THE EXTRACTION AND ELECTROPHORETIC FRACTIONATION OF SOIL HUMIC COMPOUNDS

Verhandeling ingedien ter gedeeltelike
voldoening aan die vereistes vir die
M.Sc.-graad aan die Potchefstroomse
Universiteit vir Christelike Hoër
Onderwys.

J.N. ELOFF
December 1962.

ACKNOWLEDGEMENTS

In the first place I wish to thank Dr. F.W. Pauli who aided me by his advice and example in many ways.

Without the aid and advice of Prof. Dr. J. Tiedt, head of the department of Chemistry at the P.U. for C.H.E., this work could not have been completed.

I am also grateful to the Department of Agriculture and in particular to Dr. J.H. Grobler who made the undertaking of the research project possible.

A grant from the Maize Board made possible the acquisition of the apparatus which was used in the work.

Words are not strong enough to express my gratitude towards my parents whose love and loyalty have always been an inspiration. This work is dedicated to them as a sign of my gratitude.

CONTENTS

I. Literature Review

II. Experimental Procedure

III. Discussion of Results

PART I

LITERATURE REVIEW

CHEMISTRY OF HUMUS

2/.........

INTRODUCTION.

The term "humus" was first used by the Romans to designate soil as a whole. Since that time, however, the term has been used to describe various fractions of the soil. Humus is now regarded as a hetero-polycondensation product derived from organic residues through microbiological and chemical processes in soil. Humus is, unlike the organic matter from which it is derived, fairly stable towards microbial decomposition. It appears to be a metastable form in which organic carbon is accumulated on the earth's surface(1).

The formation of humus

Various materials, such as lignin, carbohydrates and proteins, have been considered as probable starting materials for the synthesis of humus.

Lignin, the encrusting material of plant cell walls, is one of the most complicated and resistant plant materials and, next to cellulose and hemicelluloses, it is one of the most abundant constituents of plant material. It was logical, therefore, to consider lignin to be the most probable starting material for the formation of humus(2).

At one time lignin was generally accepted as a direct starting point for the formation of humus. Thus Waksman(3) formulated the well-known ligno-protein theory, while the lignin- ammonia concept was introduced by Mattson(4).

The idea according to which humus was considered to be a mere denaturated or derived product of original plant materials lost credence when it became clear that the stability of humus is far greater than that of lignin. It was also found that under certain conditions humus-like compounds were formed from plant materials even before lignin

3/.............

was attacked(5, 6).

Various investigators, e.g. Beckley(7, 8), Enders(9), Welte(10) and Marcusson(11, 12), have shown that humus could be formed from different starting materials. This disproves the view of lignin's being the sole source of humus.

A number of investigators has tried to synthesize artificial humic compounds. Eller(13) and his co-workers were the first who tried to explain the constitution of humic acids by synthesis of artificial humic acids from polyphenols. They considered humic acids to be polymers of quinones. Erdtmann(14) successfully synthesized humic acids by treatment of quinone with alkali.

A number of investigators used other means to synthesize artificial humic compounds. Welte(10) used carbohydrates whereas Enders(9) indicated the probable role played by amino-acids in the synthesis of humic compounds. Investigators such as Laatsch(15), Flaig(16), and Lindeberg(17) have shown the importance of metabolic and autolytic products of micro-organisms for the formation of humus.

From the work of investigators such as Flaig(18), and Ploetz(19) it can be deduced that microbial decomposition of lignin leads to the formation of phenolic bodies. These phenolic bodies are rapidly oxidised to quinones. The quinones may be polymerised and, when ring splitting takes place, other condensation products, the humic acids, evolve.

In 1958 Pauli(20) applied fluorescent dyestuffs to decomposing plant residues. He found no transition state between completely undecomposed structures and the newly

4/............

formed humic compounds. From his results he deduced that the plant residues were first broken down to small units which were then re-synthesized to high-molecular humic compounds.

The latest theory concerning the formation of humus is that this process takes place in two phases.

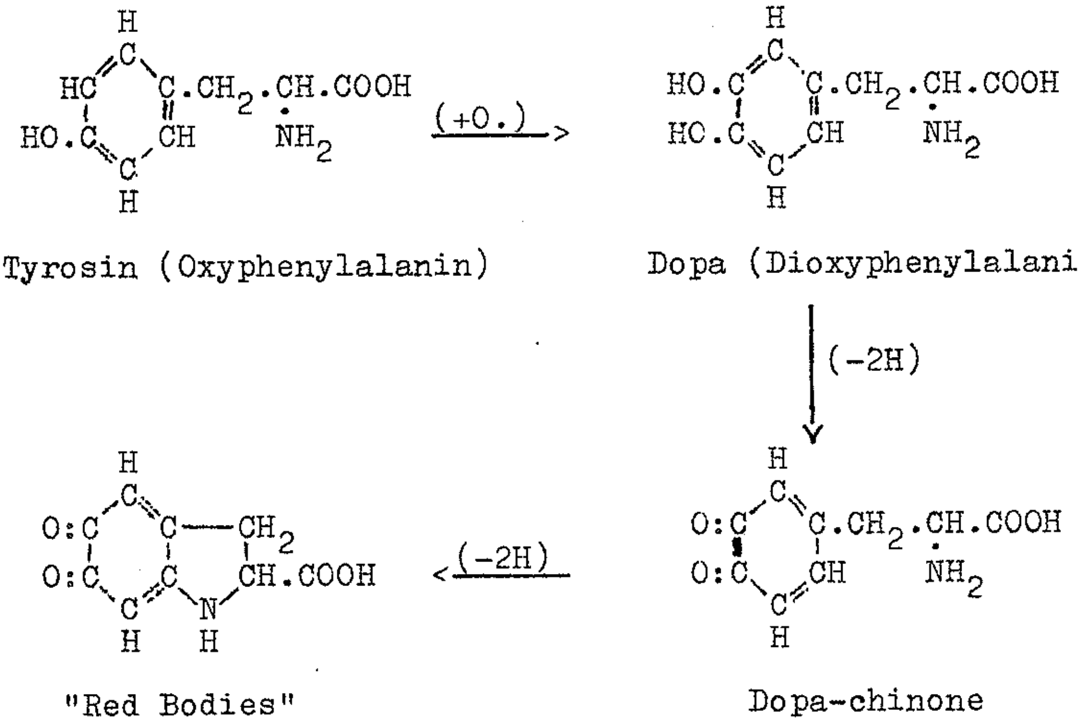
In the first phase the plant and animal residues are broken down to low molecular weight reactive compounds. This breaking-down is caused by the action of various groups of micro-organisms such as the actinomycetes(16) on undecomposed or partly decomposed organic matter in soil.

In the second phase these low molecular weight units (monomers) react upon each other to form macromolecular humic compounds.

Apparently these two phases overlap to a certain extent. After initiation of the second phase the monomers are still being changed under the influence of various enzymes produced by micro-organisms in the soil.

It is during the overlapping of the two phases that elements such as nitrogen are introduced into the monomers. The influence of the enzyme monophenol oxidase, produced by numerous actinomycetes, moulds and bacteria, on tyrosin, may be taken as an example(21). (See next page).

The monomers mentioned above are usually considered to be of a polyphenolic nature. These polyphenols polymerise and combine with nitrogenous compounds. It is generally accepted that most of these nitrogenous components arise in microbial protoplasm. It is not yet certain whether polyphenols undergo condensation with peptides and proteins in their original form or whether condensation takes place after autolytic decomposition of peptides or proteins to amino acids. Swaby[22] found that amino acids combined with polyphenols to form humic compounds.



Not only does polymerisation take place, but, as various metabolic by-products appear simultaneously, polycondensation between the numerous monomers and the by-products also have to be taken into consideration.

Humic substances are formed when polyphenolic compounds react with derivatives of peptides and they can therefore be considered to be heteropolycondensates. Consequently the composition and properties of the newly formed condensates depend to a large extent on the relative concentrations of the reacting compounds present. These condensation reactions are reversible, and in order to obtain a high yield of complex high-molecular condensation products the low-molecular by-products, e.g. water, have to be removed.

The removal of water is of the greatest importance for the formation of humus in soil. Dry periods offer the most favourable conditions for the removal of water, and therefore favour the formation of complex humic compounds. Only smaller humic compounds are formed in the presence of excess moisture, e.g. in water-logged soils.

This observation of the conditions and mechanism of humus formation leads to the conclusion that high molecular compounds in soil can be represented as a polymeric system varying in its chemical composition and in the size of its molecules. The physico-chemical characteristics of humus, such as solubility and electrolyte sensitivity, are directly affected by these variants.

Moreover from the theoretical considerations concerning the formation mechanism of polycondensates, it may be assumed that the humic compounds of soil represent a polydisperse system consisting of a large variety of molecules of differing size. Measurements of the optical density, sedimentation rate in the ultra-centrifuge and adsorption rate in column chromatography of these polycondensates support this conclusion.

Importance of humus for soil fertility

Although humus is not usually considered to be a plant food in the same sense as fertilisers are, it is nevertheless an essential requirement for plant growth.

Even though only a small percentage of humus may be present in a soil it has an immense influence on soil fertility. The following facts illustrate the importance of humus in soil fertility:-

1. Humus improves soil structure. This involves improvement of soil aeration and drainage as well as an increase of the water-holding capacity.
2. All plant nutrients, with the exception of nitrates and

sulphates, are strongly adsorbed by humus and thus protected against leaching(23).

3. The buffer capacity of soil is greatly increased by the addition of humus.

4. When humus is added to soil the exchange capacity of the soil is also increased.

5. Humus acts as a stabiliser of the organic life on earth by removing various elements necessary for plant growth when such elements are present in excessive amounts and by making these elements available when required.

6. Under certain conditions humus also increases the availability of nutrients such as inorganic phosphorus, manganese and copper.

7. Humus also plays an important part in the mobilisation of a large number of inorganic nutrients.

The breakdown of humus by micro-organisms liberates carbon dioxide. Carbon dioxide reacts with water to form carbonic acid. Various other organic acids, such as butyric, acetic and oxalic acids, are also set free. These acids then react with insoluble compounds such as phosphates, calcium and magnesium carbonates, and minerals such as kaolin and felspar. By means of these reactions a large number of nutrients is made soluble and therefore becomes available to the plant.

8. Humus also probably plays an important part in the physiology of the plant because investigations seem to indicate that humic compounds increase the permeability of the protoplasm of higher plants(24, 25, 26, 27), thereby enabling the plants to take up nutrients more quickly(28).

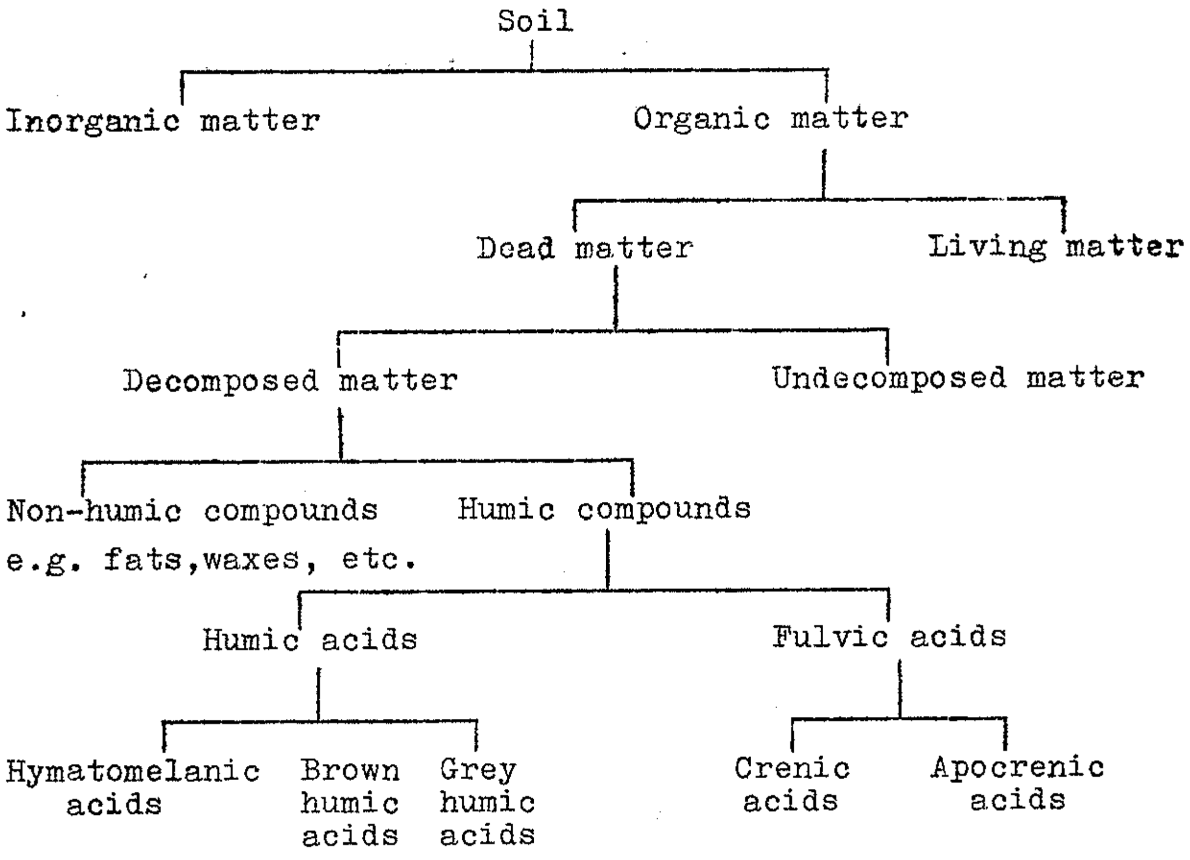
Humic compounds exert an influence on the metabolism of the plant and also stimulate the plant-enzyme sys-

8/............

tems(29, 30, 31, 32). This stimulation may be explained by the fact that humus contains a number of quinone units. These quinone units may participate in oxidation-reduction cycles which influence the respiration of the plant(33, 34).

HUMIC COMPOUNDS

Classification of various components of soil.



The main criticism which can be levelled against the above classification is that it is a purely synthetic system, since it has been shown that hymatomelanic, brown humic and grey humic acids differ mainly in their degrees of polymerisation(35).

A modern classification should therefore also take into consideration factors such as the origin, structure, etc. of the humic compounds.

9/.............

Fulvic acids

Oden(36) was the first to use the term "fulvic acids" to indicate the yellowish substances isolated from peat-waters. Tiurin and Gutkina(37) considered these compounds to be identical to the "fountain acid" isolated by Berzelius(38).

Dried fulvic acids are amorphous substances readily soluble in water, acetone and ethanol. In the presence of air the dried fulvic acids as well as their aqueous solutions slowly darken in colour until finally, in the case of the dry acids,black tarry products are formed.

Freshly dried fulvic acids give positive Molisch, phloroglucinol and orcinol tests, indicating the presence of sugars and lignin monomeric units. Aqueous solutions of fulvic acids give a red to purple colour with ferric chloride solution, indicating the presence of hydroxyl groups.

Fulvic acids are relatively strong acids. They possess an exchange capacity of 3.0-3.5 milli-equivalents/g., indicating the presence of at least four carboxyl groups per molecule. They are mobile in soil and play an important part in soil formation as a leaching agent, e.g. in podzol formation. Fulvic acids have a weak aggregating power for soil particles.

From the results of elementary analyses, ultraviolet, visible and infra-red absorption spectra it has been found that the chemical constitution of fulvic acid is constant, irrespective of the difference in the degree of humification of the corresponding humic acids. In fulvic acids the presence of aromatic nuclei, similar to those in humic acids, could be confirmed by infra-red spectroscopy.

The fulvic acids contain methylene groups and a large number of side chains with an aliphatic character. Aromatic -CH and -C=C- groups are found to be present in the molecule. The -C=C- groups in conjunction with the carbonyl groups present in the molecule may be considered to be responsible for the colour of these substances.

The abovementioned facts are in complete agreement with the physico-chemical properties of these compounds. These yellow substances are highly dispersed and practically non-precipitable. Their optical density is low and it is assumed that they can be transformed into humic acids through a process of condensation and polymerisation.

Hymatomelanic acids

Hoppe-Seyler(39) used the word hymatomelanic acid for the compounds which were extracted when "raw" humic acids were treated with warm 80-96% alcohol.

On removal of the alcohol a brittle mass was left behind. This mass could be dissolved by warming with water, but it then lost its alcohol-solubility. The hymatomelanic acids are predominantly present in rotted wood and poorly decomposed stable manure. Usually these acids possess a brown colour which indicates a higher degree of condensation and polymerisation than that found in fulvic acids.

The chemical composition and colour intensity of hymatomelanic acids indicate that they are probably simplified forms of humic acids. Shmuck(40, 41) did not regard them as special compounds, Whereas Fuchs(42) proclaimed them to be oxidation products of humic acids (dehydro-humic acids).

Consequently the question arises whether the hymatomelanic acids should be differentiated from other humic substances.

Humic acids

The composition of humic acids, the substances which are precipitated from alkaline soil extracts on acidification, is not constant. This fact, however, does not mean that humic acids are an indeterminate mixture of compounds. On the contrary, the small fluctuation in the elementary composition of humic acids isolated from various soils, indicates that organic molecules of the same type are present in varying proportions during the formation of these substances.

The dark brown to black colour of humic acids is an indication of their highly condensed state. By means of x-ray diffraction their lattice layers are found to be typical for aromatic compounds. The aromatic nature of humic acids could also be confirmed by means of infra-red spectroscopy(43, 44).

The humic compounds belong to the group of natural high-molecular compounds, e.g. starch, lignin and cellulose. Electron microscopy has revealed their considerable size, e.g. a diameter of 60-100 A for humic acids isolated from black earth(45).

The chemical composition of the humic acids explains their physico-chemical properties, e.g. exchange capacity, and also their role in the soil as well as in plant nutrition.

Springer(46) showed that there are two distinct types of "true" (insoluble in acetyl bromide) humic acids which are characterised by the difference in colour of their alkaline solutions. Humic acids from peat, brown coal, podzolic and other acid soils yield brown solutions, while those from neutral chernozems, rendzinas and fen soils yield grey-brown to black solutions. He named the two types

12/.........

brown and grey humic acid respectively.

Brown humic acid may occur by itself, but grey humic acid is always associated with small amounts of brown humic acid. The two types may be partially separated owing to the greater sensitivity of grey humic acid to electrolytes. It is generally accepted that intermediate types occur in addition to the two main types. Meyer(47) was able to separate a mixture of grey and brown humic acids by dissolving the brown humic acid with boiling concentrated sodium hydroxide and the grey component with dilute sodium hydroxide.

Grey humic acid is associated with biologically active, fertile soil types, especially chernozems and cultivated manured soils. It has a high sorptive power and occurs in conjunction with clay minerals.

According to Springer(46) grey humic acid is characterised by a much higher nitrogen content than brown humic acid, but Meyer(47) has found that this does not always apply. No clear-cut chemical distinction can be made between the two fractions and physically the sharpest distinction to be observed is in the colour of their alkaline extracts.

The humins

Sprengel(48, 49) considered the group of humic acids which could not be extracted from soils by alkaline media to be substances intermediate between humic acids and coal. He therefore coined the word "humus coal" to indicate these compounds. Later this name was replaced by humin(38).

Fuchs(42) regarded these compounds as pre-stages of coal. He considers that intra-molecular dehydrogenation, condensation and polymerisation reactions taking place in the humic acid molecule have diminished the number of functional groups in the molecule.

The latest results(35), however, indicate that humins are humic acids which are strongly sorbed onto mineral particles. The strength of this sorption depends mainly on the nature of the minerals present in the soil.

The combination of humates with the clay minerals of the montmorrilonite group, e.g. askanite and gumbrin is especially strong. The humic compounds are most probably fixed between the lattice layers of the clay mineral(35). Kaolinite, orthoclase, felspar, etc. adsorb only on the outer surface; therefore weak alkaline solutions can break this binding.

Not only the nature of the adsorbing bases and the dispersion of the minerals, but also the nature of the humic acids plays an important part in this very intricate adsorption phenomenon. The size of the humic acids is of particular importance, because the smaller low-molecular compounds with diameters of 20 A exhibit the highest activity. This is due to the fact that they can act as "true solutions".

STRUCTURE OF THE HUMIC COMPOUNDS

It has been shown, especially by means of infra-red spectroscopy, that no distinction could in principle be made between the structures of the various humic compounds(45, 46, 50).

The evaluation of the chemistry of humic compounds is made difficult by the unsatisfactory methods of extraction, purification and fractionation in use at the present

14/........

moment. The fact that these compounds do not crystallize and that their melting points cannot be determined also complicates matters.

Thiele and Kettner(51) showed that humic compounds consist of polyones which are built up of monones. Monones are synthesized from smaller units which consist of nuclei, various bridge types and reactive groups. (See plate 1 and fig. 2). The polyones exibit a tendency to conglomerate to form micelles.

The nuclei may be iso- or heterocyclic five or six ring compounds, but condensed structures are also possible. From degradation and other decomposition products which have been isolated, the following were found to be involved in the structure of humic compounds: derivatives of benzene, naphtalene, anthracene, furan, pyrrole, indole, pyridine, and chinolin. (See plate 1).

Mild oxidation of humic acids led to the formation of compounds with a fulvic acid character(52, 53). The reduction of humic acids with aluminium or sodium amalgam produces colourless products(54). In the presence of alkali these colourless products are again converted to humic acids.

(a) Bridge systems

Oxygen bridges.

There has been a certain amount of speculation on the existence of ether bridges between aromatic nuclei(55), but Ziechmann(44) finally provided proof of the presence of oxygen bridges with the aid of infra-red spectroscopy. Normally diphenyl ether bridges are relatively stable, but when new groups are introduced into the aromatic nucleus or when the conjugated double bond system is

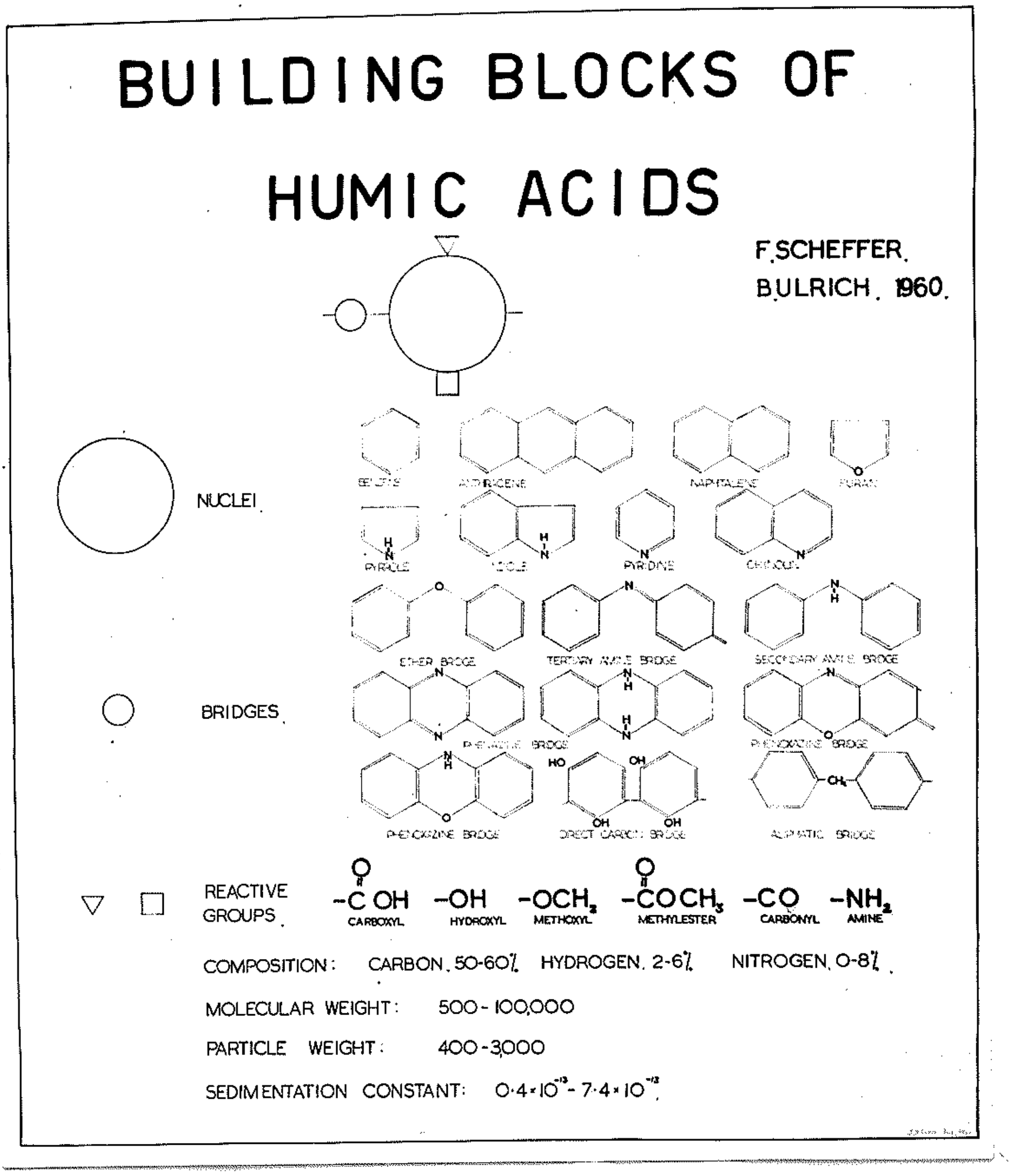
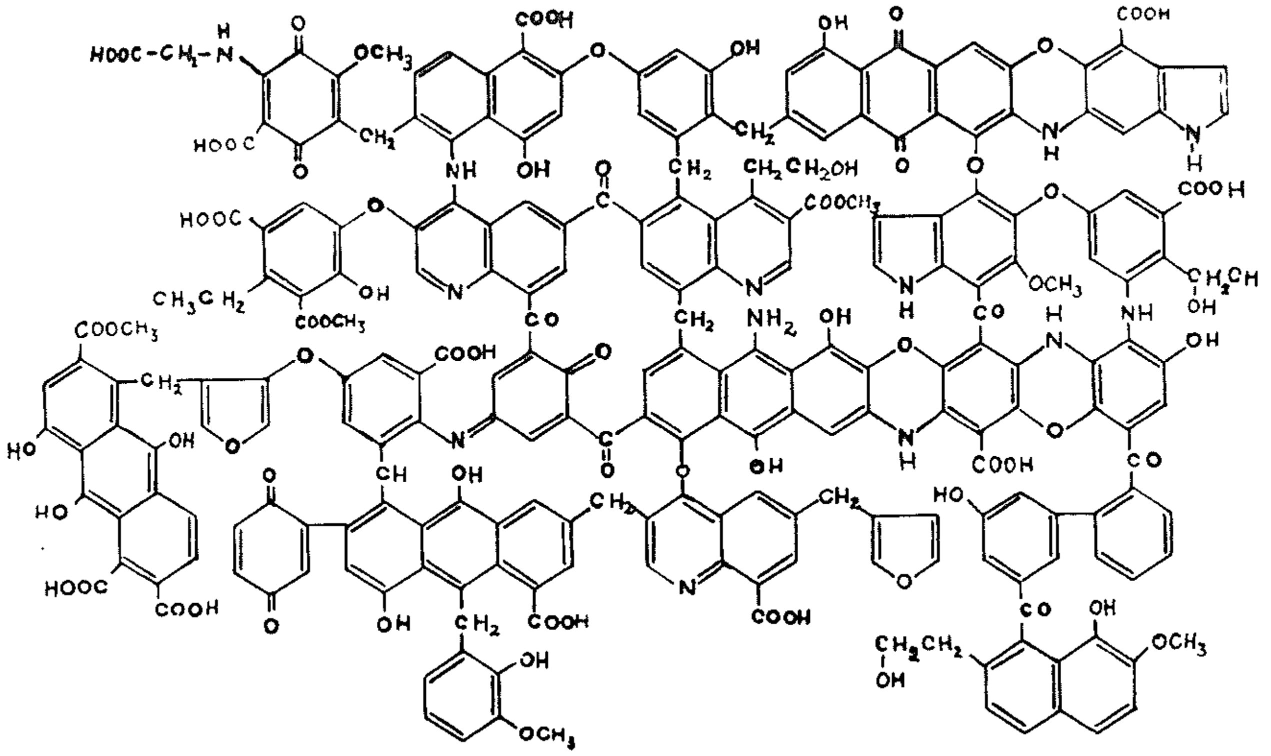
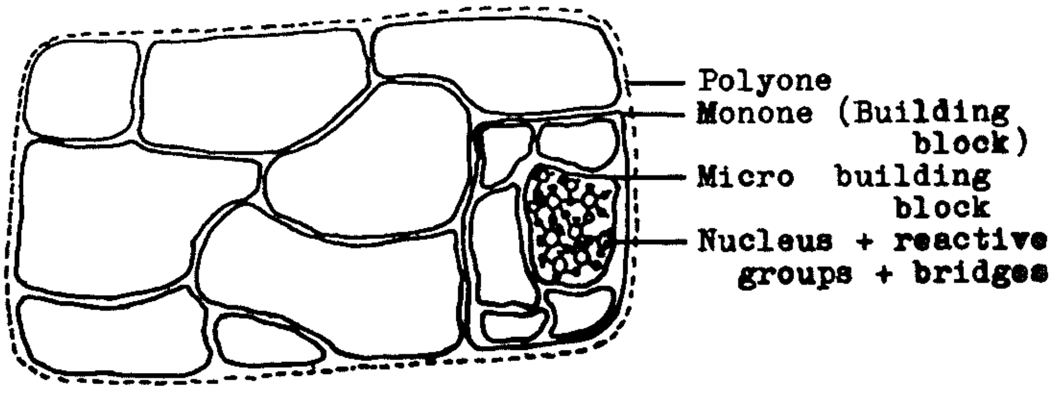


Plate 1. BUILDING BLOCKS OF HUMIC ACIDS



A HYPOTHETICAL HUMIC COMPOUND



SCHEMATIC PRESENTATION OF A HUMIC COLLOID

Fig. 2 BUILDING PRINCIPLE OF HUMIC COMPOUNDS disturbed, the ether bridge becomes unstable towards alkaline reagents. Bridges between quinone rings are also very reactive.

Nitrogen bridges:

Aminophenols and aminoquinones have a strong tendency to polymerise by forming nitrogen bridges. The nitrogen may be present in secondary or tertiary form in an open or closed system (see fig. 2). Phenoxazin systems may also be of importance as they are present in so many natural products. In quite a number of phenoxazin structures the ether bridge may be opened in alkaline and closed in acid solutions.

Aliphatic bridges:

When phenols and formaldehyde condense, methylene bridges are formed. According to Thiele(51) this process may result in the formation of alkali-soluble humic compounds. Flaig(56) and Ploetz(19) showed that aliphatic bridges could be formed when ring splitting of direct nucleus-bound humic compounds takes place. The presence of aliphatic methylene bridges could be detected by means of infra-red spectroscopy(44).

Directly bound nuclei:

According to Erdtman(14) the nuclei of quinone humic acids may be connected directly by carbon-carbon linkages. This type of bridge is present in melanin.

Other possible bridge types:

Heterocyclic five-ring compounds may also be synthesized, e.g. in the formation of tannin and lignin. In the case of dehydro-coniferyl alcohol a substituted dihydro-furan is formed. This clearly indicates the possibility of new five or six ring heterocyclic compounds being produced and that direct binding of such nuclei may result. The stability of the humic compounds may therefore increase when the direct inter-connection of nuclei increases.

(b) Reactive groups

Acidic groups:

The humic acids have an acidic character that decreases on further polymerisation. Phenolic hydroxyl and carboxyl groups are the anionic reactive groups.

Various methods for the determination of phenolic hydroxyl and carboxyl groups have been applied. Methylation with dimethyl sulphate at a high pH and with diazomethane makes possible the estimation of the phenolic hydroxyl and the carboxyl groups(57, 58). It has been shown, however that humic acids differ markedly with regard to their carboxyl group content. Welte, et al(59) found that hydroquinone humic acids contain no carboxyl groups. It was also found that humic compounds reacted polybasically with apparent dissociation constants between 10^{-4} and 10^{-9}.

Carbonyl groups:

The presence of carbonyl groups in humic compounds can be shown by infra-red spectroscopy(44). None of the analytical methods available give reproduceable values for quinone carbonyl groups. This may most probably be ascribed to steric hindrance or to secondary bindings due to chelation. It has not as yet been proved that hydroxylamine and phenylhydrazine react with all the

19/...........

carbonyl groups of a humic compound.

Methoxyl groups:

The methoxyl group content of humic acids derived from dung and compost varies from nil to as much as 8%(61). Kosaka and Honda(62) found an inverted correlation between methoxyl content and degree of humification. The presence of methoxyl groups in the nucleus retards the ease of reaction and also diminishes the degree of cross-linking. Methoxyl groups are therefore found mainly in hymatomelanic and brown humic acids. According to Springer(60) grey humic acids normally contain no methoxyl groups. The methoxyl groups attached to quinone nuclei are relatively easily demethylated in an alkaline medium, with the result that methoxyl groups are converted to hydroxyl groups and polymerisation reactions become possible.

Amino groups:

The amino-nitrogen content of natural humic compounds may be as high as 70% of the total nitrogen present.

Groups which contain amino-nitrogen are very sensitive to hydrolysis and a considerable part of these groups may be removed by the solvent used in the extraction process(63). A low amino-nitrogen content is usually associated with a low humin-nitrogen content in humic acids. The presence of amino-nitrogen may be ascribed to the coupling of protein particles such as amino acids or peptides with humic compounds.

(c) <u>Elementary analyses</u>

The elementary analyses found in the literature do not allow any valid conclusion to be drawn regarding the

20/...........

elementary composition of humic compounds because mixtures of the various humic substances, sometimes including even non-humic substances, have usually been investigated.

The percentage of carbon in the humic compounds increases with an increasing degree of polymerisation while at the same time the oxygen content is reduced. The hydrogen is usually found to be between 4 and 5%, but is seldom as low as 3% or as high as 6%. There are samples of soil humic acids and also of synthetic humic acids where values lower than 3% have been found. A low hydrogen content and a high carbon to nitrogen ratio indicate a high degree of condensation(43). Nearly all soil humic compounds contain a low percentage of nitrogen, but synthetic humic compounds without any nitrogen could be prepared.

(d) Structure and particle weights of humic compounds

It is generally accepted that humic compounds consist of three-dimensionally bound aromatic structures. Beutelspacher(64) showed by means of viscometric measurements that humic compounds are globular and therefore belong to the spherocolloids.

X-ray analysis:

Since 1938 numerous investigators have tried to use x-ray analysis for the determination of the structure of humic compounds. Jodl(65-68) calculated from interference data that the height of a crystal of a brown coal humic acid was 8 A and the diameter 25 A. He also deduced that neither water molecules nor calcium ions are present interlaminally(69).

It was afterwards shown that a 2% contamination of mineral substances may lead to the formation of interference rings(70). When electron diffraction methods were applied to natural humic acids no crystalline structure could be determined(45).

Jung(71) suggested that a small amount of crystalline carbon (for black earths up to 3% and for brown earths up to 1% of the organic substances) could be present in the amorphous to mesomorphous humic compounds. By measuring the electrical conductivity of charred humic compounds at different temperatures, it could be proved that the greater part of the humic compound is of an amorphous and not a crystalline nature. To explain the presence of crystalline sections in humic compounds Jung(71) suggested that graphite crystals might be present on the surface of the humic compounds owing to the decomposition of less stable humic compounds adsorbed on the surface of the more stable humic compound.

Strength of bonds joining the various structures:

Because humic acids are three-dimensional substances consisting mainly of spatially non-directed aromatic structures, it follows that in the smallest units still retaining a humic compound character, the binding must be by means of primary valence bindings. In the smaller units additional or secondary bindings may also be encountered. Hydrogen bonds, for example, may be formed between phenolic hydroxyl and quinone carbonyl groups when they are close enough to each other. Such an intermolecular chelate binding must influence the reactions of the humic acids(19).

According to Ziechmann(44) infra-red spectroscopy indicates both internal and external association between hydrogen and oxygen or nitrogen atoms. In the case of high

22/.........

EXTRACTION OF HUMUS

INTRODUCTION

One of the difficulties encountered in the study of humus is that of separating the organic complexes from the inorganic constituents of soil. In studies on the mineral constituents of soil it was found to be relatively easy to eliminate the organic matter by mild oxidation without altering the structure of the mineral colloids.

All previous work done on humus was performed on extracts which represented only a small part of the total organic matter present in soil and it does not, therefore, allow a satisfactory interpretation of the nature of the total organic complexes in the soil.

INSOLUBILITY OF HUMUS

The reason why humus is so difficult to extract is most probably the fact that it forms insoluble complexes with clay minerals and also with salts of certain metals, i.e. calcium, aluminium and iron.

It is still not clear in what manner the clay and organic matter are held together(106). Di- and trivalent cations may hold the two complexes together by the formation of salt bridges between the clay and functional groups of the humic compounds. According to Brydon(107) there is a possibility of amino groups being involved in the union between organic and inorganic matter. It is also possible that humic compounds may be adsorbed via reactive groups onto the cation and anion exchange locations of clay minerals. The humic substances may actually enter the crystal-lattice of the clay. Binding by means of hydrogen bonds and by van der Waals forces probably also take place, but this would not have a large effect.

33/.........

There is strong, although not conclusive, evidence of the presence of an interaction between humic compounds and polyvalent metals(108). This interaction renders the humus insoluble in water and in neutral solvents which do not form complexes with the metals involved.

There are two possible methods for the extraction of humic compounds. In the first instance the mineral matter may be removed from the humus and in the second case the humus may be removed as completely as possible from the mineral matter.

REMOVAL OF THE MINERAL MATTER FROM THE ORGANIC MATTER

In 1917 Rather (109) published a method for the determination of organic matter in soils based on the removal of hydrated clay mineral matter by treatment with a mixture of dilute hydrochloric and hydrofluoric acids prior to determination of percentage loss on ignition. The acid treatment dissolved only 4% of the organic matter, but it removed the chemically reactive inorganic material.

This pretreatment of soils may, however, quite easily remove a considerable amount of organic matter from certain soils. Ponomareva(110) found that cold hydrochloric acid dissolved a large amount of fulvic acids from certain podzolic tundra soils, but that it dissolved very little from forest and chernozem soils. Arnold, _et al_(111), found that the hydrofluoric acid used to remove silicon oxide also decomposed the humic acids present.

Bremner and Harada(112) found that practically the same amount of organic matter was dissolved from the soil by the use of 0.5 N sodium hydroxide and by pyrophosphate solutions before and after hydrofluoric acid treatment.

34/........

It is therefore clear that the removal of mineral matter from organic matter is not necessarily a very promising approach since large amounts of organic matter can be removed without increasing the efficiency of extraction. In some soils this approach may be used, because very little organic matter is dissolved by the inorganic extractants (109), although even in these cases the structure of the organic matter may be altered to some extent.

REMOVAL OF THE ORGANIC MATTER FROM THE INORGANIC CONSTITUENTS OF THE SOIL

a. Use of strong alkalies

Treatment of soil with varying concentrations of cold dilute sodium hydroxide in order to dissolve humic compounds (113) has remained the most general, and quantitatively the most effective, method for the extraction of humic compounds from soil.

Ostwald and Rödiger (77) determined the solubility of humic acid in sodium hydroxide solutions of different concentrations. They found that when the concentration of the alkali was between 0.025 and 0.001 N, peptisation of the humic acid occured in addition to chemical solution owing to the formation of soluble sodium humate.

Dragunova(114) used potassium hydroxide to dissolve humic acids. She found that the addition of compounds such as glycol, glycerol, formamide, methylformamide, urea and thiourea increases the solubility of humic acids in aqueous solutions significantly. She attributed this effect to the fact that these compounds cleave the humic acid aggregates by rupturing the hydrogen bonds that hold these aggregates together.

Although sodium hydroxide dissolves such a large part of the organic matter it is unsuitable if a thorough chemical examination of soil humic compounds is to be made because of the following:-

(i) If the chemical composition of humic substances is kept in mind, it is clear that oxidation will readily take place. It has been known for some time that soil-alkali mixtures absorb oxygen and produce carbon dioxide by oxidation of part of the organic matter (115). The uptake of oxygen and release of carbon dioxide increase with the alkalinity of the solution(82, 116). This phenomenon is due to the fact that auto-oxidation of the organic matter takes place when the alkaline solution comes into contact with air.

(ii) Not only does auto-oxidation take place during alkaline extraction(82), but other chemical changes involving condensation reactions between amino and aldehyde or phenolic groups may lead to the formation of humin type compounds through browning reactions. Hydrolysis of amino acids and sugar polymers may also occur.

(iii) Alkaline extraction contaminates the humic compound extract by dissolving protoplasmic and other components from fresh organic material, as well as silica from the mineral matter present in soil.

Choudri and Stevenson(117) suggested that stannous chloride be added to the sodium hydroxide -soil suspension. They found that an alkali-stannite solution takes up oxygen because the stannous chloride is oxidised by the air. Thus if extraction is carried out in airtight flasks, the alkaline stannous chloride solution effectively removes oxygen.

b. Use of weak alkalies

Solutions of ammoniumhydroxide (118) and sodium carbonate (119) were also used for the extraction of organic materials from soil.

Not only are the weak alkalies much less effective than the strong alkalies in extracting humus, but they are also subject to the same objections applicable to strong alkaline extractants. In addition ammoniacal solutions give side reactions with lignin and other phenolic or quinonoid compounds and are therefore unsuitable for the extraction of humic substances(120).

c. Use of "complexing" salts

Polyvalent ions, especially calcium, iron and magnesium form insoluble complexes with organic matter. If a reagent which forms insoluble precipitates or soluble complexes with these polyvalent metals are added to the insoluble soil complexes dispersion of the organic colloids in a solution containing sodium or similar cations should take place.

It was this fact that caused Simon(121) to use fluoride and oxalate solutions for the extraction of humus. Bremner and Lees(122) compared the nitrogen extracting efficiency of 18 different neutral salts of organic and inorganic acids with sodium hydroxide and sodium carbonate on six different soils. For efficient extraction the cation of the neutral salt had to be sodium potassium or ammonium because other cations caused precipitation of the organic matter. Neutral pyrophosphate was the most efficient neutral extractant, but it was still not quantitatively as effective as sodium hydroxide.

37/ ....

Of all the other neutral complexing salts tested from E.D.T.A. (123) to acetyl acetone (117) and sodium sulphite (124) none was found to be equal to sodium pyrophosphate for the extraction of humus.

d. Use of organic solvents

In comparison with aqueous solvents very little work has been done on the use of organic solvents for extracting soil organic matter.

Solvents such as pyridine (125), phenol (126), ethanol/benzene (127) and acetyl bromide (128) were used without much success in the extraction of humic compounds from soil.

Tinsley (129) found that appreciable quantities of organic matter were dissolved from a series of five soils and a compost by repeated extraction with cold 90% formic acid, while a further quantity could be extracted by boiling under reflux for 30 minutes.

Parsons and Tinsley (130) found that cold anhydrous formic acid extracted a considerable amount of organic matter from soil, but boiling under reflux for two half hour periods considerably increased the amount of organic matter extracted. When inorganic cations were added the efficiency of the extractants was increased. The dissolved material was precipitated with diisopropylether containing 1% of acetyl chloride which held the inorganic cations in solution. From 27% to 43% of nitrogen was extracted as well as from 27% to 39% of the total carbon.

The fact that the extract was boiled for an hour may be considered a very drastic method of extraction. Parsons and Tinsley (130), however, concluded that in an anhydrous system very little hydrolysis takes place. Although for-

38/ ....

mylation of the organic matter is possible, the introduced formyl groups should be removed by the hydrochloric acid formed during the precipitation and purification treatments.

Scheffer, Ziechmann and Pawelke(102) stressed extracting the organic matter in unchanged form by the use of very mild conditions, i.e. pH values below 8 and temperatures below 35°C in an inert atmosphere. By using ether, benzene, acetone, ethanol, dioxane, dimethylformamide and pyridine in a Soxhlet extraction apparatus various fractions could successively be extracted from the same soil.

A closed system allowed the extraction process to be performed under reduced pressure and in an inert atmosphere (nitrogen). By comparing the organic solvent extracts with those obtained by means of sodium hydroxide it was found that in the case of the latter the particle size was much larger and they concluded that polymerisation must have taken place. They suggested that before extraction with sodium hydroxide the soil should first be treated with the organic solvents to extract the low molecular weight humic compounds, which have the greatest tendency to polymerise.

It is clear that before deciding upon the extraction procedure to be applied the investigator should decide what is the most important, the amount of humus or the purity of the product extracted.

If the first aspect is to be stressed, sodium hydroxide should be used as extractant; if the purity of the product is considered to be more important the organic solvent extraction procedure according to Scheffer, Ziechmann and Pawelke(102) should be used.

A compromise may be made by using sodium pyrophosphate (122) or anhydrous formic acid(130) as extractant.

SCOPE OF WORK

Recognition of the fact that humus represents a poly-disperse system consisting of a large variety of compounds of differing molecular size, has changed the whole problem of humus research.

Very small amounts of humus have an immense influence on soil fertility. It is therefore important to distingquish between newly-synthesized humic compounds and the rest of the humic substance complex if this amount is to be used as an indication of soil fertility.

To contribute to the solving of this problem, it was decided to go into the matter of the extraction of humic substances from soil and to use column electrophoresis to fractionate the extracts so obtained.

PART II

EXPERIMENTAL PROCEDURE

polymers it is mainly the hydrogen bonds that cause the cross-linking of polymerised chains lying next to each other.

The question of how the polyones are kept together in the form of a micelle, can only be partially explained in the case of the humic compounds.

Oden(36) found that the equivalent conductivity of humic compounds increased with dilution and he concluded that these compounds must be basic in character. It is now generally accepted that the increase in equivalent conductivity with dilution may be caused by the action of external hydrogen binding. According to Beutelspacher(64) and Halla(72) hydrogen bonding is responsible for the binding of polyones to form micelles in acid solution.

According to Ziechmann(44) the association between the polyones depends on the concentration. With decreasing particle size a splitting of the micelles takes place and at the same time an increase in the equivalent conductance and a decrease in the viscosity occur(73). In an acid medium humic acids form "coazervaten" (multiple particles with a mutual sheath of water surrounding them)(64).

The fact that humic compounds absorb oxygen when they are dissolved may be attributed to the possibility that polyone structures may be bound by an ether linkage. It is also possible that polyvalent metals may take part in the linkage between the various polyones in the formation of a micelle. This may explain why it is so difficult to separate the inorganic constituents from the rest of the humic acids in humic acid extraction processes.

The presence of a combination of weak and strong stable bonds in humic compounds synthesized from polyones

23/.........

and other smaller units explains why humic compounds are so sensitive to chemical and physical changes in soil.

Particle size:

In dealing with humic compounds the term "particle size" or "particle weight" is very important and says much more than the term "molecular weight" does. The particle size is dependent on peptisation or breakdown effected by the solvent and is also dependent on the concentration(74, 75). Many difficulties are, however, encountered in determining the particle size because most of the methods require that the compounds to be tested should be uniform. As it is usually impossible to meet this prerequisite, comparison of the various values found for the particle size is usually not valid.

Determination of the sedimentation rate in an ultracentrifuge (10, 74, 76) allows the estimation of the diffusion coefficient and this is an excellent measure of the particle size. The polydispersity of the humic compounds may, however, cause complications in the determination of the diffusion coefficient. Therefore the sedimentation constant is used because, not only is this a direct measure of the particle size, but it may also be used to obtain comparable values for non-uniform systems.

Scheffer(74) found that humic acids with different sedimentation constants could not be shown spectroscopically to differ in structure.

24/.........

HUMIC COMPOUNDS IN SOLUTION

(a) Dissolution and peptisation

Dissolution and peptisation depends on the reaction of alkalihydroxides or alkalisalts with insoluble calcium humates or humic acids to form soluble sodium humates. It may be represented by the following non-stochiometric equations.

$$\text{Na-Oxalate} + \text{Ca-R}_{\text{Hum}} \rightleftharpoons \text{Ca-Oxalate} + \text{Na-R}_{\text{Hum}}$$

$$\text{Na-Acetate} + \text{H-R}_{\text{Hum}} \rightleftharpoons \text{Acetic acid} + \text{Na-R}_{\text{Hum}}$$

The equilibrium of the reaction will depend on the insolubility or degree of dissociation of the products.

Alkali humates may be formed in alkaline as well as in acid media e.g. by dissolving humic acids in acidic acetate or citrate buffers(77). Salt formation is always preceded by peptisation so that overlapping of peptisation and salt formation occurs to a certain extent.

When sodium hydroxide is added to a humic acid suspension, peptisation does not take place at a constant rate, but, according to viscometric measurements(36) the rate of peptisation increases after neutralisation of an acid group. Maximum peptisation takes place at a pH of just above 7 if no other electrolytes are present(78), i.e. at the equivalence point in the neutralisation curve of humic acids. Under these conditions the sodium humates are molecularly dispersed and have a particle weight of 800-1500. The occurence of peptisation is indicated by an increase in the extinction coefficient and it may therefore be determined colorimetrically. The degree of dispersion may be determined by measuring the sedimentation

25/.........

constant in an ultracentrifuge.

(b) Polymerisation and depolymerisation

In alkaline medium:

Phenolic hydroxyl groups dissociate in an alkaline medium. Oxygen and other oxidizing agents consequently attack the humic compounds under these conditions and polymerisation or depolymerisation takes place. Jansson and Valdmaa(79) made use of this phenomenon when they measured the volume of oxygen absorbed in alkaline medium to classify humic compounds.

Polymerisation reactions result in the synthesis of humic compounds from non-humic substances and also increase the degree of cross-linking in the humic compounds. It could be observed(80) that alkaline extraction of plant materials or of lignin with alkali resulted in the formation of a small amount of new humic acids. This formation of new humic acids is of no great importance because hardly any monomers capable of undergoing polymerisation are present.

On the other hand a large number of cases have been recorded where low polymeric humic compounds have been changed into high polymeric substances. Berzelius(38) and Oden(36) showed that fulvic acids changed to hymatomelanic or humic acids when left in alkaline or acid medium for a period of one month. Alcoholic solutions of hymatomelanic acids also changed to humic acids when left undisturbed for a long period. Alkaline treatment at high temperatures may also lead to the formation of alkali-insoluble humins (60). The alkaline extraction of humic compounds rich in polymerisable substances has often been critised because the extraction process may lead to an

26/............

alteration in the structure of the humic compounds. It has been shown that the humic acid yield could be doubled by extracting with alkali in the presence of air(81).

Depolymerisation reactions are of importance in the extraction of humus that has been humified to a large extent. Alkali humates and alkaline soil suspensions take up a considerable amount of oxygen after an acid pretreatment. A definite correlation exists between oxygen uptake, pH of the solution and yield of humic compounds extracted(82). If the hydroxyl groups are methylated the oxygen uptake decreases. Even when oxygen is not present oxidation of the humic compounds may still take place owing to the action of other oxidizing agents, or through the disproportionation of quinones(83).

In acid medium:

Polymerisation and depolymerisation reactions also take place in acid media. Furan and phenylpropane derivatives such as coniferyl alcohol have been known to form humic compounds by polymerisation in acid media. Fulvic acids change to humic acids in acid media in the presence of ferric and ferrous ions. Humic acids synthesized in acid media usually are high molecular weight compounds possessing a small number of active groups(84).

Protolytic decomposition usually takes place after long exposure to oxygen at low pH values. Hymatomelanic or brown humic acids may be decomposed to fulvic acids under these conditions(60). Grey humic acids are, however, resistant to protolytic decomposition. At temperatures of 50-75°C and pH values of 1-7 decarboxylation of humic compounds takes place(85). Humic compounds are oxidized in the presence of dilute nitric acid. The nitroso deri-

27/..........

vatives thus formed are more soluble in organic solvents and to a certain extent resemble fulvic acids(38, 86, 87).

Drying:

Structural changes are possible when humic substances are dried even though the temperature may not be higher than 30°C. Fulvic acids darken and hymatomelanic acids lose their alcohol solubility to a certain extent on being dried. During the drying process rearrangement and reaction of the primary particles take place. The extent of this irreversable rearrangement is dependent upon the nature of the cations. According to Baver(88) alkali humates are completely (100%) dispersable after drying, calcium humates to a lesser extent (20%),and humic acids only 10%. The dried humic acid preparations consist of crystal-like aggregates with irregular cross linking. Ostwald(89) stated that the reduced solubility was due to a greater or lesser degree of irreversable coagulation. At temperatures above 125°C decarboxylation and water elimination take place(90). This phenomenon has been noticed in soil at temperatures below 100°C.

CONTAMINATION OF HUMIC EXTRACTS

Non-humic compounds or inorganic soil constituents that were solubilized during the extraction process may be present as impurities. Springer(91) showed that a considerable amount of non-humic compounds may be present in soil extracts. Neutral reacting solvents such as sodium oxalate and sodium fluoride dissolve very small amounts of non-humin substances(92, 93), whereas alkaline solvents such as sodium hydroxide dissolve non-humic compounds such as tannin, pectin or cell-wall substances(94).

28/........

Waksman(95) found carbohydrates such as araban, xylan and pectic acids in humic acid fractions. Polysaccharide constituents such as galacturonic acid(96) and various sugars such as amino sugars, methyl sugars and sugar acids(97) were also found to be present in humic acid fractions.

Hydrochloric acid treatment causes the splitting off of amino acids. Therefore nitrogen-containing humic compounds are dissolved before non-nitrogen-containing humic substances. The presence of small amounts of carbohydrates and/or amino acids bound to reactive groups on the surface of the molecule should not actually be considered to be impurities as they are to a certain extent characteristic of humic compounds. A reasonable estimate of the contamination of humic acids by non-humic compounds may be found by comparing the value of the humic acid concentration determined colorimetrically with the values found by carbon-determination, or, alternatively, with the value of the total concentration obtained gravimetrically.

Low molecular weight colourless compounds, auto-oxidizable phenolic glycosides, polyuronic acids, hemicelluloses, etc.(98, 99, 100) as well as the majority of organic phosphorus compounds were found to be present in the fulvic acid fraction.

Welte(10) extracted humic acids by treating soil with a sodium hydroxide solution. Reprecipitation was repeated 4-6 times until the upper liquid layer was colourless. When a Kasseler Braun humic acid with an ash content of 14% was reprecipitated four times the ash content was reduced to 0.5% Further treatment could not produce any further reduction of the ash content (101). Forsyth (98) applied standard acid hydrolysis to humic acid preparations.

29/.........

He regarded the percentage of hydrolysable material as an indication of the purity of the sample. To remove acid-hydrolysable impurities such as hemicelluloses, polyuronic acids and pectins, Welte(10) recommended the addition of hydrochloric acid up to a concentration of 5%. Fats, waxes and bituminous matter can be removed from soil by prior extraction with organic solvents(98, 102).

Generally humic acid gels are very difficult to filter. An easily filterable precipitate may, however, be obtained by freezing the humic acids at -10°C(103).

The amount of inorganic soil constituents (usually determined as "ash") may be very high for certain humic acid extracts. In the case of humic acids extracted from rendzina soils the ash content may be as high as 50%. Grey humic acids usually have the highest ash content(60,61).

By using a coupled hydrochloric-hydrofluoric acid treatment or by re-precipitation with sodium fluoride the amount of mineral contaminants present may be considerably reduced. It is not possible, however, to effect a complete removal of ash by application of this method.

Stable colloid-dispersed solutions of humic acids (sols) may be formed through dialysis or electrodialysis of freshly precipitated, non-dried humic acids(104). These solutions may also be formed by passing sodium humates through a cation exchange column (H-form). This process considerably decreases the ash content. These humic acid sols show vigorous Brownian movement and have a high particle weight which might be due to hydrogen bonding.

SEPARATION OF THE CONSTITUENTS OF HUMIC COMPOUND MIXTURES

Modern chromatographic and electrophoretic techniques have been used to a large extent in the fractionation of humic compounds.

Adsorption chromatography gave the best results, e.g. in the work of Kroll(105) who was able to separate fulvic acid into seven fractions. The biggest and also disqualifying disadvantage of adsorption chromatography is the fact that humic compounds are so strongly adsorbed that when the fractions have to be eluted, such drastic conditions have to be created that the separation becomes useless since the structure of the humic compounds are altered under these conditions. Partition column chromatography has found very little application in humus research because of the limited solubility of humic compounds in the different solvents..

Paper chromatographic and paper electrophoretic techniques have been applied by a large number of workers, but, as only micro-quantities of the various fractions can be obtained, it is practically impossible to obtain sufficient amounts of the various fractions for chemical examination.

Very little work has been done on column electrophoretic fractionation of humic compounds. Scheffer(35) used a Bockemüller apparatus (U-tube filled with cellulose) to obtain a preparative fractionation of humic substances into three fractions. This separation is better than that obtained with continuous paper electrophoresis which also gives large fractions.

The difficulties encountered in the fractionation of humic compounds are due to the fact that humic compounds polymerise and depolymerise rather easily. The fact that humic compounds are very unstable in the solvents commonly used and that they tend to form micelles, further complicates matters.

31/............

EXTRACTION

INTRODUCTION

Soil humic compounds were extracted with formic acid(130), organic solvents(102) and sodium pyrophosphate(122) in order to compare the extraction efficiency of each extractant. Extraction with sodium pyrophosphate was discontinued because, in contrast to the results given in the literature (122), the pH of the solution increased to 10.3 instead of remaining constant at 7.0.

As very little information on the extraction of humus by sodium hydroxide in the presence of stannous chloride could be found in the literature, it was decided to determine the influence of the concentration of sodium hydroxide, weight of stannous chloride added, extractant/soil ratio and the time of extraction on the weight of humic acids extracted.

Because humic acids are so reactive that even during the drying of the extract a change in structure takes place, it is desireable to shorten the time lapse between the extraction of the humic compounds from soil and the transfer to a solution suitable for electrophoresis as much as possible. The logical solution, therefore, is to extract the humus from the soil directly with the buffer medium used in the electrophoretic separation.

SOILS USED FOR EXTRACTION

Humic acids were extracted from four different soils, i.e.:-

(a) Soil under a crop rotation consisting of

- (i) Kaffir corn (Sorghum spp.)
- (ii) Soya beans (Glycine hispida)
- (iii) Pearl millet (Pennisetum typhoides)

43/.........

(iv) Cow peas (*Vigna sinensis*)

(b) Soil under grass (*Eragrostis curvula*) for three years.

(c) Soil under maize (*Zea mays*) for 20 years.

(d) Soil under natural veld of the Transvaal Highveld region.

Table 1. Characteristics of soils used in extraction[☒]

	Crop rotation	Grass	Maize	Veld
Resistance (ohm)	900	820	1200	1250
Colour	brown	brown	brown	brown
pH	5.1	5.5	4.9	6.0
% P	.0006	.0001	.0001	.0001
% K	.03	.02	.04	.02
% Na	.003	.002	.002	.002
% Ca	.013	.092	.136	.080
% Mg	.045	.060	.064	.062
% Fe	0.69	1.20	1.63	1.63
% N	.08	.13	.08	.11
% C	0.96	1.15	0.90	1.45

[☒] The texture was in each case that of a slightly clayish fine sandy loam.

EXTRACTION WITH ORGANIC SOLVENTS

A variation of the method recommended by Scheffer, *et al* (102) was used to extract humic compounds from soil. The soil samples were purified by pretreatment with ether only. Instead of extracting the various soil samples consecutively with the five solvents as indicated by Scheffer, *et al* (102), each purified sample was treated with only one of the three most efficient solvents that was used by these workers. The three solvents chosen were acetone, ethyl-alcohol and dioxane. This allowed a comparison of the effects of these solvents on the same and different soil samples.

The apparatus used for the extraction of humus from soil was set up as indicated (plate 3, fig. 4). It consisted of a large-sized Soxhlet extractor set up in such a manner that the extraction could be conducted in an inert atmos-

45/...........

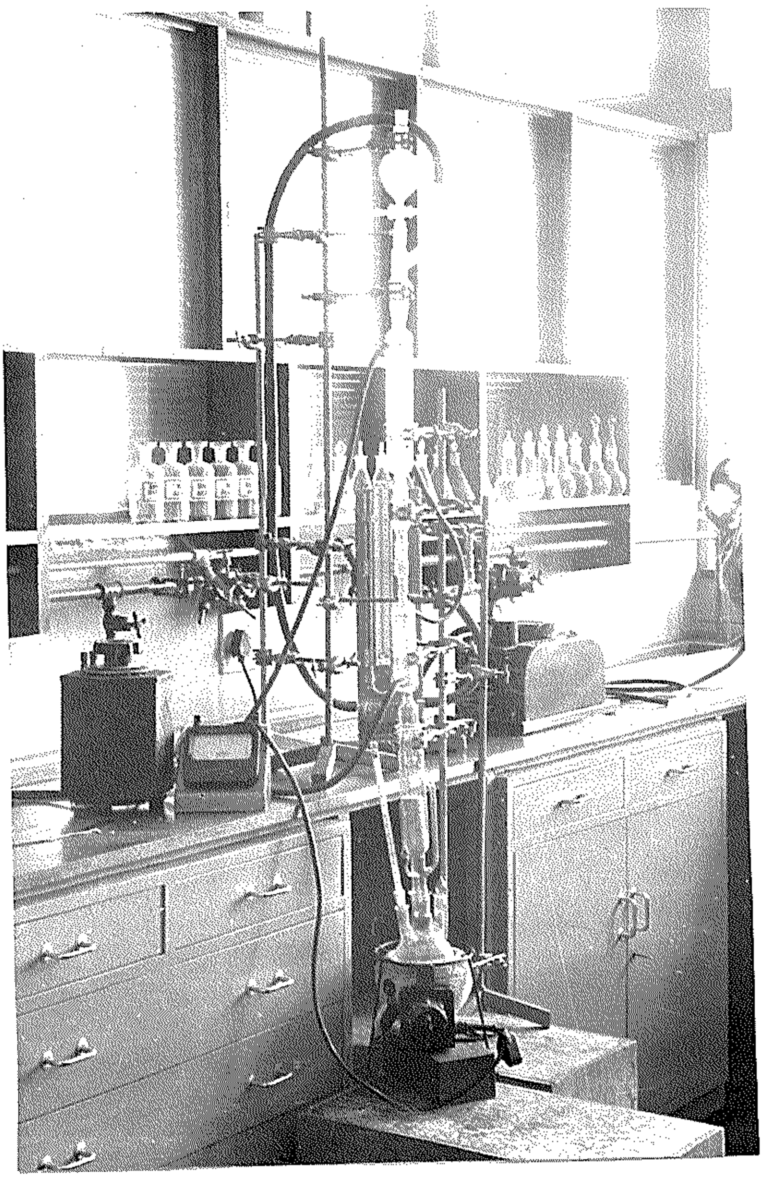


Plate 3. Apparatus for the extraction of humic compounds by organic solvents

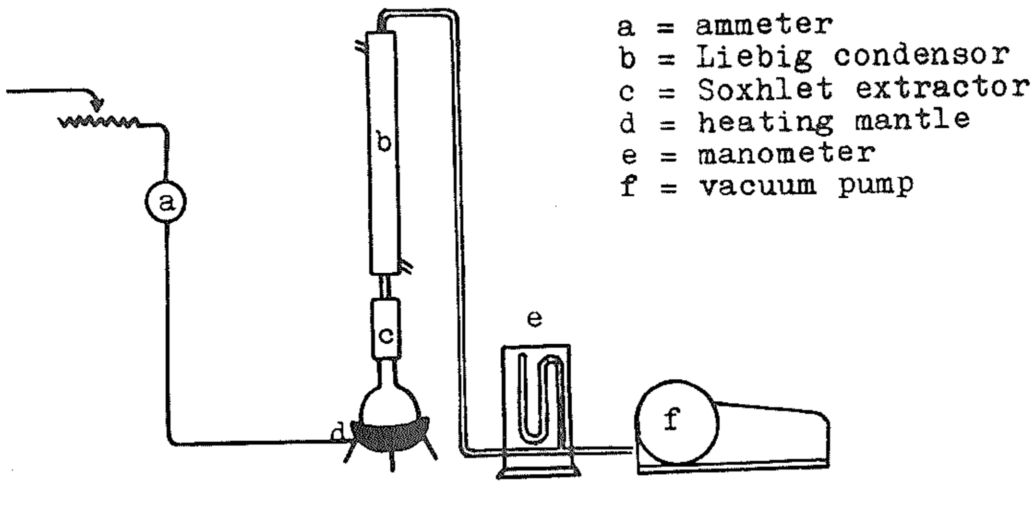


Figure 4. Set up for the extraction of humic compounds by organic solvents

phere at reduced pressure.

The soil samples were extracted for eight hours with ether to remove all fats, waxes, etc. present in the soil. Thirty grams of this pretreated soil was transferred to the extractor and extracted with the chosen solvent. The boiling point of the solvent was kept below 35°C by regulation of the pressure.

An extraction cycle of approximately 12 minutes per cycle was maintained for three hours. (Extracting for a further period of five hours did not give a significant increase in the weight of material extracted.)

After three hours the excess solvent was removed by distilling in an inert atmosphere at a temperature below 35°C. The extract was quantitatively transferred to a tared glass-stoppered flask, dried under vacuum and the weight of the dried extract determined.

Table 2. Humic compounds extracted with organic solvents (in grams).

Extractant	Soil	Ether/Benzene soluble	Residue	Buffer soluble	Residue	Total
Acetone	Crop rot.	.053	.005	.001	.005	.058
	Maize	.014	.003	-	.003	.017
	Grass	.060	.007	-	.007	.067
	Veld	.042	.012	-	.012	.054
Dioxane	Crop rot.	.144	.003	-	.003	.147
	Maize	.154	.020	.019	.002	.174
	Grass	.033	.004	-	.004	.037
	Veld	.034	.005	.004	.002	.039
Ethanol	Crop rot.	.015	.007	.004	.003	.022
	Maize	.016	.009	.006	.003	.025
	Grass	.040	.083	.078	.005	.123
	Veld	.017	.008	.006	.002	.025

The extract was washed consecutively with ether and benzene in order to remove all impurities. After determining the weight of the residue the residue was extracted,

46/............

with a borate buffer at a pH of 9.0 leaving a final insoluble residue the weight of which was again determined (table **2**).

EXTRACTION WITH ANHYDROUS FORMIC ACID

Carbon tetrachloride (150 ml.) was added to 60 g. of soil. The mixture was shaken for ten minutes and, after centrifuging, the supernatant was filtered to remove all floating organic matter. This process was repeated until the soil was free from undecomposed organic residues. The carbon tetrachloride was transferred to a tared flask and after removal of the carbon tetrachloride by distillation the weight of the fats, etc. extracted from the soil could be determined.

Two hundred ml. of a 0.2 N solution of lithium bromide in anhydrous formic acid (98-100%) was heated under reflux to boiling point. After careful addition of the air-dried purified soil to the boiling formic acid solution, the mixture was gently refluxed for 30 minutes. After cooling of the solution the extract was separated from the soil by centrifugation.

Owing to the fact that an efficient freeze-drying apparatus was not available, freeze-drying of the extract to concentrate the solution was not successful enough to justify the trouble involved.

Subsequently the whole extract was transferred to a **large** measuring cylinder. The humic compounds were precipitated by the addition of five volumes of 1% acetyl chloride in diisopropylether.

The sample of soil was again extracted with boiling formic acid plus lithium bromide for a further 30 minutes.

This extract was also recovered by centrifuging and added to the contents of the measuring cylinder. The total amount of humic acid extracted was precipitated by the further addition of the precipitating mixture. The mixture in the measuring cylinder was left overnight and the precipitate recovered by centrifuging.

Diisopropylether could be recovered for further use by washing out the formic acid with water and distilling the impure ether.

Purification of the extract was accomplished by dissolving the extract in the minimum quantity of anhydrous formic acid and precipitating with the diisopropylether mixture. The process was repeated until no ferric or calcium ions could be detected in the supernatant.

Table 3. Results of formic acid extraction

Soil	CCl_4 extract	Formic acid extract
Crop rot.	.039 g.	.084 g.
Grass	.018 g.	.102 g.
Maize	.025 g.	.130 g.
Veld	.020 g.	.250 g.

EXTRACTION WITH SODIUM HYDROXIDE + STANNOUS CHLORIDE

The influence of the sodium hydroxide concentration, weight of stannous chloride added, extractant/soil ratio, and time of extraction, on the weight of humic acid extracted from soil under grass with sodium hydroxide plus stannous chloride was determined.

Thirty grams of soil was weighed off into a 250 ml. polythene centrifuge holder with a screw-on cap. Most of the roots and other undecomposed organic matter in the soil were removed manually.

After adding the calculated weight of stannous chloride to the soil, care being taken to avoid extended contact of the stannous chloride with air, 200 ml. of 0.05 N sodium hydroxide solution was rapidly added to the contents of the polythene holder. The mixture was now shaken in a gyrotory shaker.

After shaking for 16 hours, the polythene holder with its cap still in place was centrifuged at 2,000 R.C.F. (3,000 R.P.M.) for 45 minutes to separate the extract from the remaining soil.

An aliquot of the extract was transferred to a 100 ml. weighed glass centrifuge tube containing just enough hydrochloric acid to lower the pH of the solution to pH 2.

The precipitate which formed on acidification was separated from the supernatant by centrifuging for 15 minutes at 3,000 R.P.M. After decanting the supernatant the glass tube was placed in an oven at 105°C, dried to constant weight and the weight of the extracted humic acids determined as shown in table 4. (See also figure 5.)

Table 4. Weight of humic acids extracted with 0.05 N NaOH (16 hours, 200 ml. NaOH + 30 g. soil)

$SnCl_2$ added in grams	Humic acid yield in grams
0.0	0.33
0.1	0.63
0.2	1.32
0.25	1.38
0.35	1.31
0.4	1.11
0.45	0.96
0.5	0.78
0.6	0.63
0.7	0.44
0.8	0.32
0.9	0.10

It is clear that a maximum weight of humic acid is extracted when 0.25 gram of stannous chloride is added to 30 g. of soil in sodium hydroxide solution.

In order to discover whether the quantity of stannous chloride to be added is affected by an alteration in the concentration of the sodium hydroxide used, the process was repeated with 0.1 N, 0.225 N, and 0.5 N sodium hydroxide solutions. (See tables 5, 6, 7 and also figures 6, 7 and 8).

Table 5. Weight of humic acids extracted with 0.1 N NaOH (16 hours, 200 ml. NaOH + 30 g. soil)

$SnCl_2$ added in grams	Humic acid yield in grams
0.0	0.25
0.2	0.41
0.4	1.11
0.6	1.49
0.7	1.19
0.8	0.93
1.0	0.80
1.2	0.47
1.4	0.30
1.5	0.30
1.9	0.01

Table 6. Weight of humic acids extracted with 0.225 N NaOH (16 hours, 200 ml. NaOH + 30 g. soil)

$SnCl_2$ added in grams	Humic acid yield in grams
0.0	0.48
0.2	0.66
0.5	1.00
0.7	1.20
0.8	1.30
0.9	1.40
1.1	1.44
1.2	1.52
1.4	1.43
1.6	1.37
1.8	1.28
2.0	1.20

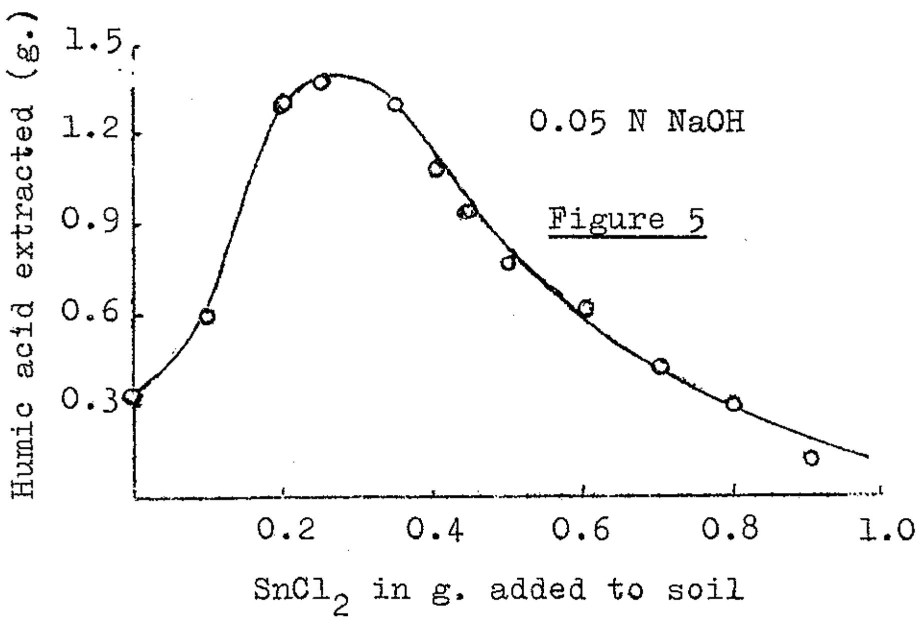


Figure 5

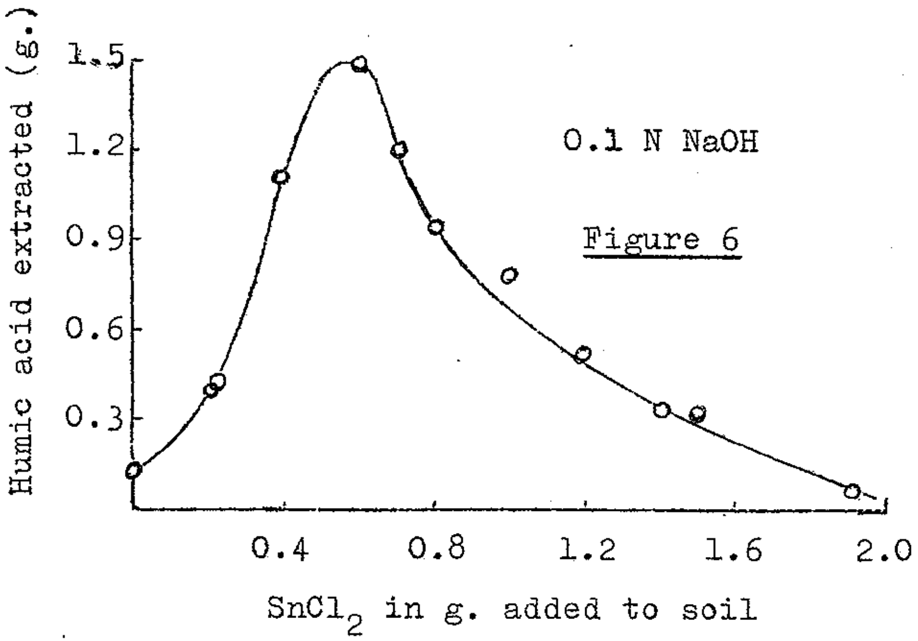


Figure 6

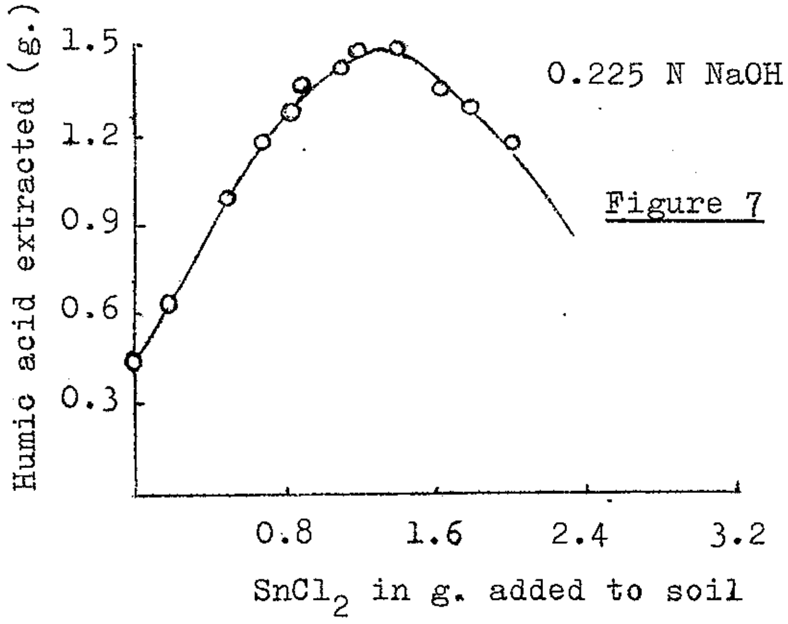


Figure 7

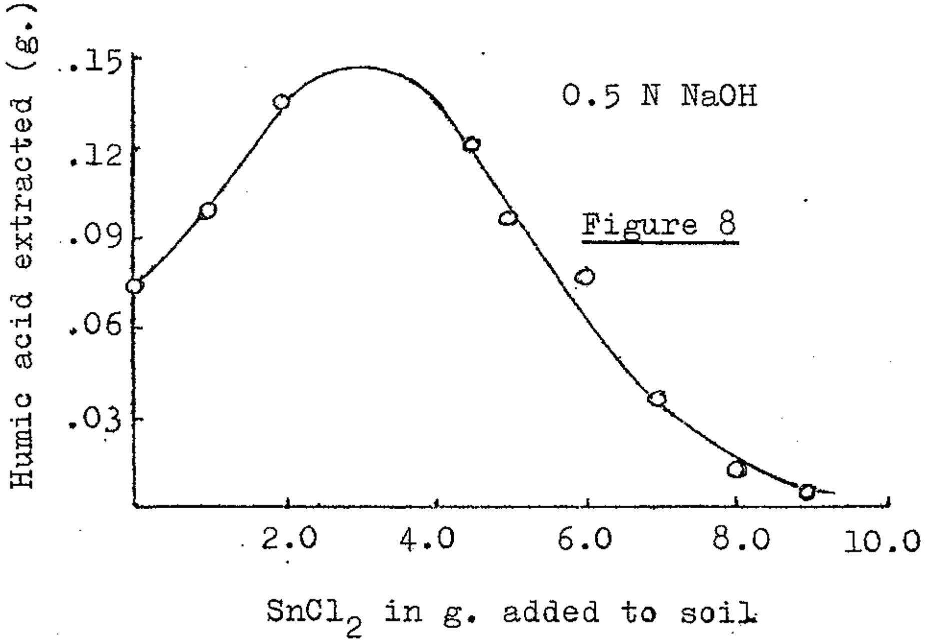


Figure 8

EXTRACTION WITH SODIUM HYDROXIDE + STANNOUS CHLORIDE

Table 7. Weight of humic acids extracted with 0.5 N NaOH (16 hours, 200 ml. NaOH + 30 g. soil)

$SnCl_2$ added in grams	Humic acid yield in grams
0.0	0.071
1.0	0.100
2.0	0.139
4.5	0.122
5.0	0.097
6.0	0.076
7.0	0.036
9.0	0.004

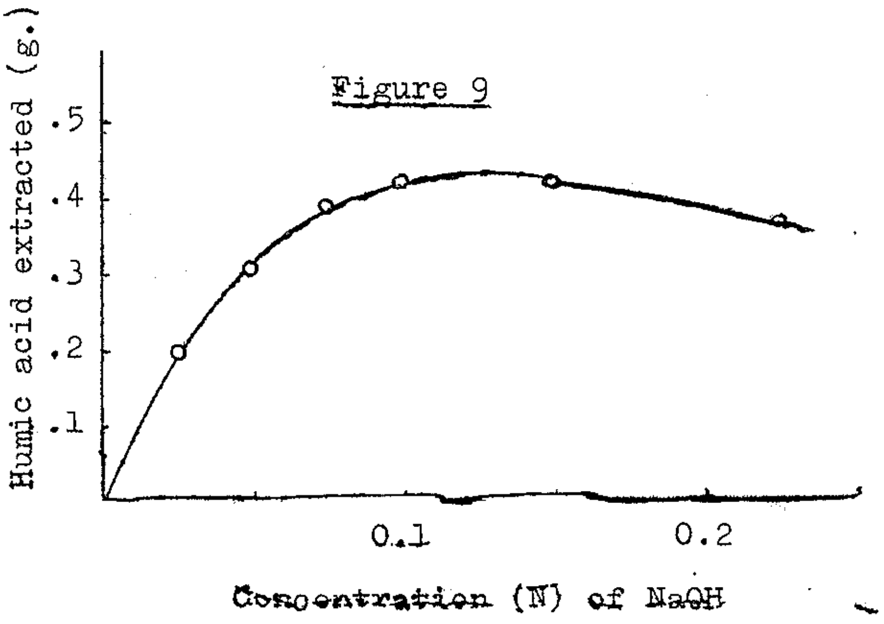
The results indicate that with all of the concentrations of sodium hydroxide used the maximum weight of humic compounds was extracted when a certain ratio of grams of stannous chloride/liter/normality of sodium hydroxide was attained. This ratio proved to be a constant, i.e. 30 : 1.

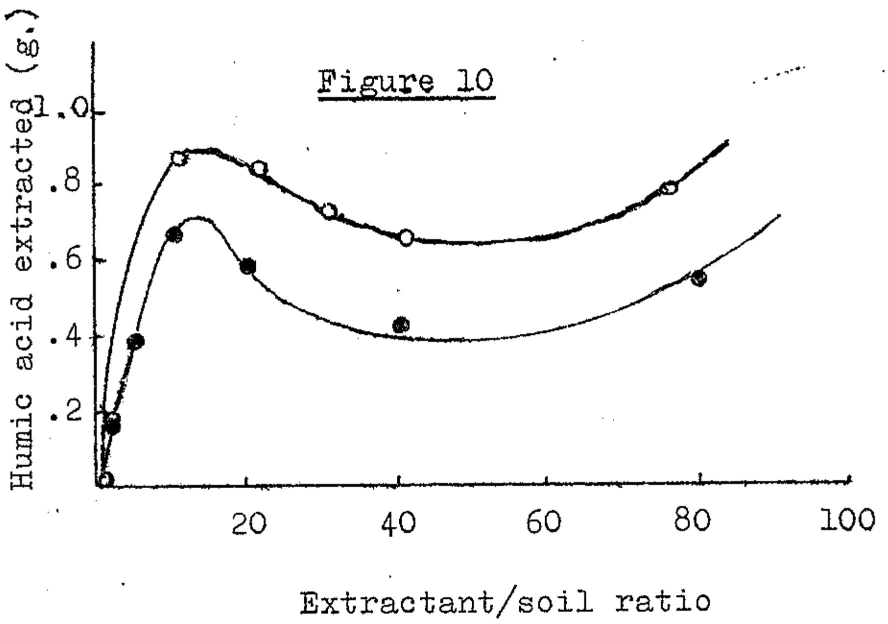
This optimum relationship was used in a series of extractions to determine the influence of the variation of the concentration of the sodium hydroxide on the weight of humic acids extracted. The extraction and precipitation process was repeated with 0.025, 0.05, 0.075, 0.15 and 0.225 N sodium hydroxide solutions, the optimum weight of stannous chloride being added in each case.

The results are given in table 8 and fig. 9.

Table 8. Weight of humic acids extracted with different concentrations of NaOH. (16 hours, 15 g. soil + 100 ml. NaOH, 30 g. $SnCl_2$/liter/norm. NaOH)

Normality NaOH	Humic acid yield in grams
0.025	0.020
0.050	0.312
0.075	0.398
0.100	0.430
0.150	0.416
0.225	0.376





EXTRACTION WITH SODIUM HYDROXIDE + STANNOUS CHLORIDE

According to figure 9 and table 8 the concentration which yields the optimum amount of humic acids is slightly higher than 0.1 N.

In order to determine the influence of the extractant to soil ratio the process of extraction was repeated by adding 0.1 N sodium hydroxide solution containing 3 g. stannous chloride per liter of sodium hydroxide to the soil. The ratio of the extractant to soil was varied and the weight of humic acid extracted in each case was determined as shown in tabel 9 (fig. 10).

Table 9. Weight of humic acids extracted with different extractant/soil ratios. (16 hours, 0.1 N NaOH, 4 g. $SnCl_2$/liter)

Extractant to soil ratio	Humic acid yield in grams [x]
1	.010
2	.180
10	.861
20	.860
30	.742
40	.661
75	.808
2	.174
5	.398
10	.663
20	.589
40	.455
80	.498

[x] Expressed in terms of 200 ml. extractant per 10 g. soil.

From figure 10 it is evident that at an extractant to soil ratio of 16:1 the maximum amount of humic acids is exracted.

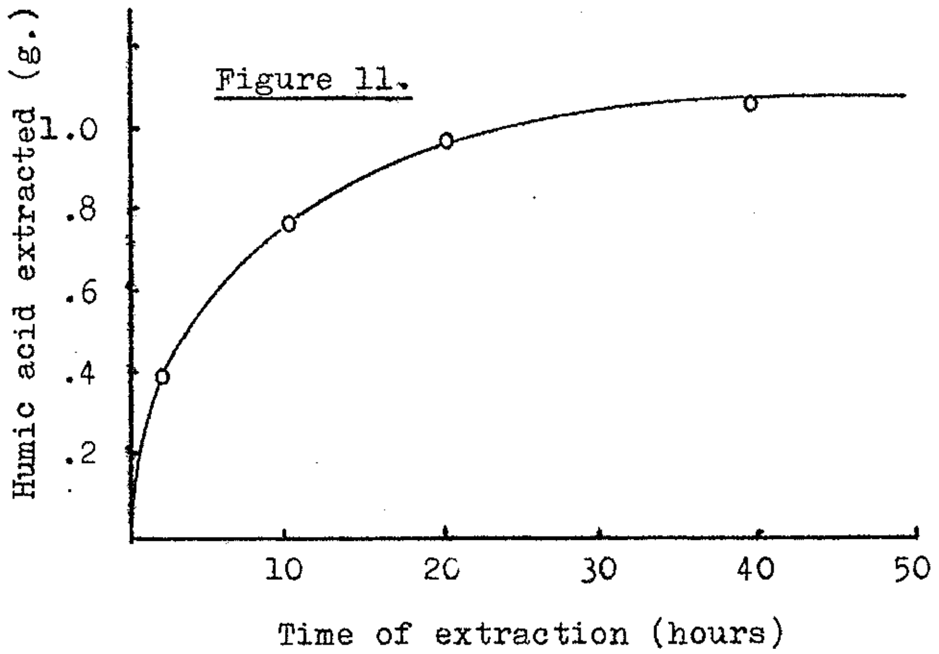
As time of extraction is also an important variable, the influence was determined of this variable in combination with all the conditions already determined, i.e. 12 g. of soil + .06 g. stannous chloride + 200 ml. 0.1 N NaOH.

54/.....

The results are shown in table 10 (fig. 11).

Table 10. Weight of humic acids extracted with different times of extraction. (100 ml. NaOH + 15 g. soil + 4 g. $SnCl_2$/liter)

Time in hours	Humic acid yield in grams
2	.394
10	.768
20	.886
40	.984



It is obvious that the time of extraction reaches an equilibrium at about 40 hours, but for all practical purposes extraction for 20 hours may be considered to be sufficient.

The optimum conditions for the extraction of humic compounds proved to be extraction for 20 hours at a sodium hydroxide/soil ratio of 12:1 using a 0.1 N sodium hydroxide solution. The weight of stannous chloride added is equivalent to 30 gram/liter/normality sodium hydroxide solution. Humic acids were subsequently extracted from the four soils under different agricultural treatments under these optimum conditions.

Table 11. Weight of humic acids extracted from different soils at optimum extraction conditions.

Soil type	Humic acid yield in grams
Crop rotation	0.954
Grass	0.822
Maize	1.013
Veld	0.538

EXTRACTION WITH BUFFER MEDIA

Humic compounds are very reactive. A change in structure even occurs during the drying of the extract. It is desireable to shorten the time lapse between the extraction of the humic compounds and the transfer of these extracts to a solution suitable for electrophoresis as much as possible. The logical solution, therefore, is to extract the humus from the soil directly with the buffer medium to be used in the electrophoretic separation.

In the search for a suitable buffer medium the following buffer solutions were tested for their extracting ability:

(i) Borate buffer (boric acid-- potassium chloride - sodium hydroxide) at pH 9.0(131).

(ii) Veronal buffer (0.05-M sodium diethylbarbiturate, 0.005-M diethylbarbituric acid, 0.05-M sodium acetate) at pH 8.6(132).

(iii) L.K.B. high resolution buffer (0.5-M trishydroxymethylaminomethane, 0.021-M ethylenediaminetetraacetic acid, 0.075-M boric acid) at pH 8.9(133).

Thirty gram soil samples were shaken in 200 ml. of a buffer solution for 16 hours in airtight containers. The humic acids were obtained by acidifying an aliquot of the extract to pH 2. The precipitated humic acids were then dried to constant weight at 105°C.

Humic acids were only extracted by the high resolution buffer. The colour of this extract compared favourably to that of a sodium hydroxide extract.

Optimum conditions for the extraction of humic acids from soil with the L.K.B. high resolution buffer were determined as follows.

The correlation between time of extraction (fig. 12, table 12) extractant/soil ratio and weight of humic acid extracted from soil under grass, was investigated by using the same procedure as was used in the extraction of humic acids with sodium hydroxide/stannous chloride solution. A white flaky solid was co-precipitated with the humic acids on acidifying the L.K.B. buffer extract. When the combined weight of humic acid and co-precipitated matter was plotted against the extractant/soil ratio, a straight line resulted (fig. 13). When, however, the white flaky material was removed manually from the black amorphous humic acids, as much as is possible, a curve similar to that obtained for the extraction with sodium hydroxide(122) was obtained (see fig. 13).

Table 12. Weight of humic acids extracted with different times of extraction. (30 g. soil + 200 ml. buffer)

Time in hours	Humic acid yield in grams
2	0.242
3	0.249
4	0.254
6	0.255
11	0.255
21	0.268

58/........

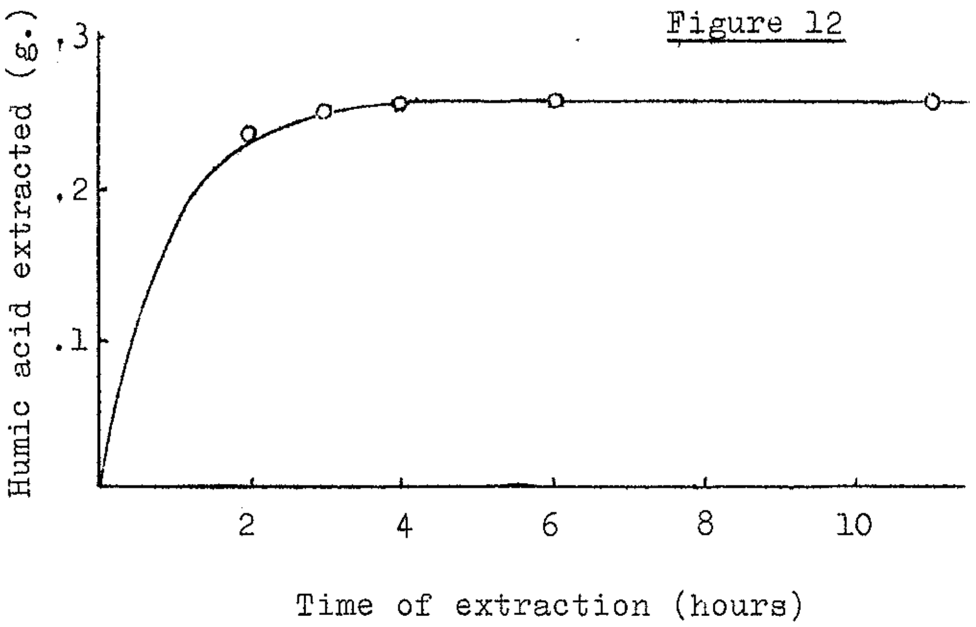


Figure 12

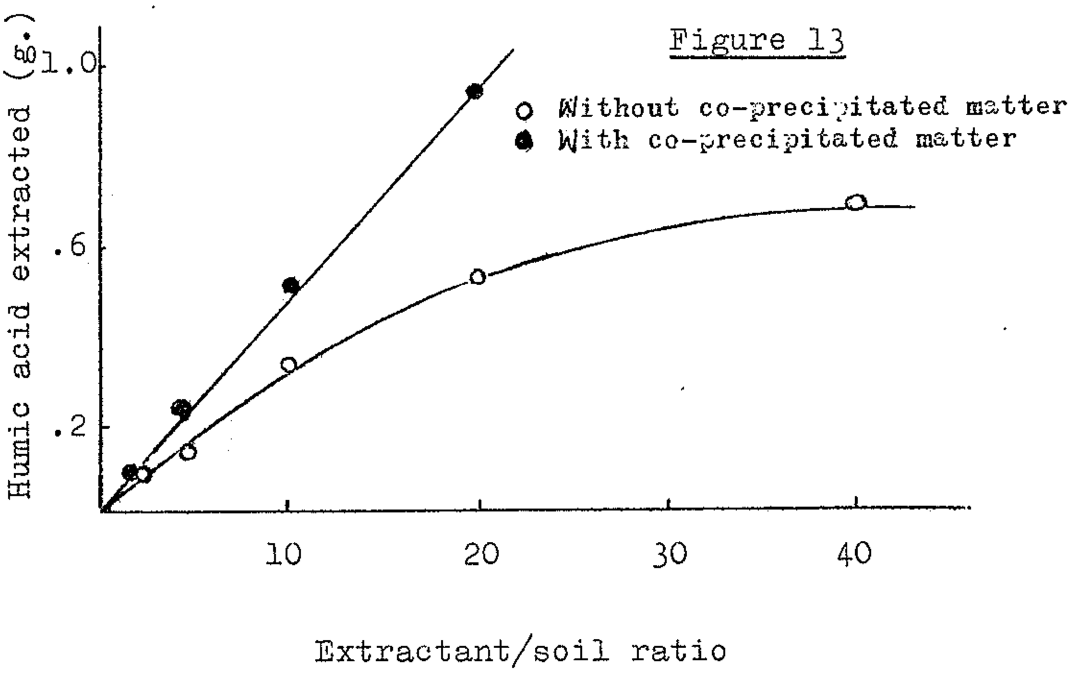


Figure 13

EXTRACTION WITH L.K.B. HIGH RESOLUTION BUFFER

Table 13. Weight of humic acids extracted with different extractant/soil ratios. (16 hours extraction)

Extractant to soil ratio	Humic acid yield in grams[⌧]	
	without co-precipitated matter	with co-precipitated matter
2	0.08	0.09
5	0.14	0.24
10	0.34	0.54
20	0.53	0.92
40	0.68	

[⌧] Expressed in terms of 10 g. of soil per 200 ml. extractant.

The four soils under consideration were purified by extracting them with ether in a Soxhlet extractor for 36 hours. When the optimum conditions for efficient extraction were determined, i.e. four hours extraction with an extractant/soil ratio of 40, humic compounds were extracted from the four purified soils (see table 14).

Table 14. Humic acids extracted from different soils. (30 g. soil).

Soil	Weight extracted by ether in grams	Humic acid yield in grams	
		by precipitation	by evaporation
Crop rot.	0.046	0.850	13.445
Grass	0.049	0.731	13.426
Maize	0.063	0.738	13.459
Veldt	0.067	0.578	13.240

ELECTROPHORETIC FRACTIONATION

The dried purified extracts obtained by organic solvent extraction(102) were dissolved in the boric acid buffer and electrophoresis carried out with this solution. When other buffer media became available, boric acid was found, in comparison, to be such a poor solvent for humic acids that all the other extracts were subsequently dissolved in, and electrophorised with L.K.B. high resolution buffer.

The column was packed by slowly siphoning a suspension of cellulose powder (Whatman, grade A, fine) in the L.K.B. high resolution buffer into the buffer-filled column of the apparatus (plate 14).

A MiniFlow precision micropump was used instead of the Mariotte flask arrangement for the elution of the fractionated compounds from the column. (See plate 15 and fig. 16).

Electrophoresis was carried out for eight hours at a potential difference of 420 volts and with a current of 50 mA. After eight hours the fractionated compounds were eluted with a buffer flow of 25 ml./hour. The elution was usually complete in eight to ten hours.

The organic solvent extracts and the sodium hydroxide/stannous chloride extracts could not be separated into fractions distinguishable under ultraviolet light. On the other hand some samples of the formic acid extracts and to a lesser degree also some of the high resolution buffer extracts could be separated into three fractions and other samples into four, as shown in figure 17.

Although the first peak (fig. 17) is much higher than the second, it does not necessarily mean that the concentration of this fraction is much higher than that of the second fraction, because the ultraviolet absorption of the two fractions differ.

63/........

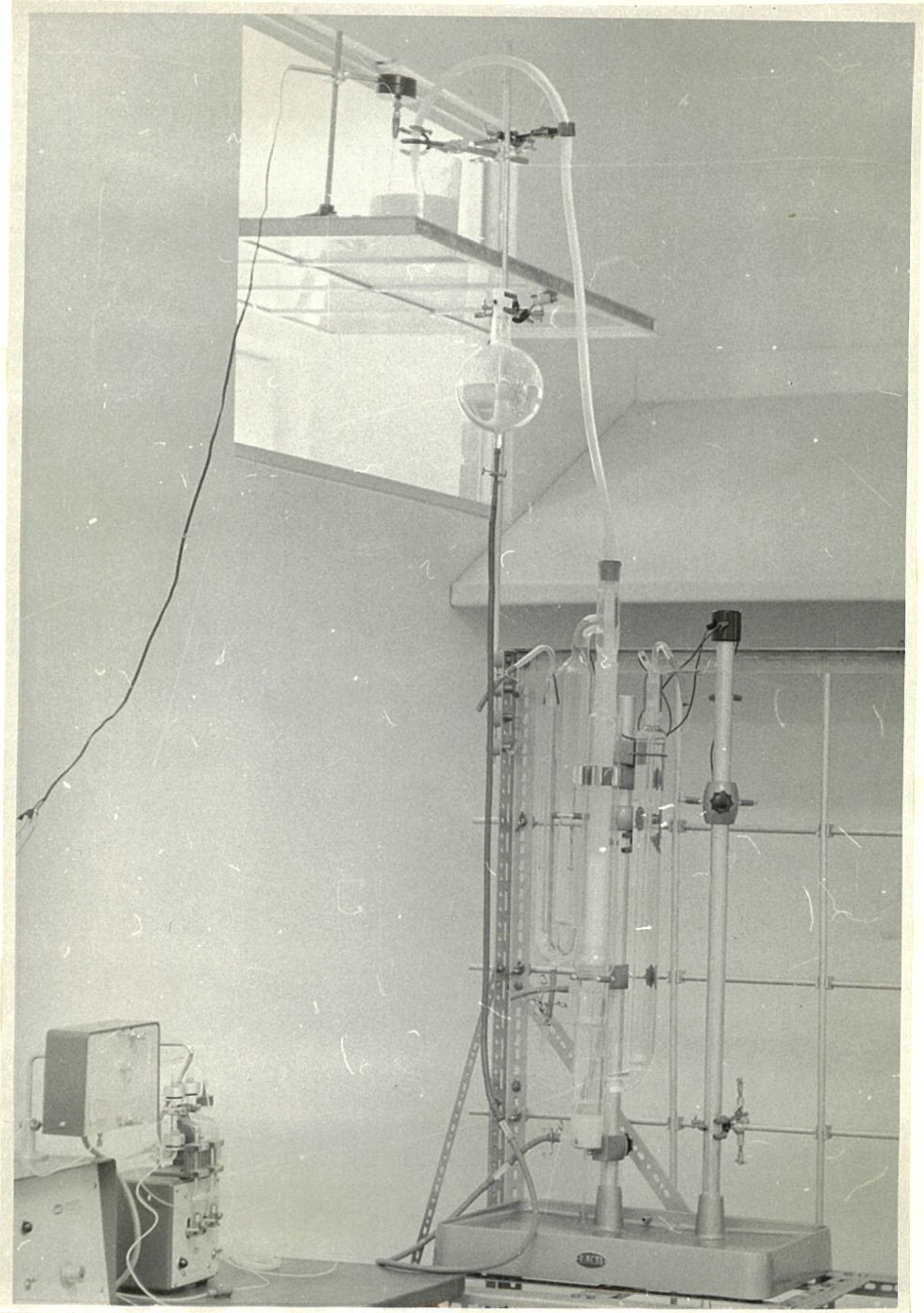


Plate 14. Packing of column

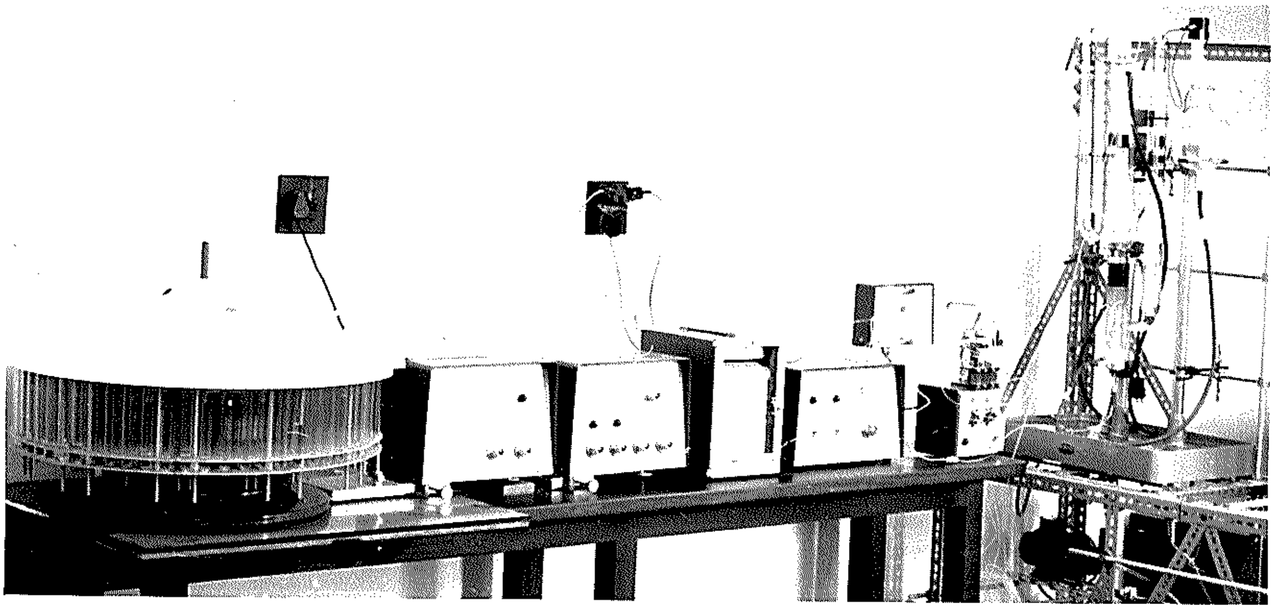
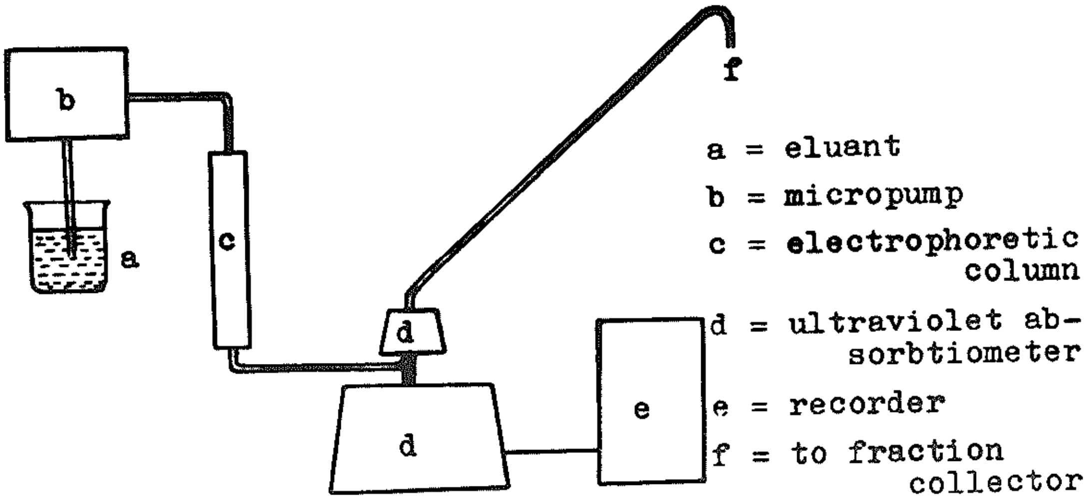


Plate 15. Set up for column electrophoresis



a = eluant
b = micropump
c = electrophoretic column
d = ultraviolet absorbtiometer
e = recorder
f = to fraction collector

Figure 16. Set up for column electrophoresis

The ratio between the height of the first and the second peak may be compared with the ratios obtained from other extracts. The ratios of the areas of the first two peaks are denoted in table 16.

<u>Table 16</u> Fractionation of formic acid extracts

Soil	Ratio of area covered by first two peaks
Grass	4.5
Veld	3.76
Crop rotation	3.5
Maize	1.8

Since the first represents the most mobile, i.e. the younger humic compounds, it may be stated that at the time of sampling, the ratio of young to old humic compounds was highest in soil under grass, then soil under veld, crop rotation and maize.

The degree of fractionation attained with the high resolution buffer extracts was found to be dependent on the time lapse between extraction and fractionation. Electrophoretic fractionation of a high resolution buffer extract was repeated one and four days after extraction. The degree of fractionation attained with the four day old extract was about 90% less than that attained with the one day old extract although the extracts were kept in glass stoppered containers.

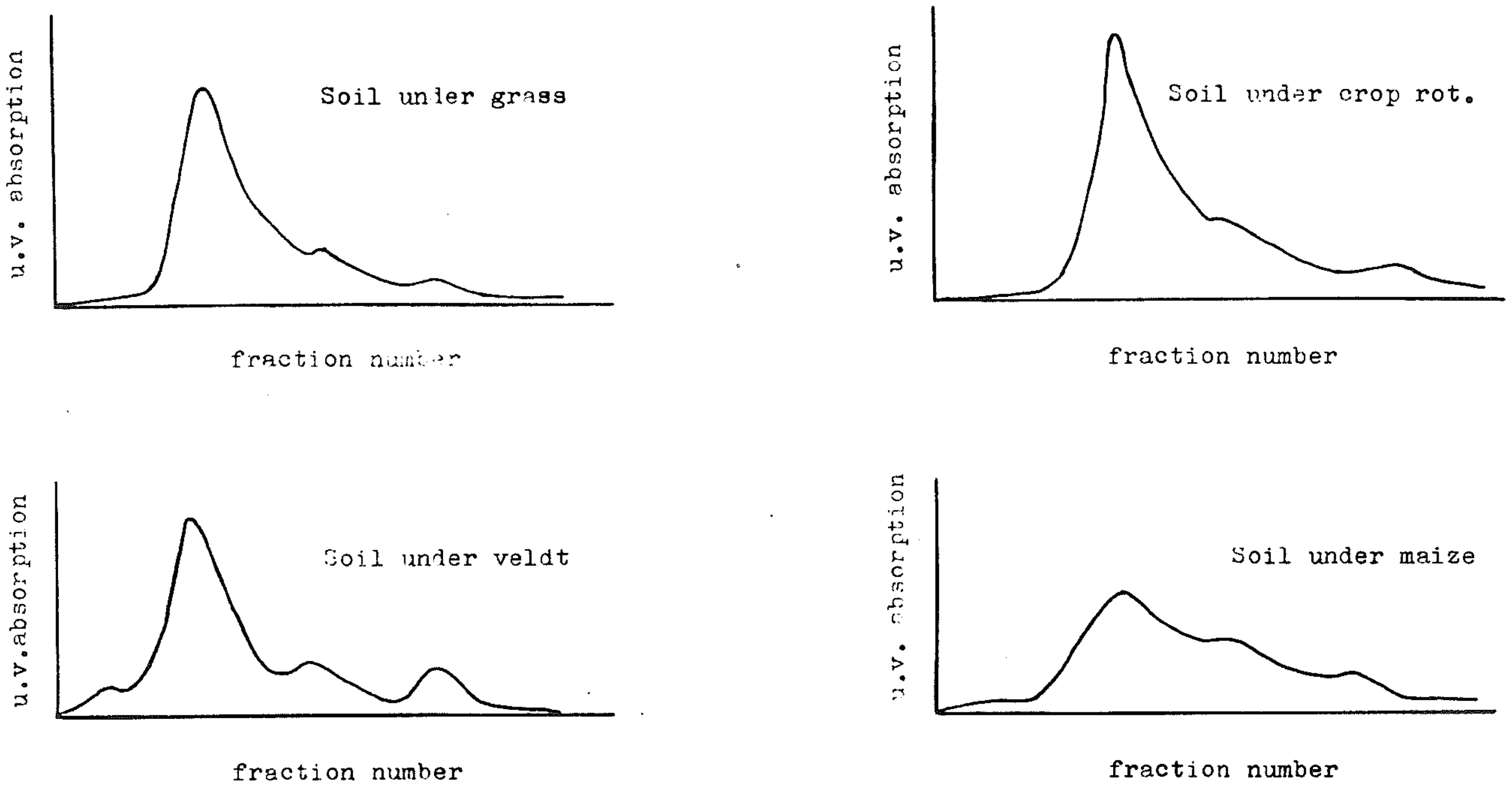


Figure 7. THE FRACTIONATION OF HUMIC COMPOUNDS EXTRACTED FROM DIFFERENT SOILS BY ANHYDROUS FORMIC ACID + LiBr.

PART III

DISCUSSION OF RESULTS

EXTRACTION

In the extraction of humic compounds by organic solvents it appears as if certain solvents preferentially dissolve humic compounds from certain soils, e.g. dioxane preferentially dissolve humic compounds from soil under maize, while acetone appears to be the best solvent for humic compounds from soil under veld and ethanol from soil under grass.

On the whole the amounts of humic compounds extracted by these organic solvents from a sandy loam is too small to allow any further work to be done on these extracts.

The extraction by means of formic acid yielded from five to ten times more humic compounds than did the different organic solvent extractants, whereas the sodium hydroxide/stannous chloride mixture extracted approximately twenty times more humic acids than in the case of the formic acid.

The weight of stannous chloride added at the optimum conditions could be directly correlated with the normality of the sodium hydroxide in that the weight of stannous chloride added per liter divided by the normality of sodium hydroxide was a constant, i.e. 30.

The addition of stannous chloride increased the amount of humus extracted considerably and the yield reached an optimum at a sodium hydroxide concentration of 0.1 N.

In the extractant/soil ratio studies a maximum was observed at a ratio of 12:1 and a minimum at 48:1 after which the yield increased with increasing extractant/soil ratio.

The high resolution buffer extracted nearly as much humic acids as the sodium hydroxide/stannous chloride mix-

67/.............

ture did, but it extracted the humic compounds at a much faster rate than did sodium hydroxide. After two hours 95% of the total weight obtained at 40 hours (fig. 12) was extracted by the high resolution buffer, whereas with the sodium hydroxide/stannous chloride mixture only 40% of this weight was extracted after two hours.

FRACTIONATION

The extracts obtained by extracting the different soils with the organic solvents and with the sodium hydroxide/ stannous chloride solution could not be separated into fractions distinguishable under ultraviolet light.

When electrophoretic fractionation methods were applied to the formic acid extracts, the presence of a number of fractions could be indicated with the aid of ultraviolet light (fig. 17). Similar results were obtained with high resolution buffer extracts, but, as already shown, the structure of the humic compounds in these extracts changed upon standing. Three to four days after extraction the extract was found to be electrophoretically homogeneous.

As the presence of non-fluorescing fractions is not indicated by this method, such fractions might have been present.

Since younger humic compounds are the most important for soil fertility, it may be concluded that the order of fertility of the four soils at the time of sampling decreased in the following order : grass, veld, crop rotation, maize.

PART IV

BIBLIOGRAPHY

BIBLIOGRAPHY.

1. Kyuma, K. Pedogenetical studies of Soil Organic Matter. Laboratory of Soil Science, Kyoto University, Japan. (1961).

2. Fuchs, W. Über die Beziehungen zwischen Huminsäure und Lignin. Z. angewandte Chem. 44, 111-118.(1931).

3. Waksman, S.A. Humus (origin, chemical composition, and importance in nature). Bailliere, Tindall and Cox. London. (1936).

4. Mattson, S.; Koutler-Andersson, E. The acid-base equilibrium in vegetation, litter and humus. VI. Ammonia fixation and humus nitrogen. Ann. agric. coll. Sweden. 11,107-134. (1943).

5. Kononova, M.M. The nature and properties of brown humus substances formed in the process of humification of plant residues (roots of alfalfa). Pochvovedeniye 7, 18-22. (1943).

6. Kononova, M. M. The process of formation of new humus substances. Pochvovedeniye 10, 456-470. (1944).

7. Beckley, V. A. The preparation and fractionation of humic acid. J. agric. sci. 11, 66-68. (1921).

8. Beckley, V. A. The formation of humus. J. agric. sci. 11, 69-77. (1921).

9. Enders, K. Wie entsteht Humus in der Natur? Angew. Chem. 56, 281 (1943).

10. Welte, E. Über die Entstehung von Huminsäuren und ihre Reindarstellung. Z. PflErnähr. Düng. 56, 105. (1952).

11. Marcusson, J. Lignin und Oxycellulosetheorie. Z. angew. Chem. 39, 898-900. (1926).

12. Marcusson, J. Lignin und Oxycellulosetheorie. Z. angew. Chem. 40, 48. (1927).

13. Eller, W. Synthetische Darstellung von Huminsäuren. Ber. deut. Chem. Ges. 53, 1469-1476. (1920).

14. Erdtman, H. Die Reaktion von Benzochinon mit Alkali. Z. PflErnähr. Düng. 69, 38-43. (1955).

15. Laatsch, W. Untersuchungen über die Bildung und Anreicherung von Humusstoffen. Beitr. Agrarwiss. Heft 3, 3. (1948).

16. Flaig, W. Die Bildung von Huminsäuren durch Streptomyceten. Z. PflErnähr. Düng. 56, 63-67. (1952).

17. Lindeberg, G. Ligninabbau und Phenoloxidasebildung der Boden Hymenomyceten. Z. PflErnähr. Düng. 69, 142-150. (1955).

18. Flaig, W. Zur bildungsmöglichkeit von Humussäuren aus Lignin. Holzforschung 9, 1-4. (1954)

19. Ploetz, Th. Polymere Chinone als Huminsäuremodelle. Z. PflErnähr. Düng. 69, 50-58. (1955).

20. Pauli, F.W. Fluorochrome absorption studies on decomposing plant residues. D.Sc. dissertation, U.O.F.S., South Africa. (1958).

21. Rippel-Baldes, A. Grundriss der Mikrobiologie. Springer Verlag. Göttingen. (1952).

22. Swaby, R. The influence of humus on soil aggregation. J. Soil Sci. 1, 182. (1950).

23. König, A. Über das Absorptionsvermögen humoser Medien. Landw. Jahrb. 11, 1-56. (1882).

24. Sladký, Z.; Tichý, V. Application of humus substances to overground organs of plants. Biol. Plant. 1, 9-15. (1959).

25. Sladký, Z. The application of extracted humus substances to overground parts of plants. Biol. Plant. 1, 199-204. (1959).

26. Pagel, H. Über den Einfluss von Humusstoffen auf das

Pflanzenwachstum. I. Einfluss von Humusstoffen auf Keimung und Wurzelwachstum. Albrecht Thaer Arch. 4, 450-468. (1960).

27. Pagel, H. Über den Einfluss von Humusstoffen auf das Pflanzenwachstum. II. Einfluss von Humusstoffen auf Ertrag und Nährstoffaufnahme. Albrecht Thaer Arch. 4, 493-506. (1960).

28. Chaminade, R. Recherche sur le role de la matière organique dans la fertilité des sols. Ann. Agron. 95. (1952).

29. Christeva, L.A. Einfluss der Huminsäure auf die Anfangsentwicklung des Pflanzenwachstums bei verschiedenem Nährstoffverhaltnis. Ber. Lenin-Akademie Landw. Wissenschaft 10. (1947).

30. Biber, V.A.; Magaziner, K.M. Effect of humic and fulvic acids on respiration of isolated plant tissue. Doklady Akad. Nauk S.S.S.R. 76, 609-612. (1951).

31. Christeva, L.A. Die rolle der Huminsäure bei der Pflanzenernährung und die Humusdüngung. Dokuchaev-Institut. (U.S.S.R.) 38, 108. (1951).

32. Christeva, L.A. Über die Teilnahme der Huminsäuren und anderer organischer Stoffe bei der Ernährung höherer Pflanzen. Soviet Soil Sci. 10. (1953).

33. Ploetz, T. Das lignin im Boden. Landb. Forsch. 4, 78-80. (1954).

34. Flaig, W. Über den Einfluss von Spuren von Humusstoffen bzw. deren Modellsubstanzen auf das Pflanzenwachstum. Landw. Forsch. 6, 133. (1955).

35. Scheffer, F.; Ulrich, B. Lehrbuch der Agrikulturchemie und Bodenkunde, III Teil: Humus und Humusdüngung. Stuttgart. (1960).

36. Odén, S. Die Huminsäuren. Leipsig. (1922).

37. Tiurin, I.V.; Gutkina, E.L. Contribution to the study of the nature of the humins of chernozems. Trans. Dokuchaev Soil Inst. 23, 41-57. (1940).

38. Berzelius, J.J. Lehrbuch der Chemie. Dresden (1839).

39. Hoppe Seyler, F. Über Huminsubstanzen, ihre Entstehung und ihre Eigenschaften. Z. Physiol. Chem. 13, 66-121.(1889).

40. Shmuk, A. The chemistry of the organic substances of the soil. Abh. Kuban Landw. Inst. 1, 1-91. (1923).

41. Shmuk, A. The problem of the chemistry of soil organic matter. Pedology (U.S.S.R.) 25, 5-48 (1930).

42. Fuchs, W. Die Chemie der Kohle. Springer. Berlin.(1931).

43. Kononova M.M.; Belt'chikova, N.P. Nature and role in soil formation of the humus of the U.S.S.R. Trans. VI. Int. Congr. Soil Sci. Paris B, 557-565.(1956).

44. Ziechmann, W. Untersuchungen über die Infrarotspektren von Huminsäuren. Brennstof Chemie 39, 353-384. (1958).

45. Flaig, W.; Beutelspacher, H. Elektronenmikroskopische Untersuchungen an natürlicher und synthetischen Huminsäuren. Z. PflErnähr. Dung. 52, 1-21. (1951).

46. Springer, U. Der heutige Stand der Humusuntersuchungsmethodik mit besonderer Berücksichtigung der Trennung, Bestimmung und Charakterisierung der Huminsäuretypen und ihre Anwendung auf charakteristische Humusformen. Z. PflErnähr. Düng. 6, 312-373.(1938).

47. Meyer,L. Ton-Humus Komplexe als Träger der Bodenfrucht-bar keit und als Bodenverbesserungsmittel. Forschungsd. 11, 344-355. (1941).

48. Sprengel, C. Über Pflanzenhumus, Humussäure und Humussäure Salze. Katner's Arch. ges. Naturlehre 8, 145-220. (1826).

49. Sprengel, C. Die Bodenkunde usw. Leipsig. (1837).

50. Kobo, K.; Tatsukawa, R. On the coloured matter of Fulvic acid. Z. PflErnähr. Düng. 84, 137-147.(1958).

51. Thiele, H.; Kettner, H. Humic acids. Koll.-Z. 130, 131-160. (1953).

52. Ahmed, M.; Kinney, C.R. Ozonization of Humic acids prepared from oxidised bituminous coal. J. Am. Chem. Soc. 72, 559. (1950).

53. Scheffer, F.; Welte, E.;Hempler, G. Über oxidimetrische Eigenschaften von Huminsäuren. Z. PflErnähr. Düng. 58, 68-88. (1952).

54. Zetzsche, F. Fossil plant materials. In G. Klein: Handbuch der Pflanzenanalyse 3, 328. (1932)

55. Flaig, W.; Schulze, H. Über den Bildungsmechanismus der Synthese Huminsäuren. Z. PflErnähr. Düng. 58, 59-67. (1952).

56. Flaig, W.; Scharrer, K.; Judel, G.K. Zur Methodik der Bestimmung des Redoxpotentials im Boden. Z. PflErnähr. Düng. 68, 97-122 + 203-218. (1955).

57. Broadbent, F.E.; Bradford, G.R. Cation-exchange groupings in the soil organic fraction. Soil Sci. 74, 447-457. (1952).

58. Flaig, W.; Beutelspacher, H. Physikalische Chemie der Huminsäuren. Landbouwk. Tijdschr. 66, 306-335. (1954).

59. Welte, E.; Niemann, A.; Ziechmann, W. Über Huminsäuren II. Molekular- und Äquivalentgewicht. Naturwisschaften 41, 334. (1954).

60. Springer, U. Compost humic acids from different vegetable initial substances. Z. PflErnähr. Düng. 69, 66-71. (1955).

61. Nehring, K.; Schiemann, E. Humus problems. 1. The reactions involved in the rotting of stable manures and composts, and a study of humic acids. 2.Humic acids.

Z. PflErnähr. Düng. 57, 193-215. (1952).

62. Kosaka, J.;Honda, C. Methoxyl content of humus. Soil Plant Food (Tokyo) 2, 59-62. (1956).

63. Sowden, F.J.; Parker, D.I. Amino nitrogen of soils and certain fractions isolated from them. Soil Sci. 76, 201-208. (1953)

64. Beutelspacher, H. Physikalisch-chemische Beitrage zur Humusforschung. Z. PflErnähr. Düng. 57, 57-65. (1952).

65. Jodl, R. Chemistry and technology of natural and synthetic humins. Brennst. Chemie. 22, 78-85. (1941).

66. Jodl, R. Röntgenographic researches on natural and artificial humic substances. Brennst. Chem. 22, 157-162. (1941).

67. Jodl, R. Carbohydrate humic acids and their relations to natural and phenol humic acids. Brennst. Chem. 22, 217-220. (1941).

68. Jodl, R. Size and shape of humic acid crystallites. Brennst. Chem. 22, 256-257. (1941).

69. Jodl, R. Röntgenographische Untersuchungen über die Wasserbindungsverhältnisse der Humusbestandteile, Lignin, Huminsäure und Kalziumhumat. Z. PflErnähr. Düng. 34, 170-174. (1944).

70. Gorbunov, N.I. X-ray and electronographic studies of humic substances and humates. Pedology (U.S.S.R.) 240-248. (1947).

71. Jung, E. The fine structure of humus substances (A) Röntgenographic investigations on humus substances. Z. PflErnähr. Düng. 37, 2-16. (1946).

72. Halla, F.; Ruston, W.R. Abnormal conductivities in humic acid sols. Z. Elektrochem. 59, 525-530. (1955).

73. Boutaric, A.; Thévenet, S. Physicochemical researches on

humic colloids. Ann. Agron. (N.S.) 7, 18-32. (1937).

74. Scheffer, F.; Ziechmann, W.; Schlüter, H. Über die Sedimentation von Huminsäuren in der Ultrazentrifuge. Koll. Z. 160, 1-7. (1958).

75. Samec, M.; Pirkmaier, B. Die Huminsäuren. Koll. Z. 51, 96-100. (1930).

76. Scheffer, F.; Ziechmann, W.; Schlüter, H. Über sedimentations Untersuchungen an Huminsäuren. Naturwiss. 44, 63. (1957).

77. Ostwald, Wo.; Rödiger, W. Solution and peptisation of humic acid by sodium hydroxide. Koll. Z. 43, 225-227. (1927).

78. Halfter, G. Cation effect on the colour values of humus materials. Z. PflErnähr. Düng. 14, 322-345. (1939).

79. Jansson, S.L.; Valdmaa, K. Attempts to differentiate humus types in cultivated soils. Nordisk Jordbruksforskning. 38, 26-275. (1956).

80. Scheffer, F.; Ulrich, B.; Meyer, B.; Oehmichen, J. Beitrage zur Huminsäure Extraktion. NaOH-Extraktion von Modellsubstanzen aus Waldhumus. Z. PflErnähr. Düng. 74, 157. (1956).

81. Kukharenko, T.A. A study of humic acids of coal of different coalification stages. Torfindustrie (Moscow) 27, 23. (1950).

82. Bremner, J.M. Some observations on the oxidation of soil organic matter in the presence of alkali. J. Soil Sci. 1, 198. (1952).

83. Schulze, H.; Flaig, W. Humic acids IV. Ring fission by the action of oxygen on polyhydric phenols in alkaline medium. Liebigs Ann. Chem. 575, 231-247 (1952).

84. Stadnikov, G. Neuere Torfchemie. Leipsig. (1930).

85. Deuel, H.; Dubach, P.; Bach, R. Dekarboxylierung der organischen Substanz des Bodens. I. Dekarboxylierung der gesamten Humusstoffe. Z. PflErnähr. Düng. 81, 189. (1958).

86. van Duuren, B.L. Nitric acid oxidation of coal. J. S. Afr. Chem. Inst. 4, 25. (1951).

87. Polansky, Th.S.; Kinney, C.R. Solvent extraction of humic acids from nitric acid-treated bituminous coal. Ind. Eng. Chem. 39, 925-929. (1947).

88. Baver, L.D.; Hall, N.S. Colloidal properties of soil organic matter. Missouri Agr. Exp. Sta. Res. Bull. 267, 3-23. ; Soil Sci. Soc. Am. Proc. 2, 75-76. (1937).

89. Ostwald, Wo.; Wolski, P. Dispersoid chemistry of peat——dispersoid-chemical changes in peat by steaming under pressure (ten Bosch process) I. + II. Koll. Z. 30, 119-133; 187-198. (1922).

90. Ubaldini, J. Huminsäuren. Brennst. Chem. 18, 273-279. (1937).

91. Springer, U. (Comment on) "Several years' observations on the decomposition and humification of organic materials in soils by O. Siegel." Bodenk. u. PflErnähr. 26, 292-298. (1942)

92. Schulte, L. Humification reactions occuring in composting straw. Bodenk. u. PflErnähr. 14, 297-322. (1939).

93. Simon, K.; Speichermann, H. Beitrage zur Humusuntersuchungsmethodik. Bodenk. u. PflErnähr. 8, 129-152. (1938).

94. Springer, U. Die Bestimmung der organischen, insbesondere der humifizierten Substanz in Böden. Bodenk. u. PflErnähr. 11, 313-359. (1928).

95. Waksman, S.A.; Reuszer, H.W. Über die chemische Natur und den Ursprung des Humus in Erdboden. Zell. Chem. 11, 209-220. (1930).

96. Stevenson, F.J.; Marks, J.D.; Varner, J.E.; Martin, W.P. Electrophoretic and chromatographic investigations of clay-adsorbed organic colloids. I. Preliminary investigation. Soil Sci. Soc. Amer. Proc. 16, 69-73. (1952).

97. Lynch, D.L.; Wright, L.M.; Hearns, E.E.; Cotnoir, L.J. Adsorption of cellulose compounds, pectins and hemi-cellulose compounds on clay minerals. Soil Sci. 84, 113-127. (1957).

98. Forsyth, W.G.C. The characterisation of the humic complexes of soil organic matter. J. Agric. Sci. 37, 132-138. (1947).

99. Forsyth, W.G.C. Carbohydrate metabolism in the soil. Chem. and Ind. 515-519. (1948).

100. Schlichting, E. Heath humus. II. The fulvic acid fraction. Z. PflErnähr. Düng. 61, 97-103. (1953).

101. Scheffer, F.; von Plotho, O.; Welte, E. Untersuchungen über die Bildung von Humusstoffen durch Aktinomyceten. Landw. Forsch. 1, 81. (1950).

102. Scheffer, F.; Ziechmann, W.; Pawelke, G. Über die schonende Gewinnung natürlicher Huminstoffe mit Hilfe milder organischer Lösungsmittel. Z. PflErnährung Düng. 90, 58-69. (1960).

103. Forsyth, W.G.C.; Frazer, G.K. Freezing as an aid in the drying and purification of humus and allied materials. Nature 160, 607. (1947).

104. Souci, S.W. Die Chemie des Moores. Stuttgart. (1938).

105. Kroll, W. Oxidativer Abbau von Huminsäuren in Gegenwart von Kieselsäure. Dissertation Landw. Fak. Göttingen. (1958).

106. Frissel, M.J. The adsorption of some organic compounds, especially herbicides, on clay minerals. Verslagen Landbouwkundige Onderzoekingen , no. 67.3 Wageningen. (1961).

107. Brydon, J.E.; Sowden, F.J. A study of the clay-humus complexes of a chernozemic and a podzol soil. Can. J. Soil Sci. 39, 136-143. (1959).

108. Bremner, J.M.; Mann, P.J.G.; Heintze, S.G.; Lees, H. Metallo-organic complexes in soil. Nature 158, 790-791. (1946).

109. Rather, J.B. An accurate loss-on-ignition method for determination of organic matter in soils. Arkansas Agr. Expt. Sta. Bull. 140. (1917).

110. Ponomareva, V.V. The determination and chemical nature of fulvic acid. Pochvovedeniye 714-723. (1947).

111. Arnold, C.L.; Lowy, A.; Thiessen, R. Isolation and the study of the humic acids from peat. U.S. Dept. Int. Bur. Mines Rep. Invest. 3258. (9 pages) (1934).

112. Bremner, J.M.; Harada, T. Release of ammonium and organic matter from soil by HF and effect of HF treatment on extraction of soil organic matter by neutral and alkaline reagents. J. Agric. Sci. 52, 137-146. (1959).

113. Achard, F.K. Chemische Untersuchung des Torfs. Crell's Chem. Ann. 2, 391-403. (1786).

114. Dragunova, A.F. Solubility of humic acids. Doklady Akad. Nauk (U.S.S.R.) 96, 77-79. (1954).

115. Shorey, E.C. Some methods for detecting differences in soil organic matter. U.St. Dept. Agric. Tech. Bull. 211. (1930).

116. Bremner, J.M. Studies on soil organic matter. III. The

extraction of organic carbon and nitrogen from soil. J. Agric. Sci. 39, 280-282. (1949).

117. Choudri, M.B.; Stevenson, F.J. Chemical and Physico-chemical properties of soil humic colloids. III. Extraction of organic matter from soils. Soil Sci. Soc. Amer. Proc. 21, 508. (1957).

118. Gortner, R.A. The organic matter of the soil. I. Some data on humus, humus carbon and humus nitrogen. Soil Sci. 2, 395-442. (1916).

119. Dyer, W.J.; Wrenshall, C.L. Organic phosphorus in soils. I. The extraction and separation of organic phosphorus compounds from soil. Soil Sci. 51, 159. (1941).

120. Sohn, J.B.; Peech, M. Retention and fixation of ammonia by soils. Soil Sci. 85, 1-9. (1958).

121. Simon, K. Über die Vermeidung alkalischer Wirkung bei der Darstellung und Reinigung von Huminsäuren. Z. PflErnähr. Düng. 18,323. (1930).

122. Bremner, J.M.; Lees, H. Studies on soil organic matter. II. The extraction of organic matter from soil by neutral reagents. J. Agric. Sci. 39,274-279. (1949).

123. Dubach, P.; Mehta, N.C.; Deuel, H. Extraktion von Huminstoffen aus dem B-Horizont eines Podzols mit Äthylendiamintetraessigsäure. Z. PflErnähr. Düng. 95, 119-122. (1961).

124. Tinsley, A.; Salam, A. Extraction of soil organic matter with aqueous solvents. Soils and Fert. 24, 81-84. (1961).

125. Piettre, M. Recherche, au moyen de la pyridine, des, matières humiques et des matières grasses du sol. C.R. Acad. Sci. Paris 176, 1329-1331.(1923).

126. Soum, M.; Podbreznik, F. The phenol solubility of humic

acids. Bull. Inst. Pin. 57, 61-64 (1929).

127. Waksman, S.A.; Stevens, K.R. A critical study of the methods for determining the nature and abundance of soil organic matter. Soil Sci. 30, 97-116. (1930).

128. Karrer, P.; Bodding-Wiger, B. Zur Kenntnis des Lignins. Helv. Chim. Acta 6, 817-822. (1923).

129. Tinsley, J. The extraction of organic matter from soils with formic acid. VI. Congr. Soil Sci. (Paris) 541-546. (1956).

130. Parsons, J.W.; Tinsley, J. Extraction of soil organic matter with anhydrous formic acid. Soil Sci. Soc. Amer. Proc. 24, 189-201. (1960).

131. Clark, W.M.; Lubs, H.A. (1916) cited by Vogel, A.I. A Textbook of Quantitative Inorganic Analysis theory and practise. (second edition) Longmans, Green and Co., London. (1959).

132. Michaelis, L. Diethylbarbiturate buffer. J. Biol. Chem. 87, 33-35. (1930).

133. Aronsson, T.; Gronwall, A. Improved separation of serum proteins in paper electrophoresis —— a new electrophoresis buffer. Scand. J. Clin. Lab. Invest. 9, 338-341. (1958).