INVESTIGATION OF ORGANIC MATERIAL EXTRACTED FROM A PODZOL

by

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INTRODUCTION

In 1947 an investigation on soil genesis was initiated at Macdonald College with the objective of obtaining information regarding the mechanism of the formation of podzolic soils under forested conditions.

A starting point in this investigation was the hypothesis that substances leaching from the litter of the forest floor would be of especial importance in this process by, (a) contributing to the organic matter content of these soils, especially in the B horizon, and (b) being involved in the translocation of iron and aluminum within the profile. Studies have been made on leaf extracts, leachates of fallen leaves produced under field conditions, leachates passing through the complete A horizon of podzol soils in the field, and solutions dripping from the canopy of different tree species.

Since the inception of this study in 1947, some of the characteristics of these materials have been determined and reported by a number of workers (23, 84, 87, 88, 115, 129). In order to relate these results to the organic matter of the soil, it is necessary to study the characteristics of the organic fraction of the soils involved, especially the organic matter of the B horizon. The present study is an attempt to characterize the organic

matter of this deposition layer of a sandy podzol. In this investigation 75 to 80 per cent of the organic matter of the soil has been studied following separation from the inorganic mass of the soil by the use of a metal-chelating agent at nearly neutral conditions.

REVIEW OF LITERATURE

Podzols constitute a great soil group, the profiles of which are characterized by marked leaching and strong acidity, by a horizon with ashy-gray colour, and by pronounced profile development. These soils develop under forest vegetation in humid, temperate climates and dominate a large portion of the habitable area of the earth's surface. They are characterized by a bleached A_2 horizon from which their name is derived; the term "podzol" being of Russian origin and meaning "ashy soil". This horizon of eluviation has a characteristically low content of organic matter and metals such as iron and aluminum. The underlying B horizon, or horizon of illuviation, usually is rust-brown or chocolate in colour, in contrast to the gray colour of the A_2 horizon. The B horizon is characterized by an accumulation of organic matter, iron, and aluminum. Presumably, the accumulated metals and organic

matter in the B horizon have originated in the bleached A_2 horizon and in the decomposing litter overlying the mineral portion of the soil.

The mode of formation of the bleached A_2 horizon and of the metal and organic matter rich B horizon in these soils has been a matter of speculation for many years. A large number of theories has been advanced in attempts to explain this process. Generally, these have been concerned primarily with the mobilization and subsequent redeposition of iron in these soils with relatively little attention being given to the mechanism of the accumulation of organic materials in the B horizon.

In the following pages some of the more realistic theories concerning the process of podzolization are reviewed and discussed. Also some of the information on the nature of soil organic matter, with special reference to podzol soils where possible, is presented.

The early Russian workers linked the process of podzolization with soil organic acids and soil organic matter in general. One example of the theories of these workers is given in the postulates of Kossovich (76) regarding the process of podzolization, which state: (a) since aerobic conditions prevail in the profile, the iron probably moves as a ferric sol protected by organic

colloids; (b) silica is usually left behind; (c) because of leaching of bases the horizon of eluviation is acid; (d) the horizon of illuviation is enriched with electrolytes causing precipitation of iron and aluminum which serve as cementing material for the formation of ortstein and of incrustations.

The theories of Mattson (94, 95) regarding the podzolization process may be summarized as follows: The process of podzolization is related to the conditions of acid hydrolysis which exist in the zone of humid, temperate climate. The net result of acid hydrolysis is the release of bases from the A horizon and their replacement with hydrogen ions, whereby a partial disruption of the silicate complexes takes place and sesquioxides are set free. Depending on the pH environment, which is periodically shifted away from the isoelectric point, the sesquioxides move downward leaving behind silica. Some of the iron and aluminum silicate complexes, saturated with hydrogen, become colloidally dispersed. Under certain conditions some silicate may be replaced by the humates and other anions, giving rise to iron and aluminum complexes of silicates, phosphates, humates, and other anionic chains, all of which possess definite isoelectric points of precipitation. As these complexes move through

the soil profile, they reach a point where a large portion precipitates, because of electrostatic forces controlled primarily by the pH of the medium and the presence of electrolytes. These precipitated materials are deposited and accumulated in the B horizon because the acid hydrolysis at that point in the profile is subdued. In the B horizon of a podzol soil, according to Mattson, the iron and aluminum exist chiefly in combination with humic acid. However, since very little is known about the chemical nature of humic acid, and since the pH of the B horizon in many podzols is not appreciably higher than that of the A horizon, it remains difficult to visualize and accept without reservation these views on podzolization as proposed by Mattson.

From the results of experiments with bentonite, gelatin, and other gels, Winters (137) suggested that iron may move in soil by surface diffusion of ferrous iron within the gel. To interpret the movement of iron in podzol soils by this means, one has to assume as Deb (39) has recently stated, that the gels are continuous throughout the profile and also that movement takes place only under reducing conditions when the soil is saturated with water.

Aarnio (1) stressed the role of humus in the

precipitation of iron and aluminum in podzol soils. He found that the amounts of humus required to coagulate one unit of ferric oxide varied from 0.82 to 2.79 units, while 1.20 to 30.12 units of humus were required to coagulate one unit of aluminum oxide. Aarnio, however, omitted to state the pH at which these values were determined. From subsequent results obtained by Deb (39), it would appear that the pH of the disperse phase is one of the chief factors controlling the amounts of humus required to precipitate iron and aluminum oxides.

Recently, Deb (39) reviewed the theories on the movement and precipitation of iron oxide in podzol soils. After discussing the various possibilities for the transportation of iron within the profile, he concluded that it was most probable that the iron moved as a negatively-charged humus-protected iron oxide sol or as a complex organic ion. He also studied, under laboratory conditions, the mutual coagulation and peptization of iron oxide sol and humus sol with special reference to concentration and pH value. He concluded that the amount of humus necessary for full peptization of an iron oxide sol having 100 parts per million of iron was not more than 30 to 40 per cent of the weight of the iron at pH values near 4. Less humus was required for more dilute iron oxide sols. Deb found that, at pH 4, over a wide range of concentration of iron

oxide sol, precipitation occurred with about seven parts of humus per 100 parts of ferric oxide. He concluded that any iron oxide sol formed by weathering in the upper horizons of podzols could be fully peptized by the humus in soil solution and carried down the profile by percolating water. Deb does not believe that precipitation of iron from humus-protected sols is effected by exchangeable calcium in the B horizon of podzols or that the adsorption of iron from complex salts of organic acids is influenced by the pH or the amounts of exchangeable bases in the soil, as suggested by Mattson. Although he advanced no evidence to support the suggestion, Deb proposed that the processes governing the precipitation were of a microbiological rather than chemical nature.

Harder (64) showed that precipitation of iron complexes from complex salts of iron and organic acids may be a microbiological process. He showed that inoculation of solutions of iron citrate, oxalate, and lactate with any of a number of soils caused precipitation of hydrated ferric oxides. No precipitation occurred in the case of similar systems which were heated to 130° C. immediately following inoculation with the same soils. Also Albrecht (2) has shown that, under laboratory conditions, the process of gleization may depend on the presence of calcium

to give a sufficient degree of saturation of the clay to serve in bacterial nutrition. However, Gray and McMaster (60) have shown that the numbers of bacteria present in the B horizons of two Quebec podzol soils were low in comparison with the numbers present in the upper horizons. In both soils the numbers of bacteria found in the B horizons were equivalent to approximately 50 per cent of the numbers found in the A_2 horizon, while 50 to 75 times as many bacteria were found in the organic A_0 horizon as in the B horizon. The numbers of actinomyces followed the same trend as the bacteria in the three horizons. Their results are principally in agreement with those reported earlier by Waksman (133), who found that the numbers of bacteria in forest soils at arbitrarily vertical intervals were progressively less with increasing depth. In the light of these results, it would seem unlikely that the deposition of iron in podzolic B horizons is due entirely to microbiological agencies.

The movement of iron as metallo-organic electronegative complexes was proposed by Jones and Willcox (69). They suggested that organic acids, derived from the decomposition of organic matter, and capable of forming complexes with iron, were responsible for the transportation of iron in podzol soils. In the B horizon, due to higher pH, the

iron would be precipitated as a basic salt. Again, it is necessary to assume that the pH of the B horizons of all podzol soils are higher than the pH of the overlying horizons, at least during the formation of the soil.

Pickering (108) showed that ferric iron could form electronegative ions in combination with citric, malic, and tartaric acids and that these would migrate to the positive pole on electrolysis. The iron in these complexes did not give the usual reactions for ferric iron and the complexes possessed great colour intensity. Smythe and Schmidt (122) found that mono-, di-, and trihydroxy carboxylic acids, inorganic acids such as phosphoric and arsenic acids, amino acids, nucleic acids, and certain proteins such as gelatin and casein were capable of forming undissociated complexes with ferric iron at pH 2.5. On the basis of experiments on the solubility of organic matter and sesquioxides of podzolized soils in oxalic acid solutions, Gallagher and Walsh (58) concluded that the movement of iron and aluminum was caused by the solvent action of simple decomposable acids, of which oxalic may be considered an example.

In relation to the role of acids of low molecular weight in the translocation of iron in podzols, the investigations of Boswall (23) and McKinley (87) on the

acid content of the leachates of decomposing leaves are of interest. These workers provided evidence that acids of low molecular weight were present only in very small quantities, usually much less than one part per million of the solution. McKinley concluded that all the organic acids isolated from leachates had been present in the original leachates in such very low concentrations that it would be unlikely that they played an important part in the translocation process in podzol soils. Boswall found small amounts of citric, malic, and oxalic acids in birch, sugar maple, and poplar leaf extracts. McKinley, studying the acidic components of sugar maple and poplar leaf leachates, identified orthophosphoric and sulphuric acids and, in agreement with Boswall, malic and citric acids.

Schreiner and Shorey (117) and Shorey (119) isolated a variety of organic acids from soils in small amounts. More recently, Schwartz et al (118) found organic acids in ether extracts of several Ohio soils. They found that only acetic and formic acids were present in appreciable amounts. The total acidic material removed from these soils was equivalent to only 1.5-2.0 m.e. of acid per 100 gm. of air-dried soil. From the small amounts of simple organic acids entering the mineral

portion of the soil, as reported by Boswall and McKinley, and from the small amounts of simple organic acids which have been found in soils, it would not seem likely that the translocation of iron and aluminum in podzol soils is caused solely by the solvent action of simple decomposable organic acids as suggested by Gallagher and Walsh. However, over very long periods of time, these simple acids could conceivably contribute to the translocation of these metals.

The role of water-soluble products of plant decomposition in the podzolization process has received considerable attention in recent years. Bloomfield (14, 15) showed that laboratory-prepared extracts of raw humus, peat, partly withered and unwithered autumn leaves and grasses, after fermentation in the presence of ammonium chloride and clay, could cause solution and reduction of ferric oxide. In further investigations, Bloomfield (16-21) studied the capacity of aqueous extracts obtained in the laboratory from Scots pine needles, leaves and bark of kauri and rimu, picked and fallen larch needles, aspen and ash leaves to dissolve iron and aluminum from co-precipitated hydrated aluminum and ferric oxides. He has suggested that iron was mobilized by such extracts as a ferrous complex and moved as such down the profile

until it was immobilized by sorption on ferric oxide in the B horizon. More recently, Bloomfield (22) has suggested that the solution and reduction of ferric oxide by these aqueous leaf extracts is apparently caused by the joint action of carboxylic acids and polyphenols.

Loissant (81) found that the titratable acidity and the presence of tannins gave a good correlation with the ability of aqueous extracts of forest litters to dissolve iron oxides in soil. Loissant (82), like Bloomfield, claims that these extracts are capable of reducing appreciable quantities of iron. In this respect, Schnitzer (115) demonstrated that the dipyridyl method for determination of iron, which was used by Bloomfield and Loissant, may give erroneously high results for ferrous iron in the presence of organic matter.

Stobbe (129) found that leaf leachates were active in the mobilization of cations, including iron and aluminum, from soil samples taken from various horizons of a variety of podzol and podzolized soils. Lutwick (84) was unable to draw any definite conclusions respecting the chemical forms of the iron present in combination with the organic materials in these leaf leachates, while MacLean (88) was unable to establish any relationship between the carbohydrate content and the iron saturation capacity of leaf

leachates. Thorp et al (130) passed solutions of organic acids and water-soluble material from leaves through columns of soil. They found that appreciable quantities of calcium, magnesium, iron, and manganese were mobilized in this way, and evidence for the redeposition of iron compounds within the soil column also was found. The investigations of Boswall (23) and McKinley (87) regarding the amounts of low molecular weight acids present in leaf leachates have previously been discussed in relation to the role of low molecular weight organic acids in the process of podzolization.

Schnitzer (115) concluded, on the basis of the results obtained by use of the Houlihan and Farina (67) thiocyanate method for the estimation of iron, that the major portion of the iron dissolved by the leaf extracts and leachates, which he investigated, was in the ferric form. This conclusion was not in agreement with the conclusions of Bloomfield and Loissant, both of whom claimed, on the basis of the results obtained by the use of a dipyridyl method for the estimation of iron, that iron was dissolved by plant extracts in the ferrous form.

One of the difficulties, which has been experienced in any study of soil organic matter, has been that of

separating the organic matter from the inorganic mass of the soil in a relatively unaltered form. The classical method of removing the organic matter has been extraction with dilute alkali solutions, commonly 0.5 N sodium hydroxide solution. The fact that oxygen is taken up during these alkaline extractions has been noted by Shorey (120), Chaminade (35, 36), and Bremner (27). The possibility that, due to this uptake of oxygen during extraction, the alkaline extracts were to some extent artifacts of the soil organic matter, has resulted in a search for methods of extraction of soil organic matter which do not require alkaline conditions. Bremner et al (30) showed that, on the whole, compounds which are good polyvalent metal extractants are also good organic matter extractants. They theorized that, in soil, part of the polyvalent metals is combined with part of the organic matter, and that the presence of metals renders the organic matter in the complexes insoluble in water and in neutral solvents that do not themselves form complexes with the metals. In an attempt to by-pass the difficulties of the classical extraction, Bremner and Lees (29) investigated the extracting ability of a number of inorganic and organic salts of sodium. Of these, the pyrophosphate proved to be the most satisfactory. The chief disadvantage of this

reagent appeared to be that it removed only a small proportion of the total organic matter; of the order of ten per cent in three of the four soils they investigated.

Martin and Reeve (91, 92, 93) have reported the extraction of large amounts of organic matter from the B horizons of Australian podzolized soils with aqueous solutions of the metal-chelating agents cupferron, 8-hydroxyquinoline, and acetylacetone. These workers preferred the use of acetylacetone mainly because of the relative ease of handling of this reagent under the desired conditions. The use of 8-hydroxyquinoline was restricted due to the very low solubility of this reagent in the nearly neutral aqueous solutions. Schnitzer et al (116) have reported the use of several extractants for the organic matter of the B horizon of a podzol soil. Dilute solutions of sodium pyrophosphate, sodium orthophosphate, sodium borate, sodium fluoride, sodium carbonate, sodium hydroxide, the disodium salt of ethylenediaminetetraacetic acid, and hydrofluoric acid removed more than 80 per cent of the organic matter from the B_{21} horizon of a podzol soil. Choudri and Stevenson (37) found that removal of calcium and free iron and aluminum oxides before extraction increased the solubility of soil organic matter in 0.5 N sodium hydroxide but had no effect on the extraction

by 0.15 M pyrophosphate. This latter extractant would be expected to complex calcium, iron, and aluminum; a process which would not be expected to occur when sodium hydroxide was employed as the extractant. These workers also found that several organic metal-complexing agents including 8-hydroxyquinoline were not as effective as neutral pyrophosphate for removing soil organic matter into aqueous solution.

In an attempt to overcome the difficulties inherent in the use of 8-hydroxyquinoline, due to its low solubility in aqueous solutions, the author (38) has reported the use of this metal-chelating agent in a water-benzene system for the extraction of organic matter from the deposition layer of a sandy podzol. In this method, the pH of the aqueous phase of the extracting medium was maintained at 6 to 7 to avoid the influences of strongly acidic or alkaline conditions upon the organic matter being extracted. This treatment removed 75 to 80 per cent of the organic material from the soil investigated. Of the organic matter removed from the soil, only one-third was present in the aqueous phase of the extracting medium, while the remainder was found in the benzene phase.

Also, in respect to the association of soil organic matter with metals, several workers (10, 13, 33, 34, 49, 65, 70, 80, 101, 114) have reported that soil organic

matter forms complexes with a variety of metals including iron, aluminum, barium, calcium, copper, magnesium, and zinc.

Most of the theories concerning the process of podzolization, which have been advanced over the years, have been concerned primarily with the mobilization and deposition of metals, especially iron. However, most of these theories have also been concerned with the role of organic matter in this process. Another feature of podzol soils, which has received less attention than the accumulation of metals in the B horizon, is the accumulation of organic matter in the B horizon of these soils. Also, as has been shown previously, much success has been achieved in the removal of organic matter from the deposition layer of podzol soils by the use of extractants capable of complexing metals, especially iron and aluminum. In view of these facts, it would seem reasonable to assume that a large portion, at least, of the organic matter of these deposition horizons is associated with metals.

Although a large amount of work has been done on the organic matter of soils, there is, even today, very little information available concerning the chemical nature of the organic matter of podzol soils, or of any soils for that matter.

According to the classical scheme of fractionation, fulvic acid is that part of the soil organic matter which is peptized by alkali and remains in solution on precipitation of the humic acids with mineral acid. Schlichting (113) found that the podzol A horizon contains a small quantity of fulvic acid. This material appeared to be concentrated in the B horizon. Tyurin (132) also reported that fulvic acid is higher in concentration at moderate depth than in the top layers in podzol soils. Similar results have been reported by Kosaka and Iseki (75). Bel'chikova (11) has stated that the ratio of fulvic to humic acid is higher in podzols than in other soil types. Ponomareva and Myasnikova (109) found that the ratio of fulvic acid to humic acid in a sod carbonate soil was approximately 1.0, whereas in a podzol soil the same ratio was 2.8.

Very little attention has been given to the identification of the components of the fulvic acid fraction of soil organic matter. Recently, Forsyth (54) introduced a method for the fractionation of the fulvic acid fraction. Fulvic acid extracts from four widely diverse types of soils were separated by selective absorption into four fractions. He succeeded in isolating from all four soils a polysaccharide containing uronic acids. He also showed the presence in all four soils of a fraction, which he

tentatively identified as a phenolic glycoside. Schlichting (113) could not isolate any polyuronides from the A and B horizon of a podzol soil by Forsyth's technique, although he found considerable amounts of uronic carbon by means of Tollen's naphthoresorcinol test in the A and B horizons of this soil. Uronic acids have also been found in soil by Dubach et al (41). Hosada et al (66) and Kosaka et al (74) found that the ratio of uronic carbon to total carbon increased markedly at the depth B_1 beyond A_2 in podzolized soils. They concluded that uronic carbon is transported to the B horizon in podzols. Norman and Bartholomew (103) observed that 10 to 15 per cent of the organic carbon of the surface soils they analysed was present in uronide groupings, and that the proportion of uronic to total carbon increased with depth. In this respect, Norman (102) states: "Profile studies have indicated that substances containing uronic carbon are to some degree mobile, since in podzolized soils there seems to be a surprising accumulation of such materials in the B horizon. These carboxyl groups are probably, in fact, the acids which have been so often postulated, but never identified as agents involved in the podzolization process."

Estimates of the quantity of uronic carbon in soils have been criticized by Bremner (28) on the grounds that

the method conventionally used is not specific for the uronic carboxyl grouping, and yields results which he considers to be impossibly high. He contended that this method, which involves prolonged boiling with 12 per cent hydrochloric acid and measurement of the carbon dioxide evolved, splits off carboxyl groups of non-uronic origin. Mattson and Koutler-Andersson (97) found that the uronic anhydride content of beech lignin increased from 2.3 to 10.1 per cent as a result of autoxidation. As Mattson and Koutler-Andersson have pointed out, the lignin complex can hardly be expected to form uronic acids by autoxidation.

By means of paper chromatography, Forsyth (56) and Stevenson et al (126) have been able to isolate and identify uronic acids in the hydrolysates of soil organic matter. Forsyth determined the constituent sugar units of the polysaccharide contained in a fulvic acid fraction representing 1 to 2 per cent of the total organic matter. Of two polysaccharides, 15.8 and 16.9 per cent were present as uronic anhydride, which would account for 0.15 to 0.34 per cent of the soil organic matter. However, Forsyth regarded his yields as minimal.

From these results, it would appear that materials containing uronic groupings do occur in soil organic

matter, and that in podzol soils they may accumulate in the B horizon. However, it would seem that they do not necessarily make up a very large portion of the soil organic matter.

Other carbohydrate materials have been obtained from soil organic matter, but usually they have not constituted any appreciable portion of the soil organic fraction. The isolation of methyl sugars from soils has been reported by Duff (42, 43), while Alvsaker and Michelson (3) found very small amounts of a number of pentoses and hexoses in cold water extracts of a pine forest soil. Also, Lynch et al (86) found a number of sugars, amino sugars, uronic acids, and methylated sugars in acid hydrolysates of the humic acid fraction of the organic matter of two soils. Several other workers have reported the presence of amino sugars in soil organic matter. This aspect will be discussed in some detail later when dealing with the nitrogenous compounds of soil organic matter.

As has been mentioned previously, Forsyth (54) showed the presence of a glycosidic fraction in the four soils which he investigated. Schlichting (113), applying Forsyth's method of separation to fulvic acid extracts from A and B horizons of a heather podzol, could not definitely prove the presence of phenolic glycosides. Because of its high methoxyl content, Forsyth's fraction

B appeared to Schlichting to be a lignin derivative. He found considerably more of this material in the B horizon extracts than in extracts from the A horizon of a podzol. A fraction of still higher methoxyl content was isolated by Gallagher (57) from the B horizon of an Irish podzol.

In the decomposition of plant residues in soil, cellulose and hemicelluloses are readily utilized by a wide variety of soil microorganisms as a source of energy, whereas lignin is broken down only slowly. As a consequence, the relative proportion of lignin in the residue increases. This fact is one of the chief supports for the belief that a large proportion of soil organic matter is either lignin or lignin-derived. Further support is provided by the presence in soil of a fraction, which, like lignin, may be dissolved in alkali and subsequently precipitated by the addition of excess acid, is resistant to hydrolysis by strong mineral acid, contains methoxyl groups, and is attacked by relatively mild oxidizing agents. Gottleib and Hendricks (59) attempted to obtain more direct evidence of a lignin fraction in soil organic matter through the application of alkaline nitrobenzene oxidation and high pressure hydrogenation; techniques which have been helpful in elucidating the structure of

wood lignin. They were unable to isolate definitely characterizable products from soil organic matter. This is in contrast to the situation with wood lignin, from which propyl benzene derivatives can be obtained in good yield.

Morrison (99) has observed the production of small amounts of vanillin, syringaldehyde, and p-hydroxybenzaldehyde by alkaline nitrobenzene oxidation of soils. In mineral soils, the yields of aldehydes accounted for only 0.5 to 1.0 per cent of the total organic carbon. Also, the yields of aldehydes decreased with depth in a pine forest soil profile. The amounts of aldehydes obtained by Morrison would not indicate a very high lignin content of the soil organic matter. However, these aldehydes are the products usually obtained by treatment of lignin with alkaline nitrobenzene.

On the basis of similarities between alkali lignin and soil organic matter preparations in their behaviour towards hydrogenolysis, Gottleib and Hendricks (59) hypothesized a condensation of demethoxylated lignin molecules with the production of fused ring structures. Scheffer and Welte (112) observed marked similarities in the ultraviolet absorption spectra of alkali lignin from

several sources and soil humic acid preparations.

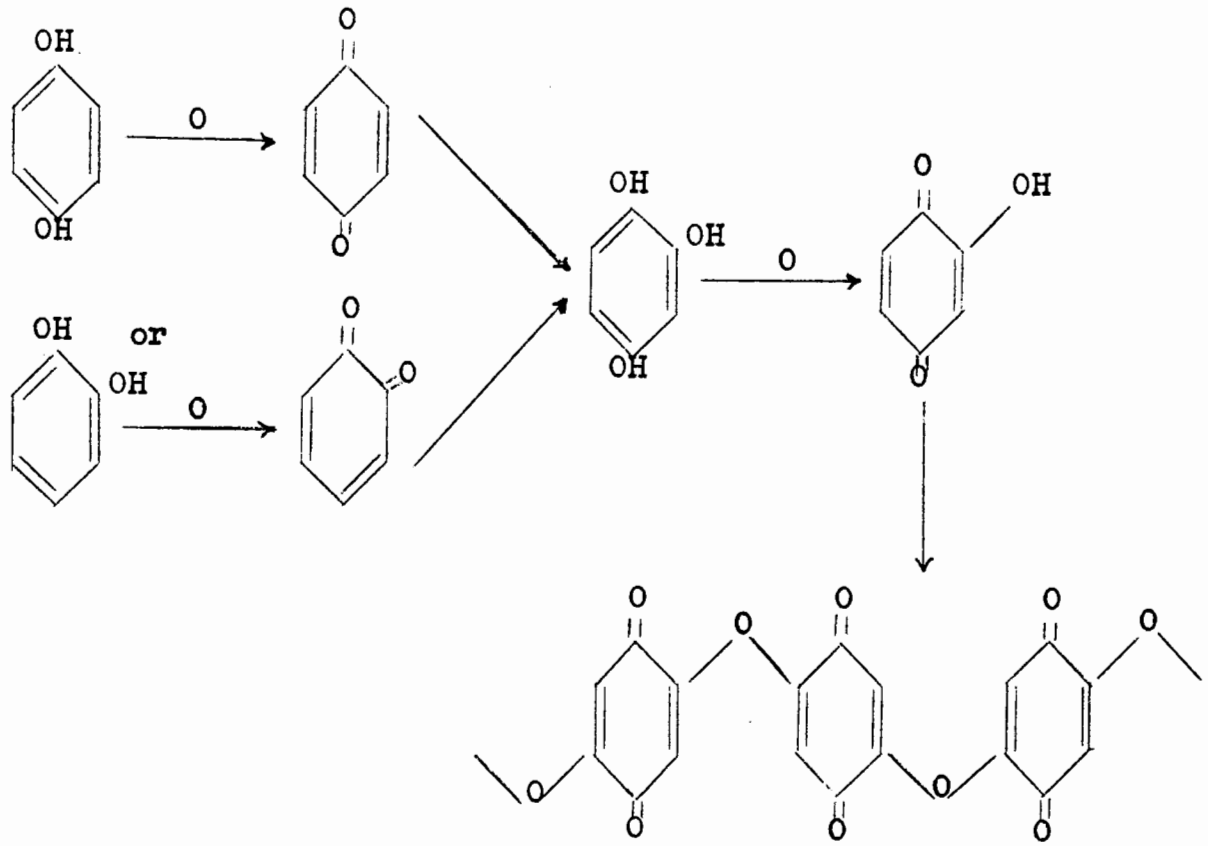
In addition to the probable conjugation of ring structures in lignin during decomposition, there is ample evidence of other changes in the molecule, which differentiate the "lignin derived" fraction in soil from the unaltered constituent in plants. Waksman and Smith (135) have shown that methyl groups are split off during the decomposition of plant residues in soils, as evidenced by the decrease of methoxyl content. Millar et al (98) and Bartlett (9) have shown that the cation exchange capacity increases during decomposition of plant residues, probably due to the oxidation of side chains to carboxyl and exposure of phenolic hydroxyl groups by demethylation. In a recent review article, Broadbent (32) states: "It appears that the resistant part of soil organic matter is somewhat unlike lignin, though resembling it in several respects. The carbon content alone seems to give an indication of this, since wood lignin usually contains more than 60 per cent, whereas organic matter of surface soils rarely contains more than 52 per cent, and the value may be much less in subsurface layers."

Kukharenko and Vvedenskaya (77) treated humic acids and lignin with 60 to 70 per cent sodium in liquid ammonia

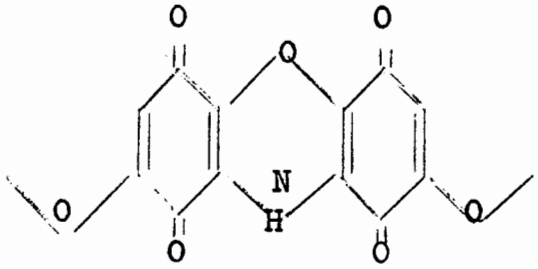
for long periods of time. They concluded, from the products obtained by this treatment, that ether linkage of large units was predominant and characteristic for both substances. However, the decomposition products of humic acid were more complex in nature than those of lignin.

On the basis of ion exchange studies on humic acids, Larina and Kasatochin (79) considered that, "The most plausible and acceptable model of soil humic acids appeared to be that of a flat aromatic carbohydrate nucleus with side radicals bearing different functional groups." Kononova (73) claims that, "The fundamental structural unit of humic acid is a flat net of polymerized cyclic carbon with side chains of linear polymerized carbon." Also, Kononova's data indicated that, fundamentally, humic and fulvic acids are similar except for their degree of polymerization. Due to the fact that only abstracts of these two papers have been available to the author, and no details of the methods and results by which these workers arrived at their conclusion are known, it is difficult to assess the significance of these conclusions.

Flaig (51) reported that the ortho- and para-dihydroxybenzenes are oxidized at pH 8-9 and polymerized to humic acid-like materials. He suggested that this took place according to the following scheme:



In the presence of ammonium hydroxide at pH 10, nearly 6 per cent nitrogen, resistant to distillation in concentrated sodium hydroxide, could be introduced. Flaig attributed this introduction of nitrogen into the polymer as due to the formation of structures of the type shown in the following formula:



These materials were found to be similar to soil humic acids in many respects. However, the pH requirements (8-10) of these reactions would prohibit their contributing, as such, to the formation of humic acid in podzol soils. It would be necessary for these reactions to occur at much lower pH levels before they could be considered as contributing to the organic matter content of podzol soils. Notwithstanding these restrictions, Flaig and Schulze (53) consider that the synthetic humic acids prepared from hydroquinone are very similar in nature to the natural humic acids. It is their opinion that the formation of humic acids goes through an oxyquinone state.

Since it is extremely difficult to obtain any soil organic matter extract or fraction thereof, which does not contain nitrogen, this element must occur as an integral part of many of the compounds present in soil organic matter. Schreiner and Shorey (117, 119) isolated a number of nitrogenous materials from soils in small amounts. Among these were a number of amino acids, purine and pyrimidine bases, and nucleic acids. Anderson (5) has found guanine, adenine, cytosine, thymine, and uracil in perchloric acid hydrolysates of the humic acid fraction of the organic matter of three representative mineral soils from northeastern Scotland. The fact that these bases were not present in the soil in the free state would

indicate the presence of nucleic acids or nucleotides in these soils. Another nitrogenous material, which has been obtained after treatment of humic acid extracts, is indole. Flaig and Breyhan (52) have reported the presence of indole after fusion of humic acid with alkali hydroxide.

As has been mentioned previously, the presence of amino sugars in hydrolysates of soils has been reported by several workers. Stevenson (127, 128) has reported that up to 24 per cent of the soil nitrogen may be accounted for as amino sugar nitrogen, with the values for surface soils usually between 5 and 12 per cent of the total soil nitrogen. Similarly, from the amounts of amino sugar nitrogen liberated by acid hydrolysis, Bremner and Shaw (31) estimated that 5 to 10 per cent of the total nitrogen of the soils examined was in the form of amino sugars. Also, Whistler and Kirby (136) detected glucosamine in hydrolysates of a water extract of soil. These results indicate that a part of the soil nitrogen is present in the polysaccharide fraction of the soil organic matter.

Since proteins are present in plant residues and microbial protoplasm, it has long been supposed that much of the soil organic nitrogen is protein derived. Kojima (71, 72) was able to account for 37 per cent of the total nitrogen in a muck soil as α-amino nitrogen, and isolated

several common amino acids in good yield. The same twenty amino acids were found in hydrolysates of each of six soils by Bremner (26). Bremner (25) also reported that approximately one-third of the total nitrogen of these soils was liberated as α-amino nitrogen by either acid or alkaline hydrolysis. Okuda and Hari (104) and Pavel et al (106) found the same fifteen amino acids in hydrolysates of humic acid preparations from several soils. Pavel et al failed to find any qualitative differences in the amino acid content of hydrolysates of humic acid preparations from chernozem, brown, and podzolic soils. Sowden (125) also has reported that the nitrogen associated with amino acids in five soils varied from 15 per cent of the total soil nitrogen to 42 per cent. Sowden (124) also failed to find any distinctive differences in the amino acid distribution in podzol, black, and brown prairie soils.

The idea of a ligno-protein complex in soil was advanced to account for the apparent resistance of soil nitrogen to microbial decomposition. It seemed reasonable to postulate a masking effect of resistant lignin upon easily decomposed protein. Certain superficial similarities were observed between a synthetic ligno-protein and soil humus by Waksman and Iyer (134). The postulated mechanism does not adequately explain the resistance to

microbial attack, as pointed out by Norman (102), nor the reactivity toward peroxide of soil organic matter, as shown by MacLean (89). An alternative explanation has been offered by Ensminger and Geiseking (45, 46) who found proteolysis to be limited in the presence of clays. In view of the evidence that the acid-resistant fraction in soils is unlike lignin in many of its properties, and that a large portion of the soil organic nitrogen is apparently of non-protein nature, it would seem that the lignin-protein complex idea, in its original form, is now obsolete.

Mattson and Koutler-Andersson (96) suggest that some of the stable nitrogen compounds in soil might be produced by interaction of oxidized lignin and ammonia, or possibly aromatic amines, at the sites of the phenolic hydroxyl groups to form amidophenols, which upon oxidation would form a polymer containing ring nitrogen. The proposed reaction is partially substantiated by the finding of Bennett (12) that when hydroxyl groups in oxidized lignin were blocked by methylation, very little nitrogen was assimilated into the molecule upon treatment with ammonia, whereas more than 7 per cent nitrogen could be fixed by an unmethylated oxidized commercial lignin. Recently, Sohn and Peech (123) have determined the capacity of

several soils to fix ammonia from anhydrous ammonia. They found that most of these soils fixed more ammonia than potassium, and that treatment of the soil with peroxide to destroy the organic matter markedly decreased the ammonia fixing capacity of the soil. The highest amounts of ammonia were fixed by acid soils containing large amounts of organic matter. These workers concluded that at least 50 per cent of the ammonia fixed by New York mineral surface soils was due to some reaction of ammonia with the soil organic matter.

Although our present knowledge concerning the chemical nature of soil organic matter is very inconclusive, it might be expected that the following types of organic material would be present in soil organic matter in appreciable amounts:

1. Carbohydrates, probably including some which contain uronic acid groupings, and some associated with nitrogen in the form of amino sugars.
2. Materials which, on hydrolysis, produce amino acids. These materials might be expected to be but are not necessarily protein in nature.
3. Materials of an aromatic nature, possibly modified lignin, but differing appreciably from plant lignin in nitrogen content, methoxyl content, and carboxyl

content. However, this material would not necessarily be lignin derived, as there is, as yet, no conclusive evidence linking the lignin structure with the structure of any fraction of the soil organic matter.

In the present study the author has devoted his attention to the fractionation and characterization of the organic materials removed from the deposition horizon of a sandy podzol by means of the metal-chelating agent 8-hydroxyquinoline.

MATERIALS

One soil was used for all of the work reported here. This was the St. Amable soil, a loamy sand podzol which has been described by Lajoie and Stobbe (78). All samples were obtained from the B_{21} horizon of this soil under a natural forest vegetation composed mainly of American beech (Fagus grandifolia Ehrh.). The B_{21} horizon soil at this location was moderately well drained, was uniformly reddish brown in colour, friable, and had an organic matter content of 5.54 per cent and a pH of 5.3. In comparison to the organic matter content of 5.54 per cent in the B_{21} horizon, the organic matter content of the horizon immediately overlying the B_{21} horizon, the A_2 horizon, was 2.42 per cent.

EXPERIMENTAL METHODS

Removal of organic matter from the soil

A mixture of 100 gm. air-dried soil and 300 ml. of water in a 1000 ml. beaker was adjusted to pH 6.5 using sodium hydroxide solution. Then 500 ml. 0.1 M 8-hydroxyquinoline in benzene was added, and the system was stirred with a mechanical stirrer for 24 hours, during which time the pH of the aqueous phase was checked periodically and readjusted to 6.5 with sodium hydroxide solution as required. At no time was the pH of the aqueous phase allowed to fall below 6. After 24 hours the aqueous and benzene phases were separated and the latter washed several times with water to remove any suspended soil particles. The aqueous phase plus washings was filtered with suction, and the filtrate was washed several times with chloroform to remove the last traces of 8-hydroxyquinoline. The residual soil was re-extracted in the same manner until 12 successive extractions had been made.

To obtain larger amounts of organic matter, in some cases a 300-gm. sample of soil was extracted in the same manner using 300 ml. of water, and 1000 ml. of 0.1 M 8-hydroxyquinoline in benzene for a 72-hour period. It was found necessary to repeat this extraction at least

five times to obtain as much organic material in the aqueous solution as was yielded by extraction of a similar amount of soil by twelve 24-hour extractions on 100-gm. samples of the soil. However, the amounts of organic matter recovered from the benzene phases were much lower from these five longer period extractions than from the twelve shorter period extractions.

Fractionation of the organic matter of the aqueous phase

Fractionation of the organic matter in the aqueous phase was obtained by the use of lauryl pyridinium chloride as a precipitating agent. The lauryl pyridinium chloride was added to the aqueous extract as a 2.5 per cent aqueous solution. Maximum precipitation required approximately three parts by weight of lauryl pyridinium chloride to one part of organic matter. The addition of a large excess of this precipitating agent caused a reduction in the amount of organic matter precipitated. The aqueous extracts were separated into the following fractions:

FRACTION A1. That portion of the organic material not precipitated by lauryl pyridinium chloride.

FRACTION A2. That portion of the lauryl pyridinium chloride precipitate which is soluble in 5 per cent sodium chloride solution and, after removal of lauryl pyridinium chloride, is precipitated by the addition of three volumes of ethanol.

FRACTION A3. That portion of the lauryl pyridinium chloride precipitate which is soluble in 5 per cent sodium chloride solution and, after removal of lauryl pyridinium chloride, is not precipitated by the addition of three volumes of ethanol.

FRACTION A4. That portion of the lauryl pyridinium chloride precipitate not soluble in 5 per cent sodium chloride solution. This fraction was soluble in water after washing the lauryl pyridinium chloride precipitate first with acetone and then with chloroform.

Lauryl pyridinium chloride was removed from the solutions of fractions A1, and the combination of fractions A2 and A3 as follows: The solution was evaporated to dryness over sulphuric acid in a vacuum desiccator. The dry residue was first washed with acetone to remove the last traces of water, and then washed with chloroform to remove most of the lauryl pyridinium chloride. After removal of chloroform, the residue was dissolved in water and the remainder of the precipitating agent was removed by extraction with chloroform. The extraction of the solutions with chloroform, without prior removal of most of the precipitating agent, was found to be impossible due to the very stable emulsions which were formed upon shaking with chloroform. The last traces of the precipitating agent also were removed from the solution of fraction

A4 by extraction with chloroform.

In some instances, the organic matter of fractions A2, A3 and A4 was precipitated as barium salts. This was accomplished by adding an excess of barium chloride to the solutions (approximately 2 gm, $BaCl_2$ per gm. of organic matter) and then adding 4, 3 and 1 volumes of ethanol to fractions A2, A3 and A4 respectively. This completely precipitated the organic matter in these solutions. After centrifugation the precipitates were washed with alcohol and water mixtures similar to those used for precipitation. The precipitates were washed until the washings failed to give any qualitative tests for either chloride or barium. Then the precipitates were washed with acetone to remove any water, and finally they were washed with ether and dried under vacuum at room temperature.

Removal of organic matter from the benzene phase

The benzene extract was shaken with approximately 10 gm. of Whatman standard grade cellulose powder per 1000 ml. of solution for 24 hours. After 24 hours, the benzene was filtered through sintered glass, and the cellulose powder was then washed with benzene to remove 8-hydroxyquinoline and 8-hydroxyquinolinates. The cellulose powder was then suspended in water and shaken for 48

hours with a 0.1 M solution of 8-hydroxyquinoline in chloroform. After this time the two phases were separated and the aqueous phase, which contained the cellulose powder, was filtered through sintered glass. The cellulose powder was then washed with water until the washings became colourless. A large portion of the organic matter was transferred to aqueous solution by this treatment. This organic matter has been called fraction B1. However, some of the organic matter remained in association with the cellulose powder. This organic material was removed from the cellulose by elution with 0.1 M sodium carbonate solution. Immediately after elution this carbonate solution was neutralized to pH 6.5 to 7.0 with acetic acid. This fraction of the organic matter has been labelled fraction B2.

In some instances, the organic matter of these two fractions from the benzene phase of the extractant has been obtained as the barium salts similarly to the fractions obtained from the aqueous phase of the extractant. The same method was used for the precipitation of the barium salts except that only two volumes of alcohol were added to the aqueous solutions of these two fractions.

A schematic representation of the extraction and fractionation of the soil organic matter is shown in Figure 1 on the following page.

Alkali fusion and preliminary fractionation of the products of fusion

Approximately 50 mgm. of dry organic material, or a weight of the barium salt equivalent to 50 mgm. of the organic material, was fused with 2.0 gm. potassium hydroxide in a nickel crucible. Heating was continued until frothing had ceased and the dark colour of the organic matter had almost completely disappeared. Heating beyond this point produced a drastic reduction in the amounts of materials recovered from the melt. The melt was cooled, dissolved in 20 to 30 ml. of water, acidified and made to 2 N with hydrochloric acid. The acidic solution was extracted twice with 25 ml. portions of ether. The ether extract was then extracted twice with 10 ml. portions of a 2 per cent solution of sodium bicarbonate to remove acidic materials. The materials remaining in ether solution were further fractionated by steam distillation of the residue remaining after evaporation of the ether. Each of these three fractions of the ether extractable materials was recovered in ether solution by acidification of the solution concerned with hydrochloric acid

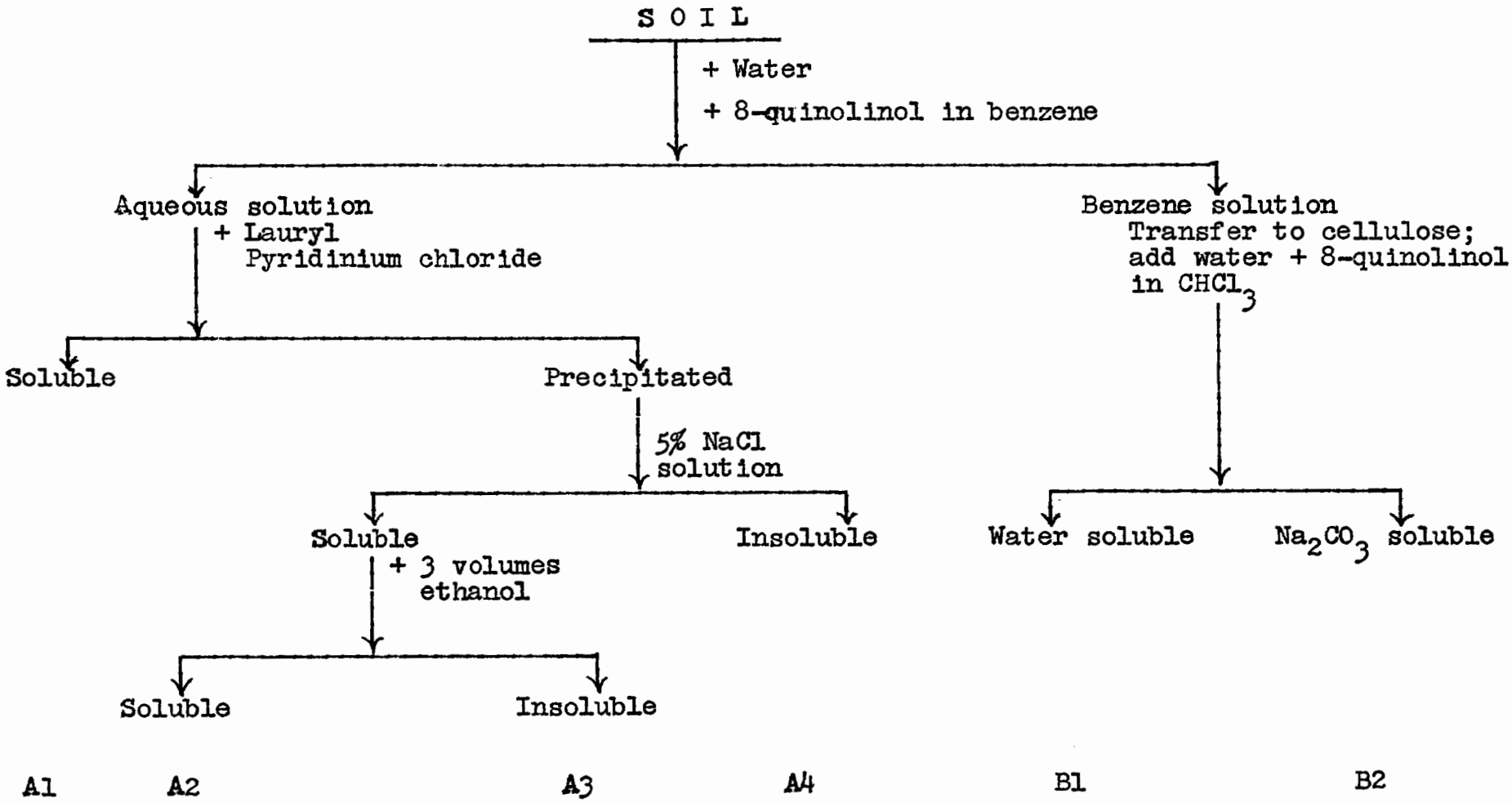


Figure 1. A schematic representation of the extraction of organic matter from the soil and the subsequent fractionation of the organic matter into six fractions.

followed by extraction with two 25 ml. portions of ether. After ether extraction of the acidified solution of the melt, this solution was further extracted with two 25 ml. portions of ethyl acetate. The ether and ethyl acetate solutions of these four fractions of the products of the fusion were dried over anhydrous sodium sulphate, and the solvents evaporated. The amount of materials present in each of these fractions was determined by weighing the residue remaining after evaporation of the solvent.

A schematic representation of the fusion and the preliminary fractionation of the products of alkali fusion is shown in Figure 2 on the following page.

Paper chromatography

Whatman No. 1 chromatographic paper was used for all paper chromatographic work, and the descending technique was employed in all instances.

Two solvents were employed for the chromatography of uronic acids. These were (a) a 5:5:3:1 mixture of ethyl acetate : pyridine : water : acetic acid (50), which was run for six hours, and (b) a 500:115:385 mixture of n-butanol : formic acid : water (44), which was run for 17 hours. Chromatography of sugars was accomplished using a solvent consisting of a 1:3:5:3 mixture of benzene : n-butanol : pyridine : water (115) and developing for a period of 24 hours.

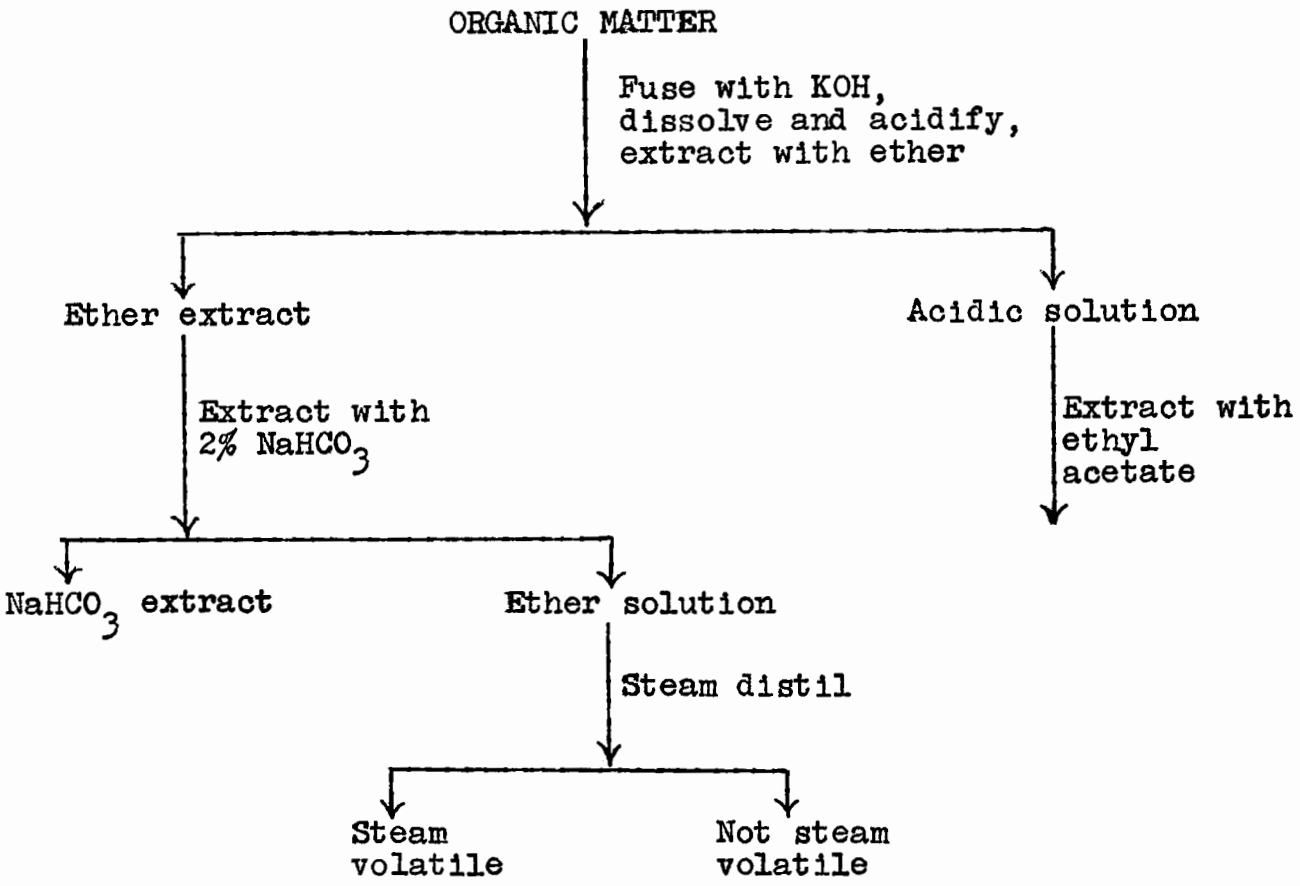


Figure 2. A schematic representation of the fusion of organic matter with potassium hydroxide and the preliminary fractionation of the products of fusion.

A solvent consisting of a 2:3 mixture of n-propanol : water was employed for the chromatography of soil organic matter extracts and of the fractions obtained from these extracts.

Basic, neutral, and acidic solvents were employed for the chromatographic separation of phenols and phenolic acids. The four solvents employed for this purpose were: (a) an 8:1:1 mixture of isopropanol : concentrated ammonium hydroxide : water (6) run for 16 hours; (b) a 6:4:3 mixture of n-butanol : pyridine : water (110) run for 12 hours; (c) 20 per cent aqueous potassium chloride solution (6) run for 3.5 hours; and (d) a 4:1:5 mixture of n-butanol : acetic acid : water (24) run for 15 hours. For the separation of phenolic materials, all chromatograms were run until the solvent front had moved approximately 35 cm. from the point of application of the sample. The times listed above are the average times required for the solvents to move this distance on paper.

Sugars were detected, on paper, by the use of p-anisidine phosphate (100) and aniline hydrogen phthalate (105) sprays. Uronic acids were detected by spraying with p-anisidine phosphate (100). The detection of acids was accomplished by staining with an alcoholic solution of bromophenol blue indicator (83). Ninhydrin (131) was

employed for the detection of α-amino acids on paper. For the detection of phenols, diazotized sulphanilic acid (4) and diazotized p-nitroaniline (110) stains were utilized. The diazotized p-nitroaniline stain was buffered at pH 4.8. A further set of staining characteristics were obtained by dipping the papers stained with diazotized p-nitroaniline into a 20 per cent solution of sodium carbonate.

Column separation of phenolic acids

Phenolic acids were separated chromatographically on a column of Whatman standard grade cellulose powder. A sample containing 50 to 100 mgm. of phenolic acids dissolved in 1 ml. of the chromatographic solvent was added to a tightly packed column of cellulose powder, which was 90 cm. high in a glass column of 12 mm. inside diameter. The column was then chromatographically developed using the solvent (8:1:1) isopropanol : concentrated ammonium hydroxide : water. The effluent from the column was collected in 3.0 gm. fractions by means of a fraction collector. The location of the individual phenolic acids in these fractions was determined by spotting samples of the effluent fractions on paper and staining with diazotized p-nitroaniline (110) and then dipping in sodium carbonate solution.

Purification of phenolic acids

The fractions containing each acid obtained from the separation on the cellulose column were combined and the solvent removed under vacuum at room temperature. The residues remaining were dissolved in 2 N potassium hydroxide solution. This solution was then acidified by the addition of hydrochloric acid. After thorough cooling, the acidic solution was extracted with ether, and the ether was then evaporated to leave a phenolic acid residue. This process of dissolving in alkali, acidifying, and extracting was repeated several times until the residue, after evaporation of the ether, was white in colour. This procedure was employed due to the fact that the small amounts of each acid present were insufficient to permit recrystallization in the usual manner without loss of all or most of the acid.

Quantitative estimation of phenolic acids

After separation on paper chromatograms using the (8:1:1) isopropanol : concentrated ammonium hydroxide : water solvent, four phenolic acids were quantitatively estimated by the following method: The chromatograms were air-dried for several hours to remove the solvent. The paper was then dipped in a solution of diazotized p-nitroaniline; 20 ml. 0.5 per cent solution of p-nitroaniline in 2 N hydrochloric acid plus 2 ml. 5.0 per cent

solution of sodium nitrite. Then the paper was dipped in a 10 per cent solution of sodium carbonate until the coloured spots developed on the paper, and the excess water was allowed to drip from the paper for approximately 10 minutes. The coloured spots were then cut from the paper, while still wet, placed in a test tube and 5 ml. of a 0.5 per cent solution of potassium hydroxide in ethanol added to extract the coloured material from the paper. The light absorption of the coloured solutions produced by the four acids were read on a Beckman Model DU spectrophotometer at the following wave lengths: m-hydroxybenzoic acid at 507 mμ; p-hydroxybenzoic acid at 507 mμ; 2,4-dihydroxybenzoic acid at 443 mμ; and 3,5-dihydroxybenzoic acid at 451 mμ. The amounts of the acids present in these solutions were determined by comparison with known amounts of standard acids which had been subjected to the same treatment.

Other methods

pH was determined using a Beckman Model G pH meter. Visible and ultraviolet absorption curves were obtained using a Beckman Model DU spectrophotometer equipped with a recording device. Iron was determined by the acid thiocyanate method of Houlihan and Farina (67), and aluminum by the use of aluminon as described by Robertson (111).

Total phosphorus was determined by the method of Dickman and Bray (40), while inorganic phosphorus was estimated by the Martin and Doty (90) method. Prior to the estimation of iron, aluminum, and total phosphorus, all samples were digested with a 1:1 mixture of nitric and perchloric acids in the presence of a vanadium catalyst (121). This digestion was continued until the volume had been reduced to a few drops. Silica was determined as the loss of weight upon hydrofluoric acid treatment following ashing and acid dehydration of silica. Organic matter was estimated by dichromate oxidation (107), and carbohydrate was estimated by the use of anthrone (47), while uronic acids were estimated with carbazole as outlined by Lynch et al (85). Nitrogen was determined by a microkjeldahl method (8), while sulphur was estimated colorimetrically as methylene blue following reduction as described by Johnson and Nishita (68). Sulphur was estimated in this manner both directly and following oxidation with magnesium nitrate (7). Neutralization equivalents were determined by titration of 10 to 15 mgm. of organic material dissolved in 50 ml. of water to pH 7.0 with 0.01 N sodium hydroxide solution.

RESULTS AND DISCUSSION

Amounts of organic matter removed from the soil

The method of extraction used, employing 8-hydroxyquinoline in a nearly neutral water-benzene system, removed 75 to 80 per cent of the organic matter from the deposition layer of a sandy podzol during 12 successive 24-hour extractions. Twenty-five per cent of the soil organic matter was removed into aqueous solution, while 50 per cent of the soil organic matter was recovered from the benzene phase of the extractant.

In comparison to these results, three successive 48-hour extractions of this soil with 0.5 N sodium hydroxide solution using a 5:1 ratio of extractant to soil removed only 60 per cent of the organic matter from this particular soil. In this instance the first 48-hour extraction removed 35 per cent of the organic matter of the soil. The second extraction removed 16.7 per cent and the third 7.6 per cent of the total organic matter of the soil. If further extractions were employed, it is possible that this extractant would remove as much of the soil organic matter as was released by the metal-chelating agent. However, from these results, it does not appear that the alkaline extraction would be any faster than the neutral system employed throughout these investigations. Also it

would be expected that less alteration of the soil organic matter would take place during extraction under nearly neutral conditions than under the high pH conditions prevailing during alkali extraction.

Forsyth fractionation of extracts

The aqueous extracts were subjected to the Forsyth method of fractionation of fulvic acid preparations. None of the materials in the aqueous extracts were precipitated by the addition of sufficient mineral acid to reduce the pH to approximately 2.5 or even to much lower pH values. The pH of the aqueous extract was adjusted to 2.5 and the extract was then filtered through a pad of charcoal. The organic matter content of the aqueous extract was almost entirely adsorbed on charcoal. The effluent, after passing the aqueous extract through a pad of charcoal, was colourless and only trace amounts of organic matter could be detected in the effluent and washings from an extract which had originally contained 4.25 gm. of organic material. The amount of organic material in this effluent was of the order of 50 mgm. No organic matter could be detected in the Forsyth B and C fractions. According to the Forsyth scheme, fraction B was eluted from the charcoal with acetone containing 10 per cent water, and fraction C was eluted with distilled water following the removal of fraction B. Also, Forsyth's fraction C was precipitated by the addition

of acetone to the water eluate. No precipitate was obtained when acetone was added to the water eluate from the aqueous extract on charcoal. Almost all of the organic matter appeared in fraction D; that is, it was eluted from charcoal by 0.5 N sodium hydroxide solution. Most of this material was eluted by the first 500 ml. of alkali solution. However, small amounts of organic matter were present in the eluates until 1000 ml. of 0.5 N sodium hydroxide solution had been passed through the charcoal. From these results, it has been concluded that the Forsyth method of fractionation is not applicable to the fractionation of these aqueous extracts.

The organic material of the benzene phase was transferred to charcoal by filtering through a pad of charcoal. The pad of charcoal was then washed with benzene until all colour of the metal chelates had disappeared from the washings. Then a 100 ml. portion of acetone was passed through the pad of charcoal to remove the excess benzene. No organic matter could be isolated from the benzene eluate and washings by adsorption on cellulose powder and elution with 0.1 M sodium carbonate, a method which had previously been employed for the removal of soil organic matter from benzene solutions (38). Also no organic matter could be detected in the acetone washings. Fraction B was a bright yellow coloured solution. However,

the organic matter of this fraction was found to be equivalent to approximately only 2 per cent of the organic material adsorbed by the charcoal. Again no organic matter could be detected in fraction C and the bulk of the organic material was present in fraction D.

Any further use of the Forsyth method for the fractionation of these soil organic matter extracts was not considered, due to the fact that practically all of the organic matter appeared in one fraction, and that fraction could be removed from charcoal only by the use of alkaline solutions.

Chromatography of organic matter extracts

Attempts were made to fractionate the organic matter of the aqueous extracts by paper chromatography. Several types of chromatographic solvents were tested. However, the only solvent which gave any significant separation of the organic material in the aqueous extracts was one composed of 2 volumes of n-propanol and 3 volumes of water. This 40 per cent propanol solvent separated the aqueous extracts into three distinct chromatographic fractions which were visible on paper, two of which could be stained with acidic stains. Three fractions of R_f values 0.00, 0.66, and 0.74 were detected. All three of these were visible on paper, the two mobile

fractions being light yellow in colour. The non-mobile fraction was coloured dark reddish brown. The spots with R_f values 0.00 and 0.74 gave yellow stains for acids with bromophenol blue, and also stained a bright blue with methylene blue (63). The third fraction did not stain with either of these reagents. Only the non-mobile fraction could be detected under ultraviolet light, while the other two fractions were not visible. None of these fractions could be stained on paper with diazotized p-nitroaniline or diazotized sulphanilic acid. Attempts to chromatograph the benzene solution in this manner proved to be very difficult due to the presence of large amounts of highly coloured metal chelates. However, after fractionation of the organic matter from the benzene phase, the two fractions obtained were mobile in the 40 per cent propanol solvent. This will be discussed later when comparing the fractions which were obtained from both the aqueous and benzene phases of the extracting medium.

Fractionation of the aqueous extracts

Treatment of the aqueous extracts with lauryl pyridinium chloride yielded three fractions of organic matter. That portion not precipitated by lauryl pyridinium chloride, fraction A1, had a very pale yellow colour

in solution, and on chromatography with 40 per cent propanol, only one constituent was visible on the paper. This had an R_f value of 0.66 and did not stain with bromophenol blue, methylene blue, or diazotized p-nitroaniline.

That portion of the organic matter precipitated by lauryl pyridinium chloride but not dispersed by a 5 per cent solution of sodium chloride, fraction A4, was, in solution, very dark brown in colour, and upon removal of the water separated in the form of shiny black needle shaped crystals. None of the other fractions, upon drying, showed any apparent crystalline structure. The dry barium salt of the organic matter from fraction A4 was very dark brown in colour. This fraction was not mobile in the 40 per cent propanol solvent. The sample remained at the point of application as a dark brown spot. This spot stained with methylene blue and bromophenol blue but did not stain with diazotized p-nitroaniline. This was the only one of the four fractions from the aqueous phase which was revealed by fluorescence in ultraviolet light, which was absorbed by this organic material.

Chromatography of the portion of the lauryl pyridinium chloride precipitate which was brought into solution in 5 per cent sodium chloride solution showed two distinct spots whose R_f values were 0.00 and 0.74. These were wine-red

and yellow in colour respectively. Both stained with bromophenol blue and methylene blue but were not stained by diazotized p-nitroaniline. These two chromatographic fractions were separated by repeated precipitation from aqueous solution, containing a small amount of sodium chloride, by the addition of three volumes of ethanol. The portion of this organic matter which was precipitated by the addition of ethanol, fraction A2, was not mobile on paper in the 40 per cent propanol solvent. This fraction was a wine-red colour in solution, and the dry barium salt of the organic matter of this fraction was a tan coloured powder. Chromatography of the fraction not precipitated by ethanol, fraction A3, showed only one spot at R_f 0.74. In solution this fraction was straw yellow in colour, while its dry barium salt was a tan coloured powder. The completeness of the separation of these two fractions was checked by chromatography with 40 per cent propanol. This check was found to be necessary as several reprecipitations were required to remove all of fraction A3 from the precipitated fraction A2.

Most of the iron and aluminum present in the aqueous extracts could be removed by extraction with 8-hydroxyquinoline in chloroform. However, the removal of these metals did not seem to influence the fractionation of

this organic matter. No differences in the amounts of organic material present in each fraction could be detected between the fractionation of aqueous extracts which had been extracted for 21 days with 8-hydroxyquinoline in chloroform, and the fractionation of aqueous extracts from which no attempt had been made to remove these metals.

Fractionation of benzene extracts

Two fractions were obtained from the benzene extracts following adsorption of the organic matter on cellulose powder. The first of these, fraction B1, was removed from the cellulose powder into aqueous solution by suspending the cellulose powder in water and extracting with 0.1 M 8-hydroxyquinoline in chloroform. This fraction of the organic matter was yellow coloured in solution and its barium salt was a light tan coloured powder.

Chromatography with 40 per cent propanol showed a yellow spot of R_f value 0.65, which did not stain with bromophenol blue or methylene blue but did stain very readily with diazotized p-nitroaniline. The organic matter remaining on cellulose was removed by elution with 0.1 M sodium carbonate solution; fraction B2. This solution after neutralization to pH 6.5 with acetic acid was brownish red in colour and its barium salt was a light

brown powder. On chromatography with 40 per cent propanol, a brownish red spot of R_f value 0.80 was observed. This spot stained readily with diazotized p-nitroaniline, but was not stained by bromophenol blue or methylene blue.

One other feature differentiating the two benzene fractions has been noticed. The amounts of organic matter removed into the benzene phase during each 24-hour extraction, reported previously (38), are portrayed in Figure 3. This figure shows that a very small amount of organic matter was removed in the third day of extraction, followed by a substantial increase on the fourth day. Also at this time, a difference in colour between the organic matter removed during the first three days and that removed after the third day of extraction was noted. It now has been found that fraction B2 was almost completely removed during the first three days of extraction, while fraction B1 did not appear until the fourth day of extraction.

These results indicate that two distinct fractions of organic matter were removed from the soil into benzene solution, and that one of these fractions was completely removed from the soil before any of the other fraction was extracted.

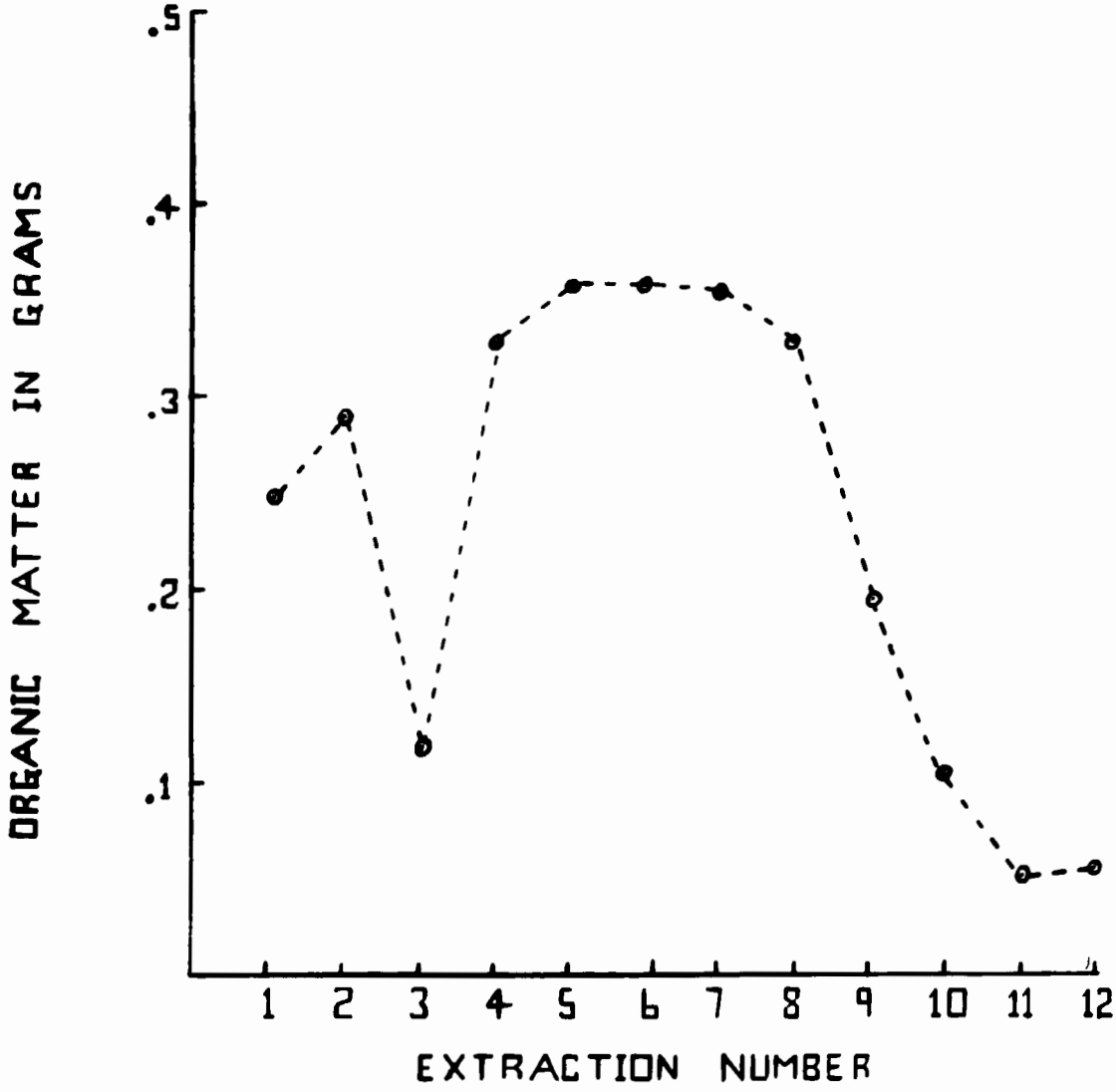


Figure 3. Amounts of organic matter recovered from the benzene phase of each of twelve successive 8-hydroxyquinoline extractions of 100 gm. of soil.

Chromatography and staining reactions of fractions

A list of the R_f values, colours on paper, and some staining characteristics of these fractions upon chromatography with 40 per cent propanol is given in Table 1. One feature which is immediately noticeable from this table is the differences in the staining characteristics of the fractions obtained from the aqueous and from the benzene phases of the extracting medium. While none of the fractions from the aqueous phase were stained by diazotized p-nitroaniline, both of the fractions obtained from the benzene phase were stained very readily by this reagent. This diazotized compound would be expected to couple with any phenols or aromatic amines, having available a free position ortho or para to the position of coupling of the hydroxyl ion or amino group, to form brightly coloured azo compounds. According to Fieser and Fieser (48), this reaction is specific to phenols and aromatic amines, and evidently depends on the powerful directive influence of the hydroxyl and amino groups. Also, the diazo coupling reaction is exhibited by aromatic compounds other than phenols and amines, which have free ortho and para positions available for coupling, in very rare instances only. Phenol ethers are distinctly less reactive than free phenols, and they do not constitute

TABLE 1

Some colour, chromatographic and staining characteristics of the fractions of soil organic matter when applied to paper

Fraction	R_f in 40% propanol solvent	Colour	Staining reaction		
			PNA	Bromophenol blue	Methylene blue
A1	0.66	pale yellow	-	-	-
A2	0.00	wine red	-	yellow	blue
A3	0.74	yellow	-	yellow	blue
A4	0.00	dark brown	-	yellow	blue
B1	0.65	yellow	red	-	-
B2	0.80	brownish red	red	-	-

PNA = diazotized p-nitroaniline.
\- = negative test.

ordinary coupling components. From the results obtained by staining with diazotized p-nitroaniline, it would appear that the material removed from the soil into the benzene phase of the extracting medium contained substances having free phenolic and/or aromatic amino groups, which have available free positions ortho or para to the position of coupling of the hydroxyl or the amino groups. The failure of this stain to produce coloured products with the fractions from the aqueous phase could possibly mean the absence of any phenols and aromatic amines in these fractions. However, any phenols or aromatic amines, not having available free ortho or para positions, also would not be stained by this reagent. Further, phenolic ethers also would fail to produce coloured products with the diazotized reagent.

All of the fractions which had been precipitated by lauryl pyridinium chloride were stained yellow by bromophenol blue and blue by methylene blue, in contrast to the remaining fractions which did not produce any coloured stains with these reagents. The yellow stains formed with bromophenol blue indicated the presence of acidic materials, while the blue stains with methylene blue probably indicated only the presence of anionic materials (63). Thus it appears that the fractions precipitated by lauryl pyridinium chloride, fractions A2, A3, and A4, were acidic in nature, while those fractions not precipitated by lauryl pyridinium chloride,

fractions A1, B1, and B2, were not acidic. This would be expected since the large cation employed to precipitate fractions A2, A3, and A4 would be expected to precipitate only anionic materials. In this connection, it is to be noted that no precipitate could be obtained by the addition of lauryl pyridinium chloride to solutions of the fractions from the benzene phase. In the case of fraction B2, containing a relatively high concentration of sodium acetate, this failure to produce a precipitate could have been due to the presence of the electrolyte. However, a similar situation did not exist in fraction B1, where the concentration of electrolytes was exceptionally low.

These results reveal some differences between the various fractions, differences evidenced by their chromatographic behaviour and by the colours of the organic materials present in these fractions. However, the major differences shown appear to be those evidenced by the staining characteristics of these materials after chromatography. Three of the four fractions obtained from the aqueous phase of the extracting medium appear to be acidic in nature, and all four of these fractions do not appear to have free phenolic hydroxyl and/or aromatic amino groups which have free ortho or para positions available

for coupling reactions. In contrast to these fractions, both of the fractions obtained from the benzene phase appeared not to be acidic and contained phenolic hydroxyl and/or aromatic amino groups having free ortho or para positions available for coupling reactions.

Chemical characteristics of the fractions

Further evidence of the differences among fractions is shown in Tables 2 and 3. Table 2 shows the proportion of the soil organic matter present in each of these fractions, and their nitrogen, apparent carbohydrate, and uronic acid contents. In this table the nitrogen, carbohydrate, and uronic acid values are expressed as a percentage of the organic matter in the fraction concerned. From this table it can be seen that fraction B1 was by far the largest of the six fractions, comprising nearly 40 per cent of the total soil organic matter. Fractions A2, A3, and B2 each contained approximately 10 per cent of the soil organic matter, while the two remaining fractions, A1 and A4, each accounted for less than 4 per cent of the soil organic matter. These six fractions accounted for very nearly 80 per cent of the total organic matter content of this soil.

The nitrogen content of the extracted organic matter was equivalent to only 2 per cent of that organic matter.

TABLE 2

The portion of the soil organic matter in each of the fractions of the organic matter removed from the soil, and the nitrogen, apparent carbohydrate and uronic acid contents of the fractions

Fraction	% of soil organic matter	* Nitrogen	* Apparent carbohydrate	* Uronic acid
A1	3.8	5.6	16.6	3.3
A2	11.2	1.5	0.0	2.8
A3	9.8	2.8	0.0	2.8
A4	3.8	2.5	1.0	4.8
B1	38.6	2.0	1.5	0.0
B2	11.4	0.5	1.5	0.0

* Expressed as per cent of the organic matter content of the fraction.

The protein contents of these extracts could account for only a small portion of the organic material present, even if all of the nitrogen was in the form of protein, which is a very doubtful assumption. Again, it has not been possible to obtain any nitrogen-free fractions of soil organic matter. Fraction B2 had the lowest nitrogen content, but even it contained 0.5 per cent. The highest nitrogen content of any of these fractions was found in fraction A1 with 5.6 per cent. The other fractions had nitrogen contents varying from 1.5 per cent to 2.8 per cent of the organic matter present in them.

It was previously concluded, from the results of staining with diazotized p-nitroaniline, that fractions B1 and B2 contained free phenolic hydroxyl and/or aromatic amino groups, which had free ortho or para positions available for coupling. Since these fractions stained very readily when only 1 to 2 µgm. of organic material was spotted on paper, their low nitrogen contents, especially that of fraction B2, would seem to eliminate the possibility of this staining capacity being due entirely to aromatic amino groups. Thus, it would appear that these two fractions contained free phenolic hydroxyl groups.

The apparent carbohydrate content of the extracted

soil organic matter has been found to be surprisingly low with only 1.8 per cent of the extracted organic matter being accounted for as carbohydrate by reaction with anthrone. Fraction A1 was the only one of the six fractions which contained an appreciable portion of carbohydrate material. The 16.6 per cent carbohydrate content of this fraction was in contrast to the 1.0 per cent carbohydrate content of fraction A4 and the complete lack of anthrone reactive materials in fractions A2 and A3. The high carbohydrate content of fraction A1 might be expected to be due to neutral carbohydrates, since only acidic materials would be expected to be precipitated by lauryl pyridinium chloride. Both of the fractions obtained from the benzene phase of the extracting medium had carbohydrate contents of 1.5 per cent.

Calculated on the basis of galacturonic acid, the uronic acid values were much higher, in some cases, than the apparent carbohydrate values for the same fractions. No uronic materials could be detected in the fractions from the benzene phase, but, according to this method, uronic acids were present in all of the fractions from the aqueous phase of the extracting medium. The two of these fractions, which did not contain any anthrone reactive materials, had apparent uronic acid contents

equivalent to 2.8 per cent galacturonic acid, while fraction A4 had an apparent uronic acid content of 4.8 per cent in contrast to a 1.0 per cent content of anthrone reactive material. Fraction A1, which contained 16.6 per cent of anthrone reactive material, had an apparent uronic acid content of 3.3 per cent.

From these results, it would seem probable that the anthrone method gave low values for carbohydrates in these fractions, or that the method employed for the estimation of uronic acids gave erroneously high values for, at least, some fractions.

Table 3 shows the organic and inorganic phosphorus contents and the results obtained for sulphur both with and without preliminary oxidation of the sample. Again, these values are all expressed as percentages of the organic matter present in the fraction concerned.

No inorganic phosphorus could be found in any of the fractions of the extracted organic matter which had been precipitated by lauryl pyridinium chloride; that is, fractions A2, A3, and A4. However, fraction A1 had an inorganic phosphorus content equivalent to 1.65 per cent of the organic matter in the fraction. Small amounts of inorganic phosphorus were found in fractions B1 and B2, which had inorganic phosphorus

TABLE 3

Phosphorus and sulphur contents of the fractions of the organic matter removed from the soil (expressed as per cent of the organic matter content of the fraction)

Fraction	Phosphorus		Sulphur	
	Inorganic	Organic	Directly	After oxidation with $Mg(NO_3)_2$
A1	1.65	0.52	4.6	0.0
A2	0.00	0.22	2.6	2.6
A3	0.00	0.18	3.2	3.2
A4	0.00	0.45	0.0	11.9
B1	0.02	0.03	11.3	0.0
B2	0.04	0.07	7.8	0.0

contents of 0.02 per cent and 0.04 per cent respectively. Organic phosphorus was found in all of the six fractions. Fractions A2 and A3 contained 0.22 per cent and 0.18 per cent respectively, while fractions A1 and A4 had organic phosphorus contents of 0.52 per cent and 0.45 per cent respectively. The organic phosphorus contents of the fractions from the benzene phase of the extracting medium were much lower than those of the fractions from the aqueous phase. Fractions B1 and B2 had organic phosphorus contents of 0.03 per cent and 0.07 per cent respectively.

Of particular interest are the results obtained for the sulphur contents of the various fractions. The sulphur contents of these fractions were first determined by a methylene blue colorimetric method, which involved the prior reduction of the sulphur present to sulphide and collection of the hydrogen sulphide gas evolved from the acidic reducing solution. By this method, hydrogen sulphide was obtained from all fractions except fraction A4. Probably the outstanding feature of these analyses for sulphur was the high concentration of this element present in the fractions obtained from the benzene phase. The estimated amounts of sulphur were equivalent to 11.3 per cent and 7.8 per cent of the organic matter of fractions B1 and B2 respectively. However, it was later noticed

that hydrogen sulphide gas was evolved upon acidification of solutions obtained from fractions A4, B1, and B2 after fusion with potassium hydroxide. Under similar conditions, no odour of hydrogen sulphide could be detected upon acidification of solutions from fractions A2 and A3. Since no sulphide had been released from fraction A4 by reduction, sulphur was again determined on each of the fractions by the same method, but following oxidation with magnesium nitrate. After this treatment, fraction A4 showed a sulphur content equivalent to approximately 12 per cent of the organic matter content of this fraction, as compared to the zero value obtained without prior oxidation of the sample. Fractions A2 and A3 had the same apparent sulphur contents when determined with and without oxidation of the sample prior to the determination of sulphur. However, after oxidation with magnesium nitrate, it has not been possible to detect any sulphur in fractions A1, B1, and B2. There are two possible explanations for this fact. The sulphur of these fractions might possibly have been converted by the oxidation to a form which could not be reduced by the method employed. This does not seem probable, as it would be expected that the sulphur would be oxidized to the sulphate form, and the method employed for the subsequent determination of sulphur is capable of

quantitatively reducing sulphates to sulphide. The second and more logical explanation for this apparent loss of sulphur would be that the sulphur present in these fractions was evolved during the evaporation of the magnesium nitrate solutions.

One possible source of sulphur, other than the soil organic matter, in these systems was the thiophene which would be expected to be present in the benzene employed for the extraction of the organic matter from the soil. For this reason, samples of thiophene equivalent to 500 µgm. of sulphur were subjected to sulphur determinations both with and without prior oxidation. In neither case could any sulphur be detected by a method capable of measuring amounts of sulphur of the order of 0-100 µgm. Thus, it would not appear that thiophene was a source of any of the sulphur detected in the various fractions.

From the values obtained for the sulphur contents of the fractions before and after oxidation with magnesium nitrate, and from the presence or absence of sulphide following potassium hydroxide fusion, it would appear that at least three classes of sulphur-containing compounds are present in these soil organic matter extracts. One of these was present in fractions A2 and A3. This type failed to produce sulphide on fusion with potassium

hydroxide, was reduced to sulphide by the reducing mixture of Johnson and Nishita (a mixture of hydriodic, hypophosphorus, and formic acids) both before and after oxidation. The second of these, present in fraction A4, produced sulphide on fusion with potassium hydroxide, and was reduced to sulphide by the reducing mixture of Johnson and Nishita only after oxidation. The third form of sulphur was found in fractions B1 and B2. These sulphur compounds produced sulphide on fusion with potassium hydroxide, released hydrogen sulphide upon treatment with the reducing mixture of Johnson and Nishita, and failed to produce hydrogen sulphide upon the same treatment following oxidation with magnesium nitrate. It would appear that fraction A1 also might belong to this group of sulphur-containing materials. However, it is not known whether or not this fraction releases sulphide upon fusion with potassium hydroxide.

Another feature of these fractions is the fact that silica was found only in fraction A1. None of the other fractions contained any detectable amounts of silica. Approximately 0.5 gm. of silica was found in the combined aqueous extracts from 600 gm. of soil. Fifty per cent of this silica was found in fraction A1. After dissolving all of the lauryl pyridinium chloride precipitate from the aqueous extracts, a gray inorganic residue containing

silica remained. The other 50 per cent of the silica extracted into the aqueous phase of the extracting medium was found to be present in this residue. No silica could be found in the fractions obtained from the benzene phase.

The removal of iron and aluminum was found to be very difficult in the case of fraction A4. Extraction of the aqueous solution of this silica-free fraction for as long as 21 days with 0.1 M 8-hydroxyquinoline in chloroform failed to reduce the iron and aluminum contents below approximately 1 m.e. each per gram of organic matter. These metals were completely removed from all other fractions, a maximum of 10 days being sufficient to remove all iron and aluminum from these fractions.

After removal of any metals remaining in the fractions precipitated by lauryl pyridinium chloride with a cation exchange resin (Nalcite H.C.R.) in the hydrogen form, fractions A2, A3, and A4 had neutralization equivalents of 163, 152, and 145 respectively.

Hydrolysis of fractions

Attempts were made to hydrolyze the fractions with hydrochloric and sulphuric acids. Solutions of the fractions were refluxed with acids in acid concentrations varying from 1.0 N to 6.0 N for periods of time varying from 6 to 48 hours. In all fractions except A1 and A2,

dark brown precipitates were formed on refluxing with the mineral acids. These precipitates were not appreciably soluble in water following removal of the acid. In none of these fractions was the colour of the solution noticeably altered or destroyed during hydrolysis. The excess acid was removed from the hydrochloric acid hydrolysates by evaporation over sodium hydroxide pellets in a vacuum desiccator, while the acid was removed from sulphuric acid hydrolysates by neutralization with barium carbonate to the congo red end point. The aqueous solutions after removal of excess acid were chromatographed for sugars and uronic acids. No indication of the presence of sugars or uronic acids in the hydrolysates could be obtained for any of the fractions other than fraction A1. A spot corresponding to galacturonic acid was obtained upon chromatography of the hydrolysate of fraction A1 in the two solvents employed, and very small amounts of four sugars, glucose, galactose, arabinose, and xylose also were detected in it. However, even in this fraction, the amounts of sugar found on chromatograms could not account for more than an extremely small portion of the organic matter present in the fraction. From the amounts of hydrolysate required to detect any sugars on the chromatogram, it has been estimated that they represented a maximum of 5 per cent of the organic matter content of this fraction.

The amounts of anthrone reactive material, the uronic acid values obtained by the use of carbazole, and the amounts of sugars and uronic acids detected in hydrolysates of the fractions all indicate that the carbohydrate contents of these organic fractions are very low, and that carbohydrate material does not constitute any appreciable portion of the organic matter removed from this soil.

Very small amounts of ninhydrin reactive materials were present in the hydrolysates of each of the fractions. However, the very faint stains developed on paper by ninhydrin did not seem to warrant any attempt to identify the α-amino acids present in these hydrolysates.

All of the fractions, after acid hydrolysis, failed to yield other than trace amounts of materials, which were mobile on paper in any of the chromatographic solvents usually employed for the separation of phenolic materials. Again, the fractions from the aqueous phase failed to form coloured products with diazotized p-nitroaniline. Similar results were obtained after refluxing with potassium hydroxide solutions for periods of up to 24 hours in alkali concentrations up to 6.0 N.

Alkali fusion of soil organic matter fractions

Since neither aqueous acidic or aqueous alkaline hydrolysis proved suitable for conversion of the original

organic fractions to simpler, more easily identifiable substances, attention was turned to a more drastic degradative procedure, namely fusion with potassium hydroxide. Preliminary experiments were required to define suitable conditions for the application of this method of degradation. In these trials the following observations were made:

1. That heating at 250°C. for 8 hours in an electric muffle furnace did not discharge the colours due to the organic materials under test;
2. that fusion at 300°C. for as short a period as 30 minutes resulted in complete decolourization of the melt, but was of little use in the production of suitable quantities of identifiable products;
3. that the weight ratio of potassium hydroxide to organic matter must be at least 40-50:1, otherwise charring of the organic material occurred;
4. that essentially the same results were obtained by the fusion of either the acidic forms, the sodium salts, or the barium salts of the various fractions; but
5. that the fusion of the barium salts, although requiring a somewhat longer period of heating, was somewhat more easily controlled, particularly with respect to

the reproducibility of the quantities of fusion products obtained.

The barium salts of the fractions obtained from the aqueous phase were conveniently at hand, having been prepared in the separation of these fractions from sodium chloride. The results of these preliminary tests indicated that the optimum temperature of fusion was in the range 250 to 300°C., and that the fusion reaction required control to a degree difficult to obtain in the furnace. Further experimentation revealed that the required degree of control could be obtained by fusion in a nickel crucible over an open flame. The fusion was begun with very gentle heating followed by a gradual increase in temperature, and then heating until frothing ceased and the colours due to the organic matter had disappeared from the melt. When heating was continued beyond this point, the quantities of fusion products obtained decreased very rapidly.

Only three of the four fractions of organic matter from the aqueous phase have been subjected to fusion. Since fraction A1 contained a very small proportion of the soil organic matter, since the removal of lauryl pyridinium chloride from this fraction required a very tedious process, and since it contained relatively large amounts of carbohydrate and nitrogen, it has not been

subjected to alkali fusion. Each of the other three fractions from the aqueous phase required different lengths of time for fusion under similar conditions, with fraction A4 requiring the longest period of heating and fraction A3 the shortest. At this point, it may be noted that it was not possible to obtain significant yields of phenolic materials after fusion of the unfractionated organic matter of the aqueous phase. Further, the yields of phenolic materials after fusion of a mixture of fractions A2 and A3 were considerably less than when either of these fractions were fused separately. These yield differences were particularly noticeable in the case of phenolic acids obtained after fusion. The organic matter of the two fractions obtained from the benzene phase required an even longer time for fusion than any of the fractions from the aqueous phase.

Preliminary fractionation of the products of fusion

Only the organic products of fusion extractable from an acidic aqueous solution of the melt by ether and by ethyl acetate have been investigated. The acidified solution was first extracted with ether. Then, after removal of the ether, it was extracted with ethyl acetate. The ether extract was divided into three portions. First, acidic materials were removed from the ether by extraction

with a 2 per cent solution of sodium bicarbonate. The substances remaining in the ether solution were further fractionated by steam distillation of the residue after evaporation of the solvent. The ether soluble fusion products present in each of these three fractions were returned to ether solution by extraction of the aqueous solutions following acidification. The amounts of material in each of the four fractions were determined by weighing after evaporation of the solvent. The quantities of fusion products obtained in this way from each of the five organic matter fractions are shown in Table 4, where the amounts of these products are given as percentages of the organic matter fused. From this table it may be seen that, in the case of fractions from the aqueous phase, the sodium bicarbonate extract contains material equivalent to approximately one-third of the organic matter fused. Smaller amounts of materials were obtained in the bicarbonate extracts after fusion of fractions B1 and B2. In these cases, the yields were equivalent to less than 20 per cent of the organic materials fused. The steam distillable portion of the ether extract accounted for approximately 7.5 per cent of the organic matter of fractions A2, A4, and B1 fused, while a value of 17.5 per cent was obtained for fractions A3 and B2. The non-steam distillable portion

TABLE 4

Amounts of materials recovered after potassium hydroxide fusion of fractions of soil organic matter (expressed as per cent of the organic matter fused)

Fraction	Ether extract			Ethyl acetate extract	Total
	$NaHCO_3$ extract	Steam distillable	Non-steam distillable		
A2	36.2	7.5	10.7	10.9	65.3
A3	31.5	17.5	17.5	4.1	70.4
A4	38.0	8.4	6.2	11.3	63.9
B1	17.3	7.3	7.5	9.8	37.7
B2	16.5	17.5	10.8	11.8	54.0

of the ether extract contained materials equivalent to 17.5 per cent of the organic matter in the case of fraction A3, while the values for fractions A2, A4, B1, and B2 varied from 6 per cent to 11 per cent. A very small amount of material, equivalent to 4 per cent of the organic matter, was found in the ethyl acetate extract from fraction A3, while fractions A2, A4, B1, and B2 had materials in this extract equivalent to 10 per cent to 12 per cent of the organic matter. The total residues from the extractions, in the case of the fractions from the aqueous phase, were equivalent to 65 to 70 per cent of the organic matter fused, while the materials recovered from fractions B1 and B2 were equivalent to 38 per cent and 54 per cent respectively of the organic matter in these fractions.

Chromatography of the products of fusion

All of these fractions of the fusion products gave very strong stains when spotted on paper and stained with diazotized p-nitroaniline or diazotized sulphanilic acid. This fact, along with the extraction procedures employed to obtain these products, indicated the presence of phenols and phenolic acids. In an attempt to separate the phenolic constituents of these residues, portions of the residues dissolved in ether or ethyl acetate were

spotted on paper and chromatographed. Although a large number of solvents have been employed for chromatography of these materials, only four of these have been employed extensively. All four of these solvents, two basic, one neutral, and one acidic, have previously been used by other workers for the separation of phenolic materials. For the phenolic materials under consideration, the best separations were obtained by the use of a basic solvent composed of 8 volumes of isopropanol, 1 volume of concentrated ammonium hydroxide, and 1 volume of water. In general, basic solvents gave better separation of the phenolic substances present in these extracts than were obtained using acidic solvents. In the acidic solvents, especially the very strongly acidic ones such as Forrestal solvent, the phenolic compounds all tended to move very rapidly. The acidic solvent used in these investigations was employed not only in an attempt to obtain separation of the phenolic substances, but also to determine which of the phenolic substances present were of an acidic nature. A bromophenol blue stain was used to detect acidic materials on paper after chromatography. This stain failed to show any acidic materials on paper after chromatography with the basic and neutral solvents. However, this reagent also failed to stain known phenolic

acids after chromatography in these solvents, but did stain these acids if only spotted on paper and also after chromatography with the acidic solvent. Before employing this stain after chromatography in acidic solvents, it was necessary to remove all of the acid component of the solvent from the paper. For this reason, papers to be stained with bromophenol blue were air-dried and then oven-heated at 105°C. for at least 30 minutes prior to staining. The failure to obtain acidic stains after chromatography with the basic and neutral solvents might possibly have been due to salt formation between the phenolic acid and the basic component of the solvent.

For adequate separation in any of these chromatographic systems, it was necessary to run all chromatograms until the solvent front had moved approximately 35 cm. beyond the point of application of the sample.

By these procedures, a total of 19 different phenolic substances have been separated. Of these 19 substances, 13 also gave acidic stains with bromophenol blue. These 13 acidic phenolic substances were all found in the sodium bicarbonate and ethyl acetate extracts. The remaining 6 non-acidic phenolic substances were found in the ether extract after removal of the acids by extraction with sodium bicarbonate solution. Two of these 6 phenolic

substances were steam distillable while the other 4 were not.

Lists of the phenolic substances separated chromatographically from the extracts are shown in Tables 5, 6, and 7. All of the substances are numbered according to the extract in which they occurred, and in the order of increasing R_f values in the isopropanol-ammonia solvent. R_f values in 4 solvents and staining characteristics of each of the 19 substances separated are given. All of the R_f values listed are average values for at least 5 chromatograms.

In Table 5 are listed the four phenolic materials which were not extracted from acidic solution by ether, but were extracted by ethyl acetate. One of these, number 2, was found in the products obtained from the organic matter fractions of the benzene phase, while number 1 was found as a fusion product of all three of the fractions from the aqueous phase. The other two products were found only in fraction A4, in which, from their staining characteristics, they would appear to be present in relatively small amounts. All of these four substances reacted positively with stains for both acids and phenols. Thus, all of these products of fusion would be expected to be phenolic acids.

TABLE 5

R_f values and staining characteristics of phenolic materials not extracted from acidic solution by ether but extracted by ethyl acetate

No.	R_f in solvent				Staining reaction			
					PNA		DSA	Bromo-phenol blue
	8:1:1	6:4:3	20% KCl	4:1:5	Acidic	Basic		
1	0.03	0.36	0.82	0.92	Y	R	O-Y	Y
2	0.17	0.73	0.21	0.89	Y	Br	P	Y
3	0.37	0.54	0.52	0.85	Y	Y	Y	Y
4	0.41	0.61	0.65	0.72	-	R	Y	Y

Y - Yellow
R - Red
Br - Brown
O - Orange
P - Purple

8:1:1 - isopropanol:concentrated ammonium hydroxide:water
6:4:3 - n-butanol:pyridine:water
4:1:5 - n-butanol:acetic acid:water

PNA - diazotized p-nitroaniline
DSA - diazotized sulphanilic acid.

TABLE 6

R_f values and staining characteristics of phenolic materials extracted from acidic solution by ether and extracted from ether by a 2 per cent solution of sodium bicarbonate

No.	R_f in solvent				Staining reaction			
	8:1:1	6:4:3	20% KCl	4:1:5	PNA		DSA	Bromo-phenol blue
					Acidic	Basic		
5	0.02	0.19	0.30	0.90	R	G	-	Y
6	0.13	-	0.32	0.79	-	-	O	Y
7	0.33	0.06	0.00	0.54	-	-	R	Y
8	0.35	0.33	0.35	0.60	-	R	R	Y
9	0.39	0.39	0.45	0.60	-	R	R	Y
10	0.43	0.50	0.54	0.76	Y	O-Y	O-Y	Y
11	0.47	0.64	0.64	0.88	-	R	Y	Y
12	0.52	0.64	0.40	0.85	Y	Br	Br	Y
13	0.61	0.58	0.64	0.88	-	R	Y	Y

Y - Yellow
R - Red
Br - Brown
O - Orange
G - Gray

8:1:1 - isopropanol:concentrated ammonium hydroxide:water
6:4:3 - n-butanol:pyridine:water
4:1:5 - n-butanol:acetic acid:water

PNA - diazotized p-nitroaniline
DSA - diazotized sulphanilic acid.

TABLE 7

R_f values and staining characteristics of phenolic materials extracted from acid solution by ether and not extracted from ether by a 2 per cent solution of sodium bicarbonate

No.	R_f in solvent				Staining reaction			
					PNA		DSA	Bromo-phenol blue
	8:1:1	6:4:3	20% KCl	4:1:5	Acidic	Basic		
14	0.24	0.40	0.56	0.96	Y	Y	Y	-
15	0.43	0.52	0.65	0.94	-	R	Y	-
16	0.79	0.88	0.00	0.89	R	R	R	-
17	0.97	0.92	0.00	0.90	Y	R	Y	-
18	0.81	0.92	0.57	0.91	Y	Br	Br	-
19	0.97	0.88	0.00	0.95	Y	R	R	-

Y - Yellow
R - Red
Br - Brown

8:1:1 - isopropanol:concentrated ammonium hydroxide:water
6:4:3 - n-butanol:pyridine:water
4:1:5 - n-butanol:acetic acid:water

PNA - diazotized p-nitroaniline
DSA - diazotized sulphanilic acid.

The phenolic substances extracted from acidic solution by ether and removed from the ether by extraction with sodium bicarbonate are shown in Table 6. Nine acidic phenolic substances have been separated chromatographically from this bicarbonate extract. Five of these, numbers 8, 9, 11, 12, and 13, have been found as products of fusion of all fractions investigated. Substance number 10 has been found as a product of fusion of all three fractions from the aqueous phase, while number 5 appeared as a product in fractions B1 and B2. Of the two remaining phenolic products of fusion, number 6 has been found in fractions A2 and A4, while number 7 was detected only in fraction A4.

The remaining 6 phenolic substances, which have been separated chromatographically, are listed in Table 7. These were the phenolic substances which were extracted from acidic solution by ether and remained in ether solution upon extraction with a 2 per cent solution of sodium bicarbonate. The first four of these substances, numbers 14-17, were not steam distillable, while substances 18 and 19 were separated from these four by steam distillation. One of the non-steam distillable phenolic compounds, number 17, has been found after fusion of all three of the fractions from the aqueous phase, while

number 16 has been obtained from fractions B1 and B2. According to their staining characteristics, the other two phenols were present in relatively small amounts, number 14 being found only in fraction A4, while number 15 was found in fractions A2 and A4. Of the two steam distillable phenolic substances, number 18 was obtained from fractions A2, A3, and A4, while number 19 was obtained from fractions B1 and B2. None of these six phenolic substances stained with bromophenol blue, so it has been concluded that these are non-acidic phenols, as also would be indicated by the failure of a sodium bicarbonate solution to extract them from ether solution.

Not all of these phenolic substances appeared as products of fusion of every fraction of the organic matter. The phenolic substances which have been obtained from each fraction of the soil organic matter are shown in Table 8. The numbers used in this table to designate the phenolic substances are those which have already been used in Tables 5, 6, and 7. One of the features, which is apparent from Table 8, is the differences between the phenolic materials obtained from the benzene and the aqueous phases of the extracting medium. For example, four of the nine phenolic substances, which were produced from fractions B1 and B2, were not obtained from any of

TABLE 8

Phenolic compounds appearing as the products of fusion in each fraction of the extracted soil organic matter

Fraction	Acidic phenolic compounds													Non-acidic phenolic compounds					
A2	1					6		8	9	10	11	12	13		15		17	18	
A3	1							8	9	10	11	12	13				17	18	
A4	1		3	4		6	7	8	9	10	11	12	13	14	15		17	18	
B1		2			5			8	9		11	12	13			16			19
B2		2			5			8	9		11	12	13			16			19

All numbers of phenolic compounds are those originally listed in Tables 5, 6, and 7.

the three fractions from the aqueous phase. The remaining five phenolic substances appearing here were common to all fractions investigated. No qualitative differences were observed between the products of fusion of the two fractions obtained from the benzene phase. Of the fifteen phenolic substances separated as products of fusion of fractions A2, A3, and A4, nine were common to all three of these fractions of the soil organic matter. Fraction A2 produced eleven phenolic substances, fraction A3 produced only the nine phenolic substances common to all three of these fractions, while fifteen phenolic substances were separated from the fusion products of fraction A4. The main features shown by Table 8 are as follows:

1. That large numbers of phenolic substances were obtained after alkali fusion of fractions of extracted soil organic matter;
2. that differences in the products of fusion of the different fractions of the organic matter of the aqueous phase, especially the large number of phenolic materials obtained from fraction A4, are shown;
3. that marked differences existed between the products of fusion of fractions from the aqueous phase of the extracting medium and those from the benzene phase; and
4. that no qualitative differences could be found between

the products of fusion of the two fractions obtained from the benzene phase of the extracting medium.

These results, in combination with the amounts of sample necessary for detection of the phenolic substances after chromatography, indicated that a large portion of the organic matter extracted from this soil was of an aromatic nature.

Separation and identification of phenolic acids

The relative R_f values of four of the acidic phenolic substances (numbers 10, 11, 12, and 13) and the staining characteristics of these compounds were similar to those which other workers (6, 110) have reported for m-hydroxybenzoic, p-hydroxybenzoic, 2,4-dihydroxybenzoic, and 3,5-dihydroxybenzoic acids. However, chromatography of samples of these known acids individually invariably resulted in higher R_f values than those of the corresponding unknown phenolic acids. But, when a mixture of these four known acids was chromatographed, the resultant R_f values coincided exactly with those obtained for the four unknown substances. The R_f values and staining characteristics of these four known phenolic acids, when chromatographed as a mixture of the four, are shown in Table 9. From these results, it would appear that acidic phenolic substances numbers 10, 11, 12, and 13 possibly were, in fact, 3,5-

TABLE 9

R_f values and staining characteristics of known phenolic acids

Acid	R_f in solvent				Staining reaction			
					PNA		DSA	Bromo-phenol blue
	8:1:1	6:4:3	20% KCl	4:1:5	Acidic	Basic		
3,5-dihydroxy-benzoic	0.43	0.50	0.54	0.76	Y	O-Y	O-Y	Y
p-hydroxy-benzoic	0.47	0.64	0.64	0.88	-	R	Y	Y
2,4-dihydroxy-benzoic	0.52	0.64	0.40	0.85	Y	Br	Br	Y
m-hydroxy-benzoic	0.61	0.58	0.64	0.88	-	R	Y	Y

Y - Yellow
R - Red
O - Orange
Br - Brown

8:1:1 - isopropanol:concentrated ammonium hydroxide:water
6:4:3 - n-butanol:pyridine:water
4:1:5 - n-butanol:acetic acid:water.

PNA - diazotized p-nitroaniline
DSA - diazotized sulphanilic acid.

dihydroxybenzoic, p-hydroxybenzoic, 2,4-dihydroxybenzoic, and m-hydroxybenzoic acids respectively.

In an attempt to separate larger amounts of these acidic phenolic constituents, 300 mgm. of organic matter from fraction A3 was fused in 50 mgm. portions with potassium hydroxide. The materials, extracted by ether from acidified solution of the combined melts, and removed from ether solution by extraction with a 2 per cent solution of sodium bicarbonate, were chromatographically separated on a column of cellulose powder, employing the 8:1:1 solvent for the separation. Three-gram fractions were collected from the column. The first phenolic substance removed from the column appeared in fractions 8 to 14, the second in fractions 16 and 17, the third in fractions 19 to 25, and the fourth in fractions 29 to 36. After removal of the solvent and purification of the residue, the melting points of these unknown phenolic acids were determined. The melting points of these four phenolic substances, the melting points of the four known phenolic acids, and the melting points of mixtures of the unknown phenolic substances and known phenolic acids are shown in Table 10.

As a further check on the identity of these four compounds, small amounts of each of the four acids obtained

TABLE 10

Melting points of known and unknown phenolic acids and melting points of mixtures of the corresponding known and unknown acids

Unknown phenolic substance number	Suspected identity	Melting point of unknown (°C.)	Melting point of known (°C.)	Mixed melting point (°C.)
10	3,5-dihydroxy-benzoic acid	233-235	234-235	233-235
11	p-hydroxy-benzoic acid	211-213	213	211-213
12	2,4-dihydroxy-benzoic acid	234-236	235-236	235-236
13	m-hydroxy-benzoic acid	199-202	202	200-202

from the chromatographic separation and the four known acids were individually dissolved in ether, and the ultraviolet absorption spectra of these solutions were obtained. The ultraviolet absorption spectra found for these compounds are shown in Figures 4, 5, 6, and 7. These figures show that (1) unknown compound number 13 and m-hydroxybenzoic acid exhibited absorption peaks at 295 mμ; (2) that unknown number 12 and 2,4-dihydroxybenzoic acid had absorption peaks at 256 and 295 mμ; (3) that unknown number 11 and p-hydroxybenzoic acid had absorption peaks at 254 mμ; and (4) that unknown number 10 and 3,5-dihydroxybenzoic acid had absorption peaks at 250 and 305 mμ.

From a combination of R_f values, staining characteristics, melting point data, and ultraviolet absorption spectra, it has been concluded that four of the acidic phenolic substances (numbers 10, 11, 12, and 13), separated by chromatography after alkali fusion of soil organic matter fractions, were 3,5-dihydroxybenzoic, p-hydroxybenzoic, 2,4-dihydroxybenzoic, and m-hydroxybenzoic acids respectively.

These are the only phenolic compounds which have definitely been identified. However, one other phenolic compound has been isolated. This compound has been listed

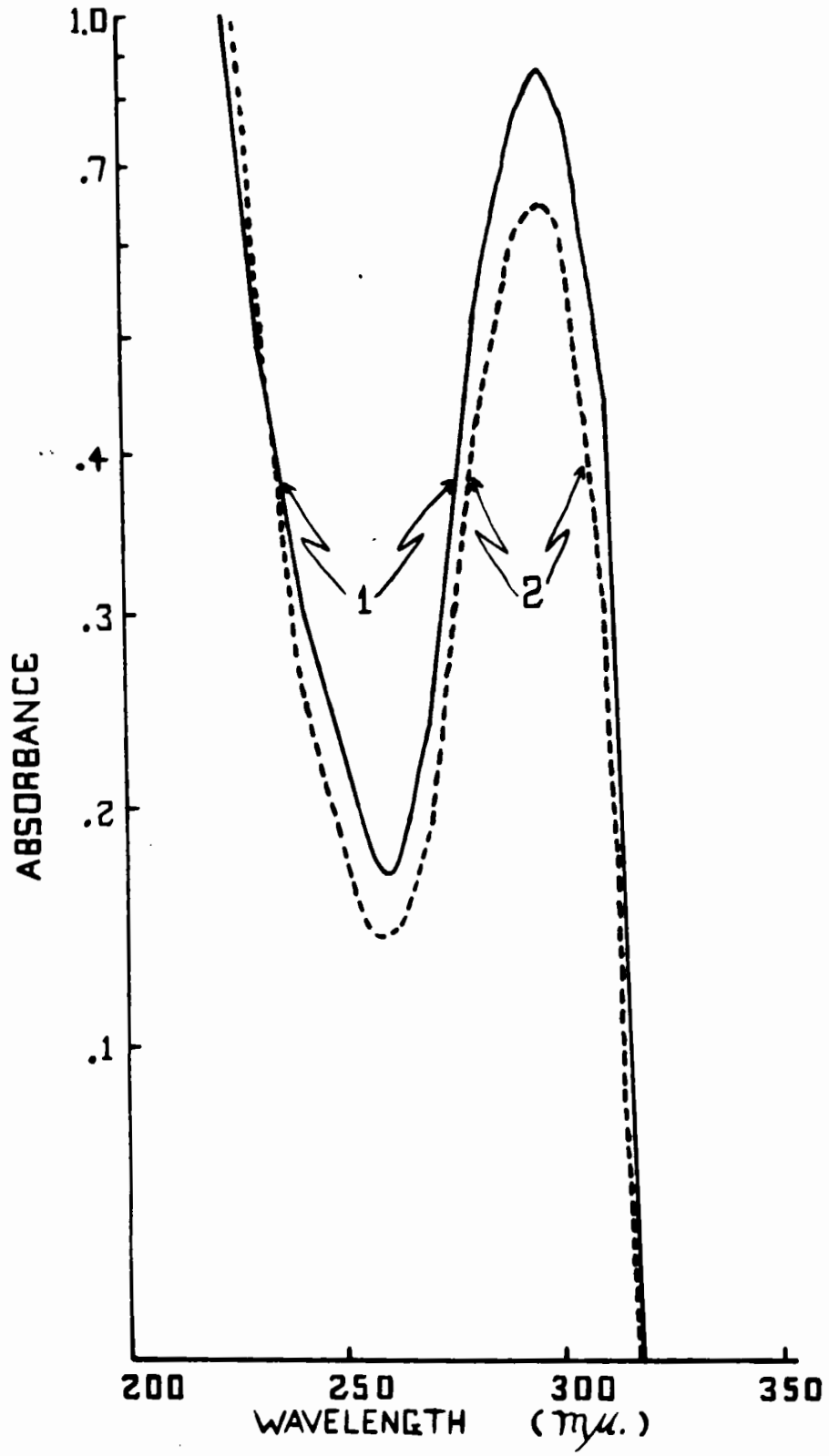


Figure 4. Ultraviolet absorption spectra of
(1) m-hydroxybenzoic acid, and
(2) unknown compound number 13.

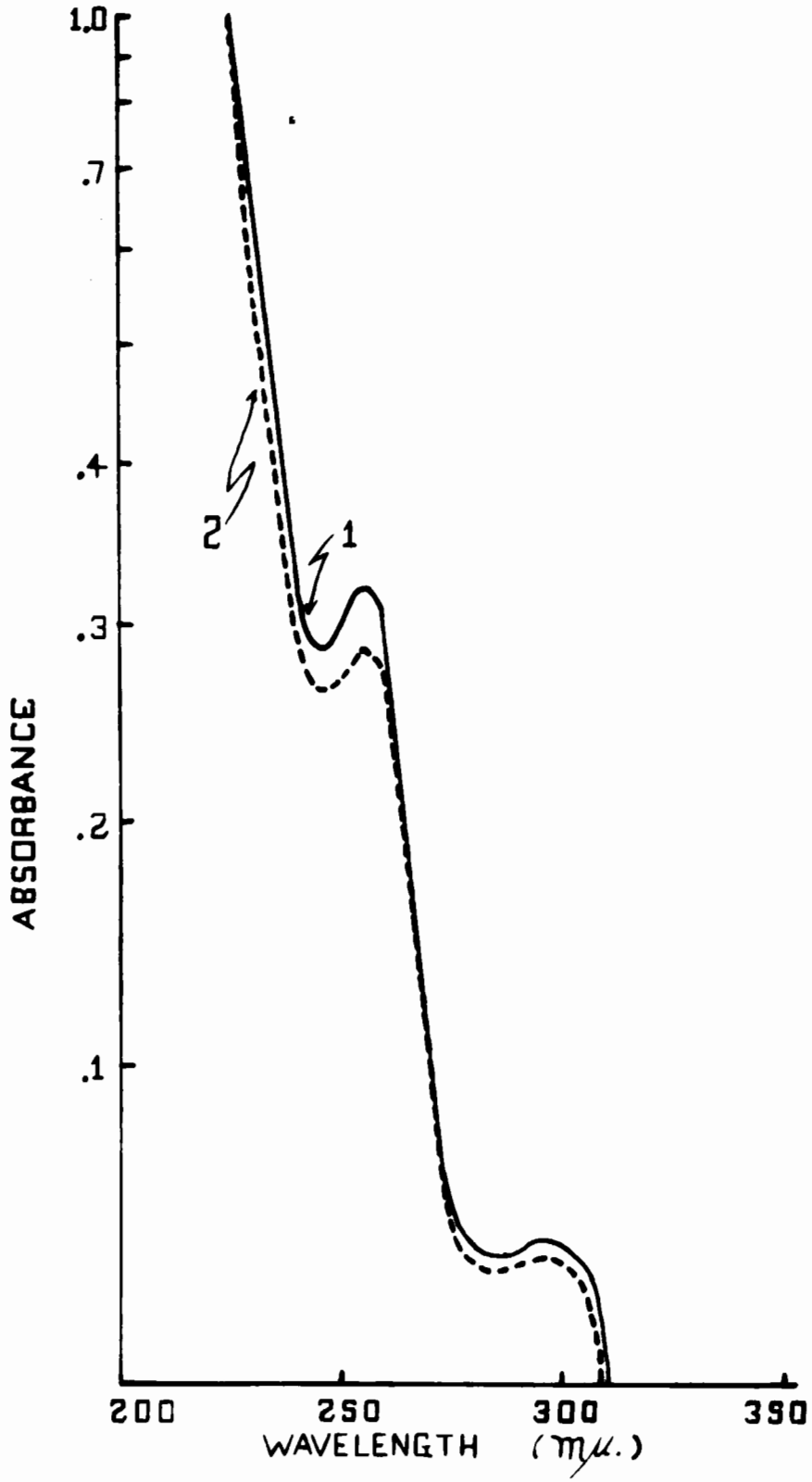


Figure 5. Ultraviolet absorption spectra of (1) 2,4-dihydroxybenzoic acid, and (2) unknown compound number 12.

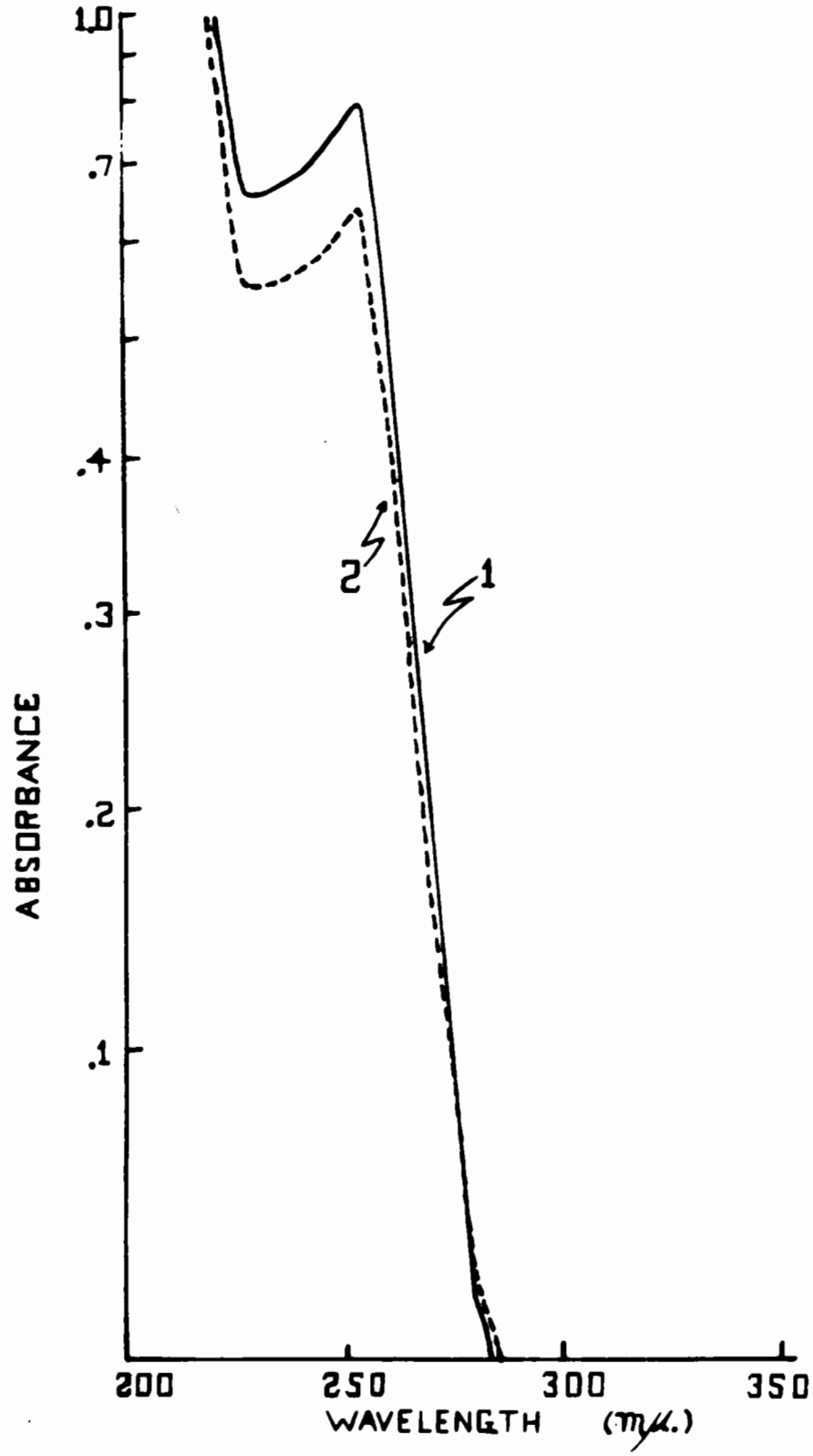


Figure 6. Ultraviolet absorption spectra of
(1) p-hydroxybenzoic acid, and
(2) unknown compound number 11.

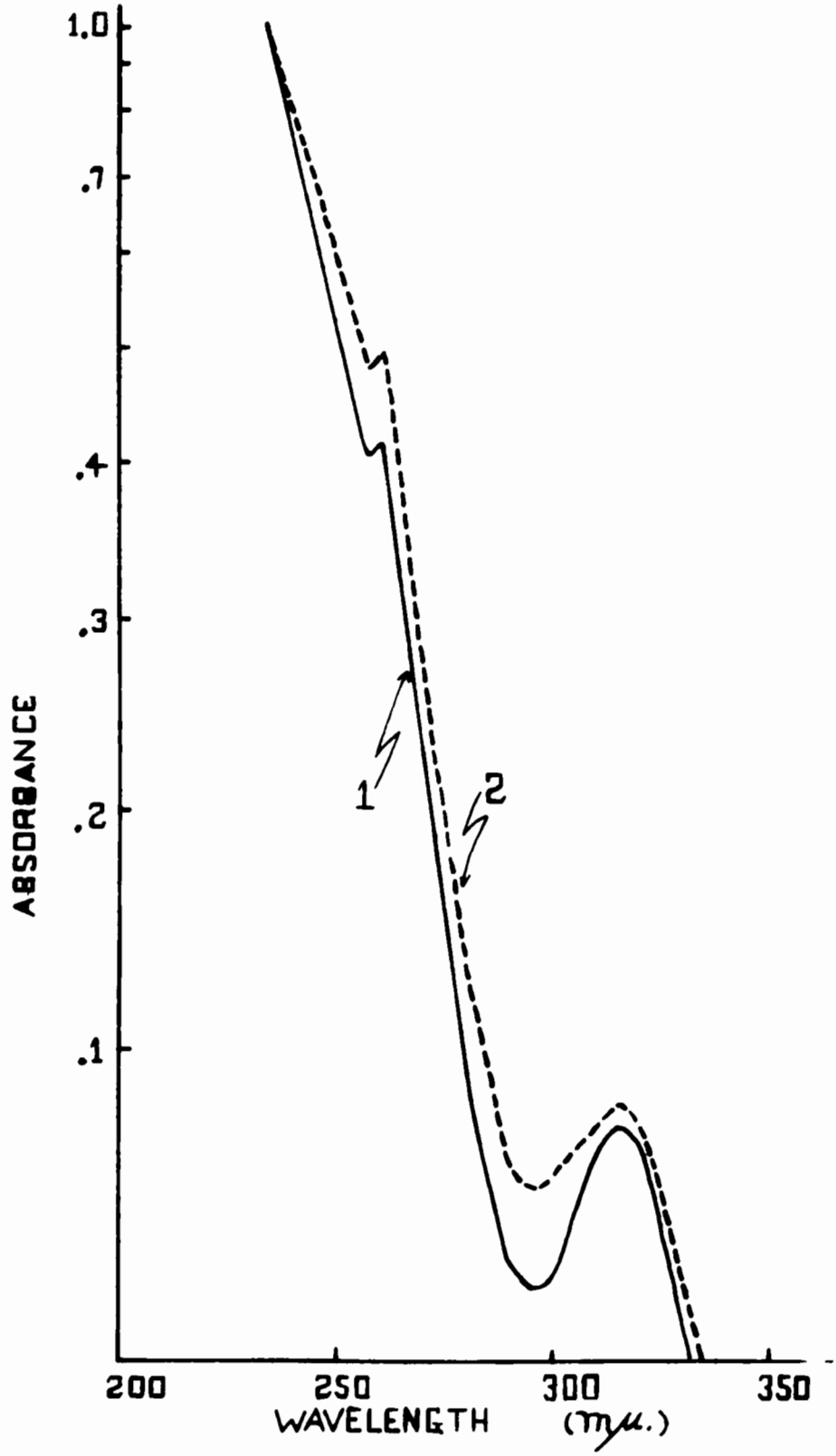


Figure 7. Ultraviolet absorption spectra of (1) 3,5-dihydroxybenzoic acid, and (2) unknown compound number 10.

in Table 7 as substance number 18. It was a non-acidic, steam distillable phenol obtained from fractions A2, A3, and A4. Small amounts of this compound have been obtained by recrystallization from water. After removal of water by filtration, a white crystalline material remained. This material melted at 35-37°C. Attempts to determine the molecular weight of this material cryoscopically failed to give reproducible results when camphor, benzene, and nitrobenzene were employed as the solvents. The values for the molecular weight obtained when these solvents were employed ranged from 200 to 300 with an average of approximately 240. But Gross and Scheurch (61) recently have found that ethylene carbonate is a good cryoscopic solvent for a large number of compounds, and Gross et al (62) found this solvent very useful in the determination of the molecular weights of lignin products. Molecular weight determinations on unknown substance number 18, using ethylene carbonate as the cryoscopic solvent, gave molecular weight values ranging from 130 to 149 with an average of 141.

A search of the available literature has shown that very few phenolic compounds melt in the same temperature range as substance number 18. It would appear that the only phenolic compounds which melt at these low temperatures are substituted phenols such as benzyl and propyl

phenols. The fact that this compound was steam volatile would suggest that it was an ortho substituted phenol. These results indicated that this compound was o-propyl phenol, which has a melting point of 35-37°C. and a molecular weight of 136. However, the positive identification of this compound requires more specific information than has been obtained.

Quantitative estimation of phenolic acids

The identification of the four phenolic acids led to an attempt to estimate these acids quantitatively. Some phenolic compounds have previously been quantitatively estimated, following chromatography, by staining with diazotized p-nitroaniline, eluting the coloured spots from paper with alkaline alcohol solutions, and determining the colour spectrophotometrically (110). In this investigation, the coloured materials were eluted from the paper with a 0.5 per cent solution of potassium hydroxide in ethanol. When lower concentrations of ethanol were employed, the elution of coloured material from the paper was very slow and it was difficult to obtain complete elution. The visible absorption spectra of the coloured coupling products of these four acids with diazotized p-nitroaniline were determined. These visible absorption spectra are shown in Figures 8 and 9. Absorption peaks

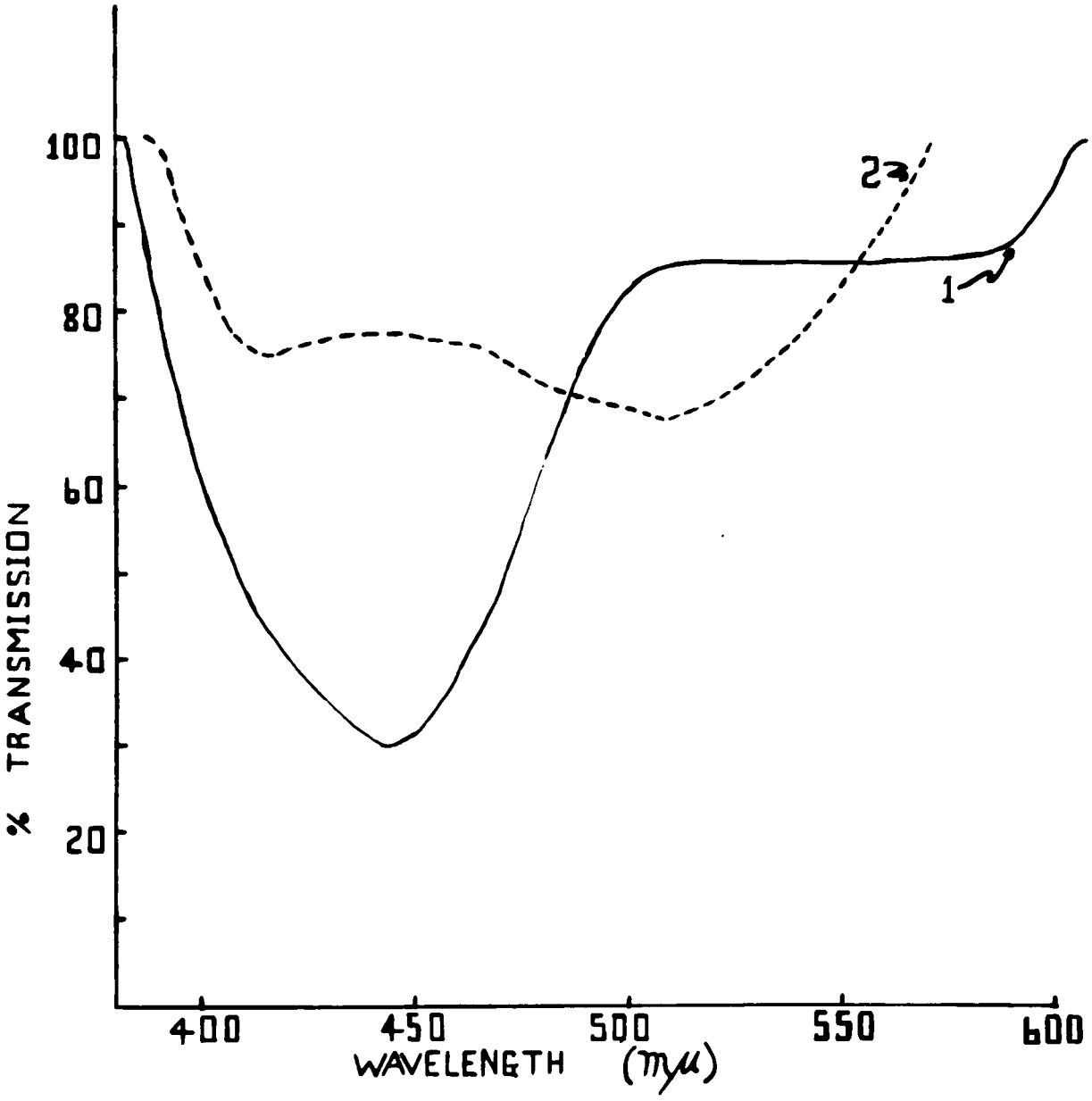


Figure 8. Visible absorption spectra of the coloured products of coupling formed with diazotized p-nitroaniline by (1) 2,4-dihydroxybenzoic acid, and (2) m-hydroxybenzoic acid.

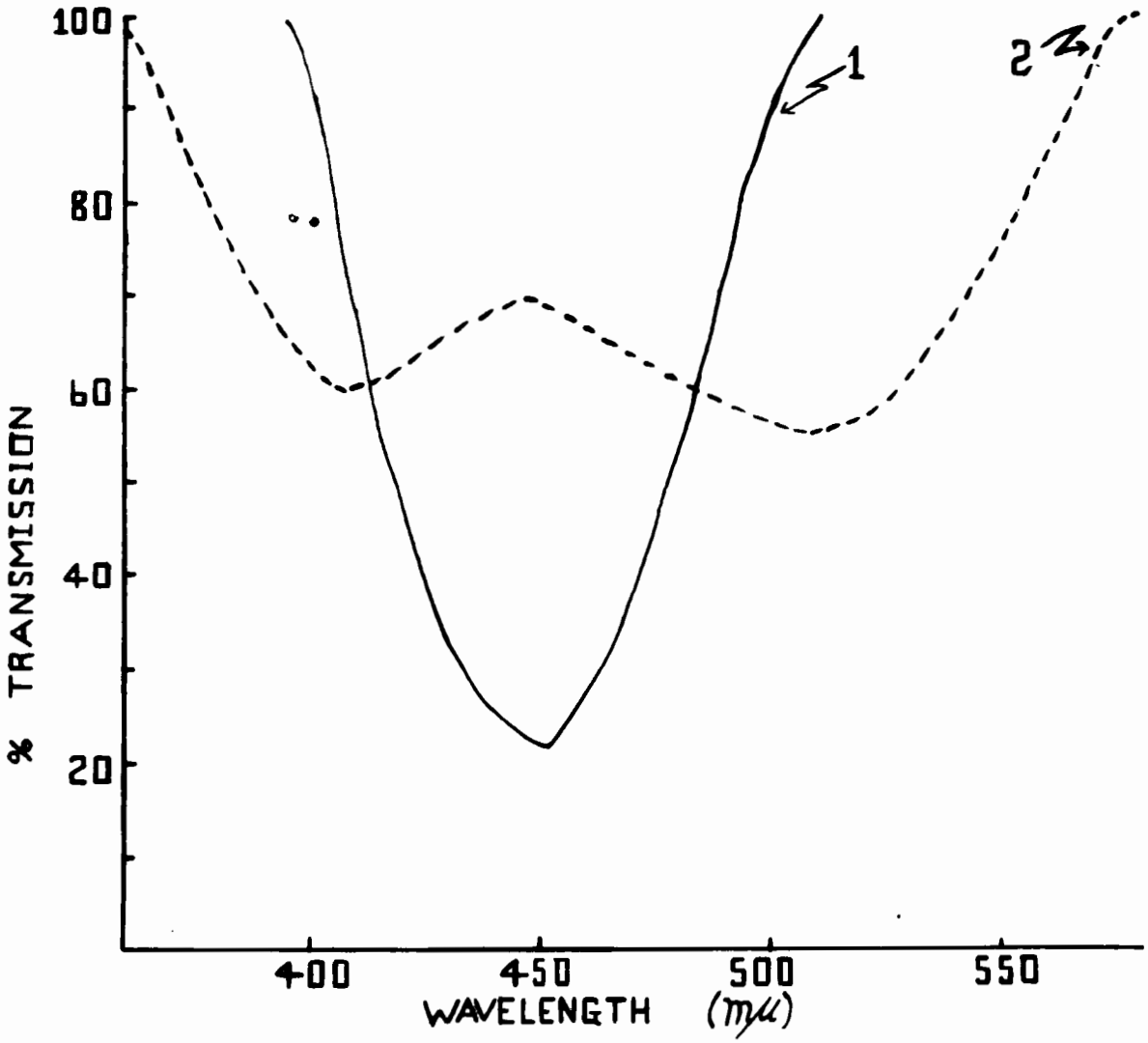


Figure 9. Visible absorption spectra of the coloured products of coupling formed with diazotized p-nitroaniline by (1) 3,5-dihydroxybenzoic acid, and (2) p-hydroxybenzoic acid.

for the coupled products of 2,4-dihydroxybenzoic and 3,5-dihydroxybenzoic acids were found at 443 mμ and 451 mμ respectively. Both m-hydroxybenzoic and p-hydroxybenzoic acids showed two absorption peaks. These were at 416 mμ and 507 mμ for m-hydroxybenzoic acid, and at 408 mμ and 507 mμ in the case of p-hydroxybenzoic acid. After examination of these spectra, the wavelengths selected for an attempted estimation of 3,5-dihydroxybenzoic, 2,4-dihydroxybenzoic, p-hydroxybenzoic, and m-hydroxybenzoic acids were 451 mμ, 443 mμ, 507 mμ, and 507 mμ respectively.

Known amounts (5-15 μgm.) of each of the known acids were chromatographed, stained, eluted from the paper, and the colour of the solutions determined spectrophotometrically. The standard curves prepared from these known amounts of standard acids are shown in Figure 10. From this figure, it can be seen that these solutions all obey Beer's Law over this range of concentrations; that is, the plot of absorbance versus concentration gives a straight line for each of the four acids. This has been found to be true for the two monohydroxybenzoic acids when quantities up to 30 μgm. of these acids were treated in this manner. These results indicated that these four phenolic acids could be estimated quantitatively by this method.

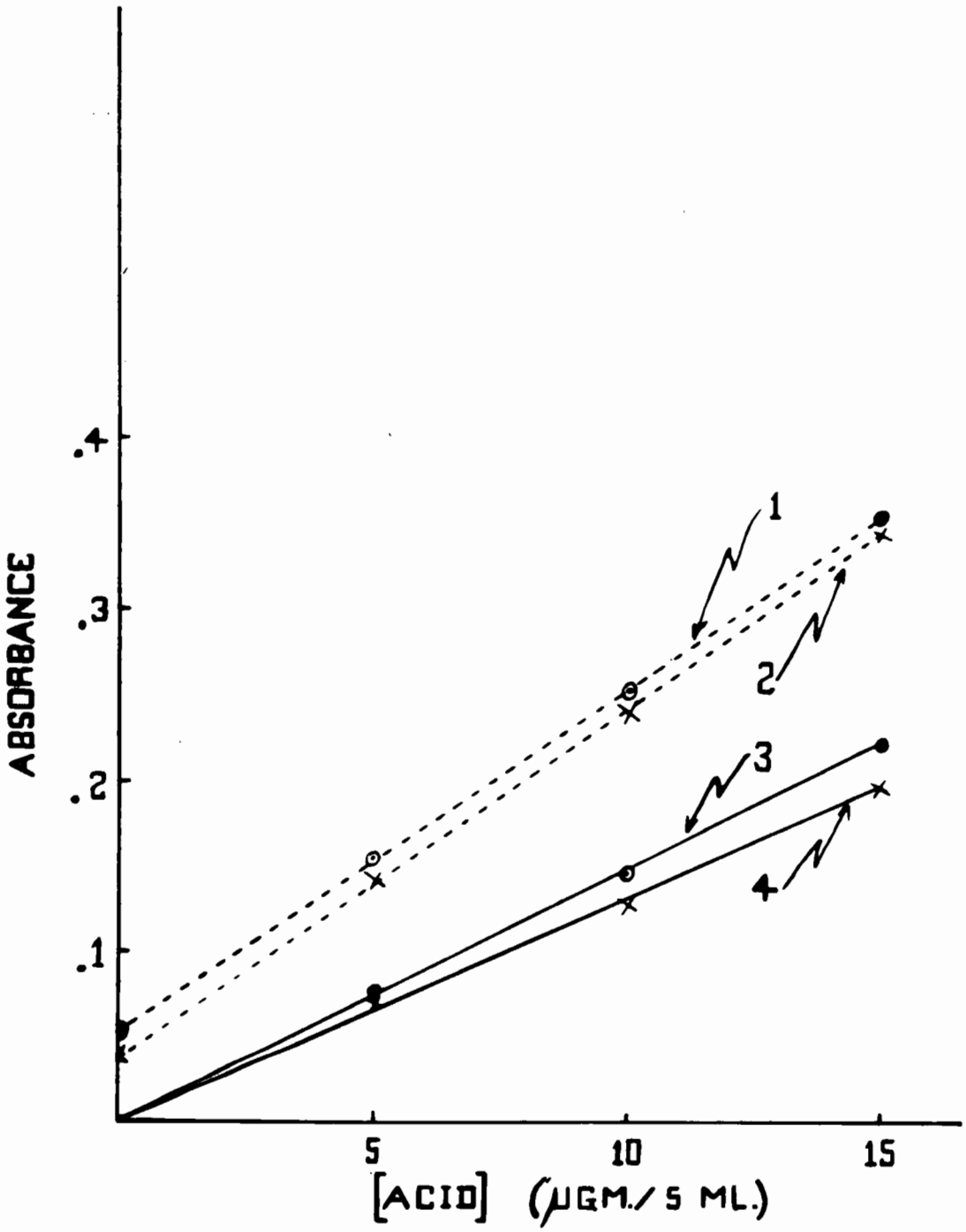


Figure 10. Standard curves for the colorimetric estimation of the products of coupling formed with diazotized p-nitroaniline by (1) 2,4-dihydroxybenzoic acid, (2) 3,5-dihydroxybenzoic acid, (3) p-hydroxybenzoic acid, and (4) m-hydroxybenzoic acid.

The amounts of the four acids present in the products of fusion extracted from acidic solution into ether and removed from ether solution by bicarbonate extraction are shown in Table 11. In this table, the amounts of each of these acids present have been expressed as a percentage of the total solid material obtained from the bicarbonate extract. In all except one of the extracted soil organic matter fractions, these four acids accounted for more than two-thirds of the material obtained from the bicarbonate extract. These acids accounted for only 28.5 per cent of the material obtained from the bicarbonate extract in the case of fraction A4. The amounts of monohydroxybenzoic acids were much larger in all cases than the amounts of dihydroxybenzoic acids obtained from the fractions of the soil organic matter.

When calculated on the basis of the total organic matter content of the soil, the amounts of these four phenolic acids found in the fusion products of the five organic matter fractions were found to be equivalent to 12 per cent of the total organic matter content of the soil. However, these were only four of the 19 phenolic substances which have been separated chromatographically, and although some of these substances appeared to be present in small quantities, others appeared, by their

TABLE 11

The amounts of some phenolic acids obtained after fusion of the fractions of soil organic matter (expressed as per cent of the material obtained from sodium bicarbonate extracts)

Fraction	m-hydroxy-benzoic acid	p-hydroxy-benzoic acid	2,4-dihydroxy-benzoic acid	3,5-dihydroxy-benzoic acid	Total
A2	27.3	35.7	13.4	10.1	86.5
A3	21.1	35.0	10.5	9.3	75.9
A4	9.5	11.9	4.7	2.4	28.5
B1	28.7	28.1	9.6	0.0	66.4
B2	33.6	34.2	10.4	0.0	78.2

staining characteristics, to be present in amounts approaching those of the monohydroxybenzoic acids. The substances, which appeared to be present in large quantities, were those numbered 1, 2, 5, 16, 17, 18, and 19 respectively. From these observations, it would appear that the residues obtained from extracts of the products of fusion of organic matter fractions were mainly phenolic in nature. Since 75 to 80 per cent of the organic matter was removed from this soil, and since the extracted fusion products were equivalent to 40 to 70 per cent of the organic matter of the various fractions, it follows that the phenolic materials obtained after fusion of the organic matter accounted for a very large portion of the organic matter of this soil. Thus, it has been concluded that the organic matter removed from this soil was mainly aromatic in nature.

SUMMARY AND CONCLUSIONS

A method, which employed 8-hydroxyquinoline in a two phase system of water and benzene for the extraction of soil organic matter under nearly neutral conditions, removed 75 to 80 per cent of the organic matter from samples of the B_{21} horizon of a sandy podzol soil. Fifty per cent of the soil organic matter was removed into the benzene phase of the extracting medium, while nearly 30 per cent of the soil organic matter was removed into the aqueous phase. In contrast to the amounts of organic matter removed from the soil by this procedure, only 60 per cent of the organic matter was removed from this soil by three successive 48-hour extractions with 0.5 N sodium hydroxide solution.

The organic materials extracted from this soil were not fractionated by the Forsyth method of fractionation of fulvic acid extracts. Essentially all of the organic matter removed from this soil by extraction with 8-hydroxyquinoline appeared in fraction D of the Forsyth scheme; that is, it was eluted from charcoal by dilute solutions of sodium hydroxide.

By paper chromatography with a 40 per cent propanol solvent, the organic matter removed into the aqueous phase of the extractant has been separated into three distinct

chromatographic fractions. This organic matter has also been separated into three fractions on the basis of the solubility of the products formed upon the addition of lauryl pyridinium chloride. One of these fractions has again been divided into two fractions by precipitation from ethanolic solution. Two of these four fractions coincided with the two mobile chromatographic fractions, while the two remaining fractions comprised the non-mobile chromatographic portion of the organic matter.

The organic matter has been removed from the benzene phase of the extractant by adsorption on cellulose, and separated into two fractions by selective elution from the cellulose. These two fractions had different R_f values, but each one separated as one distinct band, on chromatography in the 40 per cent propanol solvent. It has also been found that these fractions were extracted from the soil at different stages of the extraction, one fraction being extracted during the first three days of extraction, while the other fraction did not appear until the fourth day of extraction.

According to staining reactions following chromatography, those fractions of the organic matter precipitated by lauryl pyridinium chloride were acidic in nature and did not contain free phenolic hydroxyl or aromatic amino

groups having free ortho or para positions available for coupling reactions. The reverse was found to be true for the organic matter fractions obtained from the benzene phase of the extractant. These fractions were not acidic but contained free phenolic hydroxyl and/or aromatic amino groups having free ortho or para positions available for coupling reactions. The amounts of nitrogen found in the fractions from the benzene phase would seem to eliminate the possibility that the reactions with diazotized reagents were due entirely to aromatic amino groups, and thus indicate the presence of phenolic hydroxyl groups in the organic matter of these fractions.

The amounts of nitrogen found in these six fractions were too small for any large portion of the organic matter to be accounted for as protein. This has also been shown by the very small amounts of ninhydrin reactive material obtained from these fractions after acid hydrolysis.

The apparent carbohydrate and uronic acid contents of all fractions were surprisingly low. After acid hydrolysis, sugars and uronic acids could be chromatographically separated and detected in only one small fraction of the organic matter, in which only trace amounts of glucose, galactose, arabinose, xylose, and galacturonic acid were found.

The evolution of hydrogen sulphide gas from acidic solution after alkali fusion of some fractions, and the analytical values obtained for sulphur, with and without prior oxidation with magnesium nitrate, indicated that at least three types of sulphur-containing compounds were present in the different fractions of the organic matter extracted from the soil.

Only one of the six fractions contained a detectable amount of silica, and iron and aluminum could be completely removed from all but one of the fractions.

Acid hydrolysis and aqueous alkaline hydrolysis of the organic matter fractions failed to produce any appreciable amounts of sugars, uronic acids, amino acids, or phenols. It would appear that these treatments failed to degrade the organic matter to any appreciable extent.

A total of 19 phenolic compounds have been separated from the products of alkali fusion of the organic matter fractions by the utilization of extractive and chromatographic procedures. The amounts of materials extracted by ether and ethyl acetate from acidic solutions of the fused organic matter fractions were equivalent to 40 to 70 per cent of the organic matter of the fractions. Major differences, in the phenolic products of fusion, were found between the organic matter fractions from the

benzene phase and those fractions from the aqueous phase of the extractant. Also, differences, in the products of fusion of the different fractions obtained from the aqueous phase of the extractant, were observed. In this respect, no differences were noted between the two fractions obtained from the benzene phase.

Four of these phenolic substances have been separated chromatographically by means of a column of cellulose powder, and obtained in crystalline form. These four compounds have been identified by means of melting point, mixed melting point, chromatographic, and ultraviolet absorption data as m-hydroxybenzoic, p-hydroxybenzoic, 2,4-dihydroxybenzoic, and 3,5-dihydroxybenzoic acids. Another non-acidic steam-distillable phenol has been found to be similar to o-propyl phenol in many of its properties. The presence of o-propyl phenol might be indicative of the presence of lignin-like materials, since the basic structure of lignin is generally believed to be of a C_6 (aromatic) C_3 (aliphatic) nature.

The amounts of the four identified phenolic acids, which were obtained after fusion of five of the six fractions of the extracted soil organic matter, were equivalent to approximately 15 per cent of the organic matter extracted from this soil, or 12 per cent of the

total organic matter content of the soil. Since these were only four of the 19 phenolic compounds, which have been separated, it would appear that a large portion of the soil organic matter is of an aromatic nature.

CONTRIBUTIONS TO KNOWLEDGE

Investigations on the nature of the organic matter removed from the deposition horizon of a podzol soil by a procedure employing 8-hydroxyquinoline in a water: benzene system at pH 6.5 have shown that;

1. The organic matter could not be fractionated by the use of the Forsyth method of fractionation of fulvic acid extracts;
2. fractionation of the organic matter in the aqueous phase of the extractant was achieved chromatographically and also on the basis of the solubility of the products formed with lauryl pyridinium chloride;
3. fractionation of the organic matter in the benzene phase of the extractant was accomplished by a procedure involving selective elution from cellulose powder;
4. evidence was obtained that only a very small portion of the organic matter removed from the soil was carbohydrate and the nitrogen content calculated as protein also was small;
5. evidence was obtained indicative of the presence of three types of sulphur-containing compounds in the organic matter removed from the soil;

6. the organic matter removed into the benzene phase of the extractant was non-acidic and contained free phenolic hydroxyl and/or aromatic amino groups;
7. the organic matter removed into the aqueous phase of the extractant did not contain free phenolic hydroxyl or aromatic amino groups having free ortho or para positions available for coupling reactions. Also most of the organic matter in this phase was acidic;
8. after alkali fusion of the fractions of organic matter, a total of 19 phenolic substances were separated chromatographically and detected;
9. many of the phenolic products of fusion were common to all fractions and there were no differences in this respect between the two fractions obtained from the benzene phase of the extractant. The fractions obtained from the aqueous phase all differed with respect to their products of fusion;
10. four of the phenolic products of fusion were positively identified as p-hydroxybenzoic, m-hydroxybenzoic, 2,4-dihydroxybenzoic and 3,5-dihydroxybenzoic acids;
11. the amounts of these four acids obtained after fusion of the organic matter fractions were equivalent to 12 per cent of the soil organic matter, and semi-quantitative data indicated that a large portion of the soil organic matter was aromatic in nature.

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