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THE UNIVERSITY OF ALBERTA

CHARACTERIZATION OF Ah HUMIC ACIDS OBTAINED FROM SEVERAL SELECTED SOIL SERIES

by

© JOSEPH DOUGLAS HELGELAND

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Characterization of Ah Humic Acids Obtained From Several Selected Soil Series" submitted by Joseph Douglas Helfeland in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Soil Chemistry.

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ABSTRACT

Humic acids were extracted from samples of Ah horizons obtained from sites representative of several different soils. The soils sampled have each developed under different conditions of climate and vegetative cover. Humic acids were extracted from separate soil Ah samples with 0.5N sodium hydroxide and with the sodium form of Dowex A-1, a chelating resin. The humic acids obtained by these extractions were characterized with respect to ash content, carbon content, nitrogen content, optical properties, amino acid composition and susceptibility to enzyme hydrolysis by the proteolytic enzyme pronase. Results of these analyses showed there were differences in the ash content, total nitrogen content and quantitative amino acid composition of humic acids when they were obtained from different soils. Attempts to release amino acids from humic acids through the action of the proteolytic enzyme pronase showed humic acids from different soils varied considerably in their susceptibility to enzyme hydrolysis.

In order to further characterize humic acids a fractionation procedure was developed which uses phenol and acetone as extracting solvents to separate humic acids into three distinct humic components. Yield data showed that the proportion in which these components were present in humic acid depended on the soil series from which the humic acid originated. Comparison of optical and chemical properties of humic components recovered from different humic acids indicated that

similar components were obtained from all humic acids studied regardless of soil series or soil site from which the humic acid was obtained.

Humic components separated from the same humic acid differed from each other in optical properties, total carbon and nitrogen content, quantitative amino acid composition and ease of hydrolysis by pronase. It was concluded that phenol-acetone fractionation of soil humic acid results in the recovery of humic components which are in different relative states of humification. It was further concluded that differences between humic acids obtained from soils which have developed under different conditions of climate and vegetation, are associated mainly with differences in the relative distribution and manner of combination of individual humic components in the humic acids.

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INTRODUCTION

Levels and vertical distribution of organic matter in soils have long been known to be related to local soil-forming conditions, and are a prominent feature of some systems of soil classification. However, relatively little success has been met with in attempts to relate qualitative characteristics of the organic matter to the conditions under which the organic matter has formed.

The most representative and stable fraction of soil organic matter is humic acid, which can comprise as much as 30 to 80 percent of the total soil organic matter (Paul, 1970). Humic acid is obtained as a dark-colored amorphous precipitate when an alkaline extract of soil organic matter is acidified to pH 1.0 to 1.5 with hydrochloric or sulphuric acid. This procedure also often results in the recovery of an acid soluble component of soil organic matter called fulvic acid.

It has been postulated that the formation of humic materials in soils is not enzymatically controlled, but is the result of condensations of microbial degradation products (Flaig, 1964, 1966, 1968; Kononova, 1966). Consequently it is reasonable to assume that the physical and chemical properties of humic materials are affected by the environmental conditions prevalent at the time of formation. Thus these materials should display properties which are related to their environment both in terms of soil genesis and the plant ecosystem. In support of this contention various workers (Kononova, 1966; Tokudome et al, 1965), especially in Russia (Kononova, 1966), have reported definite relationships between the humic: fulvic acid

ratio and soil type. On the other hand, there have been only a few reports suggesting qualitative differences in these fractions that can be related to soil type, and it has been suggested (Lowe, 1969) that the distribution and manner of combination of humic acids may be of greater significance than differences in composition.

Recently some research on humic acids has concerned the biodegradation of these substances as a possible means of structure determination (Mathur and Paul, 1966; Ladd and Brisbane, 1967). Humic acids prepared in the usual manner by extraction from soils with dilute alkali or neutral salt solutions, contain varying amounts of nitrogen, of which up to 50 percent may be accounted for as α-amino acids (Bremner, 1955). The amino acid content of humic acids is thought to arise from peptides or proteins incorporated in the humic acid structure during the condensation with various phenols and quinones (Kononova, 1966; Flaig, 1964, 1966, 1968). Humic acids often differ in their total content of acid hydrolysable amino acids; analysis of these hydrolysates have generally shown only small variations in the relative proportion of amino acids present (Lowe, 1969; Brisbane et al, 1972; Khan and Sowden, 1971). Studies on the susceptibility of humic acid nitrogen to enzyme hydrolysis have shown that up to 40 percent of the acid hydrolysable amino acid nitrogen can be released by an extracellular proteolytic enzyme, pronase (Ladd and Brisbane, 1967). However very little information is available on the variability of the α-amino acid content of humic acids from different soils to hydrolysis by proteolytic enzymes.

Other studies (Ladd and Butler, 1969a, 1969b) have shown that soil humic acids can either inhibit or stimulate proteolytic enzyme

activity depending on the enzyme used. The results of these studies indicated that various humic acid fractions had a number of properties which were of overriding importance in determining the magnitude of their influence. The exact nature of these properties was not entirely clear, however relative carboxyl content of humic fractions was an important factor in determining degree of inhibition.

If the properties of humic acids are related to soil type and are affected by soil forming factors such as climate and vegetation, then the effect humic acids have on enzymes with respect to inhibition or lack of inhibition, or the relative resistance of humic acids to degradation by hydrolytic enzymes, could, together with conventional chemical analysis, provide a powerful tool for indicating structural differences among various humic acids. Such information might allow correlation of soil types with differences in mechanisms of organic matter turn-over, such as organic matter-mineral interactions which are too subtle to be detected by purely routine chemical analysis.

In view of the above considerations the present study was undertaken to provide some basic data on the nature of the humic acids associated with four different soil series located in central and southern Alberta. The soils selected for study are similar in the respect that they all have an Ah horizon, and dissimilar in the respect that they have each developed under slightly different conditions of climate and vegetation.

In order to compare humic acids from different soils it was decided to attempt to develop a fractionation procedure which would perform the separation of a nitrogen rich fraction from the bulk of humic acid material. It is hoped that once separated subsequent

characterization of such fractions by chemical and biochemical means might reveal properties which are characteristic of humic acid isolated from a particular soil type. It is also hoped that characterization of such fractions will further our insight into the susceptibility of the amino acid containing component of various humic acids and humic fractions to enzymatic hydrolysis. Thus the specific objectives of the project are:

(a) To develop an extraction procedure which results in the isolation of relatively distinct nitrogen rich fractions from the bulk of humic acid.

(b) To characterize some of the properties of the humic acids before and after fractionation and to extend such studies to the fractions obtained from humic acids.

(c) To study the susceptibility of the humic acids and humic fractions to enzymatic hydrolysis by the proteolytic enzyme, pronase.

(d) To compare the properties of similar fractions obtained from the Ah horizon of several soil types distributed throughout central and southern Alberta.

1. LITERATURE REVIEW

1.1 Soil Humic Acids

The importance of organic matter in the soil is implicit in the definition of soil, which recognizes fertility as the unique and constant feature distinguishing soil from the parent rock. In the formation of soil, organic substances play a direct part in being the sources of plant nutrients which are liberated in available forms during mineralization. Besides being a source of nutrients for plants, soil organic matter plays a direct role in soil structure formation, and has a large effect on such soil properties as exchange capacity and buffering properties.

One of the most representative and stable fractions of soil organic matter is humic acid. Humic acid is obtained as an amorphous black precipitate when the pH of alkaline solutions of soil organic matter is lowered to 1 to 1.5 by the addition of dilute mineral acids such as HCl. This procedure often also results in the recovery of an acid soluble fraction called fulvic acid. Other fractions which may be obtained are hymatomelanic acid, the portion of humic material soluble in ethanol, and humin, the residual insoluble soil fraction.

Of the humic fractions mentioned above, by far the most intensively studied is humic acid. To date, no single method has been developed that will shed light on the nature of more than a small fraction of the humic acid moiety. Two fundamental characteristics of humic acid appear to be responsible for this impasse: (a) the complex nature of the individual humic acid molecules

themselves, and (b) the large probability that there exists a great variability in the structure of humic acids so that there is no single fraction of the material that can be said to represent the characteristics of the whole substance. Despite this state of affairs, many attempts have been made to characterize soil humic acids. These have included elemental analysis, oxidative degradation, reductive degradation, and functional group analysis.

1.2 Elemental Analysis of Humic Acids

Elemental analysis of humic acids characteristically show a range of values. Typical values given by Dubach and Mehta (1963) are carbon, 45 to 65 percent; oxygen, 48 to 30 percent; nitrogen, 2 to 6 percent and hydrogen about 5 percent. Some variations in the range of these values can be found in the literature (Hurst and Burges, 1967; Kononova, 1966; Lowe, 1969). The elemental composition of humic acids from a number of different soils is shown in Table 1.1.

1.3 Aromatic Constituents of Humic Acids

Many types of benzene derivatives have been identified in products of various degradations of humic acids. Schnitzer and Wright (1960) found a total of about 6.0 percent benzene carboxylic acids plus picric acid in their nitric acid oxidation products of humic acid from a Podzol. Khan and Schnitzer (1972) oxidized humic acids, fulvic acids, and humins extracted from the Ah horizon of a Black Chernozem, a Black Solod and a Black Solonetz soil, with potassium permanganate. Total yields of oxidation products followed the order humic acids > humins > fulvic acids. The products resulting from the

TABLE 1.1 ELEMENTAL COMPOSITION OF HUMIC ACIDS FROM DIFFERENT SOILS

Source of Humic Acid	% C	% H	% N	% O	C:N	C:H	Author
Podzolic soil	52.39	4.82	3.74	39.05	14.0	10.9	
Rendzina	54.90	4.36	4.07	36.67	13.5	12.6	
Degraded Chernozem	56.34	3.54	3.58	36.65	15.7	15.9	Kononova (1966
Deep Chernozem	57.47	3.38	3.78	35.37	15.2	17.0	
Ordinary Chernozem	58.37	3.26	3.70	34.67	15.7	17.9	
Chestnut soil	58.56	3.40	4.09	33.95	14.3	17.2	
Gray Solonetz	56.92	4.66	4.25	33.90	13.4	12.2	
Gray Solod	55.80	4.75	3.49	35.70	16.0	11.8	
Orthic Gray Wooded	56.38	4.42	4.72	34.30	12.0	12.9	
Orthic Black Chernozem	55.79	3.73	4.45	35.70	13.0	15.0	Lowe (1969)
Black Solonetz	55.65	4.45	3.88	35.80	14.4	12.5	
Orthic Brown Chernozem	57.36	4.75	3.67	33.80	15.6	12.1	
Brown Solonetz	56.00	4.82	4.63	34.20	12.1	11.6	

1. 1.3 (continued)

oxidation of humic and fulvic acids averaged 63 percent benzene carboxylic, 32 percent phenolic, and 5 percent aliphatic carboxylic acids. The oxidation products from humins, averaged 76 percent benzene carboxylic, but only 20 percent phenolic and 4 percent aliphatic carboxylic acids, indicating some differences in the chemical structure of humins from those of humic and fulvic acids. The most prominent compounds produced by the oxidation of the humic acids were hydroxy benzenepentacarboxylic and benzenetetracarboxylic acids. In general, differences in the distribution of the major oxidation products between the three major fractions were greater than those within individual humic fractions. Thus, the chemical structures of humic acids extracted from three different soils appeared to be more similar to each other than to those of fulvic acid and humin fractions from the same soil. The same was true for fulvic acids and humins extracted from the different soils.

Chakrabartty et al (1974) oxidized humic acid from the Ah horizon of a Black Chernozemic soil (Malmo series) with sodium hypochlorite. Their results showed the presence of benzene carboxylic acids and aliphatic acids in the final reaction mixture similar in nature to those reported by Khan et al (1972) and Matsuda et al (1972). Based on the known chemistry of hypohalite oxidation Chakrabartty et al concluded that soil humic acid represents a special group of mixed alkyl-aryl-cycloalkyl compounds. They speculated that natural processes

1. 1.3 (continued)

of oxidation would convert the cyclo-alkyl skeletal arrangements of the component molecules of humic acid from a normal bicyclo- or hydro-aromatic system to a bridged tricyclo- or more complex system at the expense of longer alkyl chains.

Other studies of soil humic acids have shown that polyhydroxy phenols and their derivatives can be identified in sodium amalgam reductions (Stevenson and Mendez, 1967; Mendez and Stevenson, 1966; Burges et al, 1964), microbial metabolites (Mathur and Paul, 1966), acid hydrolysates (Mendez, 1967), and alkaline nitrobenzene oxidations (Morrison, 1958, 1963; Wildung et al, 1970).

Wildung, et al (1970) measured the content of p-hydroxybenzaldehyde, vanillin, and syringaldehyde released by alkaline nitrobenzene oxidations of humic acids and found total phenolic aldehydes ranging from 1.53 percent down to no detectable amounts, depending upon the particular humic acid studied. This data confirmed the results obtained earlier by Morrison (1958, 1963). Stevenson and Mendez (1967) estimated that only a small fraction of the 12 percent yield of products found to be ether soluble after sodium amalgam reduction of a humic acid occurred as phenols and phenolic acids. Mendez (1967) degraded humic acids extracted from a brunizem by acid hydrolysis and found the amount of positively identified phenols to be minimal. He concluded that the predominant material in his extracts was aliphatic in nature.

1.4 Functional Groups Associated with Humic Acids

A variety of functional groups, including COOH, phenolic OH,

1. 1.4 (continued)

enolic OH, quinone, hydroxyquinone, lactone, ether and alcoholic OH, have all been reported in humic substances (Stevenson and Butler, 1969), but there is considerable disagreement as to the amounts present. Dubach and Mehta (1963) pointed out that severe problems are encountered in the determination of functional groups because of incomplete reactions, adsorption of reagents, undesirable fractionation during manipulations, sensitivity towards acid and base at high temperatures and the proximity of groups which influence the specificity of reagents. Since the acidities of the various groups overlap (Stevenson and Butler, 1969), results obtained by methods dependent on ion exchange or pK values must be interpreted with caution. Polycarboxylic acids, for example, exhibit a whole series of dissociation constants which decrease as successive protons dissociate. On the other hand, substituted phenols are often more strongly dissociated than the unsubstituted phenol.

Despite the inherent difficulties in determining humic acid functional groups, some work has been done (Schnitzer and Desjardins, 1962; Wright and Schnitzer, 1960; Schnitzer and Gupta, 1965). The results of some of this work is summarized in Table 1.2, which has been adapted from Stevenson and Butler (1969). The data show the distribution of oxygen-containing functional groups in some humic and fulvic acids. For any specific group a considerable range of values is apparent, even with preparations obtained from the same soil type. Nevertheless,

TABLE 1.2 OXYGEN-CONTAINING FUNCTIONAL GROUPS IN SOME SOIL HUMIC AND FULVIC ACIDS (Stevenson and Butler, 1969)

Oxygen %	Total Acidity (meg/ 100 gm)	COOH (meg/ 100 gm)	Acidic OH (meg/ 100 gm)	Weakly Acidic Plus Alcoholic OH (meg/ 100 gm)	C=O (meg/ 100 gm)	OCH_3 (meg/ 100 gm)
Soil Humic Acids						
35.4	570	150	415	275	90	n.d.*
36.7	870	300	570	350	180	n.d.
34.6	570	280	290	300	300	50
Soil Fulvic Acids						
44.7	1238	908	330	355	310	n.d.
47.3	1420	850	570	340	170	n.d.
47.0	1280	610	670	330	300	n.d.
44.1	890	610	280	460	310	30

n.d.* = not determined

1. 1.4 (continued)

certain trends are evident. The total acidities of the fulvic acids are unmistakably higher than those of the humic acids. Both COOH and acidic OH groups (presumed to be phenolic OH) contribute to the acidic nature of these substances, with COOH being the most important. The concentration of exposed acidic functional groups in fulvic acids appears to be substantially higher than in any other naturally occurring organic polymer (Stevenson and Butler, 1969).

The functional groups listed in Table 1.2 were determined by a variety of analytical procedures, some of which produce results of questionable accuracy. However, not all of the variability between samples is due to differences in experimental techniques. For example, the high values shown for acidic OH in the humic preparations examined by Schnitzer and Gupta (1965), which were from a Grey-Wooded soil, were obtained using methods identical to those that Schnitzer and Desjardins (1962) employed. The latter study was conducted on material recovered from a Podzol soil.

Methoxyl and C=O groups seem to be universally present in humic substances (Stevenson and Butler, 1969). On the other hand, not all samples are reported to contain weakly acidic or alcoholic OH groups (Dubach et al, 1964). There also appears to be a major difference between the functional group content of humic and fulvic acids in that a smaller fraction of the oxygen in the former can be accounted for in COOH, OH and C=O groups. In addition the COOH content of humic substances appears to be inversely related to molecular weight (Stevenson

1. 1.4 (continued)

and Butler, 1969). Fulvic acids have lower molecular weights than humic acids (Butler and Ladd, 1969; Stevenson and Butler, 1969) and the results in Table 1.2 show that the proportion of the oxygen which occurs in the form of COOH is highest for the fulvic acids.

1.5 Relation of Soil Type to Humic Acids

The observation by Russian workers (Kononova, 1966) that changes in the amount of humic acid in various soils were zonal in character prompted studies of the state of such substances in soils in attempts to clarify some of the principles governing humic acid formation. Natural conditions that promote an accumulation of humus in soils have been discussed by Kononova (Kononova, 1966). Briefly such conditions are:

(a) A large quantity of organic matter added to the soil as a primary source of humus substances.

(b) A moderate hydrothermal regime of the soil media.

(c) An average quantity and moderate activity of micro-organisms.

(d) The presence of clay minerals with a high absorption capacity that favors the conservation of humus.

The promotion of the accumulation of humus is determined not by a single condition, but by a combination of the conditions mentioned above. Genetic soil types of various bioclimatic conditions differ in the quantity of humic and fulvic acids produced. For example, in the USSR the moderate moisture regime and near neutral pH usually prevailing in the Gray

1. 1.5 (continued)

Forest soils, Chernozems, and Chestnut soils encourage the formation of humic acids. The quantity of fulvic acids in these soils is low. On the other hand, higher moisture regimes and more acidic conditions generally associated with Podzolic, Krasnozems, and Lateritic soils encourage the formation of fulvic acid. Ratios of humic acid to fulvic acid (Ch/Cf) of Ordinary Chernozems and Krasnozem Soils in the USSR range from 2 to 5 and from 0.6 to 0.8, respectively (Kononova, 1966). Lateritic soils and Krasnozems in South China and Vietnam have relatively low Ch/Cf ratios (0.4 - 0.7) (Tokudome and Konno, 1965). Russian workers (Kononova, 1966) found that soils low in humic acid content, such as Krasnozems and Podzolic soils, were typified by humic material which is predominantly in the form of free polymeric complexes readily soluble by direct treatment with dilute alkali solutions. The content of humic acids linked with calcium in these soils and with stable sesquioxides is very small or almost negligible. Soils with a high humic acid content, such as Chernozems and Chestnut soils, are typified by humic acids linked with calcium and free humic acids are almost completely absent. Russian workers (Kononova, 1966) also found that humic acids from various soils showed differences in the ratio of light absorbed at 465 nm and 665 nm as well as differences in behavior towards precipitation by electrolytes.

Dormaar, et al (1966) and Lowe (1969) have examined humic acids from a variety of soils in Alberta and attempted to

1.5 (continued)

correlate various properties of the humic acids studied to soil types. Dormaar et al (1966) studied a biosequence of soils of the rough fescue prairie-poplar transition in southwestern Alberta. They found changes in the organic matter, because of the encroachment of trees, which were much more strikingly evident than changes in the mineral matter. Some of the changes in organic matter were related to variations in the infrared absorption spectra of humic acids in the 2500 - 1800 cm^{-1} spectral region. Dormaar claimed these could be used to differentiate between humic acids from Chernozemic organic matter versus those from Podzolic organic matter.

In a study of nine soils representing the dominant groups in the major soil zones of Alberta, Lowe (1969) found de-ashed humic fractions showed little variation between soils with respect to elemental composition, functional groups, or electrophoretic behavior. Infrared spectra indicated that samples from orthic Black Chernozemic soils contained higher proportions of aromatic to aliphatic structures. Optical properties and behavior toward electrolytes of humic acid solutions showed some differences between soil types which were similar to variations reported by Russian workers (Kononova, 1966).

In spite of studies such as those reported above, there is still no satisfactory basis for distinguishing humic acids of different soils. It has been suggested (Lowe, 1969) that the distribution and manner of combination of humic acid components may be of greater significance than differences in composition as far as their association with different soils

1. 1.5 (continued)

is concerned.

This contention is supported in part by some work of Coulson, Davis and Lewis (1960). These authors reported on some relationships between plant leaf polyphenols and soil development. They observed that there was a greater diversity and quantity of polyphenolic substances in fresh senescent and recently fallen leaves of plants grown on nutrient deficient (Mor humus) sites compared with the same species grown on nutrient rich (Mull humus) sites. In the living plant leaf polyphenols are isolated in the cell vacuoles separate from the cytoplasmic protein until senescence when autolysis occurs and the contents of the cell mix and combine. Under these conditions it is possible to have condensation of the polyphenols with leaf protein, a reaction called tanning. Such reactions produce material which tends to resist biological degradation (Riberreau-Gayon, 1972). The above authors noted there was a greater chance of tanning of the leaf proteins in fresh oak and beech green leaves from mor sites than those from mull sites. Earlier studies (Davies et al, 1964) showed that synthesis of leucoanthocyanins and tannins in plants grown in field conditions varied between mull and mor sites and, in controlled growth experiments, showed that the increase of leucoanthocyanins and tannins was associated with deficiency of soil nitrogen and phosphorus. These results served to confirm previous work by other authors (Stitt et al, 1946; Riberreau-Gayon, 1972) which had also shown that polyphenols in

1. 1.5 (continued)

growing leaves varies inversely with the nitrogen status of the soil. Extraction of surface litter collected at mor sites by tannin stripping solvents showed more extractable tanning from senescent leaves in mor than in mull sites. All these observations are consistent with the prolonged stability of litter at mor sites and with a different mode of ultimate alteration from that of the but little tanned and readily attacked litter at mull sites (Davies, 1971). This is not to suggest that mor humus is rich in tanned protein, but rather that the initial tanning plays an important role in the mode of degradation of plant material into mor humus forms. Such a view is consistent with current theories of humic acid synthesis (Flaig, 1964, 1966, 1971; Kononova, 1966) which regard humic acids as high-molecular weight products formed by condensation of phenolic and nitrogenous (amino acids, peptides) compounds of plant and microbial origin.

1.6 Soil Humic Acid Nitrogen

Observations on the possible interaction of protein with plant polyphenols as a factor in the genesis of mull and mor humus forms (Coulson et al, 1960; Davies et al, 1964) and theories of humic acid synthesis which regard nitrogen containing compounds as playing a definite role in the origin and structure of humic acids, all suggest that a study of the association of nitrogen with humic material might further our insight into the relationship between soil types and humic acids. The nitrogen content of humic acid varies with different preparations,

1. 1.6 (continued)

values between 0.4 percent to as high as 5 or 6 percent having been reported (Hurst and Burges, 1967). Humic acids from some agricultural soils still contain about 2 percent nitrogen after acid hydrolysis (Hurst and Burges, 1967)while other humic acids may contain as little as 0.4 percent nitrogen before hydrolysis. Approximately 20 to 50 percent of the nitrogen of humic acid can be accounted for as α-amino acids (Bremner, 1955; Hurst and Burges, 1967), 3 to 10 percent is amino sugar (hexosamine) nitrogen (Bremner, 1955), and a small amount appears to be purine or pyrimidine nitrogen (Anderson, 1961); the remainder has not been identified.

The variable nitrogen content of humic acids has led to much speculation on the reactions which might occur during humification of organic matter which would result in the incorporation of nitrogen into the humic acid structure. Kononova (1966) considers that two processes are involved in humification. The first involves the decomposition of plant residues by the soil micro-flora into small molecular units such as phenols and amino acids. The second process involves the polymerization of these simple subunits by the action of phenoloxidase enzymes to produce humic acids.

Swaby and Ladd (1966) have postulated a humic acid structure made up of many heterogeneous units cross-linked in an irregular fashion by covalent bonds. They suggest that a structure such as this could be formed by polymerization reactions between amino acids and enzymically formed phenolic and quinoid free

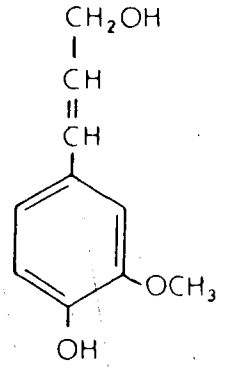
1. 1.6 (continued)

radicals present in plant and microbial cells shortly after death, but before cell lysis and attack by microbes.

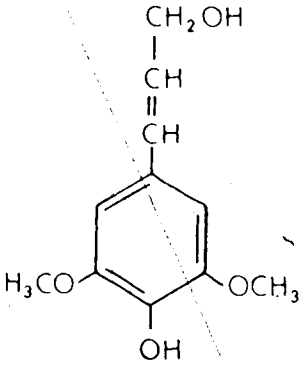
According to Flaig (1964, 1966) lignin and other plant phenolic constituents undergo a series of reactions during microbial degradation which start with oxidative degradation, followed by demethylation and dehydrogenation with concomitant increases in aromaticity, dimerization and polymerization. The polymers can be degraded into CO_2 and H_2O or transformed by ring cleavage into aliphatic compounds which may serve as energy sources for microorganisms. In the course of these processes, microbial autolysis yields amino acids, peptides and ammonia which in turn may become involved in various types of condensation reactions with other products of microbial activity such as phenolic material arising from plant constituents and/or microbial excretions.

Some of the reactions which lignin degradation products might undergo through the action of microorganisms have been summarized by Flaig (1968). These reactions are based on model studies involving compounds related to coniferyl alcohol (I), sinapyl alcohol (II), or p-coumaryl alcohol (III). These compounds are considered to be the basic structural units of lignin (Freudenberg, 1966).

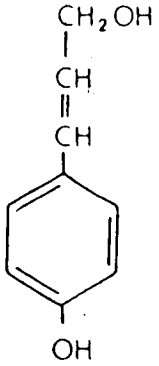
1. 1.6 (continued)



(I)
Coniferyl Alcohol



(II)
Sinapyl Alcohol



(III)
p-Coumaryl Alcohol

Briefly some of the more important reactions involved in the degradation of the above compounds are:

(a) Shortening of the side chain from three carbon atoms to one carbon atom; the carbon atoms are not always split off one after the other, because oxalic or glyoxylic acid are found as a 2C - degradation product by ^{14}C labelling (Flaig, 1968).

(b) Formation of phenols by cleavage of the methyl ether. This reaction produces phenols which have hydroxyl groups in 3,4 or 3,4,5 - positions on the benzene ring and are more reactive than their ethers.

(c) Hydroxylation introduces hydroxyl groups on to the benzene ring. Evans (1947) demonstrated that p-hydroxybenzoic acid is transformed to protocatechuic acid which in turn can be transformed to gallic acid (Haider and Martin, 1967).

(d) Oxidative decarboxylation initiated by phenoloxidases

1. 1.6 (d) (continued)

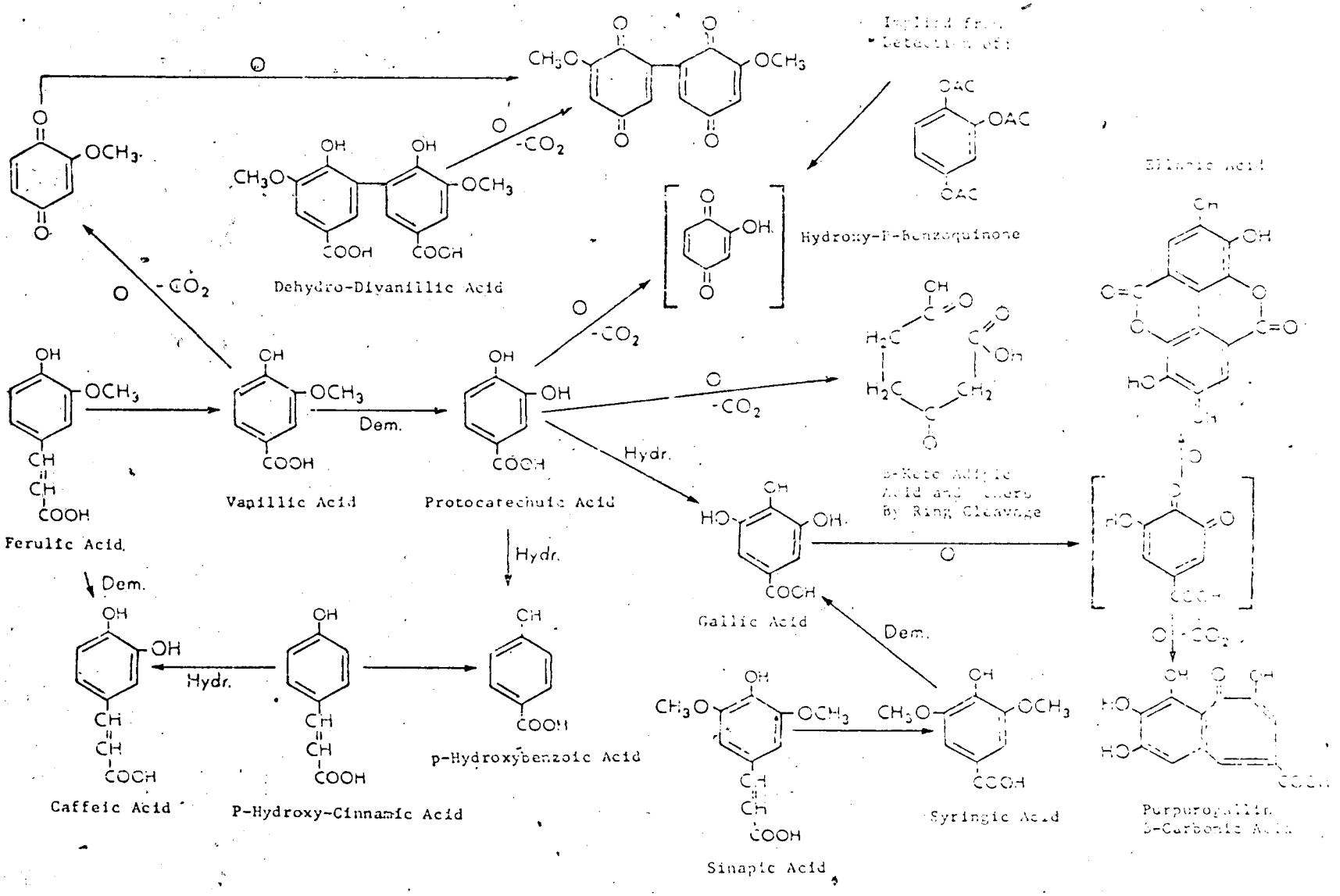
or mild oxidants leads to formation of p-benzoquinones from p-hydroxybenzene carboxylic acids. The formation of p-hydroxybenzoquinone has been verified indirectly by the isolation of the acetate of hydroxyhydroquinone (Flaig, 1968).

(e) Ring cleavage of aromatic degradation products to produce different ketonic acids such as β-ketoadipic acid. These ketonic acids can serve as readily available sources of energy for microorganisms.

(f) Dimerization and polymerization occur by dehydrogenation in the presence of phenoloxidases under oxidizing conditions. For example, vanillic acid dimerises to dehydrovanillic acid and methoxy-p-benzoquinone to 3,3' - dimethoxy-diphenyl-diquinone-2,5,2',5'. The formation of ellagic acid or purpurogallin-β-carboxylic acid may occur through an intermediate such as 3-hydroxy-5-carboxy-benzoquinone-1,2 from gallic acid.

The various reactions discussed above are shown in Figure 1.1. The products and intermediates of these reactions are thought to be the fundamental starting material for reactions which may eventually result in the formation of humic acid. Thus compounds such as p-hydroxybenzoic acid (IV) and ferulic acid (V) may undergo oxidative decarboxylation and polymerization, a reaction initiated by phenoloxidases (Figure 1.2).

Work of Burges et al (1964, 1963), Morrison (1963) and



Dem. = Demethylation
Hydr. = Hydroxylation
O = Oxidation

FIGURE 1.1 TRANSFORMATION OF LIGNIN DEGRADATION PRODUCTS BY THE ACTION OF MICROORGANISMS (TAKEN FROM FLAIG (1968)

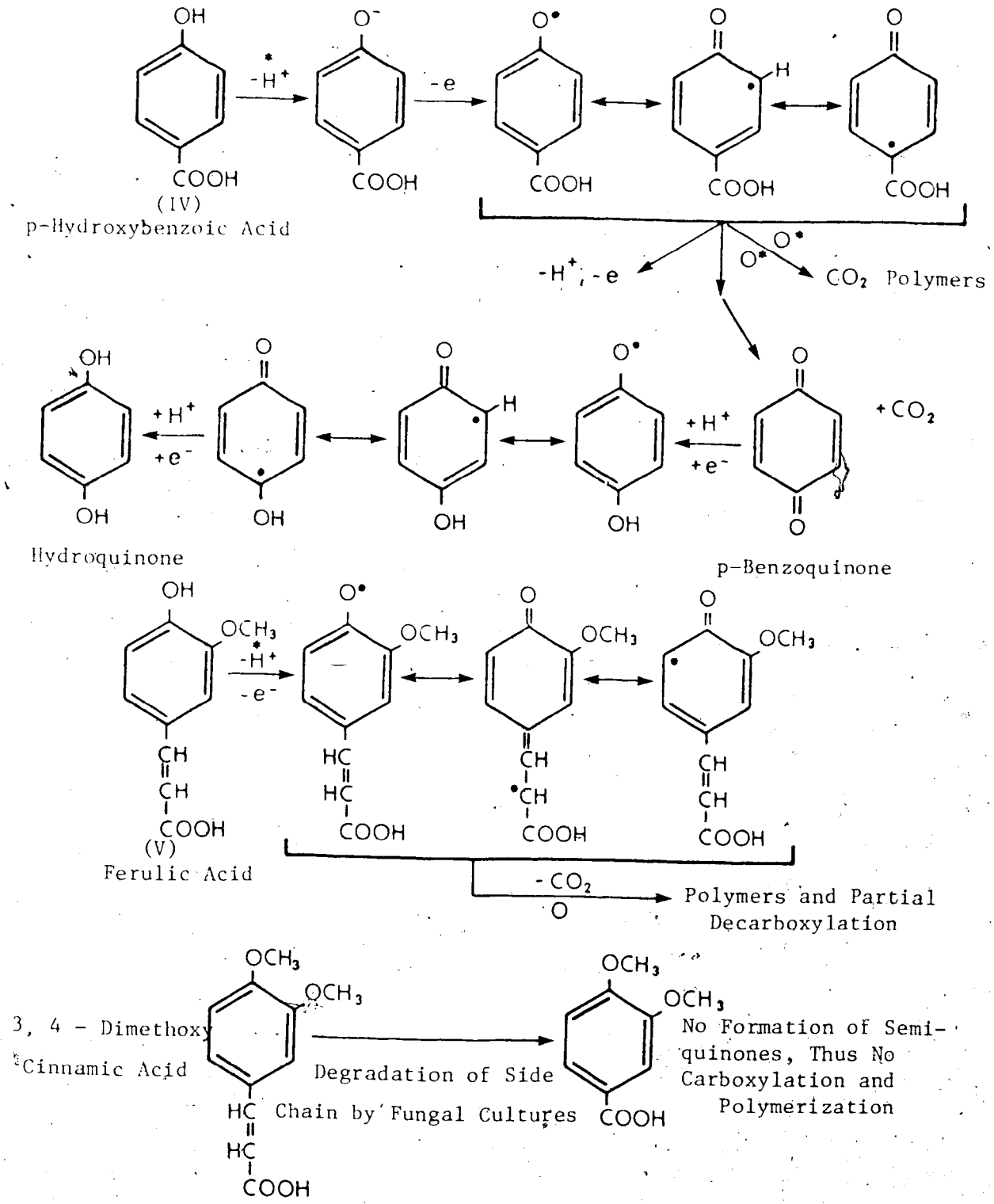


FIGURE 1.2 FORMATION OF SEMIQUINONES - MECHANISM OF OXIDATIVE DECARBOXYLATION AND POLYMERISATION (FLAIG, 1968)

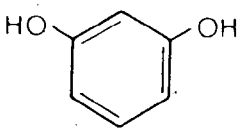
O^* - Oxidation

$\overset{*}{H}$ - Hydrogen Removal by Phenoloxidase Enzymes

1. 1.6 (continued)

Farmer and Morrison (1964) has indicated that phenols derived from flavonoids may also be important initial starting materials for humic acid formation. These authors isolated by reductive degradation or oxidative cleavage of humic acids, compounds which belong to 1,3 - di (VI), or 1,3,5 - triphenols (VII). These compounds cannot be derived-from lignin or its degradation products.

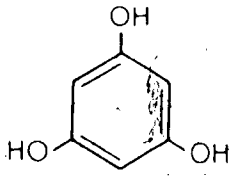
1,3-DIHYDROXY BENZENE



RESORCINOL

(VI)

1,3,5-TRI-HYDROXY BENZENE



PHLOROGLUCINOL

(VII)

Flavonoids are plant phenolics of wide spread occurrence in the plant kingdom (Riberreau-Gayon, 1972). Most flavonoids have the general structure depicted in Figure 1.3.

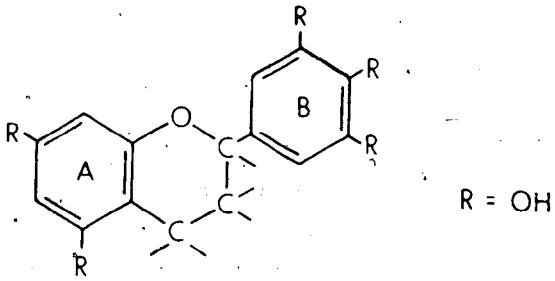
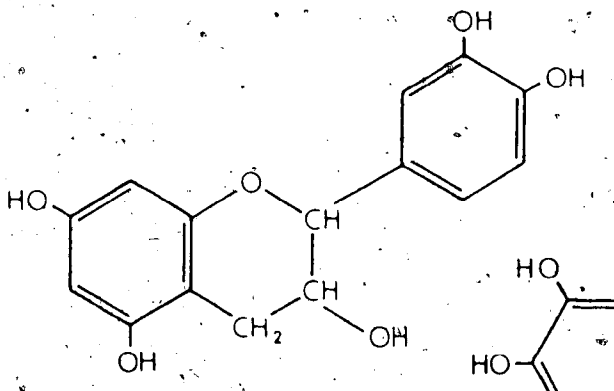


FIGURE 1.3 GENERAL STRUCTURE OF PLANT FLAVONOIDS

There exists a great many classes of plant flavonoids. These are distinguished from each other by the number and distribution of -OH groups on the A and B rings of the structure and also by differences in structure of the 3-carbon

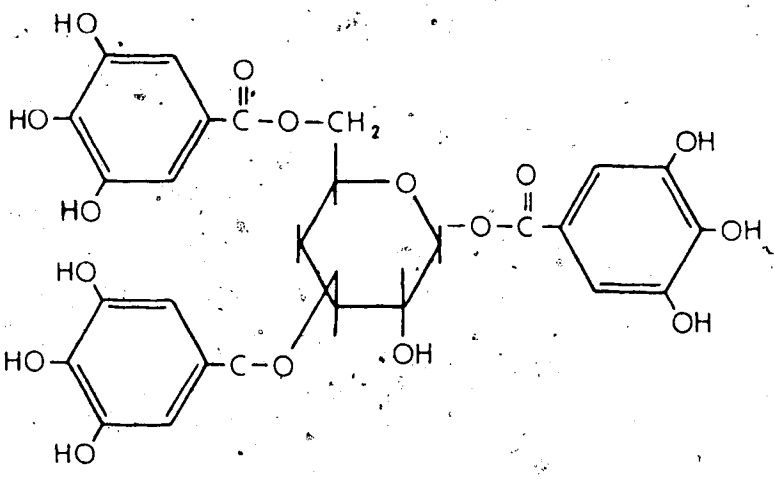
1. 1.6 (continued)

bridging group. Related to flavonoids are plant tannins, a group of high molecular weight polyphenolic materials also widely distributed throughout the plant kingdom (Riberreau-Gayon, 1972). They are generally considered to be resistant to microbial decomposition with condensed tannins more resistant than hydrolyzable tannins (Lewis and Starkey, 1969). The former are polymers of catechin (VIII, Figure 1.4) or similar flavans that are connected by carbon to carbon linkages, whereas the latter are composed of a molecule of carbohydrate, generally glucose, to which gallic acid or similar acids are attached by ester linkages (Riberreau-Gayon, 1972) (IX, Figure 1.4).



Catechin
Building Block of
Condensed Tannins

(VIII)



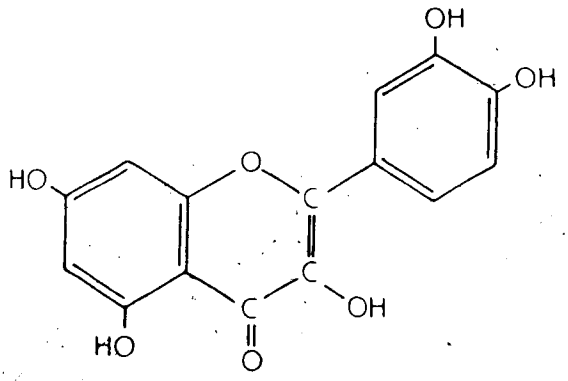
1,3,6-Trigalloylglucose
Building Block of Hydrolysable
Tannins

(IX)

FIGURE 1.4 FUNDAMENTAL CONSTITUENTS OF CONDENSED AND HYDROLYZABLE TANNINS

1. 1.6 (continued)

Little is known concerning the microbial decomposition of condensed tannins or catechin, however it has been established that catechin can be attacked fairly rapidly by fungi (Lewis and Starkey, 1969). Lewis et al (1969) did not detect intermediate substances such as phloroglucinol or protocatechuic acid in decomposition products of catechin resulting from fungal degradation. However these materials have been reported as decomposition products from quercetin (X), a flavon similar in structure to catechin (Westlake et al, 1959, 1961).



Quercetin
(X)

This may indicate that the course of catechin dissimilation differs appreciably from that of quercetin, or that if phenolics are produced they are metabolized at a rapid rate and do not accumulate.

The hydrolysable tannins are rapidly attacked by fungi and bacteria (Lewis and Starkey, 1969). Gallic acid is found to be an initial product of hydrolysis of gallotannins by fungi (Lewis and Starkey, 1969).

The importance of phenolic compounds derived either from lignin, flavonoids or related plant phenolics through microbial

activity lies in the ability of many of these substances to form quinones. Quinones can react with nitrogenous compounds to form polymers which are relatively resistant against further microbial attack and thus may eventually lead to the formation of nitrogenous humic acids. In the case of lignin degradation products, the formation of quinones is only directly possible with those compounds which have a carboxyl group as a side chain (Figure 1.5) (Flaig, 1968).

Once the quinone is formed nucleophilic addition of amino acids in 1,4 - positions can occur. In the case of vanillic acid, protocatechuic acid and gallic acid, the entire sequence of quinone formation and addition of amino acids can be initiated by the presence of phenoloxidases in the pH range 6-8 (Haider et al, 1965). These reactions are illustrated [illegible]re 1.5. Such reactions are often accompanied by deami- [illegible] of the amino acids with the resultant release of ammonia. [illegible] reactions are also accompanied by the consumption of [illegible]gen and in the case of protocatechuic acid and gallic acid, [illegible]d to the formation of dark colored nitrogenous polymers [illegible]en the pH is greater than 8 (Flaig, 1968).

The addition of amino acids to quinones depends upon the pH of the amino acids' amino group(s) (Mason, 1955a; Adams and Perry, 1973). The initial substitution and oxidation reactions go nearly to completion with amino acids with secondary amine groups such as proline and hydroxyproline. With other amino acids, these reactions are often hindered by competing quinone-

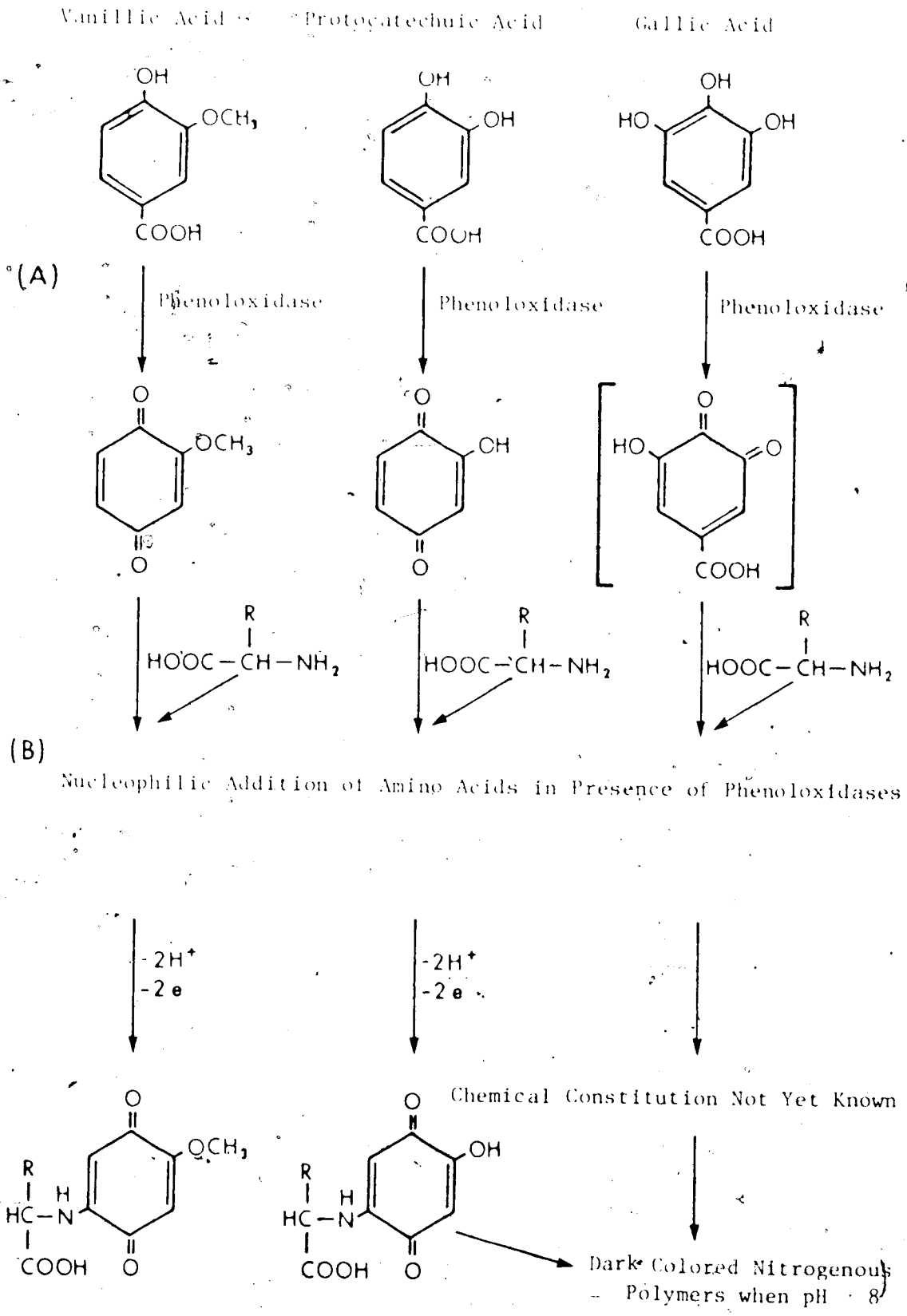


FIGURE 1.5 (A) OXIDATION OF PHENOL CARBOXYLIC ACIDS TO QUINONES BY OXIDATIVE DECARBOXYLATION; (B) NUCLEOPHILIC ADDITION OF AMINO ACIDS IN THE PRESENCE OF PHENOLOXIDASES, pH = 6-8 [MODIFIED FROM FLAIG (1968)]

1. 1.6 (continued)

quinone reactions which produce polymers with no amino acid content. With all amino acids except lysine, cysteine and proline derivatives, the initial substitution reaction occurs through the α-amino group of the amino acid. With lysine the possibility of additional reactions involving the ε-amino group also exists (Mason, 1955a).

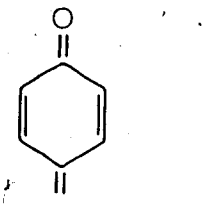
Work by Mason (1955a, 1955b) and Haider et al (1965) has shown that peptides and proteins show covalent bonding with quinones similar to reactions involving single amino acids. This work has shown that peptides react at a rate determined by the chain length. Thus in the case of glycine, the tripeptide reacts more rapidly than the dipeptide which in turn reacts more rapidly than glycine. This effect of peptide chain length has been termed the peptide effect (Mason, 1955a, 1955b). In general peptide reactions with quinones are primarily dependent on the nature of the N-terminal amino acid, although secondary reactions may differ according to the particular peptide.

Reactions between proteins and quinones involves ε-amino groups of lysine and N-terminal amino groups of residues such as aspartic acid, as well as sulfhydral groups. Peptide nitrogen and nitrogen in side chains of histidine, tryptophan and arginine are not reactive (Haider et al, 1965). Mason and Peterson (1965) indicate that N-terminal proline is particularly reactive, with bonding occurring through the secondary amino group rather than the primary amino group.

Study of the reactions involving amino acids, peptides

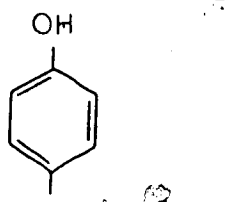
1. 1.6 (continued)

and proteins with various quinones to produce humic acid-like nitrogenous substances have involved model systems under carefully controlled conditions. Although these reactions indicate the possibilities for the incorporation of nitrogen into soil humic acids, until recently there was little evidence to indicate that such complexes actually existed in humic acid. Recent work by Piper and Posner (1972) however tends to substantiate the existence of N-phenyl-bound amino acids in humic acids. They found that additional yields of amino acids could be obtained from acid hydrolyzed humic residues by subsequent hydrolysis with alkali (Piper and Posner, 1968). The source of the additional yield of amino acids was postulated to be quinone-imine complexes such as (XI), which were produced from structures such as (XII) by alkaline hydrolysis, with the resultant release of amino acids



(XI)

QUINONE-IMINE STRUCTURE



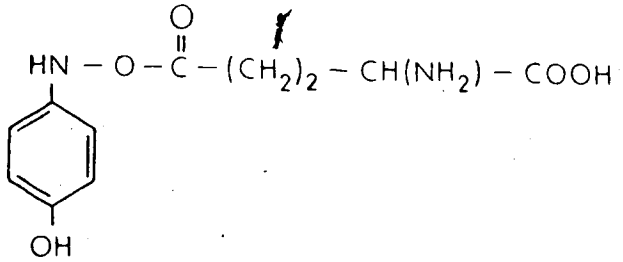
(XII)

N-p-HYDROXYPHENYLGLYCINE

from (XI) as hydrolysis continued. Since acid hydrolysis of phenol protein complexes has been shown to release all the amino acids except for the N-terminal amino acid and those amino

1. 1.6 (continued)

acids that have a free amino group when incorporated into the protein (e.g. lysine) (Haider et al, 1965), then alkaline hydrolysates should contain a preponderance of lysine and other amino acids with an extra functional group. Piper and Posner (1972) tested this hypothesis and found that alkaline hydrolysis of humic acid subsequent to acid hydrolysis did indeed yield a hydrolysate which consistently contained greater proportions of lysine and ornithine, as well as serine, glutamic acid and aspartic acid. Piper et al explained the increased proportions of glutamic and aspartic acids by suggesting that these amino acids were originally present as the corresponding amides, and that the free amido group reacted with phenolic compounds to produce structures such as (XIII).



XIII.

N-(p-hydroxyphenyl)-glutamine

Adams and Perry (1973) found that the incorporation of ^{14}C labelled asparagine, glutamic acid, glycine, lysine, phenylalanine, proline and a dipeptide, glycylglycine into humic acid was dependent upon pH. Maximum incorporation of lysine occurred at a pH which fell between the values corresponding to the dissociation constants of the free α-amino group and the ε- amino

1. 1.6 (continued)

group (i.e. between pH 9 and pH 11). Maximum incorporation of the amino acids increased with increasing pH of their isoelectric points. Lysine incorporation was very high even recognizing that both α and ε - amino groups may have been involved. In the case of glycine, between 10 and 20 percent of the total activity incorporated into humic acid was not released by 6N acid hydrolysis. Susceptibility of the other amino acids studied to release by acid hydrolysis from humic acid was not reported. The data of Adams and Perry indicate that under alkaline conditions, humic acids may react with α- amino nitrogen. Thus during alkaline extraction of humic acids from soil it is possible that amino acids may be incorporated into the humic acid.

The major portion of amino acid nitrogen associated with humic acids is not covalently bonded to aromatic constituents of humic acids, but rather occurs as peptide nitrogen. This has been established by studies using infrared spectrophotometry, (Goulden and Jenkinson, 1959; Butler and Ladd, 1969), partial acid hydrolysis (Sowden, 1966; Piper and Posner, 1968) and enzymic hydrolysis (Ladd and Brisbane, 1967; Brisbane et al, 1972). Simonart et al (1967) and Biederbeck and Paul (1973) have removed proteinaceous-like material from soil humic acids using acidic phenol solutions. Such material was thought to be linked to the more aromatic humic constituents by hydrogen bonding. Bonding of protein could also involve weak ionic bonds and Van der Waals interactions. It is also possible that

1. 1.6 (continued)

protein could be covalently bonded to humic acid constituents during the synthesis of humic acid through mechanisms previously discussed for model systems involving quinones and peptides.

If current theories of humic acid synthesis are correct, then removal of hydrogen-bonded or ionically bound proteinaceous material from the "core" of humic aromatic constituents should leave a humic residue relatively rich in covalently bound amino acid nitrogen. Amino acids most likely to be involved in such covalent bonding are lysine, proline and possibly glutamic and aspartic acids (Piper et al, 1972) when these occur as glutamine and asparagine. Covalently bound amino nitrogen has been shown to resist acid hydrolysis (Haider et al, 1965). Thus humic acids from soils in which humification processes favor the accumulation of aromatic material might be expected to show a greater proportion of covalently bound amino acids. This would result in a reduction in the quantity of these amino acids detected in acid hydrolysates of humic acids from which hydrogen bonded or ionically bonded nitrogenous material had been removed.

1.7 Amino Acid Composition of Humic Acid Hydrolysates

The amino acid composition of acid hydrolysates of soil humic acids has been the object of numerous studies (Lowe, 1969, 1973; Brisbane et al, 1972; Piper and Posner, 1972; Khan and Sowden, 1971; Huntjens, 1972; Sowden and Schnitzer, 1967). Lowe (1969) reported on the amino acid content of de-ashed humic acids from nine soils representing the major soil zones of Alberta. Lowe did not comment on the relative proportion

1. 1.7 (continued)

of individual amino acids obtained from different humic acids, however examination of his data show that there are differences, in some cases large differences, between soil humic acids in the proportion of lysine, aspartic acid, proline, valine, isoleucine, leucine and tyrosine. In a more recent study, Lowe (1973) found a consistent change in the proportions of certain amino acids with increasing humification in forest humus layers of several soils in British Columbia. Results showed that the proportion of glutamic acid, proline and leucine present in acid hydrolysates consistently decreased on passing from L to F to H or Ah horizons, an effect which appeared to be independent of vegetation. A similar but less pronounced and consistent trend was exhibited in the proportions of aspartic acid, alanine, valine and isoleucine.

Piper and Posner (1972) found increased proportions of lysine, ornithine, serine, glutamic acid and aspartic acid in alkaline hydrolysates of soil humic acid residues which had previously been acid hydrolysed. Brisbane et al (1972) extracted soil humic acids with neutral pyrophosphate, with alkali (NaOH soln) and with pyrophosphate followed by alkali. Results of acid hydrolysis showed differences in the relative proportion of valine, leucine, serine, glycine and aspartic acid present in hydrolysates of humic acids obtained by the different extractants.

Huntjens (1972) and Kahn and Sowden (1971) examined the amino acid content of acid hydrolysates of humic acids from

1. 1.7 (continued)

arable land and pasture, and from a Black Solonetz and a Black Chernozemic soil respectively. The results of both studies showed negligible differences in the proportion of amino acids present in various humic acid hydrolysates. Sowden and Schnitzer (1967) reported glycine values of 16.7, 10.7 and 14.0 percent of total amino acid nitrogen for the humic acid, fulvic acid, and humin fractions, respectively, of a Podzol B soil sample. Other amino acids which showed variation in amounts present in the humic fractions were aspartic acid, glutamic acid, proline, iso-leucine, leucine, phenylalanine and lysine.

In general, the studies reported above indicate that the relative proportions of certain amino acids can vary between humic acids from different soils and between humic fractions. The results of other studies where there was no difference in amino acid composition between humic acids may arise because of the techniques used for the preparation of humic acids prior to amino acid analysis. Thus, for example, extensive de-ashing procedures may destroy differences between humic acids by removing material of intermediate levels of humification, leaving behind material of an overall similar composition.

Reports on the amino acid composition of acid hydrolysates of whole soil samples have indicated both differences and lack of differences between various soils. Stevenson (1956) found basic amino acids to make up a somewhat larger proportion of the amino acids in soils cropped over 62 years to corn then

1. 1.7 (continued)

in plots cropped for the same period to rotations of corn, oats and clover. Similar work by Young and Mortensen (1958) showed no qualitative differences in amino compounds present in large amounts in plots having varied cropping histories. They found the total quantity of amino acids to be closely related to total amount of organic matter and little variation was found in the proportion of the various amino acids present.

In view of the great variety of organic material present in whole soil samples, it may be unreasonable to expect acid hydrolysis of such samples to yield hydrolysates which show differences in amino acid composition. Results of such studies are more likely to yield an "average" distribution of amino acids similar to what could be expected from acid hydrolysis of a mixture of proteins. Other than indicating the total amount of amino acid nitrogen present in different soils, studies of whole soil hydrolysates cannot indicate relationships between different soil organic matter fractions as these relate to possible complexing, or fractionation of particular groups of organic compounds such as amino acids or peptides. Such reactions are likely to be of considerable importance in soil organic matter mineralization reactions and their complete elucidation can only be achieved through development of techniques which will allow the extraction and separation of reaction intermediates from the bulk of soil material.

1. (continued)

1.8 Effect of Promise on Soil Humic Acids

It has been suggested that chemical degradations of humic acids in attempts to characterize the material are either too mild to be effective on the "core" of humic compounds or they cleave intermonomeric and intramonomeric bonds simultaneously due to lack of differences in their nature and strengths (Felbeck, 1965). The biodegradation of soil humic components proceeds via the action of various enzymes and enzyme systems which degrade the complex polymer-like structure of humic substances to small units which can be absorbed by soil microorganisms. Mathur and Paul (1967a, 1967b) and Mathur (1971) have used biological degradation as a technique to study the structure of humic acids on the theory that biological reactions are more specific then purely chemical techniques as well as involving milder reaction conditions. This approach was used with partial success to characterize chernozemic humic acids (Mathur and Paul, 1967a, 1967b) and fulvic acid from the Bh horizon of a Podzol (Mathur, 1971). These studies indicated that compounds such as benzoquinone, 2-methyl-1,4-naphthoquinone, salicyl alcohol and salicyl aldehyde or their homologues, may play an important role in contributing to the structure of soil humic substances.

One specific group of enzymes produced by soil microorganisms and which may be important in the biodegradation of the peptides or protein containing component of soil humic acids, is extra-cellular proteolytic enzymes. The action of

1. 1.8 (continued)

proteolytic enzymes on soil organic matter preparations have been studied by a number of workers. Enzymes used in these studies have included papain (Brisbane et al, 1972; Ladd and Brisbane, 1967), chymotrypsin, trypsin and subtilopeptidase (Brisbane et al, 1972), thermolysin (Brisbane et al, 1972; Ladd and Butler, 1969) and pronase (Brisbane et al, 1972; Ladd and Butler, 1969; Sowden, 1970; Ladd and Brisbane, 1967). From the point of view of the action of microbial extra-cellular enzymes on humic acids, studies involving pronase are most applicable. Pronase is an extra-cellular enzyme produced by the soil microorganism Streptomyces griseus. The enzyme hydrolyses a wide range of peptides and unlike most proteases, converts proteins almost quantitatively to their amino acid components.

Sowden (1970) found pronase released 20 to 50 percent of the valine, isoleucine, leucine, tyrosine and phenylalanine content of humic acids; the percentage release of aspartic and glutamic acid, threonine, serine and alanine was intermediate. Pronase released amino acids also included asparagine and glutamine indicating that aspartic and glutamic acids can occur as the bound amides in humic acids. Data for the basic amino acids were variable; lysine was not detected in all hydrolysates and amounts of histidine and arginine varied considerably.

Ladd and Brisbane (1967) found that pronase released 23.6, 33.4, 38.4 and 39.2 percent respectively of the total

1. 1.8 (continued)

acid hydrolysable amino acid content of four soil humic acids. Basic amino acids were not detected on paper chromatograms of pronase hydrolysates of humic acids, although these amino acids were present in acid hydrolysates. Ladd and Brisbane (1967) speculated that in humic acids basic amino acid residues were bound through their additional amino groups in forms not attacked by pronase. This possibility is compatible with the results of Stevenson (1956) who showed that basic amino acids were resistant to utilization under cropping conditions which reduced soil organic nitrogen. Alternatively, the apparent absence of basic amino acids in pronase hydrolysates of humic acids may reflect the specificity of pronase action on peptides, however Nomoto, Narahashi and Marakami (1960) have shown basic amino acids to be present in the products of pronase hydrolysates of proteins.

Brisbane et al (1972) investigated these alternatives more thoroughly using a gas chromatographic procedure to compare the relative release by pronase of individual amino acids from soil humic acids and a protein substrate, albumin. Their results showed that pronase released peptides as well as free amino acids from both humic acids and albumin. Further pronase preferentially released four amino acids, iso-leucine, leucine, phenylalanine and tyrosine from both albumin and the humic acids. Lysine was released in moderate precentage yields from the humic acids and albumin; the percentage releases of aspartic and glutamic acids by pronase

1. 1.8 (continued)

were also low. Acid hydrolysis of the supernatants from humic acid-enzyme hydrolysates resulted in increased yields of amino acids due to the acid hydrolysis of peptides released by pronase from the humic acids. These results showed that a disproportionately high amount of lysine in humic acids studied by Brisbane et al was not susceptible to release by pronase either as the free amino acid or in the form of TCA-soluble peptides.

In general the work of Ladd et al (1967) and Brisbane et al (1972) shows that pronase exhibits specificity in its action being most active in releasing aromatic and long chain aliphatic amino acids but releasing acidic amino acids in relatively poor yields. This work has also shown that the patterns of amino acids released from a protein and from humic acids are similar. Differences in the acid hydrolysable amino acid contents of the substrates and the presence of relatively large amounts of non-proteinaceous components in the humic acids did not alter the specificity of the pronase action. Thus the major effects of the humic components on pronase action in releasing bound amino acids or peptides appear to be quantitative rather than qualitative.

1.9 Extraction and Fractionation of Soil Humic Acids

(a) Extraction

Methods of extracting organic matter and, more specifically, the nitrogen-containing fraction, from soil have been the subject of many investigations (Goh, 1970; Choudhri and Stevenson, 1957; Evans, 1959; Tinsley and

1. 1.9 (a) (continued)

[redacted]Salom, 1961; Martin and Reeve, 1957; Bremner, 1950; [redacted]zer and Skinner, 1968; Levesque and Schnitzer, 1965, [redacted]wden, 1970; Butler and Ladd, 1968; Swift and [redacted], 1971). Reagents which have been used in these [redacted]ies include 0.5N sodium hydroxide, more dilute [redacted]olutions of sodium hydroxide, sodium pyrophosphate [redacted]olutions, sodium sulphate solutions and solutions of sodium carbonate, sodium bicarbonate, fluorides, oxalates, citrates, formic acid containing lithium bromide and hot dimethyl-formamide containing oxalic, boric, hydrofluoric, or fluoroboric acid. Organic chelating reagents such as acetylacetone, and resin bound chelating agents have also been used (Martin and Reeve, 1957; Levesque and Schnitzer, 1967).

The results of these investigations have shown that milder extractants such as aqueous solutions of various salts are much less effective than alkali in solubilizing soil organic matter. For example, of the salt solutions 0.1M sodium pyrophosphate is usually the most effective, but it generally does not dissolve more than about one quarter of the organic matter in surface soils, whereas repeated treatments with 0.5M sodium hydroxide usually led to dissolution of more than half of this organic matter (Bremner, 1967). Although organic reagents such as dimethylformamide, and acetyl-acetone solubilize organic matter, these reagents are difficult to remove and

1. 1.9 (a) (continued)

may thus contaminate the extracted organic matter. The use of bound chelating agents, such as chelating resins, eliminates this problem.

In spite of the many reagents which have been tested for their ability to solubilize organic matter, the most commonly used reagents are solutions of NaOH (usually 0.5M), sodium pyrophosphate (usually 0.1M), and more recently chelating resin such as Dowex A-1 (Bremner, 1965; Levesque and Schnitzer, 1967). Humic acids extracted from soil with sodium pyrophosphate have greater proportions of lower molecular weight material, less acid-hydrolysable amino acid nitrogen contents, but greater carboxyl contents than humic acids extracted subsequently from the same sample with alkali (Butler and Ladd, 1968). Humic acids extracted with alkali from fresh soil samples have intermediate values (Butler and Ladd, 1968).

Pyrophosphate humic acids have a higher extinction value than other humic acids extracted from the same soil (Butler and Ladd, 1968). According to Kumada (1965) the more humified the humic acid the darker the color (higher extinction value). Thus, it has been suggested (Kimber and Searle, 1970) that pyrophosphate extracts give an "older" as well as a more condensed humic acid, whereas sodium hydroxide extracts a mixture of humified material. Figure 1.6 below, shows hypothetical products of extraction with sodium hydroxide and sodium pyrophosphate (Kimber and Searle, 1970).

1. 1.9 (a) (continued) -

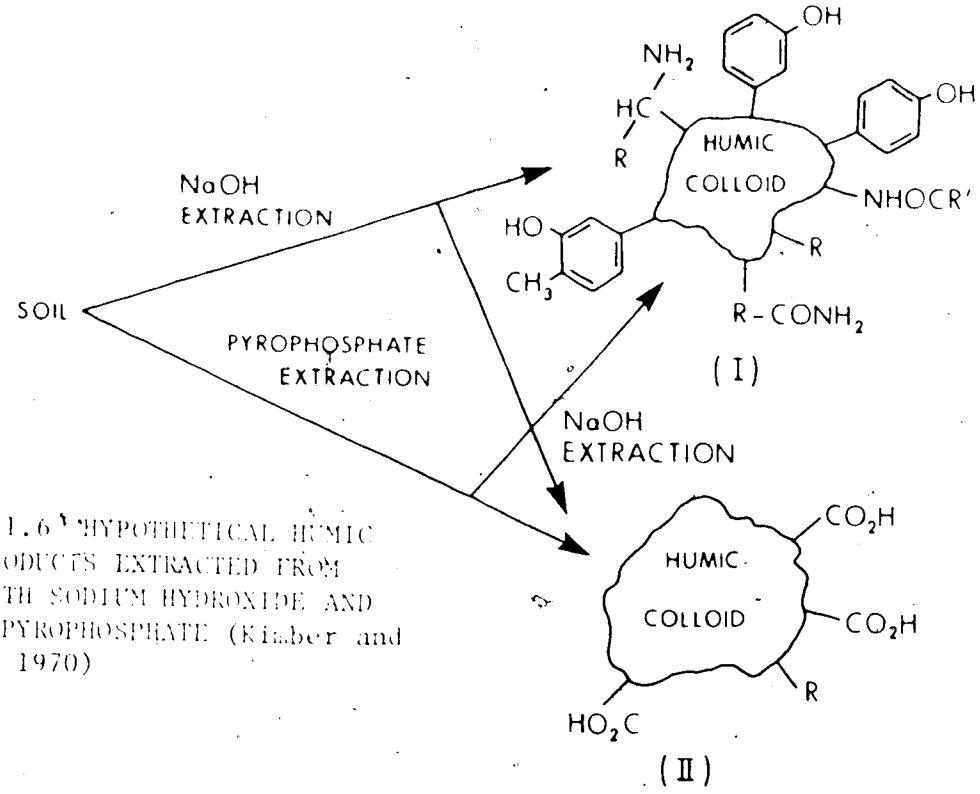


FIGURE 1.6 HYPOTHETICAL HUMIC ACID PRODUCTS EXTRACTED FROM SOIL WITH SODIUM HYDROXIDE AND SODIUM PYROPHOSPHATE (Kimber and Searle, 1970)

According to this scheme, sodium hydroxide extracts a mixture of more (I) (a mixture of condensed and not so condensed material) than (II) (more condensed material). Sodium pyrophosphate extracts mainly the more condensed material (II) and subsequent extraction with sodium hydroxide yields mainly the less condensed material (I), most of (II) having been removed by the previous pyrophosphate extraction.

The ability of salt solutions such as sodium pyrophosphate to solubilize organic matter of mineral soils is thought to be due to the complexing of polyvalent metals (Bremner, 1967). Polyvalent metals are thought to bind humified organic matter in mineral soils as clay-metal-organic matter complexes (Greenland, 1970; Evans and Russell, 1959). Thus reagents such as pyrophosphate

1. 1.9 (a) (continued)

which react with Ca^{2+}, Al^{3+} or Fe^{3+} to form relatively stable complexes, release the humified organic matter which was initially bound to clays through linkages involving these metallic cations (Edwards and Bremner, 1965).

An extraction procedure based on this principle using sodium thiosulphate in conjunction with alkali extraction results in the recovery of "mobile" and "non-mobile" humic acids (Biederbeck, 1969). The term "non-mobile" humic acid designates those humic substances which are immobilized and stabilized in soil by close association with calcium, clays and sesquioxides. This very stable type of humic acid can only be extracted by dilute alkali after a hot acid pretreatment of the soil has affected the disruption of some of the organo-mineral bonds. The term "mobile" humic acid refers to that fraction of acid insoluble humic acids which is directly extracted from soil with dilute alkali without an acid pretreatment. In soil, this type of humate is associated primarily with non-silicate forms of iron and aluminum and it is thought to be relatively mobile and unstable.

Chelating resins are another group of reagents which may solubilize organic matter by removing flocculating cations from soil suspensions. Bremner (1965) suggested the use of the chelating resin Dowex A-1 in the Na^{+} - form for this purpose. He claimed that organic matter was less

1. 1.9 (a) (continued)

altered when extracted by resin than by alkali, and that the extract was not contaminated by the extractant when resin was used. In order to investigate this further, Levesque and Schnitzer (1967) extracted organic matter from a variety of soils with dilute sodium hydroxide solution and with the Na^+ form of Dowex A-1 chelating resin. They found sodium hydroxide solution extracted a greater proportion of soil-C from four of six soils studied than did Na^+ - resin. Only in the case of an Orthic Black Chernozem soil did Na^+ - resin extract more soil-C than sodium hydroxide. With sodium hydroxide as extractant, the extractability of carbon decreased in the following order:

Humic Podzol > Organic Soil > Humic Gleysol > Gray Brown Podzolic > Brown Forest > Orthic Black

With Na^+ - resin as extractant, this order was changed to:

Humic Podzol > Orthic Black > Gray Brown Podzolic = Humic Gleysol > Brown Forest > Organic Soil

Levesque and Schnitzer (1967) found that except for the Orthic Black soil, greater proportions of extracted carbon remained in dialysis bags when Na^+ - resin was the extractant than when sodium hydroxide was used. This suggested that Na^+ - resin extracted more high molecular weight organic matter than sodium hydroxide did, however an inspection of the ash content showed that Na^+ - resin

1. 1.9 (a) (continued)

extracts contained almost twice as much ash as materials extracted by sodium hydroxide. The ash values of the non-dialyzable extracts were taken as evidence to show that sodium hydroxide was more efficient than Na^+ - resin in breaking bonds between organic matter and inorganic constituents. Thus, Levesque and Schnitzer concluded Na^+-resin extracted organic matter which was bonded to and aggregated with substantial amounts of inorganic constituents.

Many other procedures have been documented for the extraction of soil humic acids. Most of these are variations on the "classical" approach to soil humic acid studies and involve the use of alkali as the basic extracting reagent. Detailed extraction procedures are readily available and can be found elsewhere (Kononova, 1966; Stevenson, 1965).

(b) Fractionation of Humic Acid

The initial extraction of humic substances from soil is often also the first fractionation step in separating the material into relatively simpler components. This initial fractionation appears to separate the material on the basis of molecular weight. Generally, milder extracting solvents such as sodium pyrophosphate extract low molecular weight humic acids, whereas more efficient solvents such as sodium hydroxide tend to extract a greater range of molecular weight material.

The development of synthetic cross-linked polydextran

1. 1.9 (b) (continued)

gels by Porath and Flodin (1959) led to gel permeation chromatography as a technique for separating mixtures of compounds. Gel permeation chromatography separates mixtures of components largely on the basis of molecular weight, and the technique has enjoyed considerable success in the separation of complex mixtures of proteins. A number of workers in humic acid chemistry have used the technique in attempts to fractionate soil humic acids (Bailley and Margulis, 1968; Ferairi and Dell'Agnola, 1963; Posner, 1963; Swift and Posner, 1971; Piper and Posner, 1968; Butler and Ladd, 1969; Swift et al, 1969). Posner (1963), Swift and Posner (1971), and Cameron et al (1972) have indicated that humic components can interact with the gels used for permeation chromatography and thus fractions obtained by the technique are not necessarily eluted solely on the basis of molecular weight differences. However, permeation chromatography does separate humic acid into components which show differences in chemical and physical properties.

Swift and Posner (1972) fractionated humic acids which had been extracted with 0.5N sodium hydroxide from an organic soil and a lateritic Podzol, on an agar gel column. Humic acids from both soils showed a logrithmic type of elution curve from the gel column. According to Swift et al (1972), the initial material eluted represented the high molecular weight component of humic acid and subsequent fractions represented material of decreasing molecular weight.

1. 1.9 (b) (continued)

Analysis of the total nitrogen and amino acid nitrogen content of these fractions showed that the highest molecular weight components contained the greatest amount of total nitrogen and amino acid nitrogen, whereas lower molecular weight material showed a decrease in total nitrogen content which was almost entirely due to the loss of amino acid nitrogen. Although the humic acids used in this study were obtained from very different soil materials and differed significantly in their nitrogen contents, they exhibited almost identical trends in elution behavior from the agar gel column. This was interpreted by Swift and Posner as evidence that similar processes are occurring in widely dissimilar soil environments.

Tan and Giddens (1972) found that sephadex gel-filtration yielded low and high molecular weight fractions from sodium hydroxide extracted humic acids. The high molecular weight material had different chemical and spectral properties compared to the low molecular weight material. The high molecular weight humic acid fraction was insoluble in water and infrared analysis indicated that it contained considerable amounts of C-H aliphatic compounds. Low molecular weight fractions showed water solubility, and infrared analysis indicated a higher carboxyl content and fewer C-H aliphatic groups than the higher molecular weight material. Both humic acid molecular

1. 1.9 (b) (continued)

weight fractions had lower E_4/E_6 values than fulvic acid fractions, indicating higher degrees of condensation than fulvic acids (Kononoya, 1966; Biederbeck and Paul, 1973). In addition, a higher extinction coefficient was exhibited by the high molecular weight humic fraction than by the low molecular weight fraction.

Kumada and Miyora (1973) using sephadex gel chromatography found that humic acid could easily be separated into two to four fractions which showed differences in their spectrophotometric properties. Other studies (Bailley and Margulis, 1968; Piper and Posner, 1968; Butler and Ladd, 1969) have obtained similar results and Swift et al (1969) have proposed that the degree of aromaticity increases as the molecular weight of humic fractions decreases and thus the low molecular weight component separated from humic acid by gel chromatography represents end products of the humification process.

Fractional precipitation from various solutions has also been used for the fractionation of soil humic material. Kumada and Kawamura (1968) added increasing amounts of absolute ethanol to a 0.2N sodium hydroxide solution of humic acid. The procedure produced eight fractions which the authors claimed showed differences with respect to light absorption and permanganate oxidation characteristics when the fractions were compared from several soil types. German scientists (Kononova, 1966) showed that humic acid

1. 1.9 (b) (continued)

extracted by alkali could be divided into brown humic acid and gray humic acid by the addition of electrolytes such as sodium chloride to alkaline solutions of the humic acid. Theng et al (1968) obtained 19 fractions from a K-humate solution by successive additions of ammonium sulfate. Fractions obtained at low concentrations of ammonium sulfate were found to resemble 0.5N sodium hydroxide extracted humic acid fractions obtained from the least humified fraction of soil organic matter. Fractions soluble in the more concentrated ammonium sulfate solutions were found to resemble humic acid fractions generally extracted by neutral 0.1M sodium pyrophosphate solutions.

Results of gel-permeation chromatography and fractional precipitation techniques suggest that humic acid consists of a mixture of components which can be separated by means which do not require breaking covalent bonds. Small (1953) has indicated that for polymers containing functional groups such as hydroxyl, carboxyl or amide, either in the main structure or as peripheral groups, hydrogen bonding usually controls the solubility of such polymers. Thus solvents which contain hydrogen bonding functional groups should be able to disrupt and dissolve humic substances depending on the relative strength of solvent-humic and humic-humic interactions.

Simonart et al (1967) and Biederbeck and Paul (1973) used this principle to separate a protein-like fraction

1. 1.9 (b) (continued)

from the bulk of humic acid with acidic solutions of phenol. Simonart et al found phenol extracted 25.0, 32.5, and 40.0 percent of the humic nitrogen from a Podzol B horizon, a Meadow soil and a Brown Forest soil, respectively. Biederbeck and Paul fractionated sodium hydroxide extracted soil humic acids with phenol and found phenol solubilized preferentially, but not exclusively, aliphatic nitrogenous humic components. Analysis of the humic fractions by spectroscopy, elemental microanalysis, acid hydrolysis, and ultrafiltration established that phenol-insoluble humic residues consisted largely of aromatic structures and that the readily phenol-soluble portion of humic acid was predominantly proteinaceous in nature. Thus hydrogen bonding appears to play an important part in maintaining the complex structure of humic acids.

2. MATERIALS AND METHODS

2.1 Soils

Soils for the study were selected from four widely separated areas of the Province. These general areas are shown on the accompanying map (Figure 2.1). The soils selected are similar in the respect that they all have an Ah horizon and roughly similar parent materials. They are dissimilar in having developed under different conditions of climate and vegetation. More detailed soil descriptions and site locations are given below.

.11 Soil 1 - Beaverhills Loam

Classification: Orthic Black Chernozem

Location: Three widely separated soil sites were sampled, all in the general vicinity of Hay Lakes, Alberta. All sites represented virgin profiles. Locations are:

Site 1: SW-26-48-21-W4

Site 2: SW-29-48-21-W4

Site 3: NE-10-49-21-W4

Climate: Continental; characterized by relatively warm summers and cold winters. The mean summer temperature, May to September inclusive is 14°C (56°F). The mean winter temperature, November to March inclusive is -8.9°C (16°F). The mean annual precipitation is from 41 to 46 cm (16 to 18 inches). At Edmonton the past 75 years has averaged 44 cm (17.5 inches) with extremes of 23 and 76 cm (9 and 30 inches). The area can be considered as being between dry and moist sub-humid.

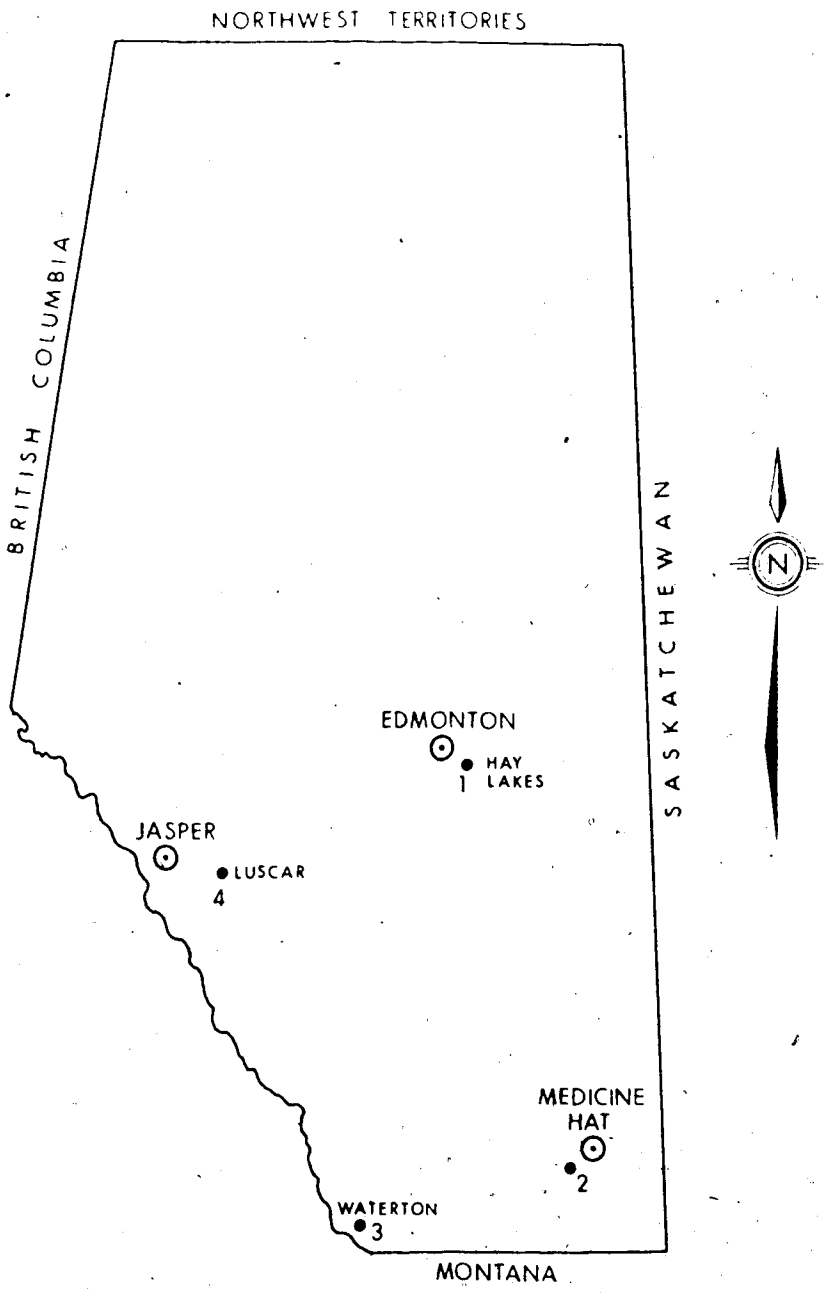


FIGURE 2.1 MAP OF ALBERTA SHOWING GENERAL AREAS OF ORIGIN OF THE FOUR SOIL TYPES STUDIED

Soil Site:
1 - Hay Lakes - Orthic Black Chernozem - Three Sites Studied.
2 - Medicine Hat - Orthic Brown Chernozem - Four Sites Studied
3 - Waterton National Park - Orthic Black Chernozem - One Site Studied
4 - Luscar Tower - Alpine Dystric Brunisol - One Site Studied

2.11 (continued)

Climate (continued)

There is no pronounced permanent water table.

Parent Material: Glacial till, derived mainly from the Edmonton formation.

Topography: Undulating to hilly. The soil sites sampled were all situated on slopes of less than 4 percent.

Drainage: Well drained

Vegetation: Grass vegetation, of which the dominant species is the rough fescue (Festuca scabrella) association. Groves of aspen poplar (Populus tremuloide) dot the area, but these are thought to be of fairly recent origin.

Profile Description: The following is a generalized description of a Beaverhills loam.

Horizon	Depth (cm)	Description
Ah1	0 - 15	10YR 2/1 m (black), loam; moderate fine granular; friable; abundant very fine random roots; clear smooth boundary.
Ah2	15 - 45	10YR 2/1 m (black), loam; moderate fine granular; friable; abundant very fine random roots; clear irregular boundary (tongued).

2.11 (continued)

Horizon	Depth (cm)	Description
AB & A & B	45 - 52	10 YR 3/3 m (dark brown), loam; weak medium prismatic and moderate fine subangular blocky; friable; abundant very fine vertical and oblique inped roots; clear irregular boundary.
Bm	52 - 75	10YR 4/4 m (brown), clay loam; weak coarse prismatic breaking to moderate medium and coarse subangular blocky; friable to firm; plentiful very fine vertical and oblique roots; gradual wavy boundary. Abundant ant activity at this site primarily in Ah and upper B horizons.
BC	75 - 95	10YR 4/3 m (dull yellowish brown), clay loam; weak coarse subangular blocky to fragmental; friable; few very fine vertical roots; gradual wavy boundary.
Cca	95 - 115	10YR 4/4 m (brown), clay loam; few very fine vertical roots; unsorted calcareous till; coal pockets; silt stones; disintegrating rocks.
Cca	115 - 137	10YR 3/4 m (dark brown); clay loam as above.

2.12 Soil 11 - Maleb loam

Classification: Orthic Brown Chernozem

Location: Four widely separated soil sites were sampled in the general vicinity of Medicine Hat, Alberta. All sites represented virgin profiles; their legal locations are:

Site 1: SW-11-11-6-W4

Site 2: SE-29-12-6-W4

Site 3: NW-11-10-8-W4

Site 4: NW-12-12-7-W4

Climate: The climate associated with Maleb loam soils is semi-arid. The mean annual precipitation of the area is about 37 cm (15 inches). Medicine Hat has been reported to have an average moisture deficit of about 28 cm (11.5 inches). Seventy-five percent of the annual precipitation falls between April 1st and October 31st. The mean annual temperature for this area is approximately 5.5°C (42°F); for the period April to September it is 14°C. The area receives a great deal of wind per year, only one percent of the days are calm. The combination of fairly high winds and high temperature results in a relatively high evaporation rate favoring desiccation.

2.12 (continued)

Parent Material: Glacial till derived primarily from Belly River formation.

Topography: The topography of these soil areas is from gently undulating to rolling; usually of the knob and kettle type.

Drainage: Well drained

Vegetation: Semi-arid prairie flora. The two main native grasses are blue gamma grass (Boutulova gracilis) and common spear grass (Stipa comata). Other native grasses include June grass (Koelecia cristata), Sandberg blue grass (Poa secunda), smooth wheat grass (Agropyron pouciflorum) and others of lesser occurrence. Common club moss (Seloginella densa) and prairie phlox (Phlox foodii) are found under very dry conditions.

Profile Description: The following is a generalized description of a Maleb loam profile.

Horizon	Depth (cm)	Description
Ah	0 - 10	7.5YR 5/3 d (dull brown), loam; weak fine granular; soft to slightly hard; abundant very fine random and vertical roots; clean smooth boundary.
Bm	10 - 25	10YR 5/3 d (dull yellowish brown), loam; strong medium and coarse prismatic breaking

2.12 (continued)

Horizon	Depth (cm)	Description
Bm (continued)		to moderate fine and medium subangular blocky; slightly hard; plentiful fine and very fine vertical and random inped and exped roots; clear wavy boundary.
Cca	25 - 50	10YR 7/2 d (dull yellowish orange), loam; upper portion has a weak coarse prismatic structure; few very fine vertical roots; gradual wavy boundary.
Ck	50 - 85	~~10YR~~ 6/2 d (grayish yellow brown), loam; structureless; no roots; calcareous glacial till.

2.13 Soil III - Waterton

Classification: Orthic Black Chernozem

Location: Waterton National Park. Only one soil site was sampled. The site was located about 100 feet northwest of the tradesmen's dump, north of Blakiston Creek and west of Knight's Lake. The site represented a virgin profile.

Climate: Semi-arid continental.

Parent Material: Glacial outwash gravels and sands with occasional boulders, mostly below the ground surface.

Topography: Outwash plain with some knob and kettle topography.

2.13 (continued)

Drainage: Rapidly drained

Vegetation: Dominantly grasses and herbs such as bluebunch fescue (Festuca idahoensis), rough fescue (Festuca scabrella), parry oat grass (Donthonia parryi), and pursh's silky lupine (Lupinus sericeus). Shrubby cinquefoil (Potentella fruticosa) covers a small amount of the area and very stunted trembling aspen (Populus tremuloides) are fairly common especially in the lee sides of hills where snow collects.

Profile Description: A profile description of the Waterton Orthic Black Chernozem soil is given below.

Horizon	Depth (cm)	Description
Ah1	0 - 8	10YR 2/1 m (black), sandy loam; weak granular; very friable; abundant very fine and fine roots; diffuse wavy boundary.
Ah2	8 - 19	7.5YR 3/2 m (very dark brown), sandy loam; moderate medium and coarse sub-angular blocky; friable; plentiful fine and very fine roots; diffuse wavy boundary.
Bm	19 - 36	5YR 3/4 m (dark reddish brown), sandy loam; moderate coarse sub-angular blocky;

2.13 (continued)

Horizon	Depth (cm)	Description
Bm (continued)		friable; plentiful fine and very fine roots; diffuse wavy boundary.
C	36 +	7.5YR 4/4 m (dark brown), sandy loam; massive; very friable; plentiful very fine and fine roots.

2.14 Soil IV - Alpine

Classification: Alpine Dystric Brunisol

Location: Luscar Mountain on the edge of the Alpine-Subalpine transition on Forest Tower Road. Only one soil site was sampled.

Climate: Alpine

Parent Material: Shallow colluvium over sandstone

Topography: Alpine. The soil site is located on a 15 percent slope with an eastern aspect near the summit.

Drainage: Well drained

Vegetation: Open stands of stunted Engelman spruce (Picea engelmani) which occurred in clusters and were discontinuous. A variety of alpine shrubs; cladonium, lichens and mosses formed the ground cover.

Profile Description: A profile description of the site sampled is given below.

2.14 (continued)

Horizon	Depth (cm)	Description
L	8 - 6	Lichens, mosses
F	6 - 0	10YR 3/1 m (very dark grey) organic matter; fibrous and matted; plentiful very fine, fine, medium random roots; abrupt smooth boundary.
Ah	0 - 5	10YR 3/2 m (very dark greyish brown) loam to clay loam; moderate granular; friable; plentiful very fine and fine random and medium horizontal inped and exped roots; clear smooth boundary.
AB	5 - 7	10YR 4/3 (brown) loam to clay loam; moderate granular to fine subangular blocky; friable; plentiful very fine and fine random and medium horizontal inped and exped roots; abrupt smooth boundary.
Bm_1	7 - 12	5 YR 4/4 m (reddish brown) loam to clay loam; weak fine subangular blocky; friable; plentiful very fine and fine random roots; abrupt smooth discontinous boundary.
Bm_2	12 - 22	10YR 4/3 m (brown) loam to clay loam; moderate fine subangular blocky; friable; plentiful very fine random roots; clear smooth boundary.
C	22 +	10YR 3/1 m (very dark grey) colluvium;

2.14 (continued)

Horizon	Depth (cm)	Description
C (continued)		large content of weathered sandstone fragments; few fine vertical roots.

2.2 Extraction of Soil Humic Acids

Two reagents were used to extract humic acids from soil. These were 0.5N sodium hydroxide and a suspension of Chelex - 100 (Dowex A-1) resin in distilled water. The resin was used in the Na^+ form. When extracting humic acids from soil the reagents were used independently on separate soil samples of each soil studied.

Sodium hydroxide solution was chosen as an extracting reagent because of its ability to solubilize a high proportion of the total soil humic matter. Na^+ - Chelex resin was used as an extractant because it appears to remove from soils humic material which contains large amounts of intact metal-organic matter complexes (Levesque and Schnitzer, 1967). It was hoped that studying properties of humic acids obtained by the different reagents would aid in detecting any qualitative or quantitative differences in humification processes occurring in the soils used for extraction.

Only samples from the Ah or Ah1 horizon of each soil studied were taken for extraction of humic acid. Prior to extraction samples of Ah horizon (air dry) were ground to pass a 2 mm sieve. The 2 mm soil was subsequently gently crushed and then sieved in order to obtain a <200 mesh size fraction for extraction. Details of the extraction procedures are given below and the general procedure is depicted in Figure 2.2. For purposes of identification in subsequent procedures and experiments humic acids extracted from soil by the following

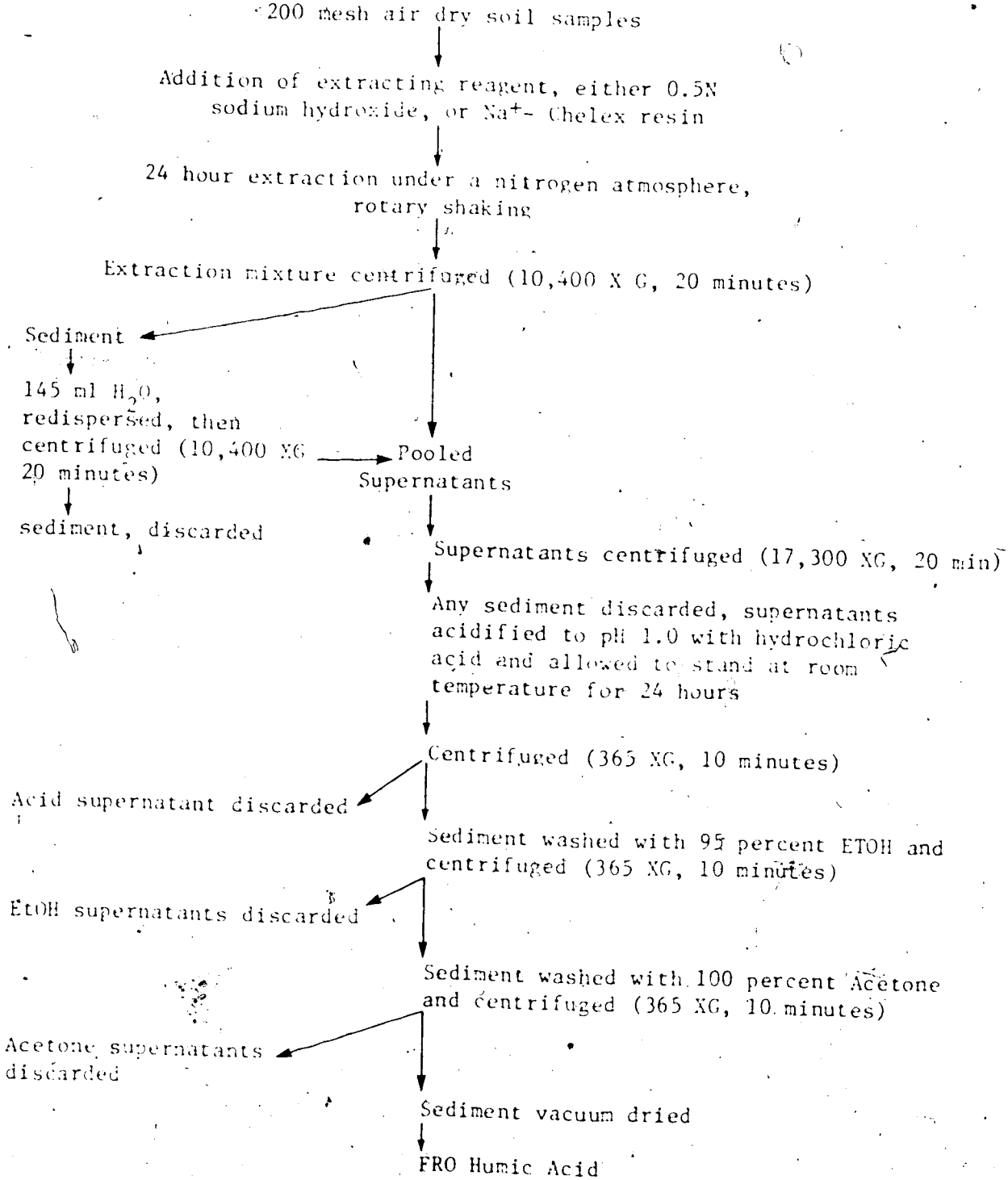


FIGURE 2.2 FLOW CHART OF EXTRACTION PROCEDURE USED TO OBTAIN FRO HUMIC ACIDS FROM SOIL

2.2 (continued)

methods will be referred to as FRO humic acids. Those obtained by sodium hydroxide extraction may often be referred to as sodium hydroxide extracted FRO humic acids and those obtained from soil by extraction with Na^+- Chelex may often be referred to as Na^+-Chelex FRO humic acids or simply as Chelex FRO humic acid.

2.21 Extraction of Soil Samples with 0.5N Sodium Hydroxide

Soil samples were placed in 1 liter erlenmeyer flasks and freshly prepared 0.5N sodium hydroxide solution was added to give a soil-solution ratio of 1:5 w/w. The flasks were flushed with dry nitrogen gas, tightly sealed and placed on a rotary shaker and shaken for 24 hours. At the end of 24 hours the contents of the flasks were transferred to centrifuge cups and centrifuged at 10,400 X G for 20 minutes. The supernatants were removed and the sediments were resuspended in an additional volume of water and centrifuged for a second time. The supernatants from the second centrifugation were combined with those obtained previously and the soil sediment remaining in the centrifuge cups was discarded.

The combined supernatants were transferred to clean centrifuge cups and centrifuged at 17,300 X G for 20 minutes to remove fine clay. The supernatants were then transferred to a large erlenmeyer flask and acidified to pH 1.0 with hydrochloric acid. Any sediment remaining in the centrifuge cups was discarded. The acidified supernatants were allowed to stand at room temperature for 24 hours after which the crude humic acids were separated by centrifugation. The acid supernatant containing fulvic acids and any other solubilized material was discarded.

2.21 (continued)

The wet humic acid sediments obtained by centrifugation were immediately washed with 95 percent ethanol and re-centrifuged. Ethanol washing removed the so-called hymatomelanic fraction of humic acids as well as excess acid and any other soluble material. Washing was continued until the ethanol supernatants were colorless. The humic sediment remaining was then washed with 100 percent acetone which removed excess ethanol and appeared to solubilize a small portion of the humic residue, although the amount removed was very small. The ethanol-acetone washed humic acids were dried under vacuum and stored in a powdered condition at room temperature until used for fractionation experiments or analysis.

2.22 Extraction of Soil Samples with Na^+ - Chelex

Soil samples were placed in 1 liter erlenmeyer flasks and Chelex-100 (200 - 400 mesh) resin was added. The resin, containing from 71 to 76 percent moisture and in the Na^+ form, was added in sufficient quantity to give a soil: resin ratio of 1:1 by weight. Distilled water was added to give a final mixture composition of 1:1:5 by weight of soil: resin: water. It was found that soil-resin suspensions prepared by this method generally had a suspension pH of approximately 10.5 both at the start and end of extraction. Extraction was carried out by flushing the flasks containing the extraction mixture with dry N_2 after which they were immediately sealed and placed on a rotary shaker and shaken for 24 hours. At the end of 24 hours the contents of the flasks were transferred to centrifuge cups and centrifuged at 10,400 X G for 20 minutes. The supernatants were removed and the sediments were resuspended in an

2.22 (continued)

additional volume of water and centrifuged for a second time. The supernatants from the second centrifugation were combined with those obtained previously and the soil-resin sediment remaining in the centrifuge cups was discarded.

The combined supernatants were transferred to clean centrifuge cups and centrifuged at 17,300 X G for 20 minutes. The supernatants were decanted through filter paper to remove any fine resin particles which were too bouyant to be removed by centrifugation. The alkaline supernatants were then acidified to pH 1.0 with hydrochloric acid and allowed to stand 24 hours at room temperature. Precipitated humic acids were recovered and washed with 95 percent ethanol and then with 100 percent acetone in the same manner described previously for sodium hydroxide extracted humic acids. Chelex extracted humic acids were dried under vacuum and stored in a powdered condition at room temperature.

2.3 Fractionation of Soil FRO Humic Acids

Studies on the fractionation of humic acids by gel-permeation chromatography (Bailley and Margulis, 1968; Ferrari and Dell'Agnola, 1963; Posner, 1963; Swift and Posner, 1971) have indicated that humic acids consist of components which can be separated by means other than those requiring breaking of covalent bonds. Work by Simonart, et al (1967) and by Biedefbeck and Paul (1973) has indicated that phenol appears to preferentially solubilize an aliphatic amino acid rich component from humic acids. These results suggested that perhaps a fractionation scheme based on solvent-humic acid interactions could be used in the present study to characterize humic acids of different

2.3 (continued)

soils. Consequently a procedure using phenol and acetone solvent extractions was developed which separated several distinct humic fractions from the bulk of FRO humic acids. It was found that treating FRO humic acids with acidic phenol separated the FRO humic acid into two components, a component soluble in acidic phenol and a component insoluble in acidic phenol. The FRO humic component insoluble in phenol was treated with sulfuric ether to remove residual phenol solvent and the phenol free humic material was then set aside and labelled FR1 humic fraction. The acidic phenol solution containing dissolved FRO humic material was treated with diethyl ether which removed the phenol solvent and allowed recovery of the phenol solubilized FRO humic component. This humic component was then extracted with 100 percent acetone in an attempt to further fractionate the humic material by means of solvent extraction. Acetone consistently dissolved some, but not all of the humic material which had previously been removed from FRO humic acids by phenol extraction. Phenol extracted humic material which was not soluble in acetone was recovered and labelled FR3 humic fraction. Phenol extracted FRO humic material which was dissolved by acetone was subsequently recovered from acetone solution by a flask evaporation technique and labelled FR4 humic fraction. Details of the various extraction and fractionation procedures referred to above are reported below.

2.31 Extraction of FRO Humic Acids with Acidic Phenol

Approximately 0.5 gram samples of FRO humic acids were mixed with 7.7 mls of 0.1N hydrochloric acid in 50 ml polyethylene centrifuge tubes and the mixtures heated at 60°C for one hour. At the

2.31 (continued)

end of the hour 33.3 mls of 90 percent liquified phenol* was added to the centrifuge tubes and the contents were then thoroughly mixed. Phenol and acidic suspensions of humic material were mixed in the amounts specified above in order to yield a final phenolic extraction mixture which contained approximately 75 percent phenol and had a pH of 4 to 4.5. According to Biederbeck (1969) these conditions of phenol concentration and acidity are most effective in causing solubilization of humic components.

Immediately after mixing phenol with acidic humic suspensions the suspensions were centrifuged at 30,900 X G for 20 minutes. The dark-colored supernatants containing phenol solubilized organic material were immediately transferred to separatory funnels and 20 ml of anhydrous diethyl ether was added to each separatory funnel. This material was saved for subsequent fractionation into separate humic components. The sediment remaining in the centrifuge tubes after decantation of the phenol solubilized material was immediately treated with 20 ml of a sulphuric acid-ether solution (1:10, H_2SO_4: $(C_2H_5)_2O$) to remove excess phenol. The sediment was thoroughly suspended, centrifuged (365 X G, 10 minutes) and the supernatant discarded.

* Analytical reagent grade

2.32 Recovery of FR1 Humic Fractions From Phenol Extracted FR0 Humic Sediments

Sulphuric-ether washed sediment from the phenol extraction procedure was mixed with 20 mls of anhydrous ether, centrifuged, the supernatant discarded and the procedure repeated once again. The ether-washed sediment was further washed with two 20 ml portions of distilled water. This was followed by acetone washing until the acetone supernatants were colorless; generally only one washing was required. The final acetone-washed sediment was vacuum dried and stored as a powder. Humic material recovered by this technique will be referred to as FR1 humic fractions. A flow chart of the procedure used to obtain this fraction is presented in Figure 2.3.

2.33 Recovery of FR3 Humic Fractions From Phenol Solubilized Humic Matter

Phenol supernatants containing solubilized FR0 humic acid material were treated with 200 ml of anhydrous diethyl ether to extract phenol. Extraction of phenol into anhydrous ether caused the phenol solubilized organic matter to precipitate. The precipitated organic matter was removed and washed three additional times with 100 ml portions of anhydrous ether to remove all traces of phenol. After the final washing any excess ether remaining in the humic matter was removed by vacuum respiration.

Forty milliliters of acetone were added to the ether free humic sediment and the mixture was allowed to equilibrate for 24 hours. The equilibrated acetone-sediment mixture was centrifuged at 365 X G for

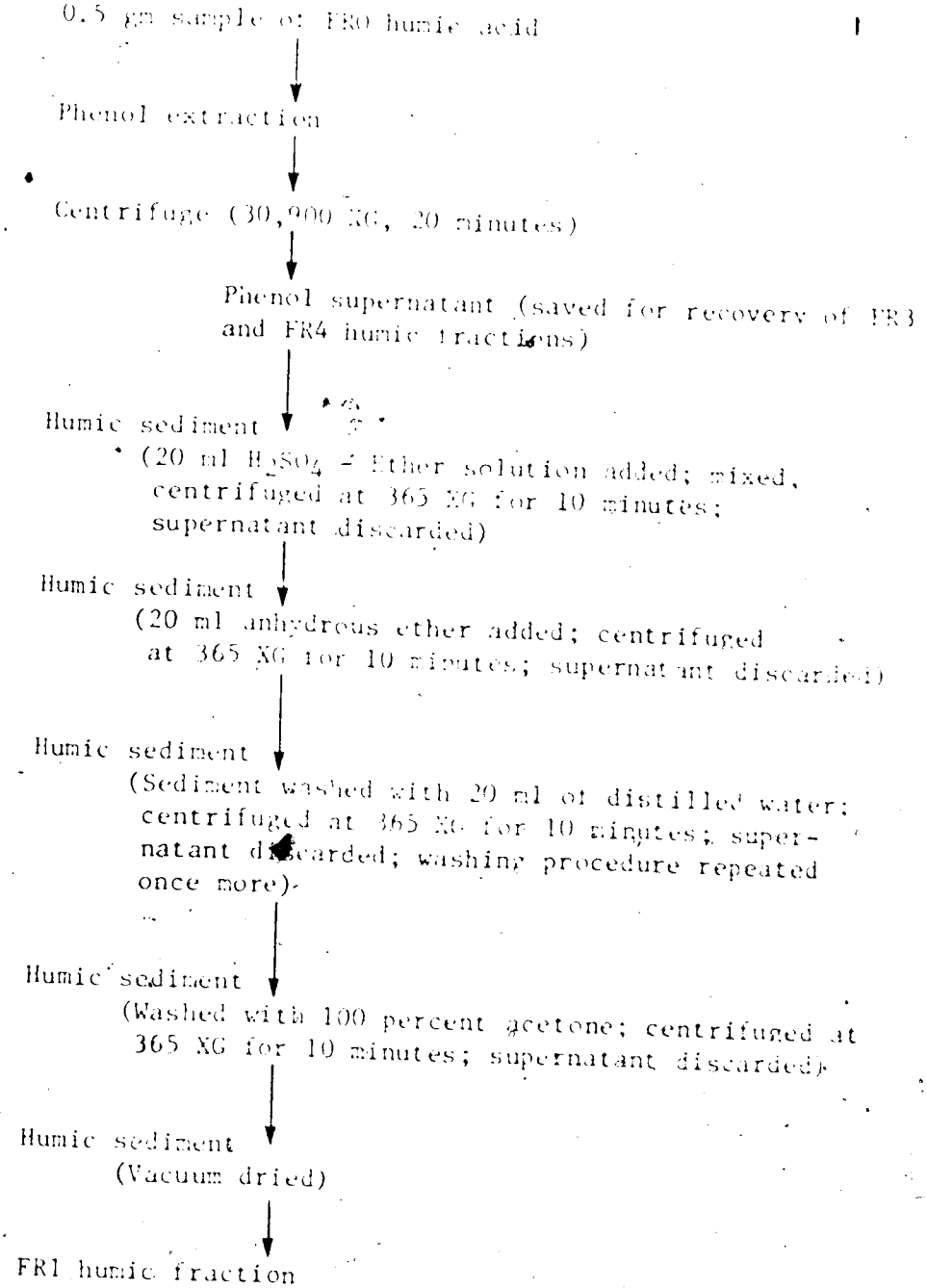


FIGURE 2.3 FLOW CHART OF PROCEDURE USED TO RECOVER FR1 HUMIC FRACTIONS FROM FR0 HUMIC ACIDS

(Continued)

10 minutes and the acetone supernatant was decanted and saved. Centrifugation and decantation was repeated with additional 40 milliliter portions of acetone until the acetone supernatants were colorless. All acetone washings from this procedure were saved and added to the original supernatant. The humic sediment remaining after all acetone soluble material had been extracted was dried under vacuum. The dried acetone insoluble humic material recovered by this procedure will be referred to as the FR3 humic fraction of FR9 humic acids.

2.34 Recovery of FR3 humic fraction from Phenol solubilized Humic Matter

Acetone supernatants used during the recovery of FR3 humic fractions, and containing solubilized humic matter, were transferred to an all-glass rotary evaporator and reduced in volume by 75 percent at a temperature of 40°C. At this point an equal volume of distilled water was added to the acetone solution remaining in the flask evaporator and distillation was continued until the acetone concentration was reduced sufficiently to initiate precipitation of the solubilized humic material. Flash evaporation was continued until the volume of the water-acetone solution was reduced by approximately 50 percent, at which point essentially all of the humic material was salted out of the solution. The humic matter precipitate was recovered by centrifugation and was dried by lyophilization. Humic matter recovered by this procedure will be referred to as the FR3 humic fraction of FR9 humic acids. A flow sheet showing the procedures used to recover FR3 and FR9 humic fractions from bovine rumen fluid is presented in Figure 2.4.

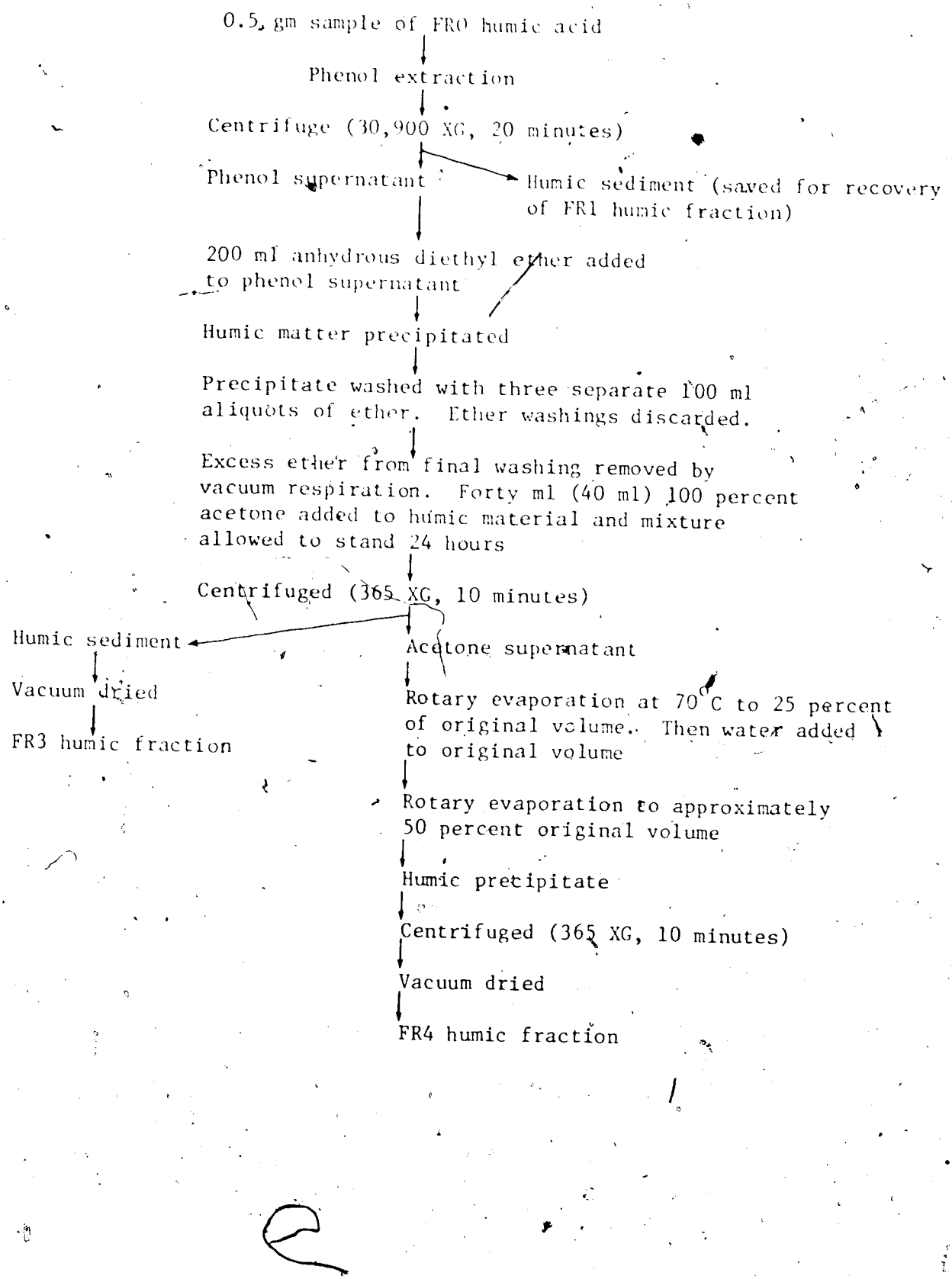


FIGURE 2.4 FLOW CHART OF PROCEDURE USED TO RECOVER FR3 AND FR4 HUMIC FRACTIONS FROM FR0 HUMIC ACIDS

2.4 Properties of FR0 Humic Acids and Associated Humic Fractions

2.41 Ash Analysis

Duplicate samples (approx. 0.5 gm) of all humic acids and humic fractions were analyzed for ash content by slowly heating the samples to 700°C in a muffle furnace. The samples were maintained at 700°C for a period of four hours to insure oxidation of all organic material.

2.42 Total Nitrogen Analysis

Approximately 2.0 mgm samples of humic acids and humic fractions were subjected to total nitrogen analysis by the semi-micro Kjeldahl procedure (Bremner, 1965). All analysis were performed in duplicate and results expressed on an ash free basis.

2.43 Total Carbon Analysis

Total carbon analysis on duplicate samples were performed using a Leco model 577-100 carbon analyzer. Standard $CaCO_3$ was used to check the calibration of the instrument (Allison et al, 1965). All results were expressed on an ash free basis.

2.44 Optical Analysis

Ultraviolet and visible spectra of humic acids and humic fractions were obtained with a Pye Unicam model SP1800 recording spectrophotometer after dissolving the material in phosphate buffer (pH = 7.0) and using the buffer solution as absorption blank. The ratio of E465/E665 (E4/E6) was taken as a measure of the degree of condensation of the various fractions (Butler and Ladd, 1969; Ladd and Brisbane, 1967; Kononova, 1966).

2.45 Yield Data

An attempt was made to determine the quantity of FR3 and FR4 humic fractions which could be extracted from the various humic

2.45 (continued)

acids. This was done by weighing the amount of FR3 or FR4 fraction that was obtained by extracting 0.5 gram samples of FR0 humic acids. The yield of FR3 and FR4 fractions obtained by this procedure was expressed as a percent of the ash free weight of FR0 humic acid which was extracted to obtain them.

2.46 Amino Acid Analysis

In order to conduct amino acid analysis of FR0 humic acids and their associated humic fractions, the following procedure was used. Approximately 2 mgm samples of humic material were placed in 12 ml glass tubes, to each of which was then added 2.0 ml portions of 6N hydrochloric acid. The tubes were then sealed with teflon caps and heated at 105°C for 18 hours. After hydrolysis the hydrolysates were centrifuged and residues were washed once with dilute hydrochloric acid. Residue washings were combined with original supernatants which were then transferred to columns (110 x 10 mm) of Dowex-50-X8H^+ resin. After addition of hydrolysates to resin columns, the columns were washed with several volumes of distilled water until the pH values of the eluates were greater than 5.0. The amino acids were recovered from the columns by elution with 3N ammonium hydroxide. The eluates were then partially dried by rotary film evaporation in an all-glass system to reduce the ammonia content. Final complete drying was achieved by lyophilization. Lyophilized material was immediately dissolved in 2.00 ml of 0.1N hydrochloric acid and 1.0 ml of the solution taken for analysis by gas chromatography.

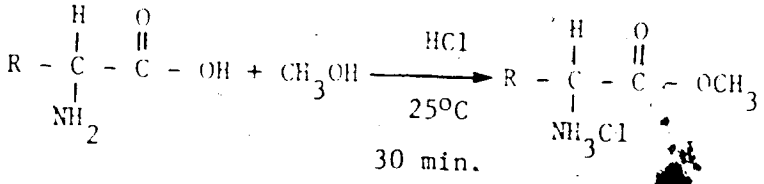
Amino acids were determined as their N-trifluoroacetyl-n-butyl esters (Lamkin et al, 1965; Gehrke et al, 1967, 1970, 1968; Coulter

2.46 (continued)

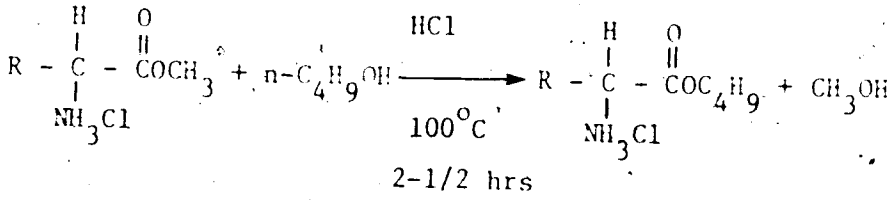
et al, 1968; Roach and Gehrke, 1969a, 1969b; Zumwalt et al, 1970; Moss et al, 1971; Hardy et al, 1972). Very briefly the derivitization procedure consisted of the following steps:

(1) Removal of water to give dry amino acids.

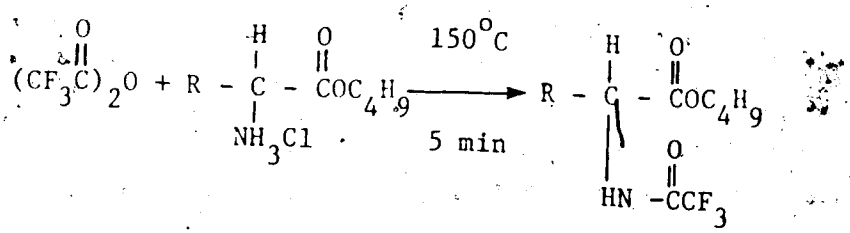
(2) Esterification of the amino acids to form methyl ester hydrochlorides:



(3) Interesterification of the methyl esters to form n-butyl ester hydrochlorides:



(4) Acylation of n-butyl ester hydrochlorides with trifluoroacetic anhydride to form N-trifluroacetyl n-butyl esters:



Methyl ester formation is necessary to achieve solubility of lysine in the n-butanol, and butyl ester formation is necessary to prevent volatilization losses and to obtain a derivative of good

2.46 (continued)

separation characteristics (Gehrke et al, 1967). Detailed steps for the preparation of the derivatives can be found in the appendix.

A Beckman GC-4 gas chromatograph, equipped with dual hydrogen flame ionization detectors was used for separation and quantification of the derivatives. Dual glass columns 1.5m x 4 mm I.D. were used. The columns were packed with Acid washed Chromasorb W 80/100 mesh loaded with 0.65 percent W/W Ethylene glycol adipate. All columns were prepared in the laboratory and the solid support Chromasorb W required heat conditioning prior to use. Details for the preparation and packing of columns are reported in the appendix.

A direct on-column injection port was used to prevent problems with derivative decomposition due to contact with metal fittings. No derivative decomposition was observed. Air and hydrogen gas flow rates to the detector were adjusted so as to obtain maximum detector sensitivity according to Beckman specifications. Helium was used as the carrier gas at a flow rate of 65 cc/minute. The inlet line was maintained at 220°C, the detector line at 250°C, and the detector at 300°C. Temperature programing was used with an initial hold of two minutes immediately after sample injection. Starting temperature of the program was 85°C increasing by 5°C/minute after the hold period, to a final temperature of 220°C. Electrometer sensitivity was adjusted to 1.6×10^{-9} amps for a full scale deflection (10") on a 1 mv recorder.

Gas chromatograms were quantified by the internal standard technique using ornithine as the internal standard (Gehrke et al, 1967). A standard amino acid chromatogram was run with all sets

2.46 (continued)

of unknowns to establish relative molar response factors for the amino acids. Results of amino acid analysis were expressed on an ash free basis.

2.5 Pronase Hydrolysis of Humic Acids and Humic Fractions

The following procedures were used to study the ability of the proteolytic enzyme pronase to release amino acids from FR0 humic acids and associated humic fractions.

Individual FR0, FR1 and FR3 humic acids and humic fractions from different sites of the same soil were pooled in order to produce a single bulk sample of each humic acid and humic fraction for each soil studied. This was done for humic material which had been obtained by 0.5% sodium hydroxide extraction of soil samples and for humic material obtained by Na^+ - Chelex extraction of soil samples.

Humic samples were prepared for enzyme hydrolysis by dissolving an appropriate weight of sample in 0.1% sodium hydroxide and adjusting the pH to 7.2 - 7.4 with hydrochloric acid (Sowden, 1970). The solubilized sample was made to volume to give a final concentration of 10 mgms humic material (ash free basis) per ml of solution. Earlier a procedure was attempted using a system buffered at pH 7.5 with 0.02% Tris (Brisbane et al, 1972), however difficulty was experienced removing Tris from the hydrolysate. Failure to remove Tris resulted in huge peaks in the gas chromatograms which interfered with amino acid peaks, consequently the procedure using Tris buffer was discontinued.

The pronase was a pronase B preparation (45,000 proteolytic units per gram) obtained from Calbiochem. For use, the pronase

2.5 (continued)

was dissolved in a .5 percent ethanol solution to give a final concentration of 0.5 mgm pronase per ml of solution. Enzyme hydrolysis was carried out by pipetting 1.00 ml aliquots of a humic solution into six 12 ml teflon capped glass test tubes. Immediately 1.00 ml of pronase solution was added to three of the tubes and 1.00 ml of pH 7.4 sodium chloride solution (same electrolyte concentration as electrolyte in humic solution) was added to the remaining three tubes. In a similar manner three pronase blanks were also prepared. Thus all analysis of humic enzyme systems were done in triplicate, including triplicate blanks.

The glass test tubes were sealed, and hydrolysis was allowed to continue for 24 hours at 37°C in a water bath. At the end of the hydrolysis period the reaction was stopped by the addition of a small amount of hydrochloric acid sufficient to lower the pH to 2-3. The tubes were immediately centrifuged, the supernatants removed, the sediments resuspended in 2.0 ml of 0.1N hydrochloric acid and recentrifuged. The second set of supernatants were pooled with the first. The supernatants were then dried at 60°C under a stream of dry nitrogen gas. Pronase liberates peptides as well as free amino acids from proteinaceous material (Brisbane et al, 1972) consequently the dried supernatants were subject to 6N hydrochloric acid hydrolysis. Hydrolysis was conducted in sealed glass tubes using 6N hydrochloric acid. The tubes were heated at 105°C for 18 hours. Corresponding blank humic sediments were also hydrolyzed to provide a measure of the acid hydrolyzable amino acid content of each fraction. Acid hydrolysates were handled in the manner described

(continued)

previously for amino acid analysis using gas chromatography (Section 2.46).

Pronase will undergo autolysis in solution and thus can produce free amino acids (Brisbane et al, 1972). In order to correct for pronase contributions to the amino acid content of humic acid hydrolysates, it was decided to subtract the total amino acid content of the pronase aliquot used for each sample from the total amino acid content of enzyme hydrolysates from humic acids and humic fractions. This amounts to a large over-correction because it is unlikely that pronase would undergo 100 percent autolysis. However the correction is necessary because it is not possible to determine the exact amounts of individual amino acids which might be contributed by varying degrees of pronase autolysis in the systems studied. Consequently, by subtracting the total possible amino acid contribution pronase could make there is no doubt that amounts of amino acids remaining in hydrolysates originated from humic acids and humic fractions and not from enzyme autolysis. The amino acid content of humic enzyme hydrolysates were also corrected for any free amino acids detected in supernatants of related enzyme free humic blanks.

After all blank corrections the moles of individual amino acids contained in pronase hydrolysates of a humic sample were expressed as a percent of the moles of the same amino acids which could be released by 6N acid hydrolysis of an equal weight of enzyme free humic blank. This procedure made possible a comparison of the relative susceptibility of the amino acid containing component of different FRO humic acids and humic fractions to pronase hydrolysis. All results of pronase hydrolysis were expressed on an ash free basis.

3. RESULTS AND DISCUSSION

3.1 General Properties of FRO Humic Acids

FRO humic acids extracted from Ah soil samples contained large amounts of mineral matter (ash). An attempt was made to reduce the ash content by centrifuging alkaline solutions of humic acids at 31,000 X G. This procedure was effective in removing fine clay, but humic acids subsequently recovered from supernatant solutions by precipitations with acid still contained a high ash content. The inability of high speed centrifugation to remove all the ash from FRO humic acids suggested a very strong association between the mineral and organic components of the humic acids. Dilute solutions containing a mixture of hydrochloric and hydrofluoric acids have often been used successfully to reduce the ash content of humic acids, however Flaig et al (1955) and Burges (1960) have suggested that the inorganic components removed by this procedure may form an integral part of the humic acid molecule. On the basis of these observations it was decided to consider the mineral content present in centrifuged FRO humic acids as part of each humic acid structure. Consequently no attempts other than centrifugation at 31,000 X G were made to remove mineral matter from FRO humic acids. Centrifuged FRO humic acids served as the starting material for all subsequent analysis and fractionation experiments.

3.11 Ash Content of Centrifuged FRO Humic Acids

The ash content of Beaverhills FRO humic acids obtained from soil samples collected at three different sites varied from 19.0

TABLE 3.1 SELECTED PROPERTIES OF FRO HUMIC ACIDS EXTRACTED FROM SOIL Ah SAMPLES WITH 0.5% SODIUM HYDROXIDE

Soil	% Ash	% C	% N	C/N	E4/E6 *
Beaverhills					
Site 1	25.6	42.9	4.0	10.7	5.4
Site 2	19.7	47.0	4.3	11.0	
Site 3	19.0	44.1	4.2	10.5	
Maleb					
Site 1	28.1	43.6	5.2	8.4	5.2
Site 2	25.9	45.3	4.8	9.4	
Site 3	33.7	41.1	4.6	8.9	
Site 4	32.1	42.8	5.0	8.6	
Waterton					
Site 1	10.7	42.9	4.4	9.8	5.2
Alpine					
Site 1	13.5	44.4	3.2	13.8	6.3

* E4/E6 ratios shown for Beaverhills and Maleb soils represent an average value for all sites.

3.11 (continued)

to 25.6 percent (Table 3.1). These humic acids were obtained by 0.5N sodium hydroxide extraction of soil samples. Sodium hydroxide extraction of Caleb soil samples collected at four different sites yielded FR0 humic acids which contained from 28.1 to 32.1 percent ash (Table 3.1). Only a single site was sampled for each of Waterton and Alpine soils. FR0 humic acids obtained by sodium hydroxide extraction from these soils contained 19.7 and 13.3 percent ash, respectively.

Extracting Beaverhills and Caleb soil samples with suspensions of Na^+ - Chelex resin yielded FR0 humic acids with very high ash contents (Table 3.2). Generally, these ash contents were about twice as high as those recovered from humic acids which had been [illegible] by sodium hydroxide extraction. The ash content of humic acids obtained by Na^+ - chelex extraction of Waterton and Alpine soil samples (Table 4.2), was almost identical to levels found in corresponding sodium hydroxide extracted humic acids.

3.12 Hydrolysis

Sodium hydroxide is able to extract large amounts of soil organic matter partly because of the reaction of hydroxyl ions with aluminum and iron with the resultant release of organic material originally bound by these metals; and partly through hydrolysis of organic-organic bonds, a reaction also likely to aid in solubilization of organic material (Bremner et al, 1946). Thus sodium hydroxide can be expected to be effective in disrupting most kinds of mineral-organic complexes. The reagent would also

TABLE 3.2 [illegible]

Soil	Ash	C	N	C/N	[illegible]
Beaverpond					
Site 1	57.1	31.1	2.6	12.1	3.9
Site 2	60.1	38.9	3.2	11.8	
Site 3	36.9	20.1	1.7	12.1	
Natel					
Site 1	55.6	34.2	3.1	11.3	4.1
Site 2	57.3	34.3	2.9	11.9	
Site 3	47.1	24.9	2.2	11.3	
Site 4	56.3	35.9	2.9	12.4	
Antelope					
Site 1	11.8	46.2	3.7	12.5	4.0
Alpine					
Site 1	14.5	43.2	3.7	11.7	4.5

* Average value for all sites based on composite sample

aluminum and/or iron ions. Such complexes are particularly stable and resist disruption by most reagents (Greenland, 1971). The bonding sites in such complexes could be effectively shielded from the disrupting action of hydroxyl ions by the bulk of the organic molecule acting as a barrier between the external environment and the mineral surface which is the site of bonding. Such

matrix.

ions,

resin is accompanied by the liberation

(Luttrell, et al, 1971). Partial hydrolysis of Na$^+$ chelating group associations in aqueous suspensions of the resin to produce Na^+ and OH^- ions explains the observed buffered alkaline pH of these suspensions.

In view of the properties of the chelating resin it could be

3.12 (continued)

expected to be effective in solubilizing organic matter associated with mineral components only by relatively weak coulombic forces or by metal-organic bonds sufficiently exposed to be susceptible to disruption by the relatively low concentration of hydroxyl ions in the system. The Na^+ ions present would aid in peptizing colloidal material as well as disrupting clay-organic complexes associated primarily by weak coulombic forces. Any cations liberated from clay-organic complexes by cation exchange with Na^+ or through the disrupting action of hydroxyl ions, would be chelated by the resin and thus removed from the system. This would aid in further peptization of colloidal material by removing the influence of flocculating cations. Consequently extraction of soils with chelating resin could be expected to disrupt weakly aggregated soil mineral-organic matter complexes while releasing little material from more stable aggregates bonded primarily by mechanisms such as ligand exchange adsorption. Thus Na^+ - Chelex extraction should yield organic matter with high ash content and containing mineral-organic complexes which are essentially intact. Data presented in Table 3.2 supports the postulated mechanism of resin extraction. Beaverhills and Maleb humic acids had very high ash contents when obtained by Chelex extraction; generally about twice as high as associated ERO humic acids obtained by sodium hydroxide extraction. Essentially identical results were obtained by Levesque and Schnitzer (1967). They extracted soil organic matter from four different soil Ah horizons with 0.5N sodium hydroxide and with Na^+ - Chelex resin in separate extractions. Their results

3.12 (continued)

showed organic material obtained by Chelex extraction consistently contained twice the ash content of similar material obtained by sodium hydroxide extraction. They attributed the ash content of Chelex extracted material to a high content of metal-organic complexes.

Humic acids extracted from Waterton and Alpine soils with Chelex resin had ash contents which were essentially the same as for corresponding sodium hydroxide extracted humic acids (Tables 3.1 and 3.2). Thus it appears that these soils are characterized by a relatively low content of mineral-organic complexes.

3.13 Total Carbon Content

Extraction of soil samples with sodium hydroxide solution yielded FRO humic acids which showed only minor variations in total carbon content between humic acids from different soil series (Table 3.1). The average total carbon content of these humic acids was 43 percent.

Chelex extraction of Beaverhills and Naleb soil samples yielded humic acids with lower carbon content than corresponding sodium hydroxide extracted humic acids (Table 3.2). Waterton Chelex humic acids had a higher carbon content than sodium hydroxide analogs, while Alpine Chelex humic acids showed little difference in carbon content compared to similar material from sodium hydroxide extractions.

3.14 Total Nitrogen Content

Nitrogen content of FRO humic acids ranged from 3.2 to 5.2 percent when the humic acids were obtained by sodium hydroxide

3.14 (continued)

extraction (Table 3.1). Maleb FRO humic acids were consistent in containing slightly greater quantities of nitrogen than FRO humic acids from other soils.

Humic acids extracted by Chelex resin from Beaverhills, Maleb and Waterton Ah soil samples (Table 3.2) contained less nitrogen than corresponding humic acids obtained by extraction with sodium hydroxide solution. Alpine Chelex humic acid contained about 15.6 percent more nitrogen than related sodium hydroxide extracted material.

3.15 E4/E6 Ratios

The ratio between extinction coefficients measured at 465 nm and 665 nm (E4/E6) is related to the structural complexity and degree of condensation in humic materials (Kononova, 1966). Low E4/E6 values are attributed to highly condensed, polynuclear, aromatic structures and wide ratios of E4/E6 to more loosely knit structures with higher aliphatic content. The E4/E6 values reported in Table 3.1 indicate that Beaverhills, Maleb and Waterton FRO humic acids have similar degrees of structural complexity. The slightly higher E4/E6 ratio of Alpine humic acid may indicate a greater proportion of aliphatic material in this humic acid relative to the other soil humic acids.

FRO humic acids obtained by Chelex extraction of soil samples (Table 3.2) were characterized by E4/E6 ratios which were consistently lower than those of corresponding humic acids obtained by extraction of soil with sodium hydroxide. Chelex resin apparently extracted humic acids which contained a high proportion of condensed aromatic

3.15 (continued)

material.

3.2 Phenol Extraction of FRO Humic Acids

A slightly acidic solution of phenol (75 percent phenol, pH 4.5) was able to solubilize portions of FRO humic acids. Humic material removed by phenol extraction was separated into FR3 and FR4 fractions using 100 percent acetone as a fractionating solvent. Material soluble in acetone and subsequently recovered from solution (Section 2.34) was labelled FR4. Material insoluble in acetone was labelled FR3.

The FRO humic residue free of FR3 and FR4 components was washed with ether to remove any extraneous phenol. The phenol free humic residue was labelled FR1. The quantity of humic fractions that could be obtained from FRO humic acids of different soils, and the distribution of total humic acid nitrogen among humic fractions, was determined.

3.21 Yield of Humic Fractions From 0.5N Sodium Hydroxide Extracted FRO Humic Acids

The yields of humic fractions obtained from different humic acids are shown in Table 3.3. The data shown for humic acids from each soil series represent averages of several independent determinations. Humic acids from the same soil series showed little variation in the proportion or amount of humic fractions which could be obtained from them.

The humic fraction recovered in greatest amount from FRO humic acids was the FR1 fraction. Beaverhills humic acid contained 57.1 percent FR1 material, Maleb humic acid, 47.1 percent, Waterton

TABLE 3.3 DISTRIBUTION OF TOTAL NITROGEN AND YIELD OF HUMIC FRACTIONS FROM 0.5N SODIUM HYDROXIDE EXTRACTED HUMIC ACIDS

Soil Humic Acid FRO	Humic Fraction	% of FRO	Nitrogen Content as % of FRO Total N
Beaverhills	FR1	57.1	54.8
	FR3	22.3	32.5
	FR4	6.2	7.9
Maleb	FR1	47.1	50.1
	FR3	35.8	52.7
	FR4	2.7	--
Waterton	FR1	55.4	54.1
	FR3	7.8	9.9
	FR4	4.1	4.5
Alpine	FR1	49.6	51.2
	FR3	6.9	8.1
	FR4	1.5	2.4

3.21 (continued)

humic acid, 55.4 percent, and Alpine humic acid, 49.6 percent. This fraction accounted for 54.8, 50.1, 54.1 and 51.2 percent of the total humic nitrogen of Beaverhills, Maleb, Waterton and Alpine humic acids, respectively.

Maleb and Beaverhills humic acids contained relatively large amounts of FR3 material. In the case of Maleb soils the FR3 fraction accounted for 35.8 percent of the FRO humic acid and contained 52.7 percent of the total humic acid nitrogen. Beaverhills FRO humic acid contained 22.3 percent FR3 material, and this fraction accounted for 32.5 percent of the total FRO humic acid nitrogen. Waterton and Alpine humic acids contained only 7.8 and 6.9 percent FR3 material, respectively. The FR3 fraction of these soils accounted for less than 10 percent of the total humic acid nitrogen.

FR4 humic fractions occurred in only small amounts in FRO humic acids (Table 3.3). Beaverhills humic acid contained 6.2 percent FR4 material, which in turn contained 7.9 percent of the total humic nitrogen. Maleb humic acid contained only 2.7 percent of FR4 humic fraction. The total nitrogen content of this fraction was not determined. Waterton humic acid contained 4.5 percent FR4 material while Alpine humic acid contained 1.5 percent. This fraction accounted for 4.5 and 2.4 percent of the total humic nitrogen of Waterton and Alpine humic acids, respectively.

3.22 Yield of Humic Fractions From Chelex Extracted FRO Humic Acids

FR1 humic material was the major component of FRO humic acids obtained by Chelex extraction of soil samples (Table 3.4). This

TABLE 3.4 DISTRIBUTION OF TOTAL NITROGEN AND YIELD OF HUMIC FRACTIONS FROM Na^+ - CHELEX RESIN EXTRACTED HUMIC ACIDS

Soil Humic Acid FRO	Humic Fraction	% of FRO	Nitrogen Content as % of FRO Total N
Beaverhills	FR1	65.6	68.6
	FR3	1.2	1.4
	FR4	2.2	2.3
Waterton	FR1	83.2	68.7
	FR3	3.5	6.1
	FR4	1.7	1.8
Alpine	FR1	70.0	68.1
	FR3	4.1	6.2
	FR4	2.0	2.4
Maleb	FR1	90.2	80.2

3.22 (continued)

fraction accounted for about 68 percent of the total nitrogen contained by Beaverhills, Waterton and Alpine humic acids. In the case of Maleb humic acid the FR1 fraction contained 80 percent of the total humic nitrogen. Only a few percent of each of FR3 and FR4 humic fractions were obtained from Beaverhills, Waterton and Alpine humic acids. Maleb humic acid contained no FR4 fraction and only trace quantities of FR3 material.

3.3 General Properties of Humic Fractions

3.31 Mineral Content (Ash)

Phenol extraction of FR0 humic acids resulted in the recovery of FR3 and FR4 humic fractions which contained less than 1 percent ash (Tables 3.5 - 3.10). FR1 humic fractions contained large amounts of ash which was intimately associated with the humic material and could not be removed by centrifugation (Tables 3.5-3.10).

During the recovery of FR1 humic fractions it was noted that some inorganic material was consistently removed from suspension during centrifugation steps involved in the procedure. Since FR0 humic acids did not contain any ash which could be separated from the humic acid prior to phenol extraction, the observation noted above suggests that phenol extraction liberated ash from the humic complex. This ash might represent inorganic material which was associated with FR3 and FR4 humic components in the original humic acid complex, and subsequently freed from this association by phenol extraction.

TABLE 3.5 SELECTED PROPERTIES OF FR1 HUMIC FRACTIONS DERIVED FROM 0.5N SODIUM HYDROXIDE EXTRACTED FRO HUMIC ACIDS

Soil	% Ash	% C	% N	C/N	E4/E6*
Beaverhills					
Site 1	32.1	39.9	3.9	10.2	4.7*
Site 2	26.2	44.3	4.1	10.8	
Site 3	25.6	44.7	4.0	11.2	
Maleb					
Site 1	22.6	39.4	5.8	6.8	
Site 2	42.1	39.8	5.0	7.9	4.6*
Site 3	36.5	33.0	4.8	6.9	
Site 4	42.4	38.7	4.9	7.9	
Waterton					
Site 1	9.3	42.4	4.3	9.9	4.6
Alpine					
Site 1	10.9	45.2	3.3	13.7	4.5

* Average value based on composite sample

TABLE 3.6 SELECTED PROPERTIES OF FR3 HUMIC FRACTIONS DERIVED FROM 0.5N SODIUM HYDROXIDE EXTRACTED FRO HUMIC ACIDS

Soil	% Ash	% C	% N	C/N	E4/E6*
Beaverhills					
Site 1	1.2	50.5	6.3	8.0	
Site 2	< 1	49.6	6.0	8.2	6.2*
Site 3	< 1	50.9	6.1	8.4	
Maleb					
Site 1	< 1	50.4	7.3	6.9	
Site 2	< 1	47.7	6.8	7.0	7.2*
Site 3	< 1	49.0	7.2	6.8	
Site 4	< 1	50.8	7.1	7.1	
Waterton					
Site 1	1.9	48.4	5.6	8.6	5.9
Alpine					
Site 1	< 1	44.1	3.8	11.6	5.3

* Average value for all sites based on composite sample

TABLE 3.7 SELECTED PROPERTIES OF FR4 HUMIC FRACTIONS DERIVED FROM 0.5N SODIUM HYDROXIDE EXTRACTED FRO HUMIC ACIDS

Soil	% Ash	% C	% N	C/N	E4/E6
Beaverhills					
Site 1	< 1	56.8	5.4	10.5	
Site 2	< 1	59.1	5.3	11.1	7.1*
Site 3	< 1	56.6	5.2	11.0	
Maleb					
Site 1	< 1	58.3	ND	ND	
Site 2	< 1	59.7	ND	ND	8.1*
Site 3	< 1	55.2	ND	ND	
Site 4	< 1	58.3	ND	ND	
Waterton					
Site 1	< 1	54.4	4.8	11.3	6.8
Alpine					
Site 1	< 1	52.8	5.2	10.1	7.4

* Average value for all sites based on a composite sample

ND= Not determined

TABLE 3.8 SELECTED PROPERTIES OF FR1 HUMIC FRACTIONS DERIVED FROM Na^+- CHELEX EXTRACTED FRO HUMIC ACIDS

Soil	% Ash	% C	% N	C/N	E4/E6
Beaverhills					
Site 1	51.6	29.6	2.3	12.8	3.9*
Site 2	41.4	36.2	3.2	11.4	
Site 3	36.7	36.3	2.9	12.5	
Waterton					
Site 1	11.3	43.4	3.1	14.0	4.8
Alpine					
Site 1	13.3	39.6	3.6	11.0	5.0

* Average value for all sites based on composite sample

TABLE 3.9 SELECTED PROPERTIES OF FR3 HUMIC FRACTIONS DERIVED FROM Na^+- CHELEX EXTRACTED FRO HUMIC ACIDS

Soil	% Ash	% C	% N	C/N	E4/E6
Beaverhills					
Site 1	< 1	50.5	4.2	12.0	5.7*
Site 2	< 1	49.5	4.5	11.0	
Site 3	< 1	50.9	4.0	12.7	
Waterton					
Site 1	< 1	54.0	6.4	8.4	6.1
Alpine					
Site 1	< 1	44.2	5.6	7.9	6.4

* Average value for all sites based on composite sample

TABLE 3.10 SELECTED PROPERTIES OF FR4 HUMIC FRACTIONS DERIVED FROM Na^+- CHELEX EXTRACTED FRO HUMIC ACIDS

Soil	% Ash	% C	% N	C/N	E4/E6
Beaverhills					
Site 1	< 1	56.8	3.6	15.7	6.2*
Site 2	< 1	56.6	3.2	17.6	
Site 3	< 1	59.1	3.4	17.4	
Waterton					
Site 1	< 1	58.9	4.0	14.7	7.1
Alpine					
Site 1	< 1	52.3	4.4	11.9	8.4

* Average value for all sites based on composite sample

3.32 Total Carbon Content

Humic fractions showed regular and consistent analysis with respect to carbon content (Tables 3.5 - 3.10). FR1 humic fractions always had a relatively low carbon content, FR3 fractions an intermediate carbon content and FR4 fractions the highest carbon content. This general pattern of carbon distribution among the humic fractions was not affected by the origin of FRO humic acids with respect to soil series, or by the extractant used to remove FRO humic acids from soil.

3.33 Total Nitrogen Content

Humic fractions soluble in phenol had a higher total nitrogen content than phenol insoluble humic material (Tables 3.5 - 3.10). Generally FR3 fractions showed the highest nitrogen content. In the case of Alpine soil the highest nitrogen analysis was obtained for FR4 humic material when sodium hydroxide was used to obtain FRO humic acid.

An examination of C/N ratios (Tables 3.5 - 3.10) shows FR3 humic fractions generally had the lowest ratios, consistent with their relatively high total nitrogen content. FR1 humic fractions had C/N ratios similar to the FRO humic acid of their origin. FR4 humic fractions often showed an increase in C/N ratio sometimes reaching values which were greater than that of the original FRO humic acid. This reflects the higher carbon content of these fractions relative to the other humic fractions and shows that phenol did not preferentially solubilize only material high in nitrogen content.

3.34 E4/E6 Ratios

Humic fractions showed a regular and consistent increase in E4/E6 ratios going from FR1 to FR4 fractions (Tables 3.5 - 3.10). This indicates that phenol dissolved material of greater aliphatic content and lower levels of condensation than material which was not solubilized. The E4/E6 ratio of FR1 humic fractions was generally lower than the original FRO humic acid indicating that FR1 humic fractions consisted mainly of relatively highly condensed and aromatic material. The E4/E6 ratios of FR4 humic fractions were consistently higher than those of any other humic fraction, indicating that this material had a relatively high aliphatic content and a structure characterized by much lower levels of condensation than other humic fractions.

3.35 Ultraviolet Spectra

Ultraviolet (UV) spectra were obtained for all humic acids and humic fractions. Representative spectra are reproduced in Figure 3.1. Spectra of FRO humic acids and FR1 humic fractions were featureless showing no well-resolved absorption maxima. FR3 and FR4 humic fractions consistently showed a well-pronounced absorption peak at about 280 nm.

Kononova (1973) obtained UV spectra similar to those shown for FR3 and FR4 humic fractions. Her spectra, however, were for humic acids extracted from incompletely humified plant residues. She attributed an absorption maxima at 280 nm to aromatic compounds of lignin origin and found that upon further humification of the plant material the absorption peak disappeared. Biederbeck and Paul (1973) also noticed an absorption peak at about 280 nm

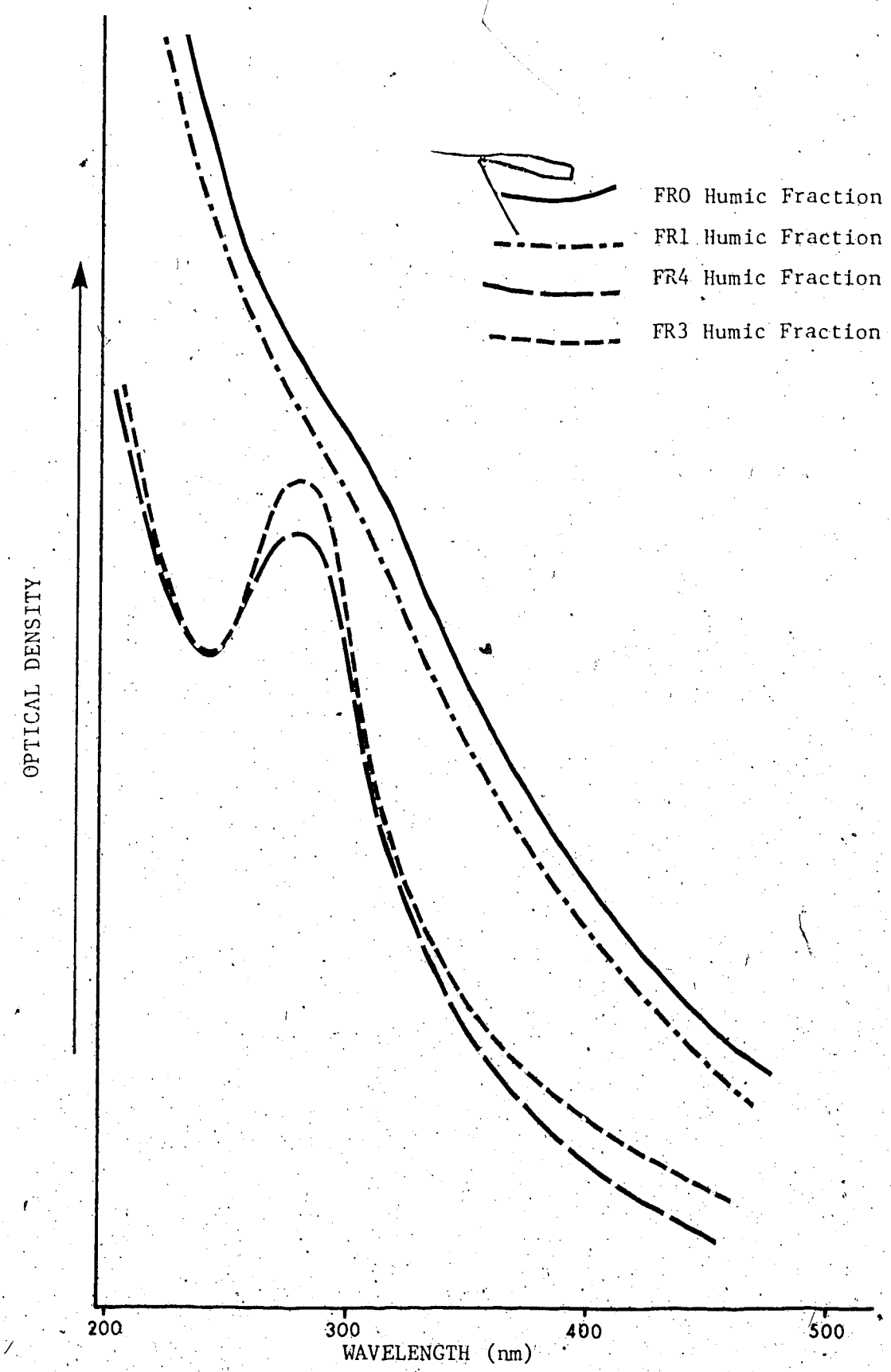


FIGURE 3.1 REPRESENTATIVE ULTRAVIOLET SPECTRA OF HUMIC ACIDS AND HUMIC FRACTIONS

3.35 (continued)

in UV spectra of purified humic fractions which had been obtained by phenol extraction of humic acid. They attributed a failure to observe this peak in the original unfractionated humic acid to the existence of hydrogen bonding between humic components which had acted to mask the absorption peak. When considered in the light of observations made by Kononova and by Biederbeck and Paul, results of UV analysis reported in the present study suggest that phenol extraction has disrupted humic complexes releasing structures of relatively simpler overall composition. These structures could contain such groups as single and polycyclic aromatic, single and polynuclear quinone structures, or aliphatic hydrocarbon moeities substituted with chromophoric groups. The structural arrangement and/or proportion of such compounds in FR3 and FR4 humic fractions could be such as to produce definite absorption maxima in the UV. The lack of well-resolved individual absorption peaks in UV spectra of FRO and FR1 humic material is probably due to both the diversity and concentration of chromaphores present in these materials, the net effect being to produce the continuous UV absorption spectra typical of soil humic acids.

3.36 Discussion

Haworth (1971) has postulated that humic acid consists of a complex largely aromatic core structure to which are attached, either chemically or through physical adsorption processes (a) polysaccharides, (b) proteins, (c) simple phenols and (d) metals. Much of this "peripheral" organic material could be associated with aromatic constituents of the humic nucleus by hydrogen bonding.

3.36 (continued)

Hydrogen bonding within the aromatic moiety of humic substances and its function in joining phenolic and benzenecarboxylic constituents was recently demonstrated by Schnitzer (1971) through exhaustive methylation of humic substances.

The exact mechanism of hydrogen bonding in humic structures is unknown, however it is reasonable to assume that such associations involve phenolic or carboxyl groups of humic constituents and receptor groups (-NH-, -CO-, or -OH) of protein residues or other polymers. Hydrogen bonds between peptide chains and polyphenols can be ruptured by treatment with phenol solutions (Biederbeck et al, 1973). The dissolution of portions of humic acids by phenol observed in the present study indicates the existence of a mixture of humic components associated by hydrogen bonding mechanisms. These results support work reported by Biederbeck and Paul (1973) and Simonart et al, (1967), who also observed the ability of phenol to dissolve portions of humic acids.

Results of the present study show that phenol effected the solubilization of humic material rich in nitrogenous components (FR3 and FR4 humic fractions, Tables 3.5 - 3.10) relative to the residual phenol insoluble FR1 humic material. Total carbon analysis of humic fractions revealed a regular increase in carbon content going from FR1 to FR4 fractions. FR1 humic fractions contained from 30 to 45 percent carbon (Tables 3.5 and 3.8), which is a much lower total carbon content than generally reported for soil humic acids (Kononova, 1966; Lowe, 1969), but is typical of analysis reported for fulvic acids (Kononova, 1966). Biederbeck

3.36 (continued)

et al (1973) noted a similar low total carbon content, as well as a high oxygen content of phenol insoluble humic residues, suggesting the humic residue consisted of highly oxidized substances. The high ash content observed for FR1 humic fractions which could not be removed by centrifugation at 31,000 X G may imply a high carboxyl content of FR1 material and thus a high state of oxidation similar to the humic fraction studied by Biederbeck et al (1973). Carboxyl groups are strong complexing ligands for aluminum and iron. Thus material with a high content of carboxyl groups could be expected to be intimately associated with soil mineral components such as hydrous aluminum and iron oxides, resulting in a high ash content.

The carbon content of FR3 fractions ranged from 44.1 to 50.9 percent (Tables 3.6 and 3.9), while the carbon content of FR4 fractions ranged from 52.3 to 59.1 percent. The progressively higher carbon content shown by FR3 and FR4 humic fractions may indicate that these fractions contain a high proportion of compounds which are in relatively low states of oxidation. According to this criteria FR4 fractions would contain the greatest amount of unoxidized material.

Phenol extraction of FRO humic acids resulted in the recovery of humic fractions which showed a consistent increase in E4/E6 ratios going from FR1 to FR4 fractions (Tables 3.5 - 3.10). In addition the E4/E6 ratio of FR1 humic fractions was generally lower than the original FRO humic acid from which the FR1 material had been obtained. According to Kononova (1966) the E4/E6 ratio

3.36 (continued)

of humic substances reflects the degree of aromatic condensation of the humic structure, low ratios indicating humic material with a relatively highly condensed aromatic structure. Campbell et al (1967), using ^{14}C dating techniques, found an inverse relationship existed between the E4/E6 ratio and the mean residence times of various humic fractions isolated from an Orthic Black Chernozem soil (Melfort) by 0.5N sodium hydroxide extraction. They found the humic fractions with the lowest mean residence times had the widest E4/E6 ratio. Campbell et al also found that humic fractions isolated by the same procedure from each of a Chernozemic soil and a Podzolic soil displayed similar E4/E6 ratios, but the humic fractions showed considerable variation between soils with respect to their mean residence time. Other workers (Butler and Ladd, 1969; Schnitzer and Skinner, 1969) have noted that increases in E4/E6 ratios corresponded to decreases in molecular weight. Butler and Ladd (1969) found that the ratio of optical density measured at 470 nm to that measured at 666 nm (E470/E666) decreased from 9.0 for humic fractions of nominal molecular weight < 5,000 to 3.6 for humic fractions with nominal molecular weight > 150,000. Molecular weights of the humic fractions were based on the results of Sephadex gel permeation chromatography. Anderson (1972) found that high E4/E6 ratios were often characteristic of low molecular weight, relatively aromatic humic acids with properties transitional to fulvic acids.

When the data reported in the present study for E4/E6 ratios of humic fractions (Tables 3.5 - 3.10) are considered in

3.36 (continued)

the light of the work reported above, it appears that FR1 humic fractions represent high molecular weight humic material, while FR3 and FR4 humic fractions represent material of increasingly lower molecular weight, with FR4 fractions apparently having the lowest molecular weight. In addition if E4/E6 ratios of humic substances do bear an inverse relationship to their mean residence time in soil (Campbell et al, 1967) then FR1 humic fractions represent the component of FRO humic acids most likely to persist for the greatest length of time in soil while the FR3 and FR4 fractions represent humic material characterized by progressively lower mean residence times, with FR4 fractions being the most labile. These observations suggest that humic components which are insoluble in phenol (FR1 humic fractions) may represent the "core" or "nucleus" of humic acids proposed by Haworth (1971). If this is the case then FR3 and FR4 humic fractions may in turn represent the "peripheral" material associated with the "core" structure (FR1 fractions) through adsorption mechanisms such as hydrogen bonding.

Kononova and Alexandrova (1973) have studied the process of humic acid formation during plant residue humification. They studied properties of these humic acids as a function of time and concluded that a common feature of all newly-formed humic acids extracted from plant residues is the low content of carboxyl groups and a weak condensation of aromatic nucleii (high E4/E6 ratio). In addition UV spectra of newly-formed humic acids showed a well-defined absorption maximum at 280 nm characteristic of aromatic compounds probably of lignin origin.

3.36 (continued)

This absorption maximum disappeared as humification proceeded to be replaced by the featureless absorption curve typical of purified de-ashed soil humic acid. Examination of ultra-violet spectra (Figure 3.1) obtained from FRO humic acids used in the present study shows the spectra to be featureless and identical in form to those reported by Kononova et al (1973) for "matured" soil humic acids. However, phenol solubilized humic material (FR3, FR4 fractions) had UV spectra which displayed well-resolved absorption maxima at 280 nm, whereas the phenol insoluble humic fraction (FR1) showed only a featureless absorption spectra, similar to that of the FRO humic acid from which it was derived. These results suggest that phenol solubilizes humic material (FR3, FR4 fractions) which is in a relatively early stage of humification. Apparently association of these fractions with FR1 humic matter results in a complex (FRO humic acid) in which the absorption maximum at 280 nm is masked in the overall high absorbance of the FRO humic acid complex.

The yield of humic fractions varied between soil series and was dependent on which extractant (i.e. sodium hydroxide vs Na^+ - Chelex) had been used to remove FRO humic material from soil samples (Tables 3.3 and 3.4). The variation in yield of humic fractions between soil series indicates differences in the intensity of humification processes occurring in the soils. A detailed discussion of some factors likely to account for these differences will be deferred until data for amino acid analysis and results of pronase hydrolysis have been discussed.

The variation in yield of humic fractions from sodium hydroxide FRO humic acids vs that from Na^+-Chelex FRO humic acids (Tables 3.3 and 3.4, respectively) is consistent with the idea that sodium hydroxide solubilizes a mixture of humic components through the disruption of mineral-organic aggregates whereas Na^+-Chelex solubilizes mainly easily-peptized colloidal material and is not effective in attacking more complex mineral-organic aggregates. Na^+-Chelex FRO humic acids consist almost entirely of FR1 material (Table 3.4) of low E4/E6 ratio indicating a relatively high content of condensed aromatic material. Total carbon content of Chelex FR1 humic fractions was also low. These results suggest that Na^+-Chelex extracted primarily "old" or well-humified organic matter from the soils studied.

3.4 Amino Acid Analysis of Humic Acids and Humic Fractions

Amino acid analysis of humic material was performed using a gas chromatographic technique. Amino acids obtained by 6N hydrochloric acid hydrolysis of the various humic acids were separated from other components of the hydrolysate by ion exchange chromatography using Dowex-50 X $8H^+$ resin. The amino acids were determined as their n-butyl ester N-trifluoroacetyl derivatives using ornithine as an internal standard.

The gas chromatographic procedure was chosen because the combination of N-trifluoroacetyl n-butyl ester derivitization with gas chromatography on ethylene glycol adipate supported on Chromosorb W gives excellent separation and peak shape. One disadvantage however, is that the derivatives of arginine, cysteine, histidine and tryptophan decompose under these chromatographic conditions. Although the problem has been under study by a number of researchers (Gehrke et al 1968, 1967, 1970; Coulter and Hann, 1968; Moss et al, 1971; Hardy et al, 1972), there is at present no combination of

3.4 (continued)

derivitization procedure and conventional packed G.C. column conditions which can give rise to a complete peak separation of all protein amino acids on a single column. However, for the amino acids which can be determined, the quantitative results compare very favorably with results obtained from an amino acid analyzer (Pereira et al, 1974).

Using the gas chromatographic procedure it was possible to quantify the following thirteen amino acids present in humic acid hydrolysates; alanine, valine, glycine, isoleucine, leucine, proline, threonine, serine, phenylalanine, aspartic acid, glutamic acid, tyrosine and lysine. Since ornithine occurs in soil hydrolysates (Khan and Sowden, 1971) and because ornithine was used as an internal standard for quantification of amino acid chromatograms, several different humic acid and humic fraction hydrolysates were derivitized and analyzed without the addition of ornithine to the reaction mixture. In general these chromatograms did not produce any readable peaks at the point where ornithine was normally eluted. Occasionally a FR3 humic fraction hydrolysate would show a trace indication of ornithine, but the peak area in such instances was negligible compared to the peak area normally produced by standard additions of ornithine.

Table 3.11 shows amino acid determinations for a mixture of pure standard L-amino acids. One millilitre of the amino acid mixture containing the amount of each amino acid shown was derivitized and the analytical procedure was carried through to determine recovery of amino acids. The results in Table 3.11 are the means

TABLE 3.11 RECOVERY OF STANDARD L - AMINO ACIDS AS DETERMINED BY GAS LIQUID CHROMATOGRAPHY

Amino Acid	Added μ moles/ml	Found μ moles/ml	Recovery %	Standard Deviation
Alanine	2.637	2.629	99.6	± 0.08
Valine	1.707	1.751	102.5	± 0.09
Glycine	3.117	3.190	102.3	± 0.15
Leucine	1.669	1.680	100.6	± 0.03
Proline	2.301	2.346	101.9	± 0.12
Phenylalanine	1.319	1.249	94.6	± 0.06
Aspartic Acid	2.028	1.974	97.3	± 0.07
Glutamic Acid	2.270	2.198	96.8	± 0.07
Lysine	1.149	1.092	95.0	± 0.02

3.4 (continued)

and standard deviation of four separate independent analysis. The overall precision shown by the technique is very good; in all cases the standard deviations are less than 5.2 percent of the mean. Recovery of amino acids is also very good.

3.41 Distribution of Amino Acid Content of FRO Humic Acids Among Humic Fractions

Table 3.12 contains data which shows the amino acid content of 0.5N sodium hydroxide extracted FRO humic acids and the distribution of this amino acid content among humic fractions recovered from FRO humic acids. Data for amino acid content is based on the sum of μmoles of each of 13 amino acids present in FRO humic acids and humic fractions, and represents average values obtained from at least triplicate independent analysis.

The amino acid content of FRO humic acids varied considerably between humic acids obtained from different soil series (Table 3.12). This variation in amino acid content reflects the difference in total nitrogen content that exists between FRO humic acids from different soil series (Table 3.12). In general the 13 amino acids determined accounted for a relatively small part of the total nitrogen content of FRO humic acids. In the case of Maleb humic acid the amino acids determined accounted for 20.0 percent of the total FRO humic acid nitrogen. For Beaverhills, Waterton and Alpine FRO humic acids the amino acids determined accounted for 12.7, 7.5 and 6.9 percent, respectively, of the total FRO humic acid nitrogen (Table 3.12).

The FR1 component of FRO humic acids was the major fraction

TABLE 3.12 HUMIC FRACTION CONTENT OF 0.5N SODIUM HYDROXIDE EXTRACTED FRO HUMIC ACIDS AND DISTRIBUTION OF FRO AMINO ACID AND NITROGEN CONTENT AMONG THE HUMIC FRACTIONS

Humic Fraction	% of FRO Humic Acid	Total FRO N %	% of FRO N	FRO Amino Acid Content* µmoles/gm	% of FRO Amino Acid Content	Amino Acid N	
						% of FRO Total N	% of FRO Total Amino Acid N *
Beaverhills							
FRO	100.0	4.2	100.0	314.4	100.0	12.7	100.0
FR1	57.1		54.8		49.0	5.5	43.0
FR3	22.3		32.5		44.9	5.0	39.0
FR4	6.2		7.9		8.1	0.9	9.1
Maleb							
FRO	100.0	4.8	100.0	649.1	100.0	20.0	100.0
FR1	47.1		50.1		34.4	8.5	41.7
FR3	35.8		52.7		58.4	11.4	58.8
Waterton							
FRO	100.0	4.4	100.0	227.3	100.0	7.5	100.0
FR1	55.4		54.1		65.6	4.9	65.5
FR3	7.8		9.9		19.1	1.5	19.4
FR4	4.1		4.5		8.7	0.7	8.8
Alpine							
FRO	100.0	3.2	100.0	152.9	100.0	6.9	100.0
FR1	49.6		51.2		52.8	3.7	54.0
FR3	6.9		8.1		7.8	0.6	8.1
FR4	1.5		2.4		4.8	0.3	5.0

* Based on sum of µmoles of each of 13 amino acids present in FRO humic acids and humic fractions

3.41 (continued)

recovered from all humic acids (Table 3.12) and generally constituted about 50 percent by weight of FRO humic acid. The proportion of FRO amino acid content recovered in FR1 humic fractions depended on the soil series from which the FRO humic acid originated. Thus for example, Beaverhills FR1 fractions contained 49.0 percent of the FRO humic acid amino acid content, while Maleb FR1 fractions accounted for only 34.4 percent of the amino acid content of Maleb FRO humic acid. Waterton FR1 fractions contained the highest proportion of FRO humic acid amino acid content, namely 65.6 percent. In the case of Alpine humic acid the FR1 fraction accounted for 52.8 percent of the FRO humic acid amino acid content.

A relatively high proportion of the amino acid content of FRO humic acids was found to be associated with the FR3 component of the humic acids. Thus Beaverhills FRO humic acids contained 22.3 percent FR3 material which in turn contained 44.9 percent of the amino acid content originally determined to be present in the FRO humic acid (Table 3.12). In the case of Maleb FRO humic acids the FR3 fraction accounted for 35.8 percent of the FRO humic acid and contained 58.4 percent of the amino acids. Waterton FRO humic acids contained only 7.8 percent by weight of FR3 material, yet this fraction accounted for 19.1 percent of the FRO humic acid amino acid content. Alpine FRO humic acids contained the smallest amount of FR3 material. This fraction made up 6.9 percent of Alpine humic acid, but contained only 7.8 percent of the amino acid content of the FRO humic acid (Table 3.12).

3.41 (continued)

Only a relatively small amount of FR4 humic material was recovered from soil FRO humic acids (Table 3.12). For Beaverhills humic acids the yield of FR4 material was less than 25 percent of the yield obtained for the FR3 fraction and amounted to only 6.2 percent by weight of the FRO humic acid. Maleb FRO humic acids contained no FR4 material. Waterton humic acids yielded 4.1 percent of FR4 material, an amount equal to about 50 percent of the yield of FR3 humic material that could be removed from these humic acids. Alpine FRO humic acids contained only 1.5 percent of FR4 material. In contrast to FR3 humic fractions, FR4 fractions accounted for less than 9 percent of the amino acid content of any FRO humic acid which had been extracted from soil with 0.5N sodium hydroxide.

Extraction of soils with Na^+- Chelex resin yielded FRO humic acids in which FR1 humic material was the major component present (Table 3.13). Amounts of this component present in humic acids depended on which soil series the humic acid was obtained from. Maleb humic acids were composed of almost 100 percent FR1 material (data not shown), while Beaverhills, Waterton and Alpine humic acids contained 65.6, 83.2 and 70.0 percent of FR1 material, respectively.

In general the amino acids determined for Na^+- Chelex extracted FRO humic acids represented 11 percent or less of the total nitrogen content of the FRO humic acid (Table 3.13). Phenol fractionation of Na^+- Chelex humic acids showed that FR1 humic material accounted for 61.7 percent of the amino acid content of

TABLE 3.13 HUMIC FRACTION CONTENT OF Na-CHELEX EXTRACTED FRO HUMIC ACIDS AND DISTRIBUTION OF FRO AMINO ACID AND NITROGEN CONTENT AMONG THE HUMIC FRACTIONS

Humic Fraction	% of FRO Humic Acid	Total FRO N %	% of FRO N	FRO Amino Acid Content* µmoles/gm	% of FRO Amino Acid Content	Amino Acid N	
						% of FRO Total N	% of FRO Total Amino Acid N *
Beaverhills							
FRO	100.0	3.0	100.0	150.6	100.0	7.3	100.0
FR1	65.6		68.6		61.7	4.4	60.0
FR3	1.2		1.4		3.4	0.2	3.2
FR4	2.2		2.3		3.3	0.3	3.7
Waterton							
FRO	100.0	3.7	100.0	259.0	100.0	10.0	100.0
FR1	83.2		68.7		71.2	7.2	71.8
FR3	3.5		6.1		6.5	0.7	6.8
FR4	1.7		1.8		1.6	0.2	1.8
Alpine							
FRO	100.0	3.7	100.0	280.0	100.0	11.4	100.0
FR1	70.0		68.1		81.5	9.3	81.6
FR3	4.1		6.2		10.9	1.3	11.2
FR4	2.0		2.4		2.5	0.7	2.6

* Based on sum of µmoles of each of 13 amino acids present in FRO humic acids and humic fractions

3.41 (continued)

Beaverhills humic acid, 71.2 percent of the amino acid content of Waterton humic acid and 81.5 percent of the amino acid content of Alpine humic acid. Na^+- Chelex humic acids generally contained less than 4.5 percent of either FR3 or FR4 humic fractions. As a consequence of their small yield, FR3 and FR4 humic fractions accounted for only a few percent of the amino acid content of Na^+- Chelex extracted FRO humic acids.

3.42 Relative Mole Percent Amino Acid Composition of Humic Acids and Humic Fractions

The complete results of amino acid analysis of humic material are quite extensive when considered on an individual soil site and humic fraction basis. Consequently the results for relative mole amino acid percent composition* have been averaged and compiled in Tables 3.14 to 3.21 for discussion purposes. In the case of Beaverhills and Maleb soils (Tables 3.14, 3.15 and 3.18, 3.19, respectively) the data represent means and standard deviations of results obtained from all soil sites sampled for each soil series. For Waterton and Alpine soils (Tables 3.16, 3.20 and 3.17, 3.21, respectively) data reported is the mean of duplicate independent analysis for each soil site. In this case standard deviations are not reported since only single soil sites were sampled; duplicate analysis for each soil were consistent. Complete

* Relative mole amino acid percent $= \frac{\mu\text{moles amino acid}}{\sum_{1}^{13} \mu\text{moles amino acids}} \times 100$

3.42 (continued)

data on an individual soil site and fraction basis can be found in the appendix.

Amino acid analysis of Beaverhills humic acids and humic fractions from 0.5N sodium hydroxide extraction (Table 3.14) show there is little variation in amino acid composition between similar humic fractions obtained from different soil sites. Of the thirteen amino acids determined, alanine, glycine and aspartic acid were present in greatest proportion; valine, leucine, proline, glutamic acid and lysine were next most abundant, with isoleucine, threonine, serine, phenylalanine and tyrosine present in smaller amounts. These general trends were noted for all humic acids and humic fractions obtained from Beaverhills soil samples.

Amino acid analysis of 0.5N sodium hydroxide derived humic acids and associated fractions of Maleb soil samples (Table 3.15) show that alanine, glycine, leucine and aspartic acid are present in greatest proportion. Valine, proline and glutamic acid are also present in high proportion relative to the remaining amino acids. Similar humic fractions obtained from different soil sites showed little variation in composition, however the data indicates (Table 3.15) that Maleb FR1 and FR3 humic fractions were characterized by greater variation in alanine, valine, isoleucine and leucine content than was the case for analogous Beaverhills fractions (Table 3.14). This was especially true of Maleb FR1 humic fractions.

Data in Tables 3.16 and 3.17 show amino acid analysis of sodium hydroxide derived humic acids of Waterton and Alpine soils, respectively. The data in these tables is generally similar to

TABLE 3.14 RELATIVE MOLE PERCENT AMINO ACID COMPOSITION OF 6N ACID HYDROLYSATES OF 0.5N SODIUM HYDROXIDE EXTRACTED BEAVERHILLS LOAM HUMIC ACID AND HUMIC FRACTIONS

Amino Acid	Humic Fraction			
	FRO	FR1	FR3	FR4
Alanine	13.7 ± 0.5	13.5 ± 1.0	13.9 ± 0.5	12.9 ± 0.2
Valine	8.2 ± 0.8	7.9 ± 0.3	8.6 ± 0.3	8.7 ± 0.6
Glycine	16.3 ± 1.6	17.3 ± 1.4	13.6 ± 1.6	12.1 ± 2.3
Isoleucine	4.6 ± 0.2	4.3 ± 0.4	5.2 ± 0.2	6.1 ± 1.0
Leucine	7.9 ± 0.4	7.3 ± 0.2	9.3 ± 0.6	11.2 ± 0.9
Proline	7.2 ± 0.8	6.5 ± 0.3	9.4 ± 1.0	8.1 ± 0.2
Threonine	4.0 ± 0.3	3.9 ± 0.4	4.7 ± 0.1	3.5 ± 0.5
Serine	4.6 ± 0.2	4.7 ± 0.4	4.8 ± 0.1	4.2 ± 0.6
Phenylalanine	4.2 ± 0.2	3.7 ± 0.2	5.0 ± 0.6	5.9 ± 0.5
Aspartic Acid	13.3 ± 0.4	14.0 ± 0.4	10.3 ± 1.0	10.2 ± 0.7
Glutamic Acid	8.7 ± 0.6	9.0 ± 0.6	8.2 ± 0.7	8.0 ± 0.2
Tyrosine	1.1 ± 0.3	1.0 ± 0.3	2.0 ± 0.6	2.6 ± 0.4
Lysine	6.0 ± 1.4	6.9 ± 1.5	5.0 ± 0.8	5.1 ± 1.4

TABLE 3.15 RELATIVE MOLE PERCENT AMINO ACID COMPOSITION OF 6N ACID HYDROLYSATES OF 0.5N SODIUM HYDROXIDE EXTRACTED MALEB LOAM HUMIC ACID AND HUMIC FRACTIONS

Amino Acid	Humic Fraction		
	FRO	FR1	FR3
Alanine	13.4 ± 0.6	12.5 ± 1.2	12.3 ± 0.7
Valine	8.7 ± 0.5	8.4 ± 0.7	8.9 ± 1.2
Glycine	15.4 ± 2.4	16.7 ± 1.8	13.0 ± 1.4
Isoleucine	5.3 ± 0.4	5.2 ± 1.3	6.1 ± 0.5
Leucine	9.9 ± 0.6	9.2 ± 1.1	10.9 ± 0.5
Proline	6.9 ± 0.3	6.7 ± 0.8	8.2 ± 0.3
Threonine	4.4 ± 0.1	4.3 ± 0.5	4.2 ± 0.4
Serine	4.7 ± 0.2	5.3 ± 0.3	4.7 ± 0.6
Phenylalanine	5.3 ± 0.4	4.0 ± 0.2	6.0 ± 0.3
Aspartic Acid	11.4 ± 0.2	12.4 ± 0.9	9.7 ± 0.7
Glutamic Acid	8.0 ± 1.6	8.1 ± 0.5	8.5 ± 0.5
Tyrosine	1.9 ± 0.4	1.6 ± 0.4	2.4 ± 0.4
Lysine	4.8 ± 2.0	5.7 ± 1.6	4.8 ± 2.6

TABLE 3.16 RELATIVE MOLE PERCENT AMINO ACID COMPOSITION OF 6N ACID HYDROLYSATES OF 0.5N SODIUM HYDROXIDE EXTRACTED WATERTON HUMIC ACID AND HUMIC FRACTIONS

Amino Acid	Humic Fraction			
	FRO	FR1	FR3	FR4
Alanine	11.9	12.1	11.8	11.7
Valine	9.1	8.8	7.8	9.6
Glycine	16.2	15.7	13.2	13.7
Isoleucine	3.8	3.3	4.1	4.5
Leucine	8.7	7.9	9.9	11.0
Proline	9.7	8.8	12.2	11.4
Threonine	4.0	4.3	4.4	3.8
Serine	5.3	5.2	5.0	4.6
Phenylalanine	2.3	2.0	2.8	3.4
Aspartic Acid	13.7	16.0	11.4	10.1
Glutamic Acid	6.8	7.6	8.4	6.7
Tyrosine	3.1	2.4	4.7	5.8
Lysine	5.5	5.8	4.2	3.5

TABLE 3.17 RELATIVE MOLE PERCENT AMINO ACID COMPOSITION OF 6N ACID HYDROLYSATES OF 0.5N SODIUM HYDROXIDE EXTRACTED ALPINE HUMIC ACID AND HUMIC FRACTIONS

Amino Acid	Humic Fraction			
	FRO	FR1	FR3	FR4
Alanine	12.3	11.5	13.1	12.6
Valine	7.1	7.8	7.4	9.1
Glycine	13.9	14.0	13.2	12.9
Isoleucine	4.0	3.8	3.8	6.2
Leucine	7.9	7.4	7.4	12.7
Proline	5.5	5.5	6.3	6.9
Threonine	4.6	4.7	4.7	4.8
Serine	5.2	5.1	5.1	4.3
Phenylalanine	2.4	2.1	2.2	3.9
Aspartic Acid	14.4	15.6	13.8	10.4
Glutamic Acid	11.5	11.0	10.3	8.8
Tyrosine	0.8	1.4	1.4	2.6
Lysine	10.2	8.8	11.4	5.1

TABLE 3.18 RELATIVE MOLE PERCENT AMINO ACID COMPOSITION OF 6N ACID HYDROLYSATES OF Na^+-CHELEX EXTRACTED BEAVERHILLS LOAM HUMIC ACID AND HUMIC FRACTIONS

	Humic Fraction			
Amino Acid	FRO	FR1	FR3	FR4
Alanine	13.1 ± 0.6	13.1 ± 0.3	14.2	11.3
Valine	7.5 ± 0.4	7.7 ± 0.9	6.5	7.2
Glycine	17.7 ± 1.4	19.2 ± 3.0	16.3	14.6
Isoleucine	4.5 ± 0.5	4.5 ± 0.4	4.7	6.4
Leucine	7.1 ± 0.4	6.5 ± 0.1	5.7	10.5
Proline	6.8 ± 0.1	6.6 ± 0.5	6.3	8.4
Threonine	5.3 ± 0.4	5.4 ± 0.2	5.7	4.8
Serine	5.4 ± 0.6	6.2 ± 0.5	7.6	5.6
Phenylalanine	3.8 ± 0.2	3.4 ± 0.1	3.7	5.8
Aspartic Acid	13.0 ± 0.3	12.8 ± 1.2	11.3	12.8
Glutamic Acid	7.9 ± 0.8	7.0 ± 1.4	6.3	7.6
Tyrosine	0.2 ± 0.1	0.1 ± 0.05	-	-
Lysine	7.1 ± 2.7	7.5 ± 2.3	6.8	0.8

TABLE 3.19 RELATIVE MOLE PERCENT AMINO ACID COMPOSITION OF 6N ACID HYDROLYSATES OF Na^+- CHELEX EXTRACTED MALEB LOAM HUMIC ACID

	Humic Fraction			
Amino Acid	FRO	FR1 *	FR3 *	FR4 *
Alanine	12.2 ± 0.7	*	*	*
Valine	8.1 ± 0.7			
Glycine	14.3 ± 0.5			
Isoleucine	4.5 ± 0.3			
Leucine	8.2 ± 0.1			
Proline	6.5 ± 0.4			
Threonine	5.4 ± 0.3			
Serine	6.6 ± 0.8			
Phenylalanine	4.1 ± 0.3			
As[illegible]	12.5 ± 3.0			
Gl[illegible]	10.9 ± 0.8			
Tyr[illegible]	1.4 ± 0.05			
Lysi[illegible]	4.7 ± 0.6			

* No[illegible]d obtained for these fractions from Maleb Na^+- Chelex humic acids

TABLE 3.20 RELATIVE MOLE PERCENT AMINO ACID COMPOSITION OF 6N ACID HYDROLYSATES OF Na^+- CHELEX EXTRACTED WATERTON HUMIC ACID AND HUMIC FRACTIONS

	Humic Fraction			
Amino Acid	FRO	FR1	FR3	FR4
Alanine	15.5	13.6	11.4	11.9
Valine	7.5	7.0	7.4	7.5
Glycine	17.5	18.6	14.2	13.8
Isoleucine	3.8	4.1	4.6	5.4
Leucine	6.5	6.4	7.2	10.6
Proline	6.3	5.6	6.6	6.5
Threonine	5.3	5.9	6.0	4.0
Serine	6.3	7.0	5.2	4.1
Phenylalanine	3.3	2.9	4.0	8.8
Aspartic Acid	14.7	13.9	10.7	15.1
Glutamic Acid	7.3	7.2	6.7	7.3
Tyrosine	0.5	0.5	1.1	0.8
Lysine	5.2	7.2	5.1	4.0

TABLE 3.21 RELATIVE MOLE PERCENT AMINO ACID COMPOSITION OF 6N ACID HYDROLYSATES OF Na^+-CHELEX EXTRACTED ALPINE HUMIC ACID AND HUMIC FRACTIONS

	Humic Fraction			
Amino Acid	FR0	FR1	FR3	FR4
Alanine	11.5	12.3	11.5	9.2
Valine	6.9	6.8	8.1	7.2
Glycine	13.8	13.3	11.5	9.3
Isoleucine	4.6	4.2	5.5	5.7
Leucine	7.2	6.5	9.2	10.3
Proline	5.6	5.4	6.6	6.8
Threonine	5.8	5.6	6.2	4.4
Serine	6.7	6.8	5.6	4.0
Phenylalanine	3.2	3.5	5.1	4.9
Aspartic Acid	14.2	14.6	11.7	11.7
Glutamic Acid	11.0	11.7	10.2	10.4
Tyrosine	0.6	0.6	1.2	0.8
Lysine	8.9	8.7	7.7	7.9

3.42 (continued)

data presented for corresponding Beaverhills and Maleb samples. However a careful comparison of the data of all four tables (3.14 to 3.17) indicates that there are small differences in quantitative amino acid composition among the four soils studied. Generally, these differences are observed for leucine, isoleucine, proline, phenylalanine, glutamic acid and lysine. Thus for example in FRO humic acids, Maleb samples contain relatively greater amounts of leucine and isoleucine than the other soils. Proline content appears to be high in Waterton samples, low in Alpine humic acid, and intermediate for both Beaverhills and Maleb samples. Phenylalanine content is low for both Waterton and Alpine soils compared to Maleb and Beaverhills. Glutamic acid content is high in Alpine humic acid; Waterton humic acid had the lowest content. Finally, lysine was found to constitute a very high proportion of the amino acid content of Alpine humic acid, almost twice as high as for the other soil humic acids.

Results of amino acid analysis of humic acids obtained by Na^+ - Chelex extraction of soil samples (Tables 3.18 to 3.21) were generally more uniform among soils for FRO humic acids than was the case when sodium hydroxide was the extractant. Differences between soil humic acids do not become apparent until FR3 humic fractions are examined. These fractions show variations in valine, glycine, leucine, serine, phenylalanine and glutamic acid content (Tables 3.18 to 3.21). In addition the amino acid composition of Waterton and Alpine FR4 humic fractions (Tables 3.16 and 3.17, respectively) appears to be different relative to corresponding fractions from

3.42 (continued)

sodium hydroxide extracted humic acids (Tables 3.20 and 3.21, respectively).

Data in Tables 3.14 and 3.21 indicates there are variations in the quantitative amino acid composition of humic fractions. Thus, for example, humic components which are soluble in phenol (FR3, FR4 fractions) contain greater proportions of proline, leucine and isoleucine than humic components insoluble in phenol (FR1 fractions). Aspartic acid, glutamic acid and glycine, on the other hand, often occur in greater proportion in FR1 fractions than in FR3 or FR4 fractions. Other amino acids, notably alanine, threonine, serine and tyrosine show relatively little variation in the proportion of each present in different humic fractions. Taken as a whole these results indicate there is some fractionation of amino acids between humic compounds.

3.43 Discussion

Lowe (1973) has reported results of a study of amino acid distribution in forest humus layers in British Columbia aimed at determining whether or not increasing decomposition of forest floor materials was associated with changes in amino acid distribution. His results showed that despite some variation between sites, there nevertheless was a consistent change in the proportion of certain amino acids with increasing humification. The proportions of glutamic acid, proline and leucine were found to consistently decrease on passing from L to F to H or Ah horizons, regardless of vegetation. A similar trend, although less consistent, was exhibited by aspartic acid, alanine, valine, and isoleucine. In

3.43 (continued)

addition Lowe observed that increased humification resulted in material with decreased levels of acid hydrolyzable amino acid content. These results are consistent with those reported in the present study. It was proposed that phenol solubilizes humic components (FR3, FR4 fractions) which are in relatively early stages of humification, and that phenol insoluble humic residues (FR1 fractions) represent "older" more highly humified material. Results of amino acid analysis (Tables 3.14 to 3.21) have shown that FR3 and FR4 humic fractions contain greater proportions of proline, leucine and isoleucine than FR1 humic fractions. According to observations made by Lowe this would indicate FR3 and FR4 material is not as highly humified as related FR1 fractions. In addition FR3 and FR4 fractions contained considerably greater amounts of acid hydrolyzable amino acids (Tables 3.12 and 3.13) than did FR1 material. Based on Lowe's work this also indicates FR3 and FR4 fractions are relatively less humified than FR1 fractions and thus lends further support to the contention that phenol separates humic components which are in different stages of humification.

The results of amino acid analysis reported here are also consistent with current theories on the incorporation of amino acid nitrogen into humic acid type structures during humification processes. According to these theories amino acids, either in the free state, or more likely as peptides or protein residues, react with polyphenols of plant or microbial origin to produce polymeric material which serves as the precursor of soil humic acid (Flaig, 1964, 1966,

3.43 (continued)

1968; Kononova, 1966). It has been proposed that many of these reactions involve active quinones derived from various organic residues through microbial activity (Flaig, 1964, 1966, 1968). Work by Mason (1955a, 1955b) and Haider et al, (1965) has shown that peptides and proteins show covalent bonding with quinones. In general peptide reactions with quinones are primarily dependent on the nature of the N-terminal amino acid, although secondary reactions may differ according to the particular peptide. Thus for example, Mason and Peterson (1965) indicate that N-terminal proline is particularly reactive, with bonding occurring through the secondary amino group apparently more readily than is the case for the primary amino groups associated with other amino acids. Reactions between proteins and quinones involve ε-amino groups of lysine and N-terminal amino groups of residues such as aspartic acid, as well as sulfhydral groups. Acid hydrolysis of peptides or protein covalently bound to aromatic constituents such as phenols has been shown to release all the amino acids except for the N-terminal amino acid and those amino acids that have a free amino group when incorporated into the protein (e.g. lysine) (Haider et al, 1965).

It is reasonable to assume that if reactions do occur during humification of soil organic matter which result in the incorporation of amino acids or peptides in humic components through N-phenyl type linkages, then as humification proceeds, amino acids most susceptible to these type of reactions, such as proline, should constitute an increasingly greater proportion of bound amino acids compared to

3.43 (continued)

other relatively less reactive amino acids. Consequently, separation of soil humic components at different relative stages of humification and subsequent acid hydrolysis of these fractions should produce hydrolysates which show differences in amino acid composition. Thus material at advanced stages of humification should yield acid hydrolysates with lower proportions of proline, lysine, aspartic acid, glutamic acid and possibly other amino acids, relative to material at less advanced stages of humification.

In the present study acid hydrolysates of FR3 and FR4 humic fractions contained greater proportions of proline and generally also of leucine, isoleucine and phenylalanine than did acid hydrolysates of FR1 humic fractions. In view of the preceeding discussion these observations are consistent with the concept that FR3 and FR4 humic fractions represent material in relatively early stages of humification compared to corresponding FR1 fractions. These results also suggest that different reactivities of amino acids with respect to the formation of N-phenyl bonds might lead to preferential fractionation of amino acids during humification processes. Thus certain amino acids such as proline may become relatively less susceptible to immediate microbial attack than other amino acids, due to differences among the amino acids in their relative ease of bonding covalently to aromatic substances which are likely to be incorporated into humic acid structures.

3.5 Pronase Hydrolysis of Humic Acids and Humic Fractions

Perhaps of more importance than the similarities or differences in amino acid composition of humic acids, is the susceptibility of

3.5 (continued)

this amino acid nitrogen to microbial utilization. Amino acids bound to humic acids as peptides or proteinaceous material can be released by the action of extra-cellular proteolytic enzymes produced by soil microorganisms (Ladd and Brisbane, 1967). One of the most powerful enzymes of this type is pronase, a proteolytic enzyme produced by Streptomyces griseus. Pronase hydrolyses a wide range of peptides and, unlike most proteases converts proteins almost quantitatively to their amino acid components (Nomoto et al, 1960). Ladd et al (1967) and Brisbane et al (1972) showed that pronase could release about one-third of the acid-hydrolysable amino acid content of soil humic acids. Sowden (1970) also found that pronase could release amino acids from soil humic acids. He found that lysine, some neutral amino acids and the acidic amino acids were released in relatively low yields. Ladd et al, Brisbane et al and Sowden studied the action of pronase on humic fractions which had been extensively treated with a number of reagents and procedures in attempts to reduce the ash content. It was decided in the present study to examine the ability of pronase to release amino acids from FR1 humic acids, and associated humic fractions (FR1, FR3), which had not been subjected to any procedures aimed at reducing their ash contents other than high speed centrifugation.

Prior to preparation for enzyme hydrolysis, all humic acids or humic fractions from a given soil were pooled with all similar fractions which had originated from the same soil. This procedure gave a composite sample for all FRO, FR1 and FR3 humic acids for each soil studied. All enzyme hydrolyses were done in triplicate,

3.5 (continued)

including triplicate blanks. The exact conditions under which hydrolysis was performed have been described earlier (Section 2.5). After hydrolysis had proceeded for the allotted time (24 hours) the amino acid composition of all hydrolysates was determined and these values corrected for any amino acids found in blank supernatants by subtracting the blank values. Pronase produces free amino acids through autolysis (Ladd et al, 1967; Sowden, 1970) which would be included in the humic acid hydrolysates. In order to correct for the pronase contribution it was decided to subtract the total amount of each amino acid which could be produced by the weight of pronase added for enzyme hydrolysis. This is a large over-correction, and produces amino acid results which are minimum numbers, however it leaves no doubt as to the origin of the remaining amino acids, i.e. from the humic acids. This procedure provides a relative measure of the susceptibility of the amino acid containing components of various humic acids to hydrolysis by pronase.

The results of pronase hydrolysis of 0.5N sodium hydroxide extracted humic acids and their associated humic fractions are shown in Table 3.22. Results reported are means of triplicate independent analysis after all appropriate blank corrections.

Examination of the data in Table 3.22 shows Beaverhills FRO humic acid exhibited a very high resistance to pronase hydrolysis. Pronase released only about 7.8 percent of the total acid hydrolysable amino acid content of Beaverhills FRO humic acid. Although the figure of 7.8 percent is low because of blank over-corrections discussed earlier, it is considerably lower than figures shown for

TABLE 3.22 AMINO ACID COMPOSITION OF PRONASE HYDROLYSATES OF 0.5N NaOH EXTRACTED HUMIC ACIDS AND HUMIC FRACTIONS, EXPRESSED AS A PERCENT OF THE AMINO ACIDS RELEASED BY 6N HCl HYDROLYSIS OF CORRESPONDING BLANKS

Humic Fraction	FRO				FR1				FR3			
Amino Acid	Beaver	Maleb	Waterton	Alpine	Beaver	Maleb	Waterton	Alpine	Beaver	Maleb	Waterton	Alpine
Alanine	6.0	62.2	43.4	49.3	28.3	40.6	39.3	44.0	33.6	54.4	48.1	43.2
Valine	22.4	72.8	46.8	74.2	58.6	46.9	48.6	54.7	78.4	43.2	57.2	39.2
Glycine	--	45.7	32.2	39.0	22.9	29.4	36.3	34.4	44.9	45.6	34.0	36.3
Isoleucine	24.4	71.4	52.1	52.2	52.3	53.3	44.4	44.0	77.7	62.8	66.1	33.3
Leucine	21.7	76.5	64.5	57.6	64.7	50.6	53.8	39.5	68.9	71.9	60.2	38.8
Proline	6.4	55.4	35.7	47.8	25.7	34.5	36.3	28.0	28.2	50.0	38.2	37.5
Threonine	--	63.2	27.2	28.0	11.7	30.9	7.1	7.4	28.3	45.7	46.5	11.1
Serine	--	55.0	36.3	25.0	28.2	27.1	42.8	15.1	30.1	53.9	44.9	20.8
Phenylalanine	25.0	77.0	56.2	50.0	42.8	53.1	42.8	33.3	29.7	73.2	50.9	33.3
Aspartic Acid	1.5	51.9	30.9	38.2	14.8	26.1	29.8	32.1	35.9	53.1	36.9	40.4
Glutamic Acid	11.6	66.9	45.0	47.7	26.1	44.5	41.0	40.0	33.5	56.2	44.2	48.1
Tyrosine	--	47.6	—	—	—	--	7.5	--	35.2	61.5	25.0	--
Lysine	9.3	40.3	48.2	37.9	15.7	39.4	34.4	24.8	18.4	43.4	26.0	57.1
*TOTAL	7.8	59.8	39.1	44.2	27.8	37.1	36.2	3[illegible]	41.3	54.3	44.1	36.1

* Shows total amino acids released by pronase as a percent of total released by 6N HCl hydrolysis of corresponding humic blanks
— Signifies blank corrections were greater than amount in hydrolysate

3.5 (continued)

Maleb, Waterton or Alpine FRO humic acids, and thus cannot be entirely due to blank over-correction. This implies that amino acids associated with Beaverhills FRO humic acid as peptidic or protein material are complexed or masked by humic components in some way which protects them from pronase hydrolysis much more effectively than is the case for the other soil FRO humic acids.

Extraction of Beaverhills FRO humic acid with phenol produced fractions (FR1, FR3) which showed increased susceptibility to pronase hydrolysis (Table 3.22). In the case of Beaverhills FR3 humic fraction the extent of pronase hydrolysis compared favorably with levels shown by FR3 fractions from the other soil humic acids. Thus it appears that phenol can disrupt the original Beaverhills FRO humic acid complex in a manner which exposes more of the amino acid containing component to enzyme hydrolysis.

The results for Beaverhills FRO humic acid contrast greatly with those obtained for Maleb FRO humic acid. Pronase hydrolysis of Maleb FRO humic acid released 60 percent of the acid hydrolyzable amino acid content of the humic acid. This is about eight times greater than the amount which could be released from Beaverhills FRO humic acid. Evidently the amino acid rich material associated with Maleb FRO humic acid is not nearly as intimately complexed with humic constituents as is the case for similar material in Beaverhills humic acid.

Waterton and Alpine FRO humic acids were less susceptible to pronase hydrolysis than Maleb FRO humic acid, but were considerably more susceptible than Beaverhills humic acid (Table 3.22). Pronase

3.5 (continued)

released 39.1 and 44.2 percent respectively of the total acid hydrolyzable amino acid content of Waterton and Alpine humic acids.

Pronase generally released a greater proportion of the acid hydrolyzable content of valine, isoleucine and leucine amino acids from humic fractions than of the acid hydrolyzable content of other amino acids present in the humic fractions (Tables 3.22, 3.23). There were also variations in the amount of the same amino acid released by pronase from corresponding humic fractions of different soils. These variations could be quite large, as for example, threonine and serine in FR1 humic fractions (Table 3.22). Other amino acids which showed variations in the amount of each released by pronase from different soil humic acids were aspartic acid, glutamic acid, leucine and proline.

A greater proportion of the acid hydrolyzable amino acid content could be released by pronase from FR3 humic fractions than from corresponding FR1 humic fractions (Tables 3.22, 3.23). This was true for FR3 and FR1 fractions from Beaverhills, Maleb and Waterton humic acids. For Alpine humic acid there appeared to be little difference between FR1 and FR3 fractions in this respect. The greater susceptibility of FR3 fractions to pronase hydrolysis seemed to be reflected mainly in increases in the yield of proline, valine, isoleucine, leucine, aspartic acid and glutamic acid amino acids detected in hydrolysates of FR3 humic fractions. Pronase hydrolysates of FR1 fractions generally contained smaller proportions of these amino acids.

Results of pronase hydrolysis of Na^+- Chelex derived humic

TABLE 3.23 AMINO ACID COMPOSITION OF PRONASE HYDROLYSATES OF Na-CHELEX EXTRACTED HUMIC ACIDS AND HUMIC FRACTIONS, EXPRESSED AS A PERCENT OF THE AMINO ACIDS RELEASED BY 6N HCl HYDROLYSIS OF CORRESPONDING BLANKS

Humic Fraction	FRO				FR1				FR3			
Amino Acid	Beaver	Maleb	Waterton	Alpine	Beaver	Maleb	Waterton	Alpine	Beaver	Maleb	Waterton	Alpine
Alanine	14.0	43.6	46.0	41.0	15.9	ND	60.0	44.4	ND	ND	56.2	60.6
Valine	20.0	48.2	47.0	52.6	16.7	ND	55.0	55.2	ND	ND	63.6	51.1
Glycine	9.1	35.1	28.6	37.2	10.2	ND	50.0	45.0	ND	ND	51.5	50.7
Isoleucine	25.0	69.6	50.0	45.4	27.3	ND	55.0	47.3	ND	ND	72.2	61.1
Leucine	36.0	73.0	65.0	58.1	44.4	ND	57.1	48.1	ND	ND	76.0	46.1
Proline	7.7	30.8	31.6	34.7	6.3	ND	25.0	42.1	ND	ND	53.8	45.0
Threonine	13.8	17.4	32.2	33.3	--	ND	--	11.7	ND	ND	66.7	30.2
Serine	7.1	48.1	25.8	31.7	10.5	ND	31.6	25.6	ND	ND	75.8	33.3
Phenylalanine	30.8	59.6	54.5	42.8	27.3	ND	42.8	38.5	ND	ND	70.6	50.0
Aspartic Acid	15.2	53.2	30.2	31.1	20.4	ND	39.6	32.6	ND	ND	60.0	55.0
Glutamic Acid	36.7	65.2	48.7	52.1	29.6	ND	48.2	46.2	ND	ND	79.4	51.4
Tyrosine	--	--	--	--	--	ND	--	--	ND	ND	--	--
Lysine	25.0	28.8	50.0	24.1	--	ND	42.8	23.1	ND	ND	72.7	62.5
*TOTAL	17.2	46.8	38.2	39.2	16.8	ND	36.2	38.9	ND	ND	63.4	48.9

* Shows total amino acids released by pronase as a percent of total released by 6N HCl hydrolysis of corresponding humic blanks
--Signifies blank corrections were greater than amount in hydrolysate
ND= not determined

3.5 (continued)

acids and associated humic fractions are reported in Table 3.23. It was noted earlier (Section 3.21) that yield of FR3 humic fractions from Na^+ - Chelex FRO humic acids was very low, Na^+ - Chelex resin appears extracting mainly FR1 humic material from soil. Comparison of FRO humic acids in Table 3.23 with data for FR1 fractions in Table 3.22 indicates that for the same soil the total amounts of amino acids released by pronase from Na^+ - Chelex FRO humic acids was nearly identical to total amounts released from 0.5N sodium hydroxide derived FR1 humic fractions. Extracting Chelex FRO humic acids with phenol did not produce any change in the total amount of amino acids subsequently released from the FR humic fraction by pronase. These results support the idea that Na^+ Chelex extracts humic material from soil which is similar in composition to FR1 fractions recovered from humic acids which have been extracted from soil with 0.5N sodium hydroxide.

Na Chelex humic acids obtained from Waterton and Alpine soils contained small amounts of FR3 humic fractions. Pronase released greater amounts of amino acids from these fractions than from corresponding FR1 humic fractions (Table 3.23). Thus FR3 humic fractions appear to be consistent in containing amino acids combined in forms which are relatively easily attacked by pronase.

3.51 Discussion

The differences between FRO humic acids in their susceptibility to hydrolysis by pronase suggests that considerable variability exists in the manner of combination of humic components present in the different humic acids. Phenol extraction of FRO humic acids

3.51 (continued)

separated FRO humic material into several individual components which, based on comparisons of E4/E6 ratios, apparently differ in their relative aromatic contents and degree of structural complexity. Thus FR1 humic fractions appear to consist primarily of condensed aromatic material, whereas FR3 humic fractions consist of more aliphatic material and have a less condensed structure. Pronase released a greater proportion of the amino acids contained in FR3 humic fractions than of the amino acids contained in FR1 humic fractions. Ladd and Brisbane (1967) found that incorporation of casein into a benzoquinone-casein polymer caused the casein to become resistant to pronase attack. They found that variations in the nature, distribution or proportion of aromatic compounds present could lead to variations in the extent of enzyme hydrolysis of the protein constituents. The observations of Ladd and Brisbane, together with results reported in the present study, imply that the greater aromatic content of FR1 fractions compared to that of FR3 fractions may be the cause of the relatively low hydrolysis of FR1 fractions by pronase.

Beaverhills FRO humic acids resisted pronase hydrolysis much more than did other soil FRO humic acids, and in addition contained large amounts of FR1 humic material. Thus it might be argued that the greater content of aromatic constituents in Beaverhills FRO humic acid resulted in a closer association of proteinaceous or peptide containing components with aromatic moieties through mechanisms such as hydrogen bonding. Such associations could effectively protect adsorbed components from enzyme attack by

3.51 (continued)

causing steric hindrance, and may thus be at least partially responsible for the inability of pronase to release amino acids from the amino acid containing components of Beaverhills FRO humic acids. The fact that phenol removed humic components from Beaverhills FRO humic acid which subsequently showed a susceptibility to pronase hydrolysis comparable to levels shown by similar fractions from other soil FRO humic acids, lends further support to the idea that steric hindrance is the mechanism responsible for inhibiting pronase hydrolysis of Beaverhills humic components complexed in FRO humic acid. Waterton and Alpine FRO humic acids, however, also contained large amounts of FR1 humic material, yet the amino acid containing component of these humic acids showed a high susceptibility to hydrolysis by pronase. Thus it appears that a variety of factors are involved in determining the extent of pronase hydrolysis of humic acids.

Results have shown (Tables 3.22 and 3.23) that pronase is more effective in releasing valine, isoleucine and leucine amino acids than other amino acids from humic acids and humic fractions. In addition there were variations in the amounts of aspartic acid, glutamic acid, and proline amino acids that could be released by pronase from corresponding humic fractions of different soil humic acids. Generally the proportion of these latter amino acids released from FR3 humic fractions was greater than the proportion released from FR1 fractions. A number of factors could explain these observations. For instance pronase may hydrolyze leucine and isoleucine peptide bonds more readily than those involving other amino acids

3.51 (continued)

and this coupled with the progressive inactivation of pronase by incubation with humic acids (Ladd and Brisbane, 1967) could account for the observed results. However, Nomoto et al (1960a, 1960b) have shown that pronase liberates leucine and other amino acids in similar proportions from proteins within a 24-hour period, the same length of time allowed for enzyme hydrolysis in the present study. Alternatively, the observed apparent specificity of pronase may indicate a closer association of amino acids such as proline and lysine with aromatic humic constituents in a manner that resists disruption by pronase. Results previously reported in this study support the latter proposal. In Section 3.42 it was shown that phenol extraction of FR0 humic acids produced FR3 fractions in which the proportions of certain amino acids, notably glycine, isoleucine, leucine, proline, phenylalanine, valine and aspartic acid were increased relative to the proportion of these amino acids present in phenol insoluble FR1 humic fractions. It was suggested that this difference was due to a greater proportion of N-phenyl bound amino acids in FR1 humic material than in FR3 humic material. N-phenyl bound amino acids have been shown to resist acid hydrolysis (Piper and Posner, 1972; Ladd and Butler, 1966; Haider et al, 1965), and certainly would also resist pronase hydrolysis. The fact that pronase generally released a greater proportion of proline, aspartic acid, leucine and glutamic acid from FR3 humic fractions than from corresponding FR1 fractions lends further support to the idea discussed in Section 3.43 that FR1 material is more strongly humified and thus contains a high proportion of amino acids intimately associated with aromatic constituents through N-phenyl covalent bonds.

4. GENERAL DISCUSSION AND CONCLUSIONS

The present study was initiated in order to compare some properties of humic acids formed in soils which have developed under different conditions of climate and vegetative cover. It was hoped that comparing properties of humic acids obtained from the soils might indicate the manner in which different environmental conditions affect humic acid formation.

Humic acids for study were extracted from samples of Ah horizon obtained from sites representative of several different soils. The soils used wereBeaverhills loam, Maleb loam, an Orthic Black Chernozem located in Waterton National Park, and a Dystric Alpine Brunisol located near Luscar, Alberta. Each of these soils has developed under different environmental conditions.

Humic acids were obtained by extracting separate soil Ah samples with 0.5N sodium hydroxide and with the sodium form of Dowex A-1, a chelating resin (Na^+-Chelex). The humic acids (FRO humic acids) obtained by these extractions were characterized with respect to ash content, carbon content, nitrogen content, optical properties, amino acid composition and susceptibility to enzymatic hydrolysis by the proteolytic enzyme pronase.

FRO humic acids extracted from soil samples obtained from different sites of the same soil series showed only small differences in ash (mineral) content. However FRO humic acids originating with different soils contained different amounts of ash. In some cases these differences in ash content were quite large. The ash content of FRO humic acids also depended on whether the humic acids were obtained from soil by extraction

with 0.5N sodium hydroxide or by extraction with Na^+- Chelex resin. FRO humic acids obtained by Na^+- Chelex extraction generally had twice the ash content of related sodium hydroxide derived FRO humic acids. However this latter effect was not observed for FRO humic acids of all soil series, although it was consistent for humic acids from the same soil series. From these results it was concluded that FRO humic acids contained greater or lesser amounts of humic-mineral complexes depending on which soil series the humic acids were obtained from. It was also concluded that the high ash content of FRO humic acids obtained by Na^+- Chelex extraction was due to the inability of the resin to break mineral-organic bonds, with the result that the resin extraction procedure yielded mainly colloidal humic-mineral complexes.

Extracting soil samples with 0.5N sodium hydroxide yielded FRO humic acids which had a higher carbon content than humic material obtained from separate samples of the same soil by Na^+- Chelex extraction. This observation was attributed to a greater proportion of highly oxidized humic material present in Na^+- Chelex extracted humic acids, than was the case for 0.5N sodium hydroxide extracted humic acids. This would result in a lower overall carbon content for Na^+- Chelex extracted humic acids. Comparison of the carbon content of FRO humic acids from different soil series showed there were only small variations between the humic acids when the same primary extractant (i.e. sodium hydroxide solution vs Na^+- Chelex) was used to obtain them from soil samples.

Extraction of soil samples with 0.5N sodium hydroxide yielded FRO humic acids which contained greater amounts of nitrogen than did FRO humic acids extracted from samples of the same soil by Na^+- Chelex. In addition the total nitrogen content of FRO humic acids also depended

on which soil series they had been extracted from. Maleb FRO humic acids consistently contained more total nitrogen than did Beaverhills, Waterton or Alpine FRO humic acids. These latter three soils yielded FRO humic acids which all contained similar amounts of total nitrogen.

Amino acid analysis of FRO humic acids showed that total amino acid content (based on determination of 13 amino acids) varied considerably between humic acids of different soil series. Comparison of data from amino acid analysis also showed there were small differences in the quantitative amino acid composition of FRO humic acids obtained from different soil series. Generally these differences were observed for leucine, isoleucine, proline, phenylalanine, glutamic acid and lysine amino acids. Thus Maleb loam FRO humic acids contained relatively greater amounts of leucine and isoleucine than did FRO humic acids obtained from the other soils studied. Proline content appeared to be relatively high in Waterton FRO humic acids, low in Alpine humic acid and intermediate for both Beaverhills and Maleb samples. Phenylalanine content was low for both Waterton and Alpine humic acids compared to Maleb and Beaverhills humic acids. Alpine FRO humic acid was characterized by a relatively high content of glutamic acid, and contained almost twice as much lysine as the other soil humic acids.

Pronase hydrolysis of FRO humic acids from different soil series indicated there were major differences between humic acids in their susceptibility to enzyme hydrolysis. Beaverhills FRO humic acid was particularly resistant to hydrolysis by pronase, whereas Maleb FRO humic acid was readily hydrolyzed. Waterton and Alpine FRO humic acids showed levels of susceptibility to enzyme hydrolysis which were intermediate to those shown by Beaverhills and Maleb humic acids.

In order to further characterize FRO humic acids an attempt was made to separate the humic acids into individual humic components. A fractionation procedure using phenol and acetone as fractionating solvents was developed which was able to separate FRO humic acids into three relatively distinct humic components. Two of these humic components (FR3 and FR4 fractions) were soluble in phenol, while the third component (FR1 fraction) was insoluble in phenol. Yield data showed that the proportion in which these components were present in FRO humic acid depended on the soil series from which the FRO humic acid originated. Comparison of optical and chemical properties of humic components recovered from different FRO humic acids indicated that similar components were obtained from all FRO humic acids regardless of soil series or soil site from which the humic acid was obtained. The properties of humic components which were soluble in phenol (FR3 and FR4 fractions) were different from properties of phenol insoluble humic components (FR1 fractions). Phenol soluble humic fractions appeared to have a lower aromatic content and a less condensed structure than phenol insoluble humic fractions, and in addition contained greater quantities of total nitrogen. The quantitative amino acid composition of phenol soluble humic components differed from that of the insoluble humic components. Phenol soluble humic material consistently contained greater proportions of proline, phenylalanine, and somewhat less consistently, of aspartic acid, isoleucine and leucine amino acids than did the related phenol insoluble humic fraction. Humic fractions obtained from different FRO humic acids were all consistent in showing the same kind of differences in quantitative amino acid composition between the phenol soluble and phenol insoluble humic components.

On the basis of variations in optical properties, total carbon and nitrogen content, and variations in quantitative amino acid composition it was concluded that phenol fractionation of soil humic acids affected the separation of humic components which are in different relative stages of humification. It was concluded that phenol insoluble humic constituents (FR1 fractions) represent highly humified and thus highly oxidized material, while phenol soluble humic components (FR3, FR4 fractions) represent less humified, less oxidized material.

Pronase hydrolysis of humic fractions indicated that phenol soluble fractions (FR3, FR4 humic material) were generally more susceptible to enzyme hydrolysis than were phenol insoluble fractions. Differences in yields of certain amino acids obtained from phenol soluble and phenol insoluble humic fractions when these were subjected to enzyme hydrolysis lent further support to the conclusion that the phenol fractionation procedure used in this study separates humic acids into humic components which are in different relative stages of humification.

According to Kononova (1968) the transformation of soil organic matter involves three principle stages: (a) the accumulation of organic residues, (b) their humification, and (c) the breakdown of humic substances. Kononova considers the process of humification to include both the decomposition of the initial organic residues with the resultant formation of intermediate and final products of mineralization, and the new formation of humic substances. It is generally accepted that in the formation of humic substances the following events occur either successively or simultaneously: (a) the formation of structural units;

(b) their condensation; and (c) polymerization of the condensation products. The result is a multi-component system of humic substances showing the same patterns but different details of structure and chemical nature, and containing molecules of different sizes. In this system the substances grouped as fulvic acids represent humic substances with a less condensed aromatic nucleus and a more highly developed peripheral component; they can be considered as precursors or as products of the destruction of numerous members of the humic acid group (Kononova, 1968). The intensity of the humification process and the nature of the resulting humic acids formed is strongly dependent on soil environmental conditions. Thus a moderate moisture regime, a neutral pH and a fairly intense microbiological activity are the main factors favoring the formation of complex humic acids. Excess moisture, an acid pH and weak microbiological activity suspend the formation of humus substances at the stage of fulvic acids and fulvic acid-like humic acids (Kononova, 1966).

In the present study it has been shown that FRO humic acids contained different amounts of FR3 and FR4 humic fractions depending on which of four soil series the FRO humic acid had been obtained from. However, FRO humic acids from different sites within the same soil series contained FR1, FR3 and FR4 humic material in approximately the same proportions. In addition results have shown that FR1, FR3 and FR4 humic fractions possess properties which imply that they each represent humic material which is at a different stage of humification. It was postulated that FR1 fractions represent more highly humified or "older" humic matter, whereas FR3 and FR4 fractions represent less humified or "younger" humic matter.

If left undisturbed the amount of organic matter in mineral soils soon reaches an equilibrium level, thus implying that the rate of formation of humic substances is equal to the rate of degradation. If this is so then the results obtained in this study suggest that the FR3 and FR4 humic fractions isolated from FRO humic acids by phenol extraction represent intermediates in the equilibrium system involving the formation and degradation of soil humus. If we accept the hypothesis that FR3 and FR4 humic fractions are indeed intermediates in humification processes and can undergo further humification reactions, to become either more complex humic acids, or conversely, simpler lower molecular weight humic substances such as fulvic acids, then the variation in yields of the fractions observed when FRO humic acids from different soils were fractionated, may be a reflection of the manner in which particular soil conditions have affected the humification processes. Thus, for example, Maleb soils have developed in a climate which is semi-arid and characterized by a high frequency of dry desiccating winds. These conditions result in an average water deficit for Maleb soils of about 25 cm (Bowser, W.E., T.W. Peters, and A.A. Kjearsgaard, 1963). The dry droughty condition common to Maleb soils is capable of supporting only a sparse grass vegetation and the dry conditions do not favor a very high level of microbial activity (Kononova, 1966). As a consequence it is not unreasonable to expect that a major part of the humus associated with Maleb soils may be in some intermediate state of humification characterized by a relatively open poorly condensed structure and a high content of peripheral aliphatic material. This postulate receives support from data reported earlier (Table 3.3, Section 3.21) which showed that Maleb

FRO humic acids contain a high proportion of FR3 humic material, a fraction which previous results have indicated is characterized by a relatively poorly condensed structure. Thus the prolonged dry periods common to Maleb soils and the resultant depressing effect such conditions have on the intensity of microbial activity appears to favor the accumulation of humic substances which are transitional between complex highly aromatic humic acids and simpler less condensed fulvic acids.

FRO humic acids from Beaverhills soils contained smaller amounts of FR3 material than did Maleb FRO humic acid. In addition Beaverhills humic acid was not as easily hydrolyzed by pronase as was the case for Maleb humic acid. This suggests that Beaverhills humic acids possess a more complex structure than do Maleb humic acids. Beaverhills soils are located in a sub-humid climatic region (Bowser, W.E., A.A. Kjearsgaard, T.W. Peters and R.W. Wells, 1962) and have formed under a grass cover of the rough fescue (Festuca scabrella) association. The Ah horizon of these soils contain on the average of about 5-6 percent total organic carbon and have a slightly acidic pH. On the whole the properties and conditions common to Beaverhills soils are such as to encourage a relatively high level of microbial activity. As a result, humification processes should be at a fairly intense level, resulting in a rapid turnover of organic matter. Under these conditions humic components in intermediate stages of humification, such as perhaps FR3 fractions, may be rapidly modified, either undergoing degradation to simpler substances such as fulvic acids, or becoming involved in reactions with products of mineral weathering to produce more complex structures which may be resistant to further microbial attack. Reactions such as these may explain why the yield of FR3 material obtained from Beaverhills soils

was lower than the yield of similar material obtained from Maleb soils. Such reactions may also explain why Beaverhills FRO humic acids are more resistant to pronase hydrolysis than are Maleb FRO humic acids.

Both Waterton and Alpine FRO humic acids contained only small amounts of FR3 and FR4 humic fractions. FRO humic acids from these soils were moderately susceptible to pronase hydrolysis. The Waterton soil sampled for extraction of FRO humic acid is classified as an Orthic Black Chernozem. The Ah horizon of this soil contains 8.7 percent organic matter and has an acid pH of 5.2. Waterton Black Chernozemic soils have developed in an area of Alberta (Waterton National Park) characterized by a summer-dry, winter-wet climate with slightly higher total precipitation and milder temperatures than other parts of the province (Coen, G.M., and W.D. Holland, 1974). The Alpine soil used as a source of Alpine FRO humic acid is classified as an Alpine Dystric Brunisol. The general area in which the soil is located (Luscar, Alberta) experiences a sub-humid, continental climate with long cold winters and moderately mild summers. The area is subject to warm chinook winds during the winter (Dumanski, J., T.M. Macyk, C.F. Veauvy, and J.D. Lindsay, 1972). The Ah horizon of the Brunisol sampled had an organic matter content of about 10 percent.

Waterton and Alpine soils have developed under environmental conditions which favor the accumulation of large amounts of organic matter. The relatively low yield of FR3 and FR4 humic fractions which can be separated from the FRO humic acid of these soils suggests that the environmental conditions are also such as to favor a rapid turnover of humic components resulting in a situation in which humic fractions in intermediate stages of humification do not accumulate, but rather are

rapidly converted to either more complex humic substances or to simpler humic substances such as fulvic acids. The observation that the major component of all FRO humic acids was FR1 material (Tables 3.3 and 3.4, Section 3.21) and that the yield of this component was generally about the same from all FRO humic acids extracted by 0.5N sodium hydroxide regardless of the soil series from which the humic acids were obtained, suggests that for Waterton and Alpine soils the low yield of FR3 and FR4 fractions is due to conversion to fulvic acids and simpler humic components. Thus it appears that differences between humic acids obtained from soils characterized by different conditions of climate and vegetation are associated mainly with differences in the manner of combination and relative distribution of individual humic components which make up the humic acids.

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APPENDIX I

Procedure for Preparation of Ethylene Glycol Adipate Gas Chromatographic Columns

(1) Heat 20 grams acid washed 80-100 mesh Chromasorb W at 140°C for 12 hours in an open container.

(2) After 12 hours, transfer heat treated Chromasorb W to a 1000 ml $24/40 round bottom flask. Stopper flask and allow Chromasorb to cool.

(3) Add 99 mole percent acetonitrite to the 1000 ml flask until the liquid level is about 1 cm above the surface of the cooled Chromasorb W.

(4) Dissolve 11.0 grams ethylene glycol adipate (EGA) in 100 ml of 99 mole percent acetonitrite. Add 10 ml of this solution to the Chromasorb W-acetonitrite suspension prepared in Step (3). This procedure adds sufficient EGA to produce a 0.65 percent W/W loading of EGA on 20 grams of Chromasorb W.

(5) Remove all acetonitrite solvent from the EGA-Chromasorb W mixture by rotary evaporation at 65-70°C. Use an all glass system and continue rotary evaporation for at least 4 hours.

(6) When rotary evaporation has removed all acetonitrite from the EGA-Chromasorb W immediately prepare two 1.5m x 4 mm I.D. glass GLC columns.

(7) Condition EGA-Chromasorb W columns at 250°C with the columns connected to a stream of He gas (20 cc/minute flow rate). Continue conditioning for at least 24 hours.

APPENDIX II

Procedure For Derivitization of Amino Acids

(1) An aqueous aliquot containing 0.1 mgm to 10 mgm total amino acids plus internal standard (ornithine) is placed in a 12 ml glass vial and the sample is evaporated just to dryness by placing the tube in a 100°C sand bath while directing a stream of dry, purified nitrogen gas into the heated tube. To ensure complete removal of H_2O, 0.5 ml of CH_2Cl_2 is added to the glass vial immediately after the contents first read dryness and the drying procedure is repeated to remove the added CH_2Cl_2. This latter procedure removes all traces of H_2O from the sample by azeotropic distillation.

(2) Add 2.0 ml of $CH_3OH.HCl$ (22.8 gms HCl/500 ml CH_3OH) to the vial containing the dried sample, cap the vial with a teflon cap and mix for 30 seconds. Allow the mixture to stand for 30 minutes at room temperature.

(3) Evaporate the $CH_3OH.HCl$ solution at 100°C using nitrogen gas and a sand bath as outlined in Step (1).

(4) Add 2.0 ml of $n\text{-}C_4H_9OH.HCl$ (22.8 gms HCl/500 mls. $n\text{-}C_4H_9OH$) to the methyl esters remaining in the vial after Step (3). Cap the vial with a teflon lined cap and mix for 30 seconds. After mixing heat the vial at 100°C for 2-1/2 hours using a sand bath. After 2-1/2 hours evaporate the $n\text{-}C_4H_9OH.HCl$ reaction mixture to dryness using a stream of dry nitrogen gas directed into the reaction vial while maintaining the vial at 100°C in a sand bath.

(5) Acylate the dried n-butyl esters by adding 0.8 ml of CH_2Cl_2 and 0.2 ml trifluoroacetic anhydride to the reaction vial. Cap the vial with a teflon cap and place the capped vial in a 150°C constant temperature

APPENDIX II (continued)

(5) (continued)

sand bath for 5 minutes. After 5 minutes the vial is cooled and the trifluoroacetyl-n-butyl amino acid esters are ready for gas chromatographic analysis.

APPENDIX III

AMINO ACID ANALYSIS OF HUMIC ACIDS AND HUMIC FRACTIONS

Amino Acid Composition of 0.5 N NaOH Extracted Beaverhills FRO Humic Acids

Amino Acid	Site 1			Site 2			Site 3			Relative Mole Percent Composition	
	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	Average For Sites	Standard Deviation
Alanine	4.04	45.36	14.2	3.93	44.16	13.6	3.50	39.33	13.2	13.7	± 0.5
Valine	2.73	23.36	7.3	3.34	28.56	8.8	2.95	25.20	8.4	8.2	± 0.8
Glycine	4.35	58.05	18.1	3.69	49.22	15.2	3.51	46.84	15.7	16.3	± 1.6
Isoleucine	1.86	14.23	4.4	2.01	15.38	4.7	1.88	14.37	4.8	4.6	± 0.2
Leucine	3.14	23.93	7.5	3.54	26.99	8.3	3.05	23.32	7.8	7.9	± 0.4
Proline	2.62	22.77	7.1	2.98	25.94	8.0	2.21	19.24	6.4	7.2	± 0.8
Threonine	1.42	11.99	3.7	1.57	13.25	4.1	1.49	12.58	4.2	4.0	± 0.3
Serine	1.53	14.57	4.5	1.57	14.95	4.6	1.51	14.46	4.8	4.6	± 0.2
Phenylalanine	2.19	13.27	4.1	2.36	14.30	4.4	2.09	12.67	4.2	4.2	± 0.2
Aspartic Acid	5.88	44.20	13.8	5.60	42.08	13.0	5.22	39.24	13.2	13.3	± 0.4
Glutamic Acid	4.34	29.50	9.2	4.30	29.24	9.0	3.51	23.92	8.0	8.7	± 0.6
Tyrosine	6.42	3.54	1.1	5.74	3.16	0.9	7.70	4.25	1.4	1.1	± 0.3
Lysine	2.84	15.59	4.9	3.20	17.53	5.4	4.13	22.63	7.6	6.0	± 1.4
TOTAL	43.35	320.36		43.83	324.76		42.75	298.05			

APPENDIX III (continued)

Amino Acid Composition of 0.5 N NaOH Extracted Beaverhills FR1 Humic Fractions

Amino Acid	Site 1			Site 2			Site 3			Relative Mole Percent Composition	
	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	Average For Sites	Standard Deviation
Alanine	3.29	36.99	14.6	3.01	33.89	13.1	3.40	38.25	12.8	13.5	± 1.0
Valine	2.26	19.35	7.6	2.48	21.16	8.2	2.73	23.36	7.8	7.9	± 0.3
Glycine	3.60	48.00	18.9	3.14	41.90	16.2	3.73	49.73	16.7	17.3	± 1.4
Isoleucine	1.48	11.34	4.4	1.56	11.90	4.6	1.53	11.70	3.9	4.3	± 0.4
Leucine	2.32	17.69	7.0	2.50	19.07	7.4	2.88	21.95	7.4	7.3	± 0.2
Proline	1.95	16.94	6.7	1.98	17.26	6.7	2.14	18.63	6.2	6.5	± 0.3
Threonine	1.04	8.75	3.4	1.26	10.62	4.1	1.49	12.58	4.2	3.9	± 0.4
Serine	1.13	10.8	4.3	1.39	13.25	5.1	1.43	13.67	4.6	4.7	± 0.4
Phenylalanine	1.47	8.90	3.5	1.60	9.74	3.8	1.89	11.48	3.8	3.7	± 0.2
Aspartic Acid	4.72	35.47	14.0	4.72	35.47	13.7	5.70	42.87	14.4	14.0	± 0.4
Glutamic Acid	3.58	24.38	9.6	3.24	22.06	8.5	3.85	26.19	8.8	9.0	± 0.6
Tyrosine	0.30	1.68	0.7	0.56	3.12	1.2	0.64	3.58	1.2	1.0	± 0.3
Lysine	2.40	13.19	5.2	3.51	19.23	7.4	4.37	23.92	8.0	6.9	± 1.5
TOTAL	29.54	253.49		30.95	258.67		35.78	297.91			

APPENDIX III (Continued)

Amino Acid Composition of 0.5 N NaOH Extracted Beaverhills FR3 Humic Fractions

Amino Acid	Site 1			Site 2			Site 3			Relative Mole Percent Composition	
	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	Average For Sites	Standard Deviation
Alanine	11.12	124.87	14.3	6.34	71.23	13.3	6.11	68.67	14.0	13.9	± 0.5
Valine	8.54	72.89	8.4	5.59	47.77	8.9	4.86	41.47	8.4	8.6	± 0.3
Glycine	10.10	134.57	15.4	5.29	70.58	13.2	4.48	59.80	12.2	13.6	± 1.6
Isoleucine	5.69	43.40	5.0	3.82	29.16	5.4	3.31	25.27	5.2	5.2	± 0.2
Leucine	10.77	82.09	9.4	6.88	52.51	9.8	5.55	42.35	8.6	9.3	± 0.6
Proline	10.37	90.16	10.3	5.91	51.34	9.6	4.77	41.47	8.4	9.4	± 1.0
Threonine	4.77	40.11	4.6	3.00	25.20	4.7	2.77	23.29	4.7	4.7	± 0.1
Serine	4.39	41.85	4.8	2.69	25.66	4.8	2.40	22.91	4.7	4.8	± 0.1
Phenylalanine	6.46	39.12	4.5	5.07	30.70	5.7	3.98	24.14	4.9	5.0	± 0.6
Aspartic Acid	11.03	82.90	9.5	7.24	54.41	10.1	7.41	55.71	11.4	10.3	± 1.0
Glutamic Acid	9.78	66.54	7.6	6.49	44.17	8.2	6.41	43.57	8.9	8.2	± 0.7
Tyrosine	2.15	11.91	1.4	1.88	10.42	1.9	2.28	12.58	2.6	2.0	± 0.6
Lysine	7.56	41.40	4.7	4.26	23.36	4.4	5.32	29.13	5.9	5.0	± 0.8
TOTAL	102.73	871.81		64.46	536.51		59.65	490.36			

APPENDIX III (continued)

Amino Acid Composition of 0.5 N NaOH Extracted Beaverhills FR4 Humic Fractions

Amino Acid	Site 1			Site 2			Site 3			Relative Mole Percent Composition	
	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	Average For Sites	Standard Deviation
Alanine	7.18	80.59	12.8	3.90	43.78	13.1	2.92	32.77	12.9	12.9	± 0.2
Valine	6.75	57.67	8.4	3.30	28.15	8.4	2.80	23.92	9.4	8.7	± 0.6
Glycine	6.83	91.10	14.5	2.96	39.53	11.8	1.91	25.48	10.0	12.1	± 2.3
Isoleucine	4.93	37.63	5.2	2.59	19.81	5.9	2.38	18.19	7.1	6.1	± 1.0
Leucine	9.53	72.66	10.6	4.78	36.44	10.9	4.07	31.05	12.2	11.2	± 0.9
Proline	7.44	64.67	8.3	3.02	26.29	7.9	2.34	20.36	8.0	8.1	± 0.2
Threonine	2.61	21.90	3.0	1.39	11.72	3.5	1.20	10.10	4.0	3.5	± 0.5
Serine	2.51	23.90	3.6	1.65	15.77	4.7	1.13	10.83	4.2	4.2	± 0.6
Phenylalanine	6.16	37.33	5.3	3.35	20.30	6.1	2.60	15.79	6.2	5.9	± 0.5
Aspartic Acid	9.19	69.10	10.2	4.86	36.58	10.9	3.26	24.54	9.6	10.2	± 0.7
Glutamic Acid	7.52	51.15	7.9	4.03	27.43	8.2	2.94	20.00	7.8	8.0	± 0.2
Tyrosine	2.63	14.56	2.8	1.34	7.42	2.2	1.33	7.35	2.9	2.6	± 0.4
Lysine	3.50	19.20	3.5	3.86	21.17	6.3	2.59	14.20	5.6	5.1	± 1.4
TOTAL	76.80	642.26		41.03	334.39		31.47	254.58			

APPENDIX III (continued)

Amino Acid Composition of Na - Chelex Extracted Beaverhills FRO Humic Acids

Amino Acid	Site 1			Site 2			Site 3			Relative Mole Percent Composition	
	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	Average For Sites	Standard Deviation
Alanine	1.46	16.46	12.4	1.81	20.34	13.4	2.04	22.92	13.4	13.1	± 0.6
Valine	1.11	9.52	7.2	1.41	12.10	8.0	1.48	12.66	7.4	7.5	± 0.4
Glycine	1.67	22.31	16.9	2.19	29.25	19.3	2.17	29.03	17.0	17.7	± 1.4
Isoleucine	0.77	5.89	4.5	0.80	6.11	4.0	1.08	8.28	4.9	4.5	± 0.5
Leucine	1.14	8.74	6.6	1.54	11.75	7.8	1.55	11.87	7.0	7.1	± 0.4
Proline	1.04	9.06	6.9	1.18	10.26	6.8	1.33	11.56	6.8	6.8	± 0.1
Threonine	0.76	6.42	4.9	0.95	8.03	5.3	1.15	9.72	5.7	5.3	± 0.4
Serine	0.68	6.55	4.9	0.95	9.13	6.0	0.96	9.20	5.4	5.4	± 0.6
Phenylalanine	0.84	5.13	3.9	0.98	5.99	4.0	1.00	6.06	3.6	3.8	± 0.2
Aspartic Acid	2.23	16.76	12.7	2.63	19.82	13.1	3.02	22.70	13.3	13.0	± 0.3
Glutamic Acid	1.48	10.10	7.7	1.62	11.03	7.3	2.20	15.00	8.8	7.9	± 0.8
Tyrosine	0.31	0.17	0.1	0.42	0.23	0.2	0.11	0.06	0.4	0.2	± 0.1
Lysine	2.43	13.34	10.1	1.38	7.56	5.0	1.95	10.72	6.3	7.1	± 2.7
TOTAL	15.92	132.02		17.86	151.60		20.04	169.78			

APPENDIX III (continued)

Amino Acid Composition of Na - Chelex Extracted Beaverhills FR1 Humic Fractions

Amino Acid	Site 1			Site 2			Site 3			Relative Mole Percent Composition	
	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	Average For Sites	Standard Deviation
Alanine	1.05	11.86	12.9	2.04	23.00	13.1	1.88	21.19	13.4	13.1	± 0.3
Valine	0.74	6.34	6.9	1.75	15.01	8.6	1.40	11.93	7.6	7.7	± 0.9
Glycine	1.19	15.94	17.4	2.98	39.77	22.6	2.06	27.56	17.5	19.2	± 3.0
Isoleucine	0.56	4.30	4.7	0.94	7.17	4.1	0.99	7.55	4.8	4.5	± 0.4
Leucine	0.86	6.55	7.1	1.40	10.70	6.1	1.28	9.76	6.2	6.5	± 0.6
Proline	0.74	6.43	7.0	1.37	11.93	6.8	1.11	9.65	6.1	6.6	± 0.5
Threonine	0.58	4.87	5.3	1.13	9.55	5.4	1.04	8.80	5.6	5.4	± 0.2
Serine	0.54	5.20	5.7	1.15	10.95	6.2	1.09	10.42	6.6	6.2	± 0.5
Phenylalanine	0.52	3.20	3.5	0.97	5.88	3.3	0.88	5.32	3.4	3.4	± 0.1
Aspartic Acid	1.46	10.99	11.9	2.88	21.69	12.4	2.96	22.25	14.1	12.8	± 1.2
Glutamic Acid	0.99	6.76	7.4	1.39	9.47	5.4	1.87	12.74	8.1	7.0	± 1.4
Tyrosine	0.02	0.12	0.13	0.04	0.20	0.1	0.02	0.10	0.1	0.1	-
Lysine	1.70	9.30	10.1	1.86	10.22	5.8	1.93	10.56	6.7	7.5	± 2.3
TOTAL	10.95	91.86		19.89	175.54		18.50	157.83			

APPENDIX III (continued)

Amino Acid Composition of Na - Chelex Extracted Beaverhills FR3 Humic Fractions

Amino Acid	Site 1			Site 3			Average Relative Mole Percent
	mgm/gm	µmoles/gm	Relative Mole %	mgm/gm	µmoles/gm	Relative Mole %	
Alanine	2.97	33.44	15.1	6.80	76.31	13.2	14.2
Valine	1.69	14.49	6.5	4.40	37.58	6.5	6.5
Glycine	2.32	30.96	14.0	8.09	107.89	18.6	16.3
Isoleucine	1.40	10.68	4.8	3.41	25.99	4.5	4.7
Leucine	1.61	12.25	5.5	4.51	34.38	5.9	5.7
Proline	1.29	11.17	5.0	5.03	43.77	7.6	6.3
Threonine	1.33	11.13	5.0	4.44	37.33	6.4	5.7
Serine	1.90	18.08	8.2	4.22	40.19	6.9	7.6
Phenylalanine	1.19	7.18	3.2	3.92	23.71	4.1	3.7
Aspartic Acid	3.22	24.16	10.9	9.01	67.76	11.7	11.3
Glutamic Acid	1.70	11.54	5.2	6.32	42.96	7.4	6.3
Tyrosine	-	-	-	0.15	0.87	0.15	-
Lysine	6.70	36.65	16.5	7.25	39.67	6.8	6.8
TOTAL	27.32	221.73		67.55	578.41		

APPENDIX III (continued)

Amino Acid Composition of Na - Chelex Extracted Beaverhills FR4 Humic Fractions

Amino Acid	Site 1			Site 3			Average Relative Mole Percent
	mgm/gm	µmoles/gm	Relative Mole %	mgm/gm	µmoles/gm	Relative Mole %	
Alanine	2.02	22.75	9.9	2.79	31.31	12.7	11.3
Valine	1.52	12.93	5.6	2.59	22.06	8.9	7.2
Glycine	2.32	30.96	13.4	2.94	39.21	15.9	14.6
Isoleucine	1.11	8.48	3.7	2.08	15.85	6.4	6.4
Leucine	3.21	24.50	10.6	3.38	25.74	10.4	10.5
Proline	2.19	19.02	8.2	2.43	21.14	8.6	8.4
Threonine	1.52	12.72	5.5	1.20	10.07	4.1	4.8
Serine	1.36	12.91	5.6	1.45	13.81	5.6	5.6
Phenylalanine	2.37	14.37	6.2	2.20	13.30	5.4	5.8
Aspartic Acid	3.78	28.39	12.3	4.37	32.85	13.3	12.8
Glutamic Acid	2.46	16.73	7.2	2.96	20.09	8.1	7.6
Tyrosine	-	-	-	0.94	0.52	-	-
Lysine	4.88	26.70	1.16	2.17	1.19	0.48	0.82
TOTAL	28.74	230.46		31.50	247.14		

APPENDIX III (continued)

Amino Acid Composition of 0.5N NaOH Extracted Maleb FRO Humic Acids

	Site 1			Site 2			Site 3			Site 4			Relative Mole Percent Composition	
	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	Average For Sites	Standard Deviation
Alanine	8.29	93.04	12.7	6.22	69.89	13.1	11.26	126.44	14.0	5.27	59.22	13.7	13.4	± 0.6
Valine	6.85	58.48	8.0	5.33	45.51	8.6	9.52	81.28	9.0	4.64	39.63	9.2	8.7	± 0.5
Glycine	6.62	88.27	12.1	6.40	85.26	16.0	12.07	160.90	17.8	5.07	67.64	15.7	15.4	± 2.4
Isoleucine	5.45	41.53	5.7	3.25	24.81	4.7	6.46	49.29	5.5	2.90	22.17	5.1	5.3	± 0.4
Leucine	10.08	76.86	10.5	6.29	47.96	9.0	11.97	91.27	10.1	5.61	42.82	9.9	9.9	± 0.6
Proline	6.04	52.43	7.2	3.99	34.73	6.5	7.31	63.58	7.0	3.47	30.14	7.0	6.9	± 0.3
Threonine	3.91	32.82	4.5	2.68	22.54	4.2	4.74	39.82	4.4	2.21	18.57	4.3	4.4	± 0.1
Serine	3.69	35.09	4.8	2.44	23.28	4.4	4.54	43.28	4.8	2.13	20.31	4.7	4.7	± 0.2
Phenylalanine	6.90	41.79	5.7	4.18	25.35	4.8	8.15	49.34	5.5	3.76	22.76	5.3	5.3	± 0.4
Aspartic Acid	10.84	81.41	11.1	7.96	59.87	11.2	13.87	104.20	11.6	6.59	49.58	11.5	11.4	± 0.2
Glutamic Acid	10.09	68.61	9.4	7.18	48.84	9.2	7.90	53.70	5.9	4.83	32.85	7.6	8.0	± 1.6
Tyrosine	1.90	10.50	1.4	2.08	11.50	2.2	3.03	16.75	1.9	1.67	9.22	2.1	1.9	± 0.4
Lysine	9.25	50.60	6.9	5.92	32.40	6.1	4.07	22.31	2.5	2.97	16.30	3.8	4.8	± 2.0
TOTAL	89.91	731.43		63.92	531.94		104.89	902.16		51.12	431.21			

APPENDIX III (continued)

Amino Acid Composition of 0.5N NaOH Extracted Maleb FR1 Humic Acids

Amino Acid	Site 1			Site 2			Site 3			Site 4			Relative Mole Percent Composition	
	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	Average For Sites	Standard Deviation
Alanine	4.28	48.13	11.3	6.48	72.77	14.3	6.43	72.20	12.3	4.14	46.55	12.3	12.5	± 1.2
Valine	4.04	34.49	8.1	4.71	40.21	7.9	6.49	55.39	9.4	3.55	30.34	8.0	8.4	± 0.7
Glycine	4.54	60.59	14.2	6.65	88.58	17.4	7.44	99.14	16.9	5.21	69.47	18.3	16.7	± 1.8
Isoleucine	3.97	30.27	7.1	2.87	21.91	4.3	3.89	29.68	5.1	2.10	16.03	4.2	5.2	± 1.3
Leucine	5.92	45.13	10.6	5.57	42.52	8.4	7.22	55.03	9.4	4.11	31.37	8.3	9.2	± 1.1
Proline	3.49	30.30	7.1	3.30	28.73	5.6	4.56	39.67	6.8	3.23	28.08	7.4	6.7	± 0.8
Threonine	2.16	18.12	4.3	2.75	23.14	4.5	3.32	27.93	4.8	1.67	14.04	3.7	4.3	± 0.5
Serine	2.18	20.75	4.9	2.98	28.36	5.6	3.42	32.60	5.6	2.07	19.70	5.2	5.3	± 0.3
Phenylalanine	2.58	15.63	3.7	3.38	20.48	4.0	3.86	23.42	4.0	2.71	16.40	4.3	4.0	± 0.2
Aspartic Acid	6.28	47.16	11.1	9.03	67.91	13.3	9.93	74.65	12.7	6.20	46.60	12.3	12.4	± 0.9
Glutamic Acid	5.39	36.69	8.6	5.55	37.76	7.4	7.11	48.33	8.2	4.44	30.24	8.0	8.1	± 0.5
Tyrosine	1.11	6.14	1.4	1.27	7.03	1.4	1.25	6.93	1.2	1.48	8.21	2.2	1.6	± 0.4
Lysine	5.86	32.07	7.5	5.43	29.73	5.8	3.85	21.12	3.6	4.02	22.04	5.8	5.7	± 1.6
TOTAL	51.80	455.47		59.97	509.13		68.77	586.09		44.93	378.67			

APPENDIX III (continued)

Amino Acid Composition of 0.5N NaOH Extracted Maleb FR3 Humic Fractions

Amino Acid	Site 1			Site 2			Site 3			Site 4			Relative Mole Percent Composition	
	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	Average For Sites	Standard Deviation
Alanine	9.65	108.30	12.3	12.47	139.98	12.1	15.31	171.90	13.2	9.24	103.73	11.5	12.3	± 0.7
Valine	7.58	64.76	7.4	11.74	100.22	8.7	15.77	134.50	10.3	9.89	84.40	9.4	8.9	± 1.2
Glycine	8.34	111.16	12.7	11.74	156.49	13.6	14.19	189.00	14.5	7.63	101.70	11.3	13.0	± 1.4
Isoleucine	6.87	52.35	6.0	8.62	65.77	5.7	10.30	78.55	6.0	8.08	61.58	6.8	6.1	± 0.5
Leucine	13.05	99.45	11.3	16.71	127.37	11.0	19.19	146.20	11.2	12.03	91.72	10.2	10.9	± 0.5
Proline	8.62	74.86	8.5	11.17	97.05	8.4	11.79	102.40	7.8	8.57	74.50	8.3	8.2	± 0.3
Threonine	4.10	34.44	3.9	6.51	54.71	4.7	6.96	58.40	4.4	4.32	36.33	4.0	4.2	± 0.4
Serine	3.90	37.14	4.2	6.27	59.74	5.2	7.23	68.80	5.3	3.84	36.62	4.1	4.7	± 0.6
Phenylalanine	8.89	54.41	6.2	11.03	66.76	5.8	12.47	75.50	5.8	9.39	56.84	6.3	6.0	± 0.3
Aspartic Acid	10.78	80.97	9.2	16.42	123.30	10.7	15.72	118.10	9.1	11.73	88.15	9.8	9.7	± 0.7
Glutamic Acid	11.10	75.45	8.6	14.19	96.46	8.4	15.18	103.20	7.9	11.92	81.01	9.0	8.5	± 0.5
Tyrosine	4.46	24.60	2.8	5.69	31.41	2.7	5.01	27.60	2.1	3.64	20.10	2.2	2.4	± 0.4
Lysine	11.02	60.32	6.9	6.40	35.06	3.0	5.53	30.29	2.3	11.77	64.43	7.2	4.8	± 2.6
TOTAL	108.47	878.21		138.96	1154.30		154.65	1304.40		112.05	901.11			

APPENDIX III (continued)

Amino Acid Composition of Na-Chelex Extracted Maleb FRO Humic Acids

Amino-Acid	Site 1			Site 2			Site 3			Site 4			Relative Mole Percent Composition	
	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	Average For Sites	Standard Deviation
Alanine	1.62	18.18	13.2	1.17	13.13	11.4	1.42	15.93	12.2	1.41	15.82	12.3	12.3	± 0.7
Valine	1.21	10.32	7.5	1.24	10.58	9.18	1.19	10.15	7.7	1.22	10.41	8.1	8.1	± 0.5
Glycine	1.55	20.64	15.0	1.25	16.65	14.4	1.35	17.98	13.7	1.38	18.38	14.3	14.3	± 0.5
Isoleucine	0.75	5.71	4.2	0.66	5.03	4.4	0.85	6.47	4.9	0.75	5.71	4.4	4.5	± 0.3
Leucine	1.48	11.28	8.2	1.23	9.37	8.1	1.45	11.05	8.4	1.39	10.59	8.3	8.2	± 0.1
Proline	1.02	8.86	6.4	0.80	6.95	6.0	1.06	9.20	7.0	0.96	8.34	6.5	6.5	± 0.4
Threonine	0.89	7.47	5.4	0.70	5.87	5.1	0.93	7.80	5.9	0.84	7.05	5.5	5.5	± 0.3
Serine	1.02	9.70	7.1	0.67	6.37	5.5	1.01	9.60	7.3	0.90	8.56	6.7	7.0	± 0.3
Phenylalanine	0.57	5.26	3.8	0.76	4.60	4.0	0.98	5.93	4.5	0.87	5.26	4.1	4.1	± 0.3
Aspartic Acid	2.10	15.77	11.4	2.57	19.30	16.7	1.68	12.62	9.6	2.13	16.03	12.5	12.5	± 3.0
Glutamic Acid	2.19	14.88	10.8	1.68	11.42	9.9	2.32	15.77	12.0	2.06	14.00	10.9	10.9	± 0.9
Tyrosine	0.35	1.93	1.4	0.28	1.54	1.3	0.34	1.87	1.4	0.33	1.82	1.4	1.4	± 0.1
Lysine	1.33	7.27	5.3	0.81	4.43	3.8	1.19	6.51	4.9	1.11	6.07	4.7	4.7	± 0.6
TOTAL	16.38	137.27		13.82	115.24		15.77	130.83		15.35	128.04			

APPENDIX III (continued)

Amino Acid Composition of Waterton 0.5N NaOH Extracted FRO Humic Acids and FR1 Humic Fractions

Amino Acid	Site 1 Fraction 0(R-1)			Site 1 Fraction 0(R-2)			Average Mole %	Site 1 Fraction 1(R-1)			Site 1 Fraction 1(R-2)			Average Mole %
	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %		mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	
Alanine	2.40	27.00	11.9	2.42	27.20	11.9	11.9	2.46	27.60	11.9	3.39	38.10	12.3	12.1
Valine	2.43	20.80	9.2	2.44	20.80	9.1	9.1	2.35	20.10	8.6	3.27	27.90	9.0	8.8
Glycine	2.72	36.30	16.0	2.81	37.50	16.4	16.2	2.63	35.10	15.1	3.82	50.90	16.4	15.7
Isoleucine	1.13	8.62	3.8	1.09	8.38	3.7	3.8	0.98	7.50	3.2	1.44	10.90	3.5	3.3
Leucine	2.50	19.10	8.4	2.66	20.30	8.9	8.7	2.38	18.10	7.8	3.30	25.20	8.1	7.9
Proline	2.49	21.70	9.6	2.58	22.40	9.8	9.7	2.29	20.00	8.6	3.19	27.70	8.9	8.8
Threonine	1.06	8.92	3.9	1.08	9.10	4.0	4.0	1.13	9.50	4.1	1.65	13.80	4.4	4.3
Serine	1.24	11.80	5.2	1.30	12.40	5.4	5.3	1.32	12.60	5.4	1.63	15.50	5.0	5.2
Phenylalanine	0.84	5.09	2.2	0.88	5.35	2.3	2.3	0.71	4.30	1.8	1.11	6.70	2.2	2.0
Aspartic Acid	4.16	31.30	13.8	4.12	31.00	13.6	13.7	5.13	38.60	16.6	6.36	47.80	15.4	16.0
Glutamic Acid	2.31	15.60	6.9	2.22	15.10	6.6	6.8	2.56	17.50	7.5	3.46	23.50	7.6	7.6
Tyrosine	1.42	7.86	3.5	1.12	6.21	2.7	3.1	1.16	6.40	2.8	1.11	6.10	2.0	2.4
Lysine	1.79	12.30	5.4	1.83	12.50	5.5	5.5	2.23	15.30	6.6	2.28	15.60	5.0	5.8
TOTAL	26.49	226.39		26.55	228.24		227.36	27.33	232.60		36.01	309.70		

APPENDIX III (continued)

Amino Acid Composition of Waterton 0.5N NaOH Extracted FR3 and FR4 Humic Fractions

Amino Acid	Site 1 Fraction 3(R-1)			Site 1 Fraction 3(R-2)			Average Mole %	Site 1 Fraction 4(R-1)			Site 1 Fraction 4(R-2)			Average Mole %
	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole		mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	
Alanine	6.11	68.70	12.5	5.57	62.60	11.0	11.8	4.99	56.00	11.4	5.11	57.39	12.1	11.7
Valine	5.65	48.30	8.8	4.54	38.80	6.8	7.8	5.21	44.60	9.5	5.57	47.60	9.7	9.6
Glycine	5.54	73.80	13.4	5.49	73.10	12.9	13.2	4.89	65.20	13.4	5.07	67.47	14.1	13.7
Isoleucine	2.99	22.80	4.2	2.88	21.90	3.9	4.1	2.64	20.20	4.6	3.06	23.28	4.4	4.5
Leucine	6.87	52.40	9.6	7.62	58.10	10.2	9.9	6.12	46.69	12.0	7.92	60.41	10.1	11.0
Proline	7.61	66.20	12.1	8.09	70.30	12.4	12.2	5.83	50.70	11.8	6.84	59.39	11.0	11.4
Threonine	2.69	22.60	4.1	3.09	26.00	4.6	4.4	2.31	19.41	3.5	2.10	17.66	4.2	3.8
Serine	2.67	25.50	4.6	3.23	30.80	5.4	5.0	2.30	21.88	4.4	2.31	21.90	4.7	4.6
Phenylalanine	2.40	14.50	2.6	2.86	17.40	3.1	2.8	2.57	15.59	3.3	2.71	16.40	3.4	3.4
Aspartic Acid	8.39	63.10	11.5	8.56	64.30	11.3	11.4	6.10	45.79	10.4	6.95	52.18	9.9	10.1
Glutamic Acid	5.53	37.60	6.8	8.33	56.60	10.0	8.4	4.64	31.60	6.6	4.90	33.26	6.8	6.7
Tyrosine	5.35	29.60	5.4	4.12	22.70	4.0	4.7	4.99	27.58	5.6	5.11	28.19	6.0	5.8
Lysine	3.42	23.40	4.3	3.51	24.00	4.2	4.2	2.44	16.66	3.3	2.45	16.80	3.6	3.5
TOTAL	65.22	548.50		67.89	566.60		575.55	55.03	461.90		60.10	501.93		

APPENDIX-III (continued)

Amino Acid Composition of Na-Chelex Extracted Waterton FRO Humic Acid and FR1, FR3 and FR4 Humic Fractions

Amino Acid	Site 1 Fraction 0			Site 1 Fraction 1			Site 1 Fraction 3			Site 1 Fraction 4		
	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %
Alanine	3.58	40.26	15.5	2.69	30.25	13.6	5.50	61.78	11.4	2.72	30.59	11.9
Valine	2.29	19.55	7.5	1.82	15.51	7.0	4.67	39.89	7.4	2.26	19.29	7.5
Glycine	3.40	45.34	17.5	3.09	41.22	18.6	5.80	77.22	14.2	2.66	35.51	13.8
Isoleucine	1.30	9.93	3.8	1.19	9.06	4.1	3.23	24.66	4.6	1.80	13.77	5.4
Leucine	2.22	16.94	6.5	1.86	14.16	6.4	5.09	38.86	7.2	3.57	27.26	10.6
Proline	1.88	16.39	6.3	1.44	12.49	5.6	4.11	35.77	6.6	1.91	16.68	6.5
Threonine	1.62	13.65	5.3	1.55	13.04	5.9	3.85	32.33	6.0	1.21	10.17	4.0
Serine	1.72	16.44	6.3	1.63	15.55	7.0	2.97	28.29	5.2	1.10	10.50	4.1
Phenylalanine	1.40	8.48	3.3	1.08	6.56	2.9	3.61	21.89	4.0	3.73	22.57	8.8
Aspartic Acid	5.08	38.18	14.7	4.11	30.90	13.9	7.69	57.77	10.7	5.16	38.83	15.1
Glutamic Acid	2.80	19.03	7.3	2.33	15.87	7.2	5.32	36.20	6.7	2.76	18.81	7.3
Tyrosine	0.22	1.25	0.5	0.22	1.19	0.5	1.07	0.59	10.9	0.10	0.21	0.8
Lysine	2.47	13.53	5.2	2.92	15.96	7.2	5.07	27.78	5.1	1.88	10.31	4.0
TOTAL	29.98	258.97		25.93	221.76		57.98	483.03		30.86	254.50	

APPENDIX III (continued)

Amino Acid Composition of Alpine 0.5N NaOH Extracted FRO Humic Acids and FR1 Humic Fractions

Amino Acid	Site 1 Fraction 0(R-1)			Site 1 Fraction 0(R-2)			Site 1 Fraction 1(R-1)				Site 1 Fraction 1(R-2)			
	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	Average Mole %	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	Average Mole %
Alanine	1.77	19.80	12.3	1.59	17.90	12.3	12.3	1.55	17.40	11.8	1.81	20.40	11.2	11.5
Valine	1.64	14.00	8.7	1.21	10.30	7.1	7.9	1.17	10.00	6.8	2.17	18.60	8.7	7.8
Glycine	1.80	24.00	14.9	1.51	20.10	13.9	14.4	1.56	20.80	14.2	1.99	26.60	13.8	14.0
Isoleucine	0.82	6.30	3.9	0.76	5.80	4.0	4.0	0.72	5.50	3.7	0.82	6.20	3.3	3.8
Leucine	1.66	12.70	7.9	1.51	11.50	7.9	7.9	1.40	10.70	7.3	1.69	12.90	7.4	7.4
Proline	0.98	8.60	5.4	0.92	8.00	5.5	5.5	0.93	8.10	5.5	1.08	9.40	5.4	5.5
Threonine	0.77	6.50	4.0	0.79	6.70	4.6	4.3	0.90	7.60	5.2	0.94	7.90	4.2	4.7
Serine	0.84	8.00	5.0	0.80	7.60	5.2	5.1	0.83	7.90	5.4	0.91	8.60	4.8	5.1
Phenylalanine	0.56	3.40	2.1	0.58	3.50	2.4	2.3	0.51	3.10	2.1	0.59	3.60	2.0	2.1
Aspartic Acid	2.94	22.10	13.8	2.79	20.90	14.4	14.1	3.15	23.70	16.1	3.79	28.50	15.1	15.6
Glutamic Acid	[illegible]	17.00	10.6	2.46	16.70	11.5	11.1	2.52	17.10	11.6	2.79	19.00	10.4	11.0
Tyrosine	[illegible]	1.80	1.1	0.23	1.20	0.8	1.0	0.43	2.40	1.6	0.42	2.30	1.2	1.4
Lysine	[illegible]	16.50	10.3	2.16	14.80	10.2	10.3	1.85	12.60	8.6	2.18	14.90	8.9	8.8
TOTAL	19.03	160.70		17.31	145.00		152.85	17.52	146.90		21.18	178.90		

APPENDIX III (continued)

Amino Acid Composition of Alpine 0.5N NaOH Extracted FR3 and FR4 Humic Fractions

Amino Acid	Site 1 Fraction 3(R-1)			Site 1 Fraction 3(R-2)				Site 1 Fraction 4(R-1)			Site 1 Fraction 4(R-2)			
	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	Average Mole %	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	Average Mole %
Alanine	2.17	24.30	14.0	1.92	21.60	12.2	13.1	6.61	74.30	13.4	4.51	50.70	11.7	12.6
Valine	1.55	13.20	7.6	1.50	12.80	7.2	7.4	5.65	48.20	8.7	4.81	41.20	9.5	9.1
Glycine	1.65	22.00	12.6	1.84	24.50	13.9	13.2	4.86	64.70	11.6	4.57	60.80	14.1	12.9
Isoleucine	0.86	6.60	3.8	0.86	6.60	3.7	3.8	4.93	37.60	6.8	3.15	24.10	5.6	6.2
Leucine	1.70	12.90	7.4	1.72	13.10	7.4	7.4	9.57	73.00	13.1	6.95	53.00	12.3	12.7
Proline	1.23	10.70	6.1	1.32	11.50	6.5	6.3	4.73	41.10	7.4	3.16	27.50	6.4	6.9
Threonine	0.95	7.90	4.5	1.00	8.40	4.8	4.7	3.16	26.60	4.8	2.48	20.80	4.8	4.8
Serine	0.94	9.00	5.2	0.90	8.60	4.9	5.1	2.68	25.50	4.6	1.75	16.70	3.9	4.3
Phenylalanine	0.65	3.90	2.2	0.65	3.90	2.2	2.2	3.59	21.80	3.9	2.79	16.90	3.9	3.9
Aspartic Acid	3.23	24.30	14.0	3.18	23.90	13.5	13.8	8.04	60.50	10.9	5.71	42.90	9.9	10.4
Glutamic Acid	2.52	17.10	9.8	2.78	18.90	10.7	10.3	6.54	44.50	8.0	6.12	41.60	9.6	9.8
Tyrosine	0.48	2.70	1.6	0.39	2.20	1.2	1.4	2.74	15.20	2.7	1.86	10.30	2.4	2.6
Lysine	2.75	19.30	11.1	3.01	20.60	11.7	11.4	3.38	23.20	4.2	3.72	25.50	5.9	5.1
TOTAL	20.68	173.90		21.07	176.60		175.20	66.48	556.20		51.58	432.00		

APPENDIX III (continued)

Amino Acid Composition of Alpine Na-Chelex Extracted FR0 Humic Acid and FR1, FR3 and FR4 Humic Fractions

Amino Acid	Site 1 Fraction 0			Site 1 Fraction 1			Site 1 Fraction 3			Site 1 Fraction 4		
	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %	mgm/gm	µmoles/gm	Rel. Mole %
Alanine	2.87	32.27	11.5	3.57	40.07	12.3	7.63	85.67	11.5	3.13	35.22	9.2
Valine	2.27	19.42	6.9	2.59	22.13	6.8	7.09	60.53	8.1	3.22	27.52	7.2
Glycine	2.89	38.61	13.8	3.26	43.40	13.3	6.41	85.49	11.5	2.67	35.63	9.3
Isoleucine	1.68	12.85	4.6	1.82	13.83	4.2	5.36	40.83	5.5	2.87	21.87	5.7
Leucine	2.63	20.04	7.2	2.79	21.29	6.5	8.97	68.39	9.2	5.19	39.54	10.3
Proline	1.78	15.53	5.6	2.03	17.61	5.4	5.66	49.14	6.6	3.00	26.13	6.8
Threonine	1.94	16.31	5.8	2.17	18.20	5.6	5.49	46.13	6.2	1.99	16.78	4.4
Serine	1.96	18.67	6.7	2.33	22.15	6.8	4.42	42.05	5.6	1.62	15.45	4.0
Phenylalanine	1.46	8.87	3.2	1.87	11.34	3.5	6.23	37.73	5.1	3.10	18.77	4.9
Aspartic Acid	5.29	39.80	14.2	6.33	47.57	14.6	11.61	87.20	11.7	5.97	44.82	11.7
Glutamic Acid	4.54	30.89	11.0	5.59	37.98	11.7	11.21	76.17	10.2	5.91	40.18	10.4
Tyrosine	0.28	1.57	0.6	0.35	1.94	0.6	1.58	8.71	1.2	0.58	3.21	0.8
Lysine	4.52	24.79	8.9	5.15	28.21	8.7	10.47	57.35	7.7	5.54	30.36	7.9
TOTAL	34.11	279.62		39.85	325.72		92.13	745.39		44.79	355.48	