—The acronym for a computer program used for the determination of chemical equilibrium constants from experimental data.

*fluorescence spectrometry.*—The absorption of ultraviolet to visible radiation causes a molecule to change from the ground electronic and vibrational states to excited electronic and vibrational states. Most molecules return to the ground state by dissipating the additional energy in the form of heat. In some molecules, however, only a fraction of this energy is dissipated as heat and the residual energy is emitted as electromagnetic radiation of longer wavelength than the incident radiation. This is fluorescence.

*fluorophores.*—The functional groups that fluoresce, most commonly conjugated double bonds or aromatic rings.

*freezing-point lowering.*—A technique for the molecular-weight determination of dissolved substances. A weighed quantity of a solute of unknown molecular weight is dissolved in a weighed quantity of a liquid whose freezing-point-depression constant is known. The freezing point of the solution is measured, the freezing-point depression and the molality of the solution are calculated, and the molecular weight of the solute is determined from the weights of solute and solvent and the molality.

*fulvic acid.*—That fraction of humic substances that is soluble in water under all pH conditions.

*Guinier plot.*—In a small-angle, X-ray scattering experiment, a Guinier plot is a plot of the natural logarithm of the scattered intensity, I, versus $h^2$, where $h=(2\pi\sin 2\theta)/\lambda$; in this equation, $2\theta$ is the scattering angle and $\lambda$ is the wavelength of the impinging X-radiation.

*heat of combustion.*—The quantity of heat measured when a known quantity of a substance is reacted with an oxidizing agent, such as oxygen, in a calorimeter. Heat of combustion is expressed as E, in calorie units.

*heat of formation.*—The quantity of heat measured at standard temperatures and pressures when a compound is formed by reaction of its elements. Heat of formation is expressed as $H^o$, in calorie units, and is related to heat of combustion by the equation $H^o = E+(PV)$, where (PV) is the difference of the pressures and volumes of the products from the reactants.

*hydrodynamic molar volume.*—The volume of a mole of a substance including bound water measured in the dynamics of an aqueous system.

*hysteresis.*—A lag or delay between a changing force and its resulting effect. In chemistry, hysteresis is commonly manifested in titrimetry or sorption isotherms when data obtained in one direction are not reversible in the opposite direction.

*inductively coupled plasma–mass spectrometry.*—A mass-spectrometric technique used primarily for inorganic elemental analysis whereby elements in aqueous solution are dissociated to isotope ions in an inductively coupled plasma followed by separation of isotope ions in a mass spectrometer.

*intensity factor.*—Used in the context of determining the pH of a fulvic-acid solution; the pH of a fulvic-acid solution is based on a combination of the $pK_a$ of the constituent acid groups and on the concentration of these acid groups.

*ion-selective electrode potentiometry.*—The metering of specific ion concentrations, such as cupric ion, by the potential developed by an ion-specific electrode.

*ionic strength.*—The concentration of ions in solution where ionic strength, I, is equal to $1/2\Sigma m_i z_i$, where $m_i$ = solution molality of ions and $z_i$ = ionic charge.

*isotope-dilution analysis.*—Quantitation of an element by determining the change in the isotopic ratio when the same element of different isotopic ratio is added in known concentration to the sample.

*$K_{dom}$.*—Known as the dissolved organic-matter partition coefficient. $K_{dom}$ is equal to the concentration of

solute at equilibrium partitioned into organic matter dissolved in water divided by the concentration of solute at equilibrium in water.

*lactone.*—A cyclic ester. Hydroxy acids may exist as lactones if the hydroxyl groups are situated so that the lactone formed has a five- or six-membered ring.

*lignin.*—The most abundant, natural, aromatic organic polymer that is a major structural component of wood. There is no general agreement about the structure of lignin; however, it is known to lack a regular sequence of monomers. Lignin contains phenolic, hydroxyl, and methoxyl groups; phenols are formed when lignin decomposes.

*Maillard reaction.*—The reaction of amino groups of amino acids, peptides, or proteins with the "glycosidic" hydroxyl group of sugars resulting in the formation of pigments. Also known as the "Browning" reaction.

*melanoidin.*—Brown pigments produced in Maillard reactions.

*methoxyl.*—The functional group $CH_3O$-.

*methylation.*—An organic synthesis or derivatization procedure whereby a proton attached to a carbon or oxygen atom, as a hydroxyl or carboxyl functional group, is replaced by a methyl (-$CH_3$) group.

*MINEQL.*—The acronym for a computer program used for the determination of mineral equilibrium; the program computes the chemical-equilibrium composition of aqueous systems.

*monodisperse.*—A condition whereby particles in solution, such as fulvic-acid molecules, do not interact with each other to form particle aggregates.

*Nernst equation.*—The equation used to calculate the potential, E, of any electrode that is used to measure a redox reaction. The generalized form of the Nernst equation is:

$$E=E^{o}+RT/nFln(a_{ox}/a_{red})$$

where

$E^o$ is the standard electrode potential,

R is the molar gas constant,

T is the absolute temperature,

n is the number of electrons transferred in the electrode reaction,

F is the Faraday constant,

$a_{ox}$ is the solution activity of the oxidized species, and

$a_{red}$ is the solution activity of the reduced species.

*nuclear-magnetic-resonance spectrometry.*—A form of spectrometry in which, as a result of the magnetic properties of nuclei arising from their axial spin, radio-frequency radiation is absorbed in a magnetic field.

*octanol-water partition coefficient, $K_{ow}$.*—$K_{ow}$ is defined as the concentration of solute at equilibrium in octanol divided by the concentration of solute at equilibrium in water.

*organic free radical.*—An unpaired electron on an atom in an organic molecule.

*partial specific volume.*—The volume of a monodisperse solute, in milliliters per gram. The reciprocal of the partial specific volume is the density of the solute.

*permethylation.*—The process of derivatizing all hydroxyl groups (-OH) in an organic molecule to methoxyl groups (-$OCH_3$).

*pH-stat experiment.*—A pH titration in which the sample solution is titrated to a specific pH, and acid or base is added as required at this pH to monitor the rates of a reaction, such as hydrolysis, oxidation, or reesterification, which consumes acid or base at a fixed pH.

*phase-resolved emission spectra.*—The single fluorescence spectrum in a mixture of spectra with different fluorescence lifetimes calculated by using the phase shift between the excitation source and the emission species.

*phenolic.*—Of, relating to, or containing a phenol group, which is a hydroxyl group bonded directly to an aromatic ring structure; for example, naphthol is a phenolic compound.

*photolysis.*—Chemical reaction (synthesis or degradation) induced by absorption of ultraviolet or visible radiation.

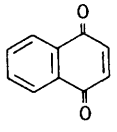
*polydisperse.*—Characterized by particles of varying size in a dispersed phase.

*polyelectrolyte.*—A macromolecule containing multiple ionic functional groups (either cationic or anionic).

*polysaccharide.*—A carbohydrate polymer composed of monomeric sugar subunits, such as glucose, mannose, and fructose; commonly used as a form of energy storage in living organisms. Cellulose and starch are polysaccharides.

*pycnometry.*—A method that measures densities of liquids by precise determination of liquid weight and volume at a specific temperature in a container called a pycnometer.

*quinone.*—A conjugated cyclic diketone with one to several conjugated six-membered carbon rings, for example, napthoquinone:




*radius of gyration.*—The radius of a rotating particle in an aqueous system that scatters electromagnetic radiation, such as X-rays, which are scattered by humic substances in solution.

*Rayleigh scattering.*—The scattering of the excitation light off the diffraction grating of a spectrophotometer at the same wavelength as the excitation light.

*saponification.*—The hydrolysis of an ester especially by alkali (for example, NaOH) into the corresponding alcohol and the sodium salt of the corresponding acid. The process is usually applicable to fats, and the sodium salt so formed is called a soap.

*semiquinone.*—A partially reduced quinone having an unshared electron on one of its oxygen atoms.

*Sephadex.*—Trademark name for a chromatographic gel used in gel permeation chromatography; Sephadex is composed of an extensively cross-linked gel derived from dextran and epichlorohydrin.

*solubility enhancement.*—The increase of saturation solubility of a solute in a water sample compared to its solubility in pure water. Solubility enhancement of nonpolar organic solutes commonly occurs by partitioning of these solutes into humic substances dissolved in water.

*suspended organic carbon.*—That portion of total organic carbon in a water sample that is removed by filtration through a 0.45-micrometer porosity filter.

*ultracentrifugation.*—A method for determining molecular weight of molecules based on their sedimentation characteristics in solutions in the large gravitational fields developed by the ultracentrifuge.

*vapor-pressure lowering.*—A colligative property used to determine molecular weight of solutes by the reduction in vapor pressure of the solution.

*vapor-pressure osmometry.*—A colligative property used to determine molecular weight of solutes by the vapor-pressure difference between solution and solvent; this vapor-pressure difference is determined by measuring the temperature increase associated with condensation of vapor into the solution.

*viscometry.*—A method used to determine molecular weight and molecular configuration of organic solutes based on measurements of solute effects on solution viscosity.

*XAD resins.*—A series of nonionic resins of macroporous structure, manufactured by Rohm and Haas, which have been used to adsorb humic substances from water; the XAD-2, XAD-7, and XAD-8 resins are most commonly used to isolate humic substances from water.

## APPENDIX OF DATA ON HUMIC SUBSTANCES FROM THE SUWANNEE RIVER

### I. Properties of water from the Suwannee River during isolation of dissolved humic substances

| Measurement | Value | Method | Chapter |
|---|---|---|---|
| Dissolved organic carbon (DOC) | 35–50 mg/L range (38 mg/L average) | Wet oxidation, Technicon Autoanalyzer. | B |
| Suspended sediment organic carbon | 2–4 mg/L (range) | Sealed vial, wet oxidation of sediment on silver filter, Oceanographic International Analyzer. | B |
| Percent humic substances of DOC | 75 percent | Sorption on XAD–8 resin. | B |
| Percent fulvic acid of DOC | 65 percent | Sorption on XAD–8 resin after separation of humic acid. | B |
| Percent humic acid of DOC | 10 percent | Precipitation at pH 1. | B |
| Specific conductance | less than 50 µS/cm | Conductivity bridge. | B |

### II. Interactions of humic substances from the Suwannee River with certain organic contaminants and metals

| Measurement | Value | Method | Chapter |
|---|---|---|---|
| Fulvic-acid-water partition coefficient (log $K_{dom}$) of: | | Solubility enhancement of organic contaminant by fulvic acid in water followed by solvent extraction and gas chromatographic assay of organic contaminant. | C |
| pp′-DDT | 4.13 | | |
| 2,4,5,2′5′-PCB | 3.83 | | |
| 2,4,4′-PCB | 3.30 | | |
| 1,2,3-trichlorobenzene | 1.7 | | |
| Lindane | 1.2 | | |
| Humic-acid-water partition coefficient (log $K_{dom}$) of: | | Solubility enhancement of organic contaminant by humic acid in water followed by solvent extraction and gas chromatographic assay of organic contaminant. | C |
| pp′-DDT | 4.12 | | |
| 2,4,5,2′,5′-PCB | 3.80 | | |
| 2,4,4′-PCB | 3.17 | | |
| 1,2,3-trichlorobenzene | 1.7 | | |
| Lindane | 1.2 | | |
| Fulvic-acid cupric-ion formation constants and ligand (L) concentrations with various concentrations of background electrolytes | | Potentiometric titration. | D |
| (1) $Ca(NO_3)_2$, $CaCl_2$ background electrolytes | $\log K_1$ = 5.9–6.3<br>$\log K_2$= 7.2–8.3<br>$L_1$ = 0.39–0.74 mmol/g<br>$L_2$ = 0.07–0.13 mmol/g | | |
| (2) $KNO_3$ background electrolyte | $\log K_1$ = 5.9<br>$\log K_2$ = 7.8–8.1<br>$L_1$ = 0.64–0.74 mmol/g<br>$L_2$ = 0.14–0.17 mmol/g | | |

## APPENDIX OF DATA ON HUMIC SUBSTANCES FROM THE SUWANNEE RIVER—Continued

III. Composition of humic substances isolated from the Suwannee River

A. Major constituents

1. Organic elements in fulvic acid

| Measurement | Value (calculated on ash-free basis) | Method | Chapter |
|---|---|---|---|
| Carbon | 53.49 percent | Combustion, measurement of $CO_2$. | I |
| Hydrogen | 4.29 percent | Combustion, measurement of $H_2O$. | I |
| Oxygen | 41.02 percent | Reductive pyrolysis; measurement of CO. | I |
| Nitrogen | 0.70 percent | Dumas method. | I |
| Sulfur | 0.56 percent | Combustion, measurement of $SO_4^=$. | I |
| Phosphorus | < 0.1 percent | Acid digestion. | P |
| Ash | 0.85 percent | Residual after combustion. | I |

2. Organic functional groups

(a) Fulvic acid

| Measurement | Value | Method | Chapter |
|---|---|---|---|
| Carboxyl group | 6.1 mmol/g | Potentiometric titration. | L |
| | 6.8 mmol/g | Methylation–$^1$H-NMR. | M |
| Ester (linear and cyclic) plus amide groups | 2.0 mmol/g | $^{13}$C-NMR of carboxyl plus ester minus titrimetry value of carboxyl. | N |
| Linear and cyclic esters | 2.7 mmol/g | Infrared spectrometry. | P |
| Ketone group | 2.6 mmol/g | $^{13}$C-NMR spectrometry. | N |
| Phenol group | | | |
| Reactive to methylation | 1.5 mmol/g | Diazomethane methylation–$^{13}$C-NMR spectrometry. | N |
| Reactive to acetylation | 1.4 mmol/g | Acetylation under Schotten-Baumann conditions, $^1$H-NMR. | M |
| Titrated at pH 10 | 1.2 mmol/g | Titrated at pH 10 times 2. | L |
| Nonreactive phenol | 2.7 mmol/g | Infrared spectrometry after acetylation under Schotten-Baumann conditions. | M |
| Alcoholic hydroxyl group | 1.5 mmol/g | Infrared spectrometry after acetylation under Schotten-Baumann conditions. | M |
| Total hydroxyl group (noncarboxylic) | 4.4 mmol/g | Acetylation–$^1$H-NMR. | M |
| Total hydroxyl group (noncarboxylic) | 7.0 mmol/g | Permethylation with NaH, $^{13}CH_3I$, $^{13}$C-NMR. | N |
| Total exchangeable hydrogen (total acidity) | 10.5 mmol/g | $^1$H-NMR in dioxane–$d_8$. | M |

(b) Humic acid

| Measurement | Value | Method | Chapter |
|---|---|---|---|
| Carboxyl group | 4.9 mmol/g | Potentiometric titration. | N |
| Ester (linear and cyclic) plus amide groups | 2.3 mmol/g | $^{13}$C-NMR determination minus potentiometric titration value for carboxyl group. | N |
| Ketone group | 3.2 mmol/g | $^{13}$C-NMR spectrometry. | N |
| Phenol group (reactive to methylation) | 2.9 mmol/g | Diazomethane methylation–$^{13}$C-NMR spectrometry. | N |
| Total hydroxyl (noncarboxylic) | 9.0 mmol/g | Permethylation with NaH, $^{13}CH_3I$, $^{13}$C-NMR spectrometry. | N |

## APPENDIX OF DATA ON HUMIC SUBSTANCES FROM THE SUWANNEE RIVER—Continued

III. Composition of humic substances isolated from the Suwannee River—Continued

A. Major constituents—Continued

3. Structural carbon distributions

(a) Fulvic acid

| Measurement | Value | Method | Chapter |
|---|---|---|---|
| Aliphatic -C-H | 27 percent | Quantitative $^{13}$C-NMR spectrometry. | N |
| Aliphatic H-C-O- | 15 percent | -do- | N |
| Aromatic/Acetal-C | 5 percent | -do- | N |
| Aromatic-C | 28 percent | -do- | N |
| Carboxyl/Ester-C | 19 percent | -do- | N |
| Ketone-C | 6 percent | -do- | N |

(b) Humic acid

| Measurement | Value | Method | Chapter |
|---|---|---|---|
| Aliphatic -C-H | 17 percent | Quantitative $^{13}$C-NMR spectrometry. | N |
| Aliphatic H-C-O- | 12 percent | -do- | N |
| Aromatic/Acetal-C | 6 percent | -do- | N |
| Aromatic-C | 42 percent | -do- | N |
| Carboxyl/Ester/Amide-C | 16 percent | -do- | N |
| Ketone-C | 7 percent | -do- | N |

4. Structural (nonexchangeable) proton distributions

(a) Fulvic acid

| Measurement | Value | Method | Chapter |
|---|---|---|---|
| Aliphatic -C-H | 32 percent | Quantitative $^{1}$H-NMR spectrometry. | N |
| Aliphatic H-C-C=O/H-C-O | 30 percent | -do- | N |
| Aliphatic H-C-O- | 23 percent | -do- | N |
| Aromatic ϕ-H | 15 percent | -do- | N |

(b) Humic acid

| Measurement | Value | Method | Chapter |
|---|---|---|---|
| Aliphatic -C-H | 17 percent | Quantitative $^{1}$H-NMR spectrometry. | N |
| Aliphatic H-C-C=O/H-C-O | 26 percent | -do- | N |
| Aliphatic H-C-O- | 34 percent | -do- | N |
| Aromatic ϕ-H | 22 percent | -do- | N |

B. Minor constituents

1. Inorganic elements

(a) Fulvic acid

| Element | Standard fulvic acid value[1] (μg/g) | Reference fulvic acid value[1] (μg/g) | Method | Chapter |
|---|---|---|---|---|
| Ag | 9 | 3 | Inductively-coupled plasma/ | E |
| Al | 30 | 20 | mass spectrometry. | E |
| B | 200 | 80 | -do- | E |

[1]Values not in parentheses are semiquantitative; values in parentheses are quantitative measurements determined by isotope dilution analyses.

## APPENDIX OF DATA ON HUMIC SUBSTANCES FROM THE SUWANNEE RIVER—Continued

III. Composition of humic substances isolated from the Suwannee River—Continued
B. Minor constituents—Continued
1. Inorganic elements—Continued
(a) Fulvic acid—Continued

| Element | Standard fulvic acid value[1] (µg/g) | Reference fulvic acid value[1] (µg/g) | Method | Chapter |
|---|---|---|---|---|
| Ba | 0.3(6.8) | 0.4(7.8) | Inductively-coupled plasma/ | E |
| Ca | 100 | 90 | mass spectrometry. | E |
| Cd | <0.1(0.06) | <0.1(0.09) | -do- | E |
| Cr | 4 | 2 | -do- | E |
| Cu | 3(1.7) | 2(1.7) | -do- | E |
| Fe | 20 | Not detected | -do- | E |
| Hf | 0.1 | 0.1 | -do- | E |
| Hg | 1 | <0.1 | -do- | E |
| Mg | 2 | Not detected | -do- | E |
| Mo | 505 | 2 | -do- | E |
| Na | 50 | 60-do- | -do- | E |
| Ni | 1(0.92) | 2(1.6) | -do- | E |
| Pb | 4(1.2) | 5(1.8) | -do- | E |
| Sc | 3 | 1 | -do- | E |
| Sn | 10 | 20 | -do- | E |
| Sr | 0.4(0.12) | 0.4(1.8) | -do- | E |
| Th | 10 | 20 | -do- | E |
| Ti | 1 | 1 | -do- | E |
| Zn | 3 | 3 | -do- | E |
| Zr | 7 | 6 | -do- | E |

(b) Humic acid

| Element | Standard humic acid value[1] (µg/g) | Reference humic acid value[1] (µg/g) | Method | Chapter |
|---|---|---|---|---|
| Ag | 200 | 20 | Inductively-coupled plasma/ | E |
| Al | 200 | 100 | mass spectrometry. | E |
| B | 300 | 200 | -do- | E |
| Ba | 0.1(10.5) | 0.2(3.9) | -do- | E |
| Ca | 90 | 200 | -do- | E |
| Cd | <0.1(0.07) | <0.1(0.14) | -do- | E |
| Cr | 10 | 10 | -do- | E |
| Cu | 10(9.6) | 4(5.0) | -do- | E |
| Fe | 400 | 200 | do- | E |
| Hf | 2 | 2 | -do- | E |
| Hg | 20 | 20 | -do- | E |
| Mg | 4 | 4 | -do- | E |
| Mo | 30 | 20 | -do- | E |
| Na | 40 | 100 | -do- | E |
| Ni | 10(7.2) | 8(5.4) | -do- | E |
| Pb | 2(1.4) | 3(1.6) | -do- | E |
| Sc | 2 | 3 | -do- | E |
| Sn | 20 | 20 | -do- | E |
| Sr | 0.1(0.26) | 0.1(0.83) | -do- | E |

[1]Values not in parentheses are semiquantitative; values in parentheses are quantitative measurements determined by isotope dilution analyses.

## APPENDIX OF DATA ON HUMIC SUBSTANCES FROM THE SUWANNEE RIVER—Continued

III. Composition of humic substances isolated from the Suwannee River—Continued
B. Minor constituents—Continued
1. Inorganic elements—Continued
(b) Humic acid—Continued

| Element | Standard humic acid value[1] (µg/g) | Reference humic acid value[1] (µg/g) | Method | Chapter |
|---|---|---|---|---|
| Th | 7 | 6 | Inductively-coupled plasma/ | E |
| Ti | 10 | 10 | mass spectrometry. | E |
| Zn | 4 | 4 | -do- | E |
| Zr | 90 | 80 | -do- | E |

2. Amino acid residues
(a) Fulvic acid

| Amino acid | Value (nmol/mg) | Method | Chapter |
|---|---|---|---|
| Basic: | | | |
| Arginine | 0 | Acid hydrolysis, amino-acid analyzer. | F |
| Ornithine | 0 | -do- | F |
| Lysine | 0.5 | -do- | F |
| Histidine | 0.2 | -do- | F |
| Acidic: | | | |
| Aspartic acid | 5.7 | -do- | F |
| Glutamic acid | 3 | -do- | F |
| β-Alanine | 0 | -do- | F |
| Hydroxy-imino: | | | |
| Proline | 1.8 | -do- | F |
| Hydroxyproline | 0 | -do- | F |
| Serine | 1.5 | -do- | F |
| Threonine | 1.7 | -do- | F |
| Neutral: | | | |
| Glycine | 10.5 | -do- | F |
| Alanine | 2.8 | -do- | F |
| Leucine | 0.9 | -do- | F |
| Isoleucine | 0.7 | -do- | F |
| Valine | 1.2 | -do- | F |
| Sulfur: | | | |
| Cysteine | 0 | -do- | F |
| Methionine | 0 | -do- | F |
| Aromatic: | | | |
| Phenylalanine | 0.4 | -do- | F |
| Tyrosine | 0.2 | -do- | F |

Values not in parentheses are semiquantitative; values in parentheses are quantitative measurements determined by isotope dilution analyses.

## APPENDIX OF DATA ON HUMIC SUBSTANCES FROM THE SUWANNEE RIVER—Continued

III. Composition of humic substances isolated from the Suwannee River—Continued

B. Minor constituents—Continued

2. Amino acid residues—Continued

(b) Humic acid

| Amino acid | Value (nmol/mg) | Method | Chapter |
|---|---|---|---|
| Basic: | | | |
| Arginine | 1.4 | Acid hydrolysis, amino-acid analyzer. | F |
| Ornithine | 0.4 | -do- | F |
| Lysine | 2.4 | -do- | F |
| Histidine | 1.3 | -do- | F |
| Acidic: | | | |
| Aspartic acid | 11.9 | -do- | F |
| Glutamic acid | 9.1 | -do- | F |
| β-Alanine | 2.9 | -do- | F |
| Hydroxy-imino: | | | |
| Proline | 7.8 | -do- | F |
| Hydroxyproline | 16.2 | -do- | F |
| Serine | 5.3 | -do- | F |
| Threonine | 5.8 | -do- | F |
| Neutral: | | | |
| Glycine | 22 | -do- | F |
| Alanine | 9.7 | -do- | F |
| Leucine | 4.3 | -do- | F |
| Isoleucine | 2.9 | -do- | F |
| Valine | 5.3 | -do- | F |
| Sulfur: | | | |
| Cysteine | 0.7 | -do- | F |
| Methionine | 0.7 | -do- | F |
| Aromatic: | | | |
| Phenylalanine | 2 | -do- | F |
| Tyrosine | 1.1 | -do- | F |

3. Free-radical content

| Sample | Value (spin content per gram) | Method | Chapter |
|---|---|---|---|
| Standard fulvic acid (solid phase) | $5.41\times10^{17}$ | Electron-spin-resonance spectrometry. | G |
| Standard fulvic acid (liquid phase, pH 12.5) | $1.06\times10^{18}$ | -do- | G |
| Reference fulvic acid (solid phase) | $7.85\times10^{17}$ | -do- | G |
| Reference fulvic acid (liquid phase, pH 12) | $1.29\times10^{18}$ | -do- | G |
| Standard humic acid (solid phase) | $3.93\times10^{17}$ | -do- | G |
| Standard humic acid (liquid phase, pH 12.5) | $2.62\times10^{18}$ | -do- | G |
| Reference humic acid (solid phase) | $4.87\times10^{17}$ | -do- | G |

## APPENDIX OF DATA ON HUMIC SUBSTANCES FROM THE SUWANNEE RIVER—Continued

III. Composition of humic substances isolated from the Suwannee River—Continued

B. Minor constituents—Continued

3. Free-radical content—Continued

| Sample | Value (spin content per gram) | Method | Chapter |
|---|---|---|---|
| Reference humic acid (liquid phase) | $2.04 \times 10^{18}$ | -do- | G |

IV. General chemical and physical properties

A. Molecular weight

| Sample | Value (daltons) | Method | Chapter |
|---|---|---|---|
| Standard fulvic acid | Number average $M_n$=756 | Vapor pressure osmometry in tetrahydrofuran. | J |
| Reference fulvic acid | $M_n$=781 | -do- | J |
| Standard fulvic acid | $M_n$=734 | Vapor pressure osmometry in water. | J |
| Reference fulvic acid | $M_n$=823 | -do- | J |
| Reference fulvic acid | $M_n$=655<br>Weight average $M_w$=1,335 | Equilibrium ultracentrifugation in tetrahydrofuran. | J |

B. Molecular size

| Sample | Measurement | Value | Method | Chapter |
|---|---|---|---|---|
| Standard fulvic acid | Radius of gyration (pH 9) | 7.7 angstroms | Low-angle (X-ray scattering). | J |
| Reference fulvic acid | -do- | 7.0 angstroms | -do- | J |
| Standard humic acid | -do- | 11.4 angstroms | -do- | J |
| Reference humic acid | -do- | 11.3 angstroms | -do- | J |
| Standard fulvic acid | Molar volume in water, pH 6.5 | 3,950 cubic centimeters | Fluorescence depolarization. | K |
| Standard fulvic acid | Molar volume in water, pH 4.0 | 4,390 cubic centimeters | -do- | K |
| Standard fulvic acid | Molar volume in dimethylsulfoxide | 2,860 cubic centimeters | -do- | K |

C. Density of fulvic acid

| Solvent | Sample form | Value (grams per milliliter) | Method | Chapter |
|---|---|---|---|---|
| Water containing 0.2 N KCl, pH 8 | Potassium salt | 2.198 | Pycnometry. | O |
| 95 percent tetrahydrofuran 5 percent $H_2O$ | Free acid | 1.827 | -do- | O |
| 94 percent tetrahydrofuran 4 percent $H_2O$, 1 percent HCl | Free acid | 1.757 | -do- | O |
| Tetrahydrofuran | Free acid | 1.755 | -do- | O |
| N,N-dimethylforamide containing 1 percent trifluoroacetic acid | Free acid | 1.731 | -do- | O |

## APPENDIX OF DATA ON HUMIC SUBSTANCES FROM THE SUWANNEE RIVER–Continued

IV. General chemical and physical properties—Continued

C. Density of fulvic acid—Continued

| Solvent | Sample form | Value (grams per milliliter | Method | Chapter |
|---|---|---|---|---|
| Water | Free acid | 1.679 | -do- | O |
| Water containing 0.1 N hydrochloric acid | Free acid | 1.670 | -do- | O |
| Dimethylsulfoxide containing 0.1 N trifluoroacetic acid | Free acid | 1.658 | -do- | O |
| N,N-dimethylforamide | Free acid | 1.616 | -do- | O |
| N,N-dimethylforamide | Methyl ester | 1.533 | -do- | O |
| 94 percent dimethylsulfoxide, 5 percent $H_2O$, 1 percent HCl | Free acid | 1.465 | -do- | O |
| Dimethylsulfoxide | Methyl ester | 1.379 | -do- | O |
| Anhydrous N,N-dimethylforamide | Methyl ester | 1.379 | -do- | O |

D. Heat of combustion of fulvic acid

| Determination | Value | Method | Chapter |
|---|---|---|---|
| Experimental measurement | 3,375 kcal/mol | Calorimetry. | I |
| Calculated measurement | 3,295 kcal/mol | Additivity of bond heats of combustion from number-average model. | P |

E. Fluorescence properties of fulvic acid

| Measurement | Value | Method | Chapter |
|---|---|---|---|
| Fluorescence lifetime in water | 1.05 and 6.54 ns of the two fluorophores | Fluorescence spectrometry. | K |
| Fluorescence lifetime in dimethylsulfoxide | 1.31 and 6.78 ns of the two fluorophores | -do- | K |
| Excitation and emission maxima of fluorophores | 350 nm excitation 410 nm and 470 nm emission maxima | -do- | K |



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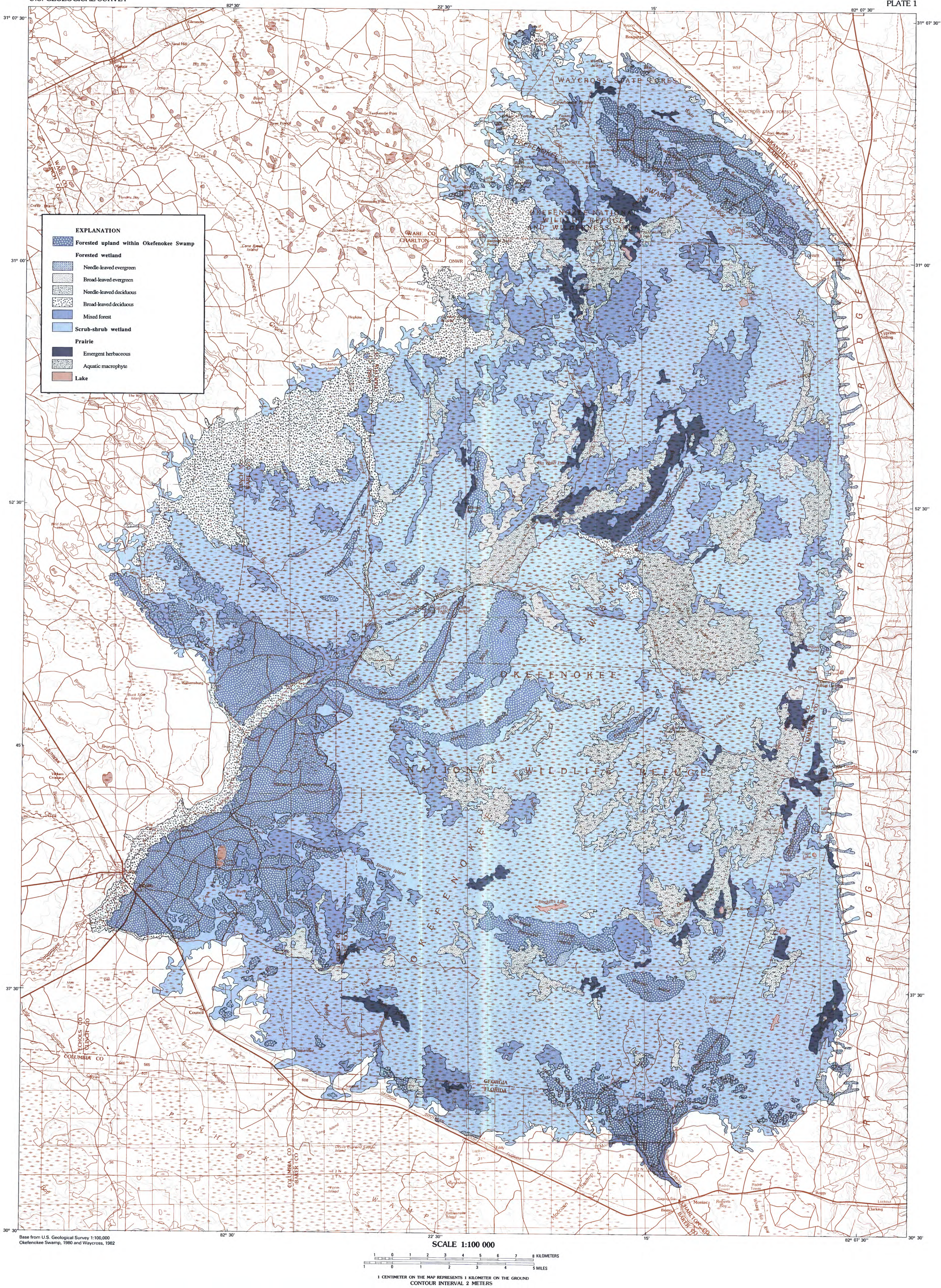
WATER-SUPPLY PAPER 2373
PLATE 1




# MAP SHOWING DISTRIBUTION OF VEGETATION IN THE OKEFENOKEE SWAMP, SOUTHEASTERN GEORGIA AND NORTHEASTERN FLORIDA