THE NITROGEN FRACTIONATION OF A CLAY TREATED SANDY SOIL

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Juan Colom
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This is to certify that the

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THE NITROGEN FRACTIONATION OF
A CLAY TREATED SANDY SOIL

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A.R. Wolcott
Major professor

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THE NITROGEN FRACTIONATION OF

A CLAY TREATED SANDY SOIL

By

JUAN COLOM

A THESIS

Submitted to the College of Agriculture
Michigan State University of Agriculture and
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AN ABSTRACT

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The distribution of nitrogen in a sandy soil which had received applications of clay three years previously was studied.

The addition of Wyoming Bentonite to the sand increased the electrophoretic mobility of its colloidal fraction. The C:N ratio was found to decrease with increasing application levels of clay, and this was due primarily to increases in total nitrogen. There was no relation between clay treatment and the hydrolyzable ammonia fraction, where inorganic nitrogen would appear. However, the close inverse relationship between cataphoretic mobility and carbon content suggested that organic, rather than inorganic, nitrogen compounds were principally effective in altering the charge on the clay.

The increase in nitrogen was due to increases in nitrogenous materials which were hydrolyzed in 6N-HCl and were soluble in the presence of excess $Ca(OH)_2$.

Fractionation of the hydrolyzable nitrogen into basic and non-basic constituents with phosphotungstic acid showed that both constituents increased with clay treatments. However, no relationship between Van Slyke amino nitrogen and clay treatment was found.

The acid hydrolyzable materials were then fractionated into functional constituents by the process of column separation recommended for protein hydrolysates by Fromageot and Lederer. Basic materials such as diamino acids were retained on a silica column, the acidic or

dicarboxylic on an alumina column, the aromatic on a charcoal column, and the final perfusate contained neutral non-aromatic materials. The recovery of basic and non-basic forms of nitrogen by this method agreed very closely with the phosphotungstic acid procedure.

A considerable proportion of the hydrolyzable nitrogen in these soils was found in the form of neutral non-aromatic constituents. They showed a marked tendency to increase with clay treatments. Aromatic forms of nitrogen were essentially unrelated to clay treatments, while the basic nitrogen constituents increased and the acidic decreased with increasing clay content.

Attempts were made to characterize amino acids in the soil separates by paper partition chromatography. Good separation of standard amino acids was achieved. However, no comparable separation was obtained with any fraction or separate from any of the soils. The ninhydrin-reactive compounds in these fractions were not present as free amino acids. Rather, the amino acids were complexed with other organic compounds which appeared to be aromatic in nature. It was not clear whether these complexes had been formed in the soil or whether they had arisen as artifacts during chemical treatment in the laboratory. However, their mobilities in phenol and in butanol-acetic acid on paper chromatograms were clearly influenced by the level of clay application.

The possibility that clay could have been present in the soil preparations was rejected on evidence from X-ray analyses. Glycerated

soil hydrolysates proved to be X-ray amorphous. X-ray patterns of unglycerated preparations showed no diffraction angles within the limits of the native soil clay or of Wyoming Bentonite.

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INTRODUCTION

Practice can never get far ahead of basic principles except accidentally (77). A basic understanding of the principles governing the scientific management of soils must take into consideration the transformations undergone by organic matter in the soil. The role of organic matter in fixing and liberating nutrients, as well as its ability to complex metals and react with clay colloids during its decomposition, have drawn the attention of many soil scientists. Numerous proximate fractionation procedures have been devised in an effort to isolate constituents of soil organic matter which could be further characterized and related significantly to soil properties.

The nitrogenous fractions of soil organic matter have been studied in greatest detail. We are still in the initial stages of learning about their importance in humification processes and their role in organic-inorganic soil colloid interactions. The interactions between clay minerals and various organic compounds have been studied in the laboratory. Effects of clay on the retention of carbon and nitrogen from organic materials decomposing in soils in the greenhouse have also been studied. The present study was concerned with the effects of clay under field conditions on the retention of carbon and nitrogen and upon the distribution of nitrogen among a number of chemically distinct fractions.

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REVIEW OF THE LITERATURE

For convenience, this review is divided into three general areas: Humus formation and chemical nature of humus, soil organic nitrogen, and evaluation of some of the newer methods used in soil organic matter investigations.

Humus Formation and Chemical Nature of Humus

From the standpoint of quantity added and residual material remaining in the soil, residues from higher plants predominate over animal residues and microorganisms as the principal source of soil organic matter. Largely by biochemical processes, organic matter is gradually transformed into a uniform, dark-colored, amorphous mass, designated as humus, in which the products of decomposition can no longer be identified with the original plant and aminal materials from which they came. Waksman (148) has enumerated some properties of humus that distinguish it from the plant and animal materials from which it was formed. Some of the outstanding properties mentioned by Waksman are its insolubility in water, considerable solubility in dilute alkali, and its partial solubility in acid. Chemically, it contains about 3 to 6% nitrogen and a C:N ratio close to 10:1. Humus is not a definite organic entity. It is a complex of substances in a state of more or less dynamic equilibrium. Its chemical composition depends, in part, upon the chemical nature of its precursors and, in part, upon the degree of decomposition and the environmental conditions under which decomposition has occurred. Humus is,

therefore, chemically not always the same, but changes constantly as a result of the continuous processes of decomposition brought about mainly by microorganisms (149).

The extent of decomposition of organic matter in soil is found to depend not only on the nature of the decay population, but also on the nature and amount of available nitrogen (148). Accordingly, for every unit of carbon decomposed as a source of energy, a certain amount of carbon and nitrogen is assimilated into microbial tissues. Nitrogen is present in fresh plant residues largely in the form of proteins. In the process of decomposition, these are hydrolyzed through a series of intermediary complexes with the final liberation of surplus nitrogen as ammonia which, in turn, is oxidized in normally aereated soils to nitrites and then to nitrates (148).

Apparently, the rate of decomposition of organic matter in soil is a function of the amount of available energy material added to the soil (31, 117). This viewpoint has been questioned by Allison (3). The latter believes that plant material composition is more important than C:N ratio per se. Maillard (84) claims that microorganisms are of minor importance in the formation of humus in nature, except for their hydrolytic action in converting proteins to polypeptides and amino acids and complex carbohydrates to sugars. Rubins and Bear (116) are also of the opinion that it is not the C:N ratio of the material as much as it is the nature of the specific constituent carbon compounds which, ultimately, decide the rate of decomposition of organic matter p

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in soil. Birch and Friend (17) have observed that it is possible that the so called "priming action" of fresh organic matter on the breakdown of soil humus, generally ascribed to microbial activity is, in fact, a displacement effect caused by an exchange between products of organic matter decomposition and organic compounds previously adsorbed and protected by clay.

Although proteins, nucleic acids, and other nitrogenous organic compounds in plant residues are rapidly mineralized when added to soils, not much more than about 1% of the organic nitrogen of the soil is made available during the growing season (24).

The low availability of soil organic nitrogen has been the subject of much speculation. Two general types of mechanisms have been proposed to account for the formation of humus of a resistant nature. The first of these includes various organic condensation reactions. The second includes several types of clay-organic interaction. The former theory and the different modalities of it which have been expressed (6, 10, 57, 58, 84) are best represented by the concepts of Waksman & Iyer (150, 151, 152, 153) and those of Mattson and Koutler-Andersson (91). Waksman and Iyer advanced the theory that the availability of soil proteins is reduced through combination with lignins. They advanced evidence in support of this theory from studies on the properties of complexes prepared by mixing and then acidifying alkaline solutions of lignin and protein (150, 151, 152, 153). Such complexes were found to be highly resistant to biological decomposition. From a,

these results they postulated that "lignoprotein complexes" are formed in the soil by a gradual condensation process.

As pointed out before, an entirely different explanation of the low availability of soil organic nitrogen has been provided by the proponents of the clay-organic interaction theory. Although the interaction was originally observed by Demolon and Barbier (42), no theoretical basis on which the reduced availability of organic nitrogen, in association with clays, could be explained appeared until Ensminger and Gieseking (50, 51, 52, 53) showed that hydrolysis of proteins by proteolytic enzymes was markedly reduced in the presence of certain clay minerals. They also found that the 001 spacing of the clay lattice increased with increase in gelatine adsorption and decrease in pH. Similar interactions have been found for clays and organic molecules other than proteins (19, 29, 48, 68, 80).

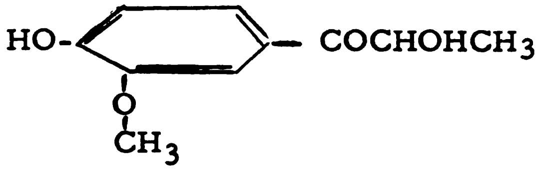
Lynch and Cotnoir (80) and Birch and Friend (17) observed that organic substrates adsorbed on clay minerals were protected from breakdown by soil microorganisms. Two leguminous crop residues, alfalfa meal and soybean leaf meal, produced, respectively, 29.0 and 40.0% less CO_2 in the presence of Bentonite over a short period. The inactivation of microbial enzymes by clay minerals, as well as the adsorption of substrates by the clay, were proposed as two hypotheses explaining the observed behavior.

Chemical interpretations regarding the manner in which both mechanisms of humus formation, i.e. the organic condensation and the

clay-organic interaction, take place have been proposed by several investigators. Waksman and Iyer (154) suggested that there is a reaction between the carbonyl groups of the lignin and the amino groups of the protein to give a Schiff's base: $\text{Lignin} - CO + H_2N - R - COOH \xrightarrow{\text{condensation}} \text{Lignin } C = N - R - COOH$. They considered that this complex is stable and has a high exchange capacity. Norman (105) suggested that such a complex may be one of physical adsorption, since some of Waksman's data suggest this and that Waksman's proposed reaction would not explain the high resistance to decomposition. Mattson and Koutler-Andersson (91) have postulated a process of autoxidative ammonia fixation by different organic matter fractions. They concluded that the nitrogenous humus complex is a chemical product resulting from autoxidation and simultaneous ammonia fixation. Alkaline conditions were found to enhance the process.

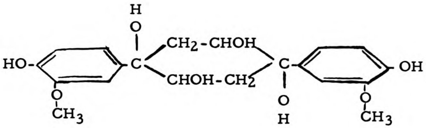
The following reactions have been suggested by Mattson and Koutler-Andersson(91) as possible reactions which take place in the oxidative fixation of ammonia by humus:

> Lignins in plant material are considered to be composed of C_6-C_3 units or C_6-C_6-C_6 units in varying degrees of polymerization. The C_6-C_3 units are related to degradative lignin derivatives of the type:



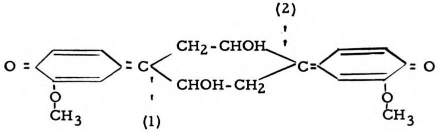
(a)

The C_6-C_6-C_6 units are of the type:



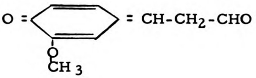
(b)

Mattson and Koutler-Andersson found that autoxidation of lignin resulted in a loss of phenolic hydroxyls. Oxidation of compounds (a) and (b) to quinones would account for this. For example, oxidation of (b) would form:



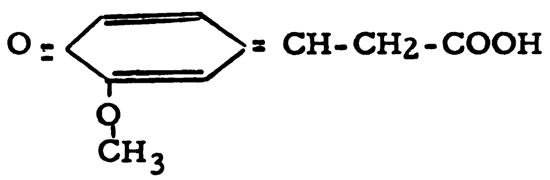
(c)

Fission of the central six carbon ring in compound (c) at (1) and (2) would give two moles of the aldehyde (d).



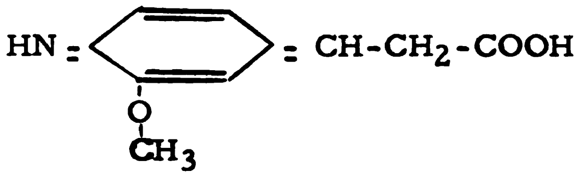
(d)

The aldehyde (d) would be readily oxidized to an acid:



(e)

The formation of organic acids as in (e) would account for the increasing cation exchange activity of organic materials which accompanies chemical autoxidation or normal microbial decomposition. Mattson and Koutler-Andersson (91) found an approximately linear relationship between nitrogen retained in litter or humus and its acidoid content. They observed that this relationship could be accounted for, if it were assumed that ammonia fixation occurred simultaneously with the above fission and oxidation reactions to give quinone - imid carboxylic acids of the type:



The interactions taking place between organic compounds and clay minerals have been described by several workers (3, 48, 68, 94, 102). As Allison (3) has observed, there is no complete agreement as to the exact nature of the clay-organic combination. Postulated mechanisms

vary from mere mechanical mixtures, to close unions that approach the nature of chemical compounds. Several investigators (43, 87, 126) claim to have demonstrated the esterification of the Si - OH groups of clay minerals by reactive organic compounds. Duel and his collaborators (43) observed that the charge on the clay was reduced in an amount equivalent to the degree of esterification. Greenland and Russell (68) have accumulated X-ray and chemical data tending to show that the interaction of clays with thionyl and acetyl chloride reported by other workers (87, 126) is to be explained in terms of hydrolysis of the reagent with adsorbed water, exchange between the cations initially present and the hydrogen formed by hydrolysis, and adsorption of the organic reagent or organic hydrolysis product. In any event, Glaeser (62) has shown that simple organic compounds adsorbed on the surface of montmorillonite by physical adsorption forces are extremely difficult to remove.

The foregoing observations and concepts relating to the origin of humus and its chemical nature, attest the complexity of the organic constituents of soil. Broadbent (29) believes that a sizable portion of soil nitrogen may be in forms which are not closely related to compounds produced either by plants or by microorganisms but, instead, are produced in the soil. It is obvious, as Bremner (24) has pointed out, that "present information regarding the chemical nature of the nitrogenous organic complexes of soil is extremely unsatisfactory and that this problem deserves high priority in any scheme of research on soil organic matter".

Soil Organic Nitrogen

For a long time, it was assumed that organic nitrogen in the soil was largely protein (79, 123, 136, 149, 152). Quite a large volume of recent evidence has accumulated tending to show that soil organic nitrogen contains considerable amounts of nitrogenous constituents very dissimilar to proteins. The fact that a considerable fraction, as much as 30%, of the soil nitrogen is resistant to acid or alkali hydrolysis has been taken to indicate that much of this nitrogen is of non-protein nature and has led to the suggestion that part of the organic nitrogen of soils is in the form of heterocyclic nitrogen compounds (24). Bremner (20) found that as much as 60% of the nitrogen in some humic acid preparations was not dissolved by hydrolysis with strong acids. He has also reported (26) that the amount of ammonia liberated by hydrolysis of humic acids with 6N - HCl was about maximum after six hours; whereas the amount of ammonia released by hydrolysis of soils increased steadily with time. Anderson (5) has identified purine and pyrimidine bases in hydrolysates of soil humic acid. Dyck and McKibbin (45) reported that not all organic soil nitrogen is determined by the Kjeldahl method; in every sample tested, the Dumas method (155) gave a considerably higher percentage of nitrogen. "This discrepancy in nitrogen values could be attributed to the presence of certain heterocyclic nitrogen compounds or of compounds containing nitrogen linked directly to nitrogen" (24).

Mattson and Koutler-Andersson (91) suggest that some of the organic nitrogen of soil that is stable to hydrolysis may be in the form of nitrogenous complexes formed in soil by interaction between oxydized lignin and ammonia. They showed that lignin was able to fix ammonia in a non-exchangeable form under soil conditions. Studies on the fixation of ammonia by organic compounds of known constitution led them to the view that fixation of ammonia-nitrogen by lignin takes place at the site of phenolic hydroxyl groups and is preceeded by the oxidation of these groups. Bennett (13) obtained evidence in support of this view. He was able to show that the nitrogen content of a complex prepared by autoxidation of lignin in NH_4OH was reduced from 7.22 to 2.66% by a somewhat drastic, prior methylation.

Sohn and Peech (128) attributed the capacity of mineral soils to fix ammonia, partly to a neutralization of the exchangeable hydronium and aluminum ions, and partly to the formation of organic nitrogen compounds by autoxidation of soil organic matter and simultaneous ammonia fixation. They estimated that at least 50% of the ammonia fixed was taken up by the latter mechanism.

Bremner and Shaw (27) found that the nitrogen in soil humic acid preparations was mineralized at a rate intermediate between that of nitrogen in the form of lignin-ammonia complexes and that in lignin-protein complexes. They proposed that neither the lignin-protein theory advanced by Waksman and Iyer (152, 153) nor the lignin-ammonia theory of Mattson and Koutler-Andersson is adequate, and that the observed properties of the humic acid fraction of soil organic matter is more readily explained by a concept incorporating both of these theories.

From hydrolytic fractionation studies it appears that from 20 to 50% of soil organic nitrogen is amino nitrogen, the rest is presumed to be associated with lignin or in heterocyclic combinations (21, 24, 79, 114). The isolation of soil amino compounds is hindered by the interference of other substances present in the hydrolysate and by induced alterations which the analytical procedure imposes by fission and polymerization reactions (20, 25, 91, 108, 120, 156). Bremner (25) briefly reviewed the amino acids which have been isolated as hydrolysis products of soil organic matter by different investigators. Adding to his list others reported in the literature (107, 112, 136), the following amino acids have been reported in hydrolysates of soil or soil organic fractions: Leucine, isoleucine, alanine, proline, arginine, histidine, lysine, aspartic acid, tyrosine, phenylalanine, valine, glycine, threonine, serine, glutamic acid, dihydroxyphenylalanine, ornithine, α -aminobutyric acid, methionine sulfoxide, and methionine sulfone. This list is of the order of a first approximation since, in a number of instances, reported amino acids were not rigorously identified (87, 135).

Bremner (25), and Stevenson (106), agree that the proteinaceous materials in different soils are similar in their amino acid composition. The evidence in the literature tends to show that the chemical

nature of soil organic nitrogen differs considerably from that of plants. In this connection Parker and Sowden (106) have compared the amino acid composition in soils and plants and have pointed out striking qualitative and quantitative differences. Bremner (25) and Stevenson (135) have verified the presence in the soil of α , ϵ -diaminopimelic acid; an amino acid previously identified in bacteria only.

Amino acids in soils do not appear to exist as free amino acids (20, 25, 130). Nonetheless, the question arises as to whether failure to show free amino acids may not be due to alterations during the isolation procedure. Payne (108) was able to show that soil leachates concentrated by freeze-drying permitted the detection of free ninhydrin reactive compounds, which he designated as free amino acids by comparing their Rf values in one dimensional paper chromatograms with standard amino acids. He failed to show these spots when concentration was effected in vacuo at 40°C. Putnam and Schmidt (112) also claimed to have identified free amino acids in the soil by column chromatographic separation.

On the other hand, amino acids do not appear to exist in soils as normal proteins (27, 73, 106, 136). Sowden and Parker (130), using the Sanger dinitrofluorobenzene procedure for tagging free amino groups, recovered large quantities of dinitrophenol derivatives of amino acids only from hydrolysates of soil humic acid preparations, none from the whole soil and relatively small amounts from the humic acid preparations themselves. From this, they concluded that soil amino

acids are essentially associated with humic acid. The same authors have pointed out (106) that it seems probable that protein itself is altered during humus formation. This concept is consistent with Bremner's finding (21) that as much as 60% of the nitrogen in some humic preparations he examined was non-acid-hydrolyzable.

Furthermore, soil humates, variously prepared, do not appear to be uniform organic entities. Sowden's work (130) clearly showed that amino acids in their humic acid preparations were distributed among several fractions which showed striking differences in solubility and in their susceptibility to acid hydrolysis. In the water phase of the unhydrolyzed material, the DNP derivatives of aspartic acid, glycine, ϵ-lysine, valine, and leucine, were identified; the ether phase of the same material contained DNP-glycine, DNP-valine, and DNP-leucine. Interestingly enough, no DNP derivatives were identified in the water phase of the hydrolyzed material; but DNP-valine and DNP-threonine were found in the ether phase.

Stevenson (136), contradicting an opinion expressed by Bremner (23), felt that it was not essential to regard amino acids isolated from soil as structural constituents of proteins. "Evidently, amino acids can be protected from microbial decomposition, either by adsorption on clays or by association with soil organic colloids" (135). Stevenson pointed out that his own data was consistent with the concept that humic acid may fix amino compounds in soils.

Amino acid reactions with other organic molecules as well as with

clay minerals have been proposed by several investigators (17, 29, 30, 91, 102, 103, 136).

Beside the amino acids, other nitrogenous organic compounds have been found in the soil. Anderson (5) identified purine and pyrimidine bases from hydrolysates of humic acid from Scottish soils. From his data he concluded that the soil nucleic acid derivatives appear to be of microbial rather than plant or animal origin. Bremner (24) has estimated that not more than 10% of the total nitrogen is present as nucleic acid. Adams, Bartholomew and Clark (1) consider that there is very little nucleic acid in soils. Stevenson (133, 134, 137) and Bremner and Shaw (28) have reported amino sugar nitrogen in soils in amounts equivalent to 1 to 10% of the total nitrogen. Glucosamine, chondrosamine, and their polymers, are some of the amino sugars, which have been identified in soils. Stevenson (134) found that hexosamines had accumulated preponderantly in the B horizon of some profiles studied.

It has recently been recognized that clay minerals have high sorptive capacity for ammonia (101, 122, 128). Some of the sorbed ammonia is held in non-exchangeable form by entrapment within the clay lattice. Scott, et al. (122) found that drastic treatments with NaOH and heat failed to release considerable quantities of the ammonia present in vermiculite. Stevenson, et al. (138) has presented data which indicates that non-exchangeable, clay-fixed ammonia is included in standard Kjeldahl procedures for determining total nitrogen. This fixed ammonia was found to increase in deeper soil horizons and accounted for the narrowing of soil C:N ratios with depth.

Evaluation of Some of the Newer Methods Used in Soil Organic Matter Investigations

Chromatographic Procedures

According to Cramer (40), chromatography was originally intended for use solely on colored substances, but as a result of numerous improvements and modifications, it has been possible, especially during the last few years, to adapt the process for employment with colorless materials. In 1941, '42, and '43, Martin, Gordon and Synge (37, 64, 86) developed a process of partition chromatography for the separation of hydrophilic substances, especially amino acids. In 1944, Consden, Gordon, and Martin (36) developed the paper partition chromatographic technique, now extensively used for amino acid analyses.

Regarding the experimental conditions desirable for resolving amino acids on paper, Consden et al. (36) have found that the most satisfactory solvents are those which are partially miscible with water; solvents completely miscible with water can be employed provided that the water content is not too high. In this case, presumably, the cellulose of the paper, by a 'salting out' effect, allows the system to function as a partition chromatogram. The main effect of temperature is on the rate of movement of amino acids but is also explained partly in terms of changes in composition of the phases (36). Normally, the paper chromatographic method may be applied to amino acid quantities ranging up to a maximum of 600 micrograms for each amino acid (40).

Inorganic salts and ions, if present in fairly high concentration, lead to considerable variance in Rf-values and also to "tail" formation. This is due to the fact that ions act as hydrophilic particles and attract water, thus upsetting the equilibrium of the water between the solid and liquid phases of the system (40). In rendering visible the amino acids in the chromatogram, the ninhydrin (triketo-hydrindene hydrate) reaction is recommended (18, 37, 40, 144).

Chromatographic procedures are finding considerable application in the study of soil organic matter problems. As Bremner (22) has pointed out; "the failure of attempts to achieve useful fractionation of humus by chemical methods, suggests that the time is now ripe for an intensive application of modern chromatographic methods of fractionation". Before Bremner (25) first made a qualitative study of the amino acids in soils using the technique of paper chromatography, others (79, 115, 121, 124, 125) had isolated soil amino acids by chemical means but with limited results as far as yield and number of amino acids identified is concerned. Beside Bremner's (25) report, the following workers, among others, have used paper chromatographic techniques in identifying and semi-quantitatively evaluating amino acids in soil hydrolysates: Davidson et al. (41); Sowden & Parker (130); Stevenson (139). Chromatographic analysis of soil preparations has been extended by the use of column separation as in the Moore & Stein technique (95, 112, 129, 137). The latter procedure is supposed to eliminate interfering substances and separate the amino acids into groups.

The procedure is involved and expensive. Furthermore, paper chromatography or specific chemical tests have to be used for positive identification purposes.

Encouraging results have been obtained by the application of paper chromatographic analyses to soil hydrolysates. More information must be gained regarding the absolute distribution of soil amino nitrogen and its chemical associations in the soil. In this connection, it is interesting to recall that Stevenson (135), after observing that basic amino acids appeared to be more resistant to deamination reactions in soils than mono-amino acids, suggested that they are held more firmly by humic colloids and, hence, become less accessible for enzymatic action. He also suggested that diamino molecules could form points of attachment of humic colloids to clays, hence decreasing their availability. Accordingly, the ability of basic amino acids to resist decomposition in soils may also depend upon the kind and amount of clay mineral present.

Another point of interest is the reported presence (25, 129) of interfering materials in the chromatographic fractionation of soil hydrolysates. Sowden (129) found an interfering material which did not seem likely to be one of the more common amino acids. It reacted readily with the Moore and Stein reagents (96) but very slowly with a ninhydrin-in-butanol spray on paper chromatograms. Full color intensity did not develop for more than 24 hours after spraying and was pink rather than the blue or reddish blue of most amino acids. Because of the

interference of this material, they were unable to separate tyrosine and phenylalanine properly.

Other studies (5, 17, 20, 94, 106, 108, 112) on soil amino acids and their unknown soil combinations suggest that difficulties encountered in isolating specific amino acids are associated with their origin in the soil.

Electrophoretic Procedures

Electrophoretic or cataphoretic mobilities, among other electrodynamic properties, have been used for the determination of the sign and the magnitude of the electrokinetic potential on colloidal surfaces. The electtophoretic motion of colloidal particles is due to their electrical charges, i.e., to the presence of ionized groups (9, 89). Electrophoretic mobilities can be measured either by observing the motion of the particles directly with a microscope, or by observing the motion of the boundaries that the colloidal solution forms when the latter is placed in a U tube (33). The direction of migration indicates the sign of the charge on the colloidal particle. The velocity of migration is proportional to the electrokinetic potential (ζ potential) existing across the Hemholtz-double layer*. The potential difference in the double layer depends on the number of ions and on the thickness of the atmosphere (89).

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* "From the principle of the constant ion product, it follows that a Donnan equilibrium must be established between the outer layer surrounding the particles, in which the ions of one sign of charge predominate over the ions of the opposite charge, and the outside solution which contains an equal number of both kinds of ions" (90).

It has been established (85) that the Z potential is the most important factor governing the stability of dilute lyophobic solutions. Marshall (85) has summarized average cataphoretic velocities for different pure clays. He has given an average value of 2.75 μ/sec/volt/cm. for H-bentonite.

Mattson (89) has shown that increasing amounts of polyvalent anions associated with clay colloids increase their Z-potential. Thus, polyvalent anions function as stabilizers of clay suspensions (85). Mattson (89), after studying the behavior of electrodyalized bentonite, concluded that the resulting saturation of the clay with hydrogen leads to a low potential difference between the interior of the atmosphere and the outside water, as well as a low ionic concentration in the micellar atmosphere; thus, to a low Z potential.

As far as the author is aware, only Mattson (88) has studied the effect of polyfunctional ionic molecules, like proteins and humic acid, on the electrophoretic behavior of clay minerals. He observed that the isoelectric point of a protein is lowered by bentonite and explained that change as being due to the formation of a non-ionized compound between bentonite and proteins. He concluded that "there is no doubt that the proteins enter into the make-up of the soil colloidal complex, combining with the basic and acidic constituents according to the pH of the soil solution. But the reactions in the soil are so complex that a discussion of the subject must be postponed..."

X-Ray Diffraction

When an X-ray strikes an object that is made up of repeating units whose distances of separation are commensurate with the wavelength of the X-ray, we obtain diffraction of the X-rays. Diffraction involves the reinforcement of rays whose waves are in phase and the cancellation of rays whose waves are out of phase.

X-ray diffraction studies of fatty acid crystals (55, 111) have shown three principal lattice spacings. The two small similar spacings are nearly independent of the carbon chain but are dependent on the thickness of two parallel fatty acid molecules, while the long ones do depend on the carbon chain and approximately correspond to two molecular lengths of fatty acids. In a crystal, the carboxyl groups form the main reflecting planes and are packed in double layers alternating with double layers of methyl groups; the double layers of methyl groups do not reflect. The individual hydrocarbon chains have parallel orientation.

Regarding X-ray diffraction studies with peptides, repeating distances along the fibre axis of 7.0Å have been reported. This is the distance occupied by two amino acid residues. The side chain spacing was 5.2 Å and that of the "back bone" of the peptide of silk fibroin was 4.6 Å(33).

Astbury and Street (7), as cited by Bull (33), were able to show that there is a definite relation between the molecular structure and the extent of stretching of a protein fiber. Thus ordinary hair or wool is reported to exist as an α-Keratin with a repeating distance along the fiber axis of 5.5 Å. When the hair is extended sufficiently, it is changed into β-Keratin with a repeating distance along the fiber of 3.32 Å, a backbone spacing of 4.6 Å, and a side chain spacing of 9.8 Å. The structure of β-Keratin is that of a stretched peptide chain (4, 39).

Bernal (14), in an extensive survey, tabulated the results of preliminary studies of fifteen amino acids and related compounds, including diketopiperazine, by means of X-ray.

No information about X-ray diffraction by glycerated amino acids was found in the literature. The information presented in this work appears to be the first.

Regarding X-ray diffraction studies with clay minerals, extensive work has been published, and principal diffraction peaks are well known. Quite reliable techniques for differentiating one clay mineral from another are available (69, 92).

Ensminger and Gieseking found that the 001 spacing of bentonite increased with the adsorption of proteins and with decrease in pH (52, 53, 61). They inferred that the large protein molecules were adsorbed within the variable portion of the 001 spacing of the crystal lattice. When the amino groups were destroyed by treating the protein with nitrous acid, the clay with untreated gelatin gave a 001 spacing 8.0 Å higher than that with treated gelatin.

No X-ray diffraction patterns have been published of amino acid-clay complexes as such.

Infra Red Absorption

The infra red region of the electromagnetic spectrum may be div-

ided into four sections (71); the photographic (visible to 1.2 microns), overtone (visible to 3 microns), near infra red (2.5 to 25 microns), and far infra red (25 to 300 microns). In the photographic region the principal absorbancies observed are associated with electronic transitions. Rotational and vibrational effects are also superimposed. The overtone region is so called because transitions can occur at energy levels which are multiples of the "finger print" section. We find rotational and vibrational absorption in this region. Absorption in the near infra red is caused by the vibration of atoms about an equilibrium position in the molecule and the combination of these vibrations with rotation of the atoms to produce vibrational spectra. This is also called the "finger print" infra red region. Last, absorption in the very far infra red derived from transitions from radiant to rotational energy only.

The region of the spectrum of interest for analysis is from 2.5 to 25 microns, equivalent to wave numbers of 4,000 to 400 cm.$^{-1}$ (71).

Infra red absorption has been successfully applied to clay minerals and soil organic fractions (2, 74, 93). Bertramson (15) has observed that with some improvements this method will, probably, be the best single method for clay mineral identification.

As far as the infra red spectra of amino acids are concerned, Bellamy (12) has presented a detailed review of the literature on this subject. He points out that no absorption in the usual NH stretching region of 3,500 to 3,300 cm.$^{-1}$ (2.9 to 3.0μ) is shown by any amino

acid or hydrochloride. This is supported by the work of many investigators cited by Bellamy (12). According to Bellamy, all amino acids capable of possessing the NH_3^+ structure and their hydrochlorides, show two characteristic absorptions in the region of 1,600 to 1,500 cm.$^{-1}$ (6.3 to 6.7μ). In addition, an ionic carbonyl absorption also takes place in this region. The first of these, 1,660 to 1,610 cm.$^{-1}$ (6.0 to 6.2μ), is often weak while the second, 1,550 to 1,485 cm.$^{-1}$ (6.5 to 6.7μ), is usually more intense.

The NH vibration for amido acids (NH_2 present in an amide instead of an amine) falls in the range 3,390 to 3,260 cm.$^{-1}$ (3.0 to 3.1μ).

No infra red absorption bands characteristic of amides, proteins, polypeptides, and amino acids are reported in the literature (12, 113) for frequencies greater than 4,825 cm.$^{-1}$ (2.1μ) or less than 880 cm.$^{-1}$ (11.4μ). There is, nevertheless, information regarding infra red absorption in this region by aromatic compounds, including aromatic amines. Bell (11), studying the absorption spectra of organic derivatives of ammonia, particularly anilines, found that the band occurring in the region 2.8 microns may be regarded as due to amino groups. Alkyl and aryl substitutions on the amino group of aniline caused the band to become shallower and further additions (yielding tertiary amines) caused the 2.8 micron band to disappear. A 2.3 micron (4,348 cm.$^{-1}$) band is present in aniline, as well as substituted anilines. Ellis (46) observed bands in toluene, xylene, and mesitylene, in the region 2.3 to 2.4 microns (4,348 to 4,167 cm.$^{-1}$) while Glatt and Ellis (63)

reported absorption bands in the 4,216, 4,322, 5,671 cm.$^{-1}$ (2.4, 2.3, 1.8 μ) and higher frequencies for polythene and Parowax. They also found a 4,291 cm.$^{-1}$ (2.3 μ) band in nylon, a polyamide.

Colthup, as cited by Bellamy (12), found that the band within the range 1,625 to 1,575 cm.$^{-1}$ (6.2 to 6.3 μ) was characteristic for most aromatic materials. Again, according to Bellamy, these bands are highly characteristic of the aromatic ring itself and, taken in conjunction with the C-H stretching band near 3,030 (3.3 μ), they afford a ready means of recognition for this structure. The following remarks are cited from Bellamy (12).

> "On theoretical grounds, the main 1,600 and 1,500 cm.$^{-1}$ (6.3 and 6.7 μ) bands of the phenyl ring can be expected to occur also in polycyclic materials where there should be expected some broadening of the ranges over which they can occur.
>
> ' The 1,600 to 1,500 cm.$^{-1}$ (6.3 to 6.7 μ) absorption bands are notorious for very wide fluctuations in intensity. Frequently, the bands are weak in conjugated structures, and are often shown only as shoulders on other bands.
>
> 'However, heterocyclic aromatics such as pyridine and pyrimidines also give a similar pair of bands in the 1,600 cm.$^{-1}$ (6.3 μ) region from the C = C and C = N links, although in this case the third skeletal

> vibration is usually at appreciably lower frequencies. In certain cases in which the carbonyl absorption is capable of shifting toward the 1,600 cm.$^{-1}$ (6.3μ) region under the influence of strong hydrogen bonds, this sometimes makes it difficult to differentiate between the two."

Bellamy has presented a detailed discussion of the infra red absorption of aromatic molecules and their derivatives in the region of 1,000 cm.$^{-1}$ (10.0μ) but, as he pointed out, the lower intensity of these bands renders them less generally useful than the higher frequency absorptions. "No information is available in the literature on these absorptions, and the correlations depend almost entirely on the unpublished work of those workers who have drawn up correlation charts" (12).

METHODS OF ANALYSIS

Total Carbon

Total carbon was determined by the dry combustion method (110).

Carbon Dioxide Evolution During Incubation

Carbon dioxide evolved during incubation at 35°C was collected in 0.5N sodium hydroxide. One-hundred-gram samples of soil were placed in two-quart glass fruit jars. Water was added to bring the soil to the moisture equivalent. The alkali for CO_2 absorption was introduced in small vials, placed on the soil surface, and the jars were sealed. The jars were thoroughly aereated and the vials of alkali replaced at one to four-day intervals. Unused alkali was titrated with standard hydrochloric acid in the presence of phenolphthalein and an excess of $BaCl_2$, and in a CO_2-free atmosphere.

Total Nitrogen

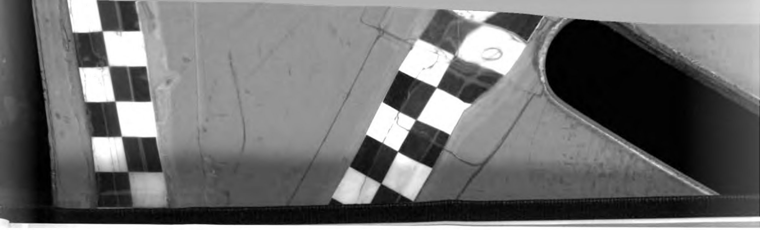
Total nitrogen in soils and in soil fractions was determined by the micro-Kjeldahl method using a 1:1:8 mixture of $SeO_2:CuSO_4:K_2SO_4$. Ammonia was collected in a 2% boric acid solution and titrated with standard acid in the presence of bromcresol green-methyl red indicator (81).

Nitrifiable Nitrogen

Nitrate released during incubation of moistened soils at 35°C was determined by the Iowa incubation procedure (131).

Primary Hydrolytic Fractionation of Nitrogen

The nitrogen fractionation used in the present work has been des-



cribed elsewhere (26, 98, 114, 142). The diagram on Figure 1 shows the detailed procedure followed in the fractionation and the determinations which were performed on every fraction.

Van Slyke Nitrogen

Amino nitrogen was estimated using 5 ml. aliquots and strictly following Van Slyke's nitrous acid technique (109, 141, 146, 147). Standard amino acids run prior to the determination of the unknowns demonstrated recoveries of 100 ± 0.1% of theoretical amino nitrogen.

Photometric Ninhydrin Procedure

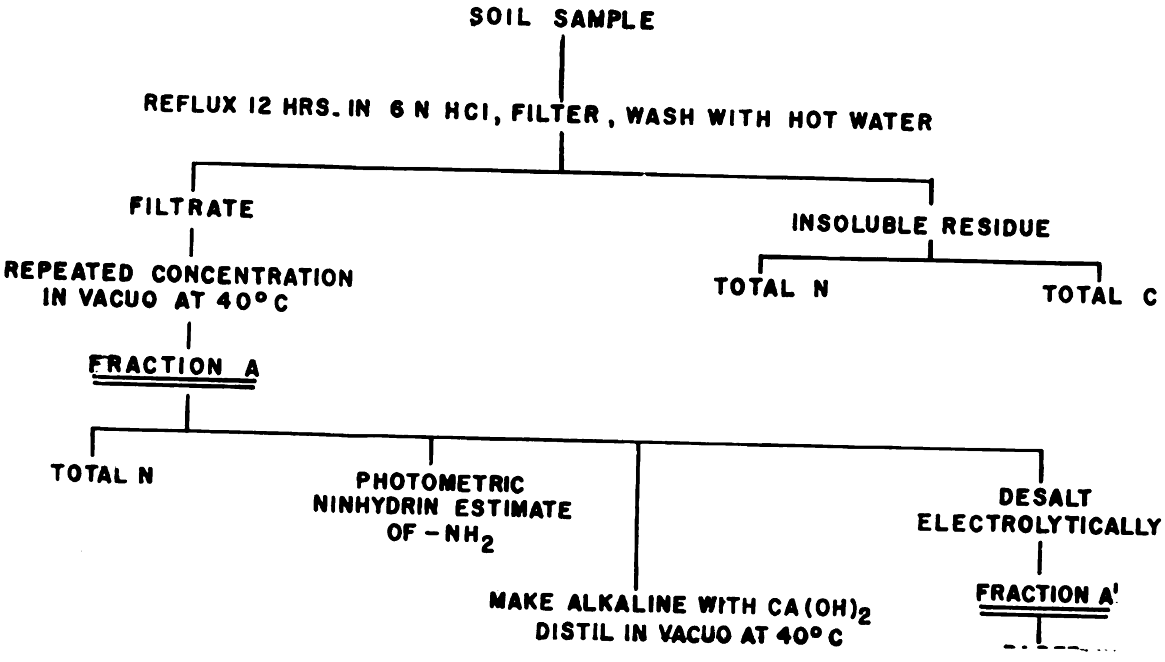
Alpha amino nitrogen was estimated photometrically with ninhydrin as described by Troll and Cannan (140).

Desalting

Soluble fractions were desalted electrolytically, prior to spotting on paper, with a commercial desalter manufactured by the Research Equipment Corporation, Oakland, California, Model X-100 (157).

Paper Chromatography

Two-dimensional ascending paper chromatography was employed, using Whatman No. 1 paper, 30 cm. square; 80% phenol was used in the first direction and butanol-acetic acid-water (40:10:50) in the second direction through the fastest grain of the paper (18, 40, 72). The phenol was purified by distilling at reduced pressure and a constant temperature of 84°C, and was never used more than once; the butanol solvent was always prepared fresh immediately before using it. A desalted aliquot of 10 microliters was always spotted


SOIL SAMPLE
REFLUX 12 HRS. IN 6 N HCl, FILTER, WASH WITH HOT WATER
FILTRATE
INSOLUBLE RESIDUE
REPEATED CONCENTRATION IN VACUO AT 40° C
TOTAL N
TOTAL C
FRACTION A
TOTAL N
PHOTOMETRIC NINHYDRIN ESTIMATE OF $-NH_2$
DESALT ELECTROLYTICALLY
MAKE ALKALINE WITH $Ca(OH)_2$ DISTIL IN VACUO AT 40° C
FRACTION A'

from a micropipete held at 3 cm. distance from the same corner of the paper while drying with a hair drier and an infra red lamp. The papers were dried at 90°C in the oven after each solvent and after spraying with a 0.2% alcoholic ninhydrin solution.

Mixtures of pure amino acids of considerably different Rf values were chromatographed by the above described procedure to provide standard reference chromatograms.

Separation of the Phosphotungstic Acid Fractions

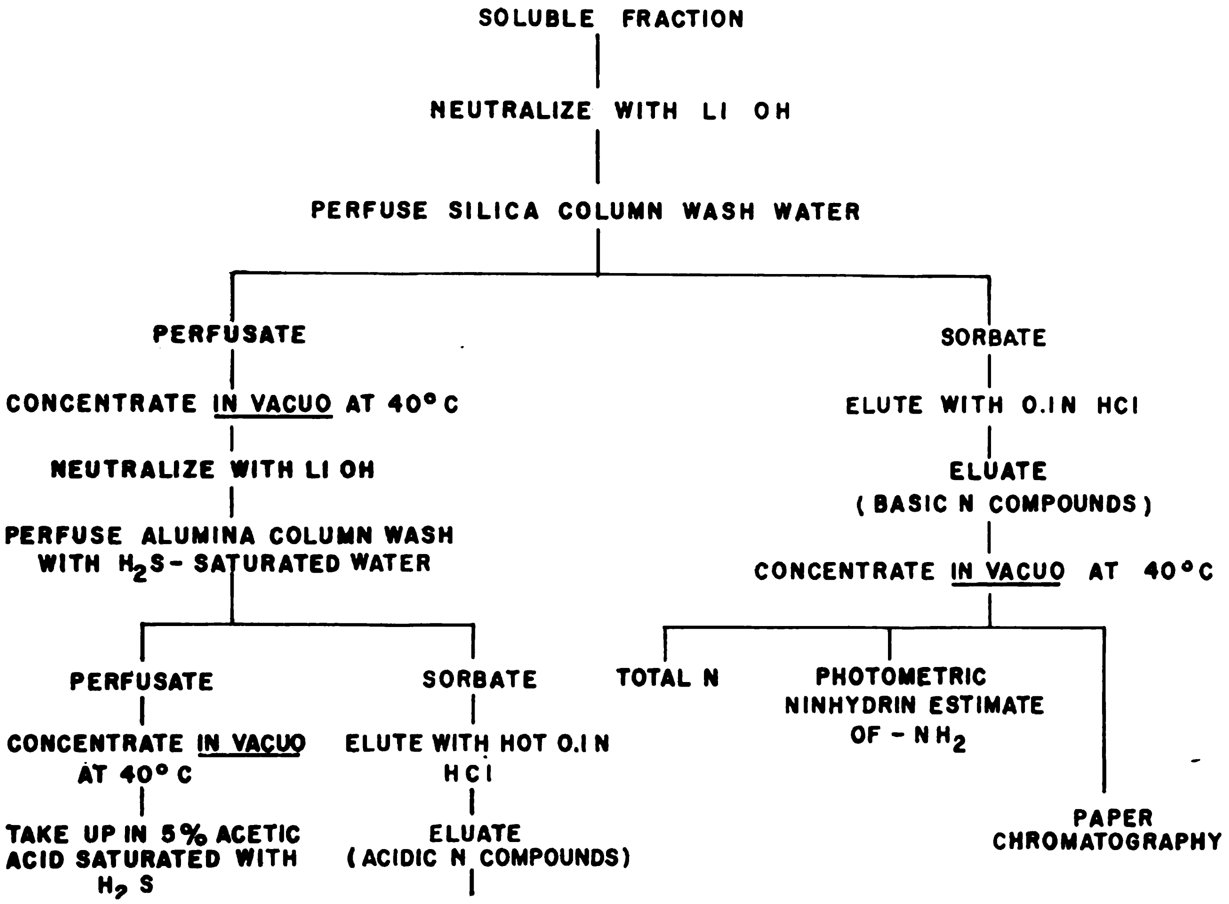
Basic and non-basic nitrogen compounds in soil hydrolysates were separated using purified phosphotungstic acid (145) and following the procedure outlined by Dunn and Drell (44) for removing the phosphotungstic acid with a 1:1 mixture of amyl alcohol and diethyl ether (143).

Column Separation Procedure

Hydrolyzable nitrogen compounds were separated into basic, dicarboxylic, aromatic, and neutral non-aromatic fractions by closely following Fromageot and Lederer's chromatographic separation procedure (59). Aliquots of the desalted fraction B^1 (see Figure 1) or intact portions of fraction A were used, since the latter could not be directly desalted because of foaming and decomposition shown on trial runs. Figure 2 shows the sequence followed during this part of the work.

Electrophoresis

Electrophoretic mobilities were observed on 10 ml. aliquots of


SOLUBLE FRACTION
NEUTRALIZE WITH LI OH
PERFUSE SILICA COLUMN WASH WATER
PERFUSATE
CONCENTRATE IN VACUO AT 40° C
NEUTRALIZE WITH LI OH
PERFUSE ALUMINA COLUMN WASH WITH H_2S- SATURATED WATER
PERFUSATE
CONCENTRATE IN VACUO AT 40° C
TAKE UP IN 5% ACETIC ACID SATURATED WITH H_2S
SORBATE
ELUTE WITH HOT 0.1 N HCl
ELUATE (ACIDIC N COMPOUNDS)
SORBATE
ELUTE WITH 0.1 N HCl
ELUATE (BASIC N COMPOUNDS)
CONCENTRATE IN VACUO AT 40° C
TOTAL N
PHOTOMETRIC NINHYDRIN ESTIMATE OF $-NH_2$
PAPER CHROMATOGRAPHY

.03% clay suspensions containing particles with diameter less than 0.7 microns, using the moving boundary technique (33). A potential gradient of 5.25 volt/cm. was used and the medium was prepared by adding 0.1N NaCl to distilled water until the specific conductance equaled that of the clay suspension.

X-Rays

In the X-ray and infra red analyses, aliquots of the same preparations employed in the paper chromatographic analyses were used. Diffraction patterns of the soil fractions were obtained with a Phillips Norelco X-ray spectrometer (1950 model) equipped with a Copper target. One mililiter of the sample was served over a microscope slide and completely dried under an infra red lamp, except where otherwise indicated.

Infra red

The infra red absorption studies were conducted with a model-21, double beam, self recording spectrophotometer, using NaCl plates, and Nujol or hexachlorobutadiene as mounting medium whenever the sample was not thick enough to smear directly on the salt plates.

Glyceration

Glyceration was accomplished by mixing the sample with C.P. glycerine (4:1 sample to glycerine ratio). After glyceration, the samples were dried inside a vaccum oven at 70°C for 4 days in the case of infra red analysis.

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CHARACTERIZATION OF THE SOILS

Field Description*

The soils used in the present work were taken from a field experiment on Plainfield sand at the Rose Lake Experiment Station, Michigan State University. Various plots in this experiment were treated in 1954 with Wyoming Bentonite at rates of 0, 6.25, 12.5, 25, and 50 tons per acre. After application, the clay was mixed into the top six inches of the soil, using a rototiller. One ton of limestone per acre and half-a-ton of 10-10-10 fertilizer was also applied. The following cropping and fertilization sequence was followed after treatment (99): Alfalfa was seeded with oats in the Spring of 1954. Oats were harvested in 1954 and one cutting of alfalta in 1955. Wheat was planted in September 1955 with 640 pounds of a 12-12-12 fertilizer broadcast ahead of planting. Forty pounds of nitrogen per acre was applied as a top dressing of ammonium nitrate on wheat in the Spring of 1956.

The plots were sampled to a depth of 6 inches in March 1957. The samples were air dried, sieved through a 10 - mesh screen, and composited by mixing 300 g. from each of the five replicates.

Significant increases in yields of oats, alfalfa, wheat, and corn with the clay treatments in the plots described above have been reported (99). Although there were significant increases in the yield of wheat

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* The field experiment described was established by Dr. M. M. Mortland and Dr. A. E. Erickson, Soil Science Department, Michigan State University.

and alfalfa above the check plots for all clay treatments, oat and corn yields showed significant increases for the low and intermediate levels of clay application only. In fact, the yield of oats decreased with the two high clay increments; the yield of the check being higher than the 50 tons per acre clay treatment, but lower than the rest.

Laboratory Determinations

Total nitrogen and total carbon were determined in the composited soil samples. It was found that C:N ratio decreased with increasing clay treatment (Table 1).

Table 1.

Carbon and Nitrogen in the Soils of the Experiment

Clay applied tons/acre	% Carbon	% Nitrogen	C:N
0	0.30	0.0245	12.5
6.25	0.37	0.0295	12.3
12.5	0.36	0.0290	12.4
25	0.34	0.0305	11.3
50	0.33	0.0310	10.6

These results are in harmony with those of Allison et al. (3), Bower (19), and of Ensminger and Gieseking (51). It should be pointed out that only trace amounts of exchangeable ammonia or of nitrate-nitrogen were found in the soils studied here. Although the carbon content was always higher for the clay-treated soil than for the check, there was, nevertheless, a tendency for the carbon to drop again with the heavier clay treatments. The latter tendency was accompanied by a steady increase in total soil nitrogen. As a result, C:N ratio decreased with successive clay increments. It is highly interesting to note that these findings were evident three years after the sandy soil was treated with clay. Using artificial sand-colloid preparations Allison showed that certain clay colloids, especially montmorillonite, protect organic matter against microbial attack (3).

Because of the field plot arrangements and cropping history of the experimental field, it can be assumed that the whole area was in contact with the same type of plant residues. Differences in quantity of residues returned, however, were undoubtedly associated with the recorded differences in yield of the various crops (99). Nevertheless, it does appear that nitrogenous compounds were preferentially retained by clays during the biochemical transformation of these residues.

Ensminger and Gieseking (51) have suggested that soil organic matter occurs in combination with inorganic colloids. Previously, Mattson (88) had suggested that the tendency of proteins to combine with inorganic colloids may account for the high nitrogen content of humus and

of the B horizons of soils. He observed that "silicate of gelatin", aluminum "proteinates", and leather ("protein tannate") were very resistant to decay. In some respects these substances are comparable to clay-protein or clay-humus complexes. Mattson found that a steady increase in positive cataphoretic mobility of protein "bentonates" was associated with increasing protein content (88). These observations led us to attempt the characterization of the treated soils in terms of the cataphoretic mobilities of their colloidal fraction.

The electrokinetic behavior of 0.03% clay suspensions extracted* from the composited sandy soil treated with the indicated levels of Wyoming Bentonite in the field for three years, is shown in Table 2.

Since it was considered possible that the observed positive migration could have been due to the presence of crystalloidal substances in the suspensions, the clay samples were then dialyzed in collodion bags, against running distilled water for a period of seven days, until first free of chlorides and then calcium, as detected by the silver nitrate and ammonium oxalate tests, respectively.

Table 2 contains the migration velocity and the corresponding values of the Z potential for both the dialyzed and the undialyzed clay suspensions. The 15 minute interval was chosen for comparing the Z potential of the clay suspensions because this was estimated to be a good average for most treatments. Besides, some determinations had

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* Particle sizes smaller than 0.7 microns were obtained by the pipette method, following dispersion in distilled water.

Table 2.

Migration Velocities and Calculated Values of ζ Potential for the Clay Suspensions After 15 Minutes

	Clay treatment tons/acre	Migration velocity microns/sec.	ζ potential millivolts
Undialyzed	0	9.4	22.34
	6.25	14.7	34.70
	12.5	13.1	31.02
	25	15.6	36.80
	50	18.0	42.58
Dialyzed	0	0	--*
	6.25	10.0	23.66
	12.5	5.6	13.14
	25	11.1	26.29
	50	12.22	28.91

* Precipitation after closing the circuit for running the trial invalidated this determination.

to be discontinued after 15 minutes because of lack of uniformity in the ascending boundary.

It should be noted that figures on Table 2 are relative rather than absolute. The Z potential has never been measured directly, but always calculated from observed movements which, in turn, are dependent on factors such as viscocity and dielectric constant of the medium, particle size and shape, and the electrical potential imposed. These figures show the comparative electrophoretic behavior of the colloidal fractions of the different soils.

There was, in fact, a reduction in Z potential when the suspensions were dialyzed, indicating that adsorbed ions had contributed to the high mobility of the undialyzed clays. The native soil clay in the check was completely precipitated at pH 6.1 (Table 3). There was a general trend for increasing Z potential with increasing level of clay treatment. At first glance, it might appear that such behavior could be attributed solely to dilution by bentonite of the native soil clay. As will be shown later, the native clay was largely chlorite, a slow moving clay.

However, there was an inversion of mobilities in the dialyzed suspensions between the 6.25 and 12.5 ton per acre levels of clay treatment. The low Z potential for the dialyzed clay from the 12.5 ton treatment was associated with a relatively high carbon content of the dialyzed suspension (Table 4). A generally inverse relationship between Z potential and carbon content existed for the whole group of soils, which suggests that organic materials associated with the clay

Table 3.

The pH of Soil Suspensions and of 0.03% Dialyzed Clay Suspensions

Clay treatment tons / acre	pH 1:1 Soil Suspension	 0.03% Dialyzed clay suspension
0	5.3	6.1
6.25	5.3	6.0
12.5	5.5	6.0
25	6.0	6.1
50	6.0	6.1

Table 4.

Carbon and Nitrogen Analyses* of the Dialyzed Clay Suspensions

Clay treatment tons / acre	% Carbon	% Nitrogen	C:N
0	0.32	not detectable	∞
6.25	0.20	0.84	0.24
12.5	0.34	0.84	0.40
25	0.15	0.92	0.16
50	0.18	1.00	0.18

* The assumption is made that the clay suspensions were .03% $^{w}/_{v}$ and the percentage figures are based on air dry weight of the initially added clay.

influenced its electrophoretic behavior. Associated carbon appears to have been more significant than nitrogen, since nitrogen content increased rather consistently with clay treatment and did not reflect any corresponding deviation for the erratic 12.5 ton treatment.

The deviating behavior of the soil from the 12.5 ton treatment does not appear to be due to sampling error, since the clay content was in line with the level of treatment. Moisture equivalents also showed the expected trend: 3.1% for the check soil and 3.1, 3.6, 4.1, and 10.3% for successive clay applications. The high carbon content of this soil appears to be related to the fact that maximum crop yields have been the highest with this treatment, from which it may be inferred that larger quantities of residues have also been returned to the soil with this treatment.

The low C:N ratios for the clay suspensions (Table 4) indicate that only a portion of the nitrogen in the dialyzed clay could have been organic. Since it was not removed by dialysis, much of it may have been ammonia entrapped within the clay lattice.

The results of carbon dioxide evolution during a thirty-day incubation study are shown on Table 5. In contrast with the observed decomposition of the organic matter after two weeks, the initial decomposition of the organic matter in these soils was independent of the clay or nitrogen content. Recalling that the C:N ratio for the whole soil at the three lower clay application levels was rather similar (Table 1), the observation is made that the increased evolution of CO_2 with increased

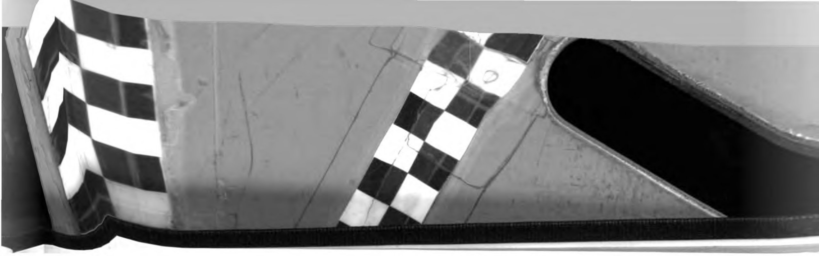


Table 5.

Carbon Mineralized During Incubation of the Soils of the Experiment

Clay applied tons / acre	Milligrams of carbon evolved as CO_2 per 100 grams of soil						
	2 days	3 days	5 days	7 days	11 days	16 days	30 days
0	4.0	6.8	10.2	12.8	17.6	21.9	27.5
6.25	4.3	7.3	11.1	12.9	18.2	24.3	31.7
12.5	4.5	7.4	11.0	13.6	18.9	24.0	34.2
25	4.6	7.2	11.9	14.3	20.5	25.5	36.8
50	4.9	7.5	11.1	13.2	19.3	26.1	37.3

clay treatment after two weeks appears to be associated with the chemical nature of the organic constituent rather than with its C:N ratio or its nitrogen content. These results are further supported by the fact that the NO_3-nitrogen accumulation for two weeks' incubation was 15 parts per million for all soils except the check. The latter nitrified 6 parts per million NO_3-nitrogen during the same period. The protection of soil organic matter by montmorillonite has been demonstrated by several investigators (3, 51, 88). The results reported here indicate that nitrogen is stabilized more effectively by this protective action of clay than is carbon. It would also appear that residual carbon retained during previous decomposition in the presence of clay is more readily available to microbial attack as the proportion of clay is increased.

The X-ray diffraction data for the clay suspensions is shown in Table 6 and Figures 3, 4, and 5. Chlorite appears to be the principal native clay mineral (Figure 3). This is supported by its non-expanding behavior demonstrated by its non-collapsing lattice when heated to 500°C after KCl treatment. It shows high intensity of its second and third order reflections. Figure 4 shows that all clay mineral preparations, except the check, show an expanded lattice above the limits of pure Bentonite. Interstratification of the Wyoming Bentonite was evident from the behavior of the clay mineral suspensions (other than the check) after heating to 500°C (Figure 5). Bands, rather than peaks, having a very low intensity were observed; diffraction hardly occurred,

Table 6.

X-Ray Diffraction Peaks of the Dialyzed Clay Suspensions and Dialyzed Wyoming Bentonite (in Å Units)

X-ray pre-treatment	Clay Treatment in Tons per Acre					Wyoming Bentonite
	0	6.25	12.5	25	50	
None	14.0	18.8, 14.5	18.3, 14.5	19.2, 14.5	19.2, 14.9	18.4
Dried in dessicator for 24 hours	14.0	18.3, 14.5	17.3, 14.2	18.8, 16.5, 14.5	18.8, 14.5	18.4
Heated to 110°C for 6 hours	13.6, 14.0, 6.8, 4.2	15.0, 14.3	15.0, 14.0	18.8, 14.2	15.8, 14.5	14.0
KCl - heated to 110°C	14.0	14.5	14.0	14.0	Lost	10.5
KCl - heated to 500°C	14.0	14.5, 8.5-12.5	14.0, 8.5-12.5	14.0, 8.5-12.5	Lost	10.0

FIGURE 3.

X-RAY DIFFRACTION PATTERN OF DIALYZED NATIVE CLAY

ISOLATED FROM THE CHECK SOIL

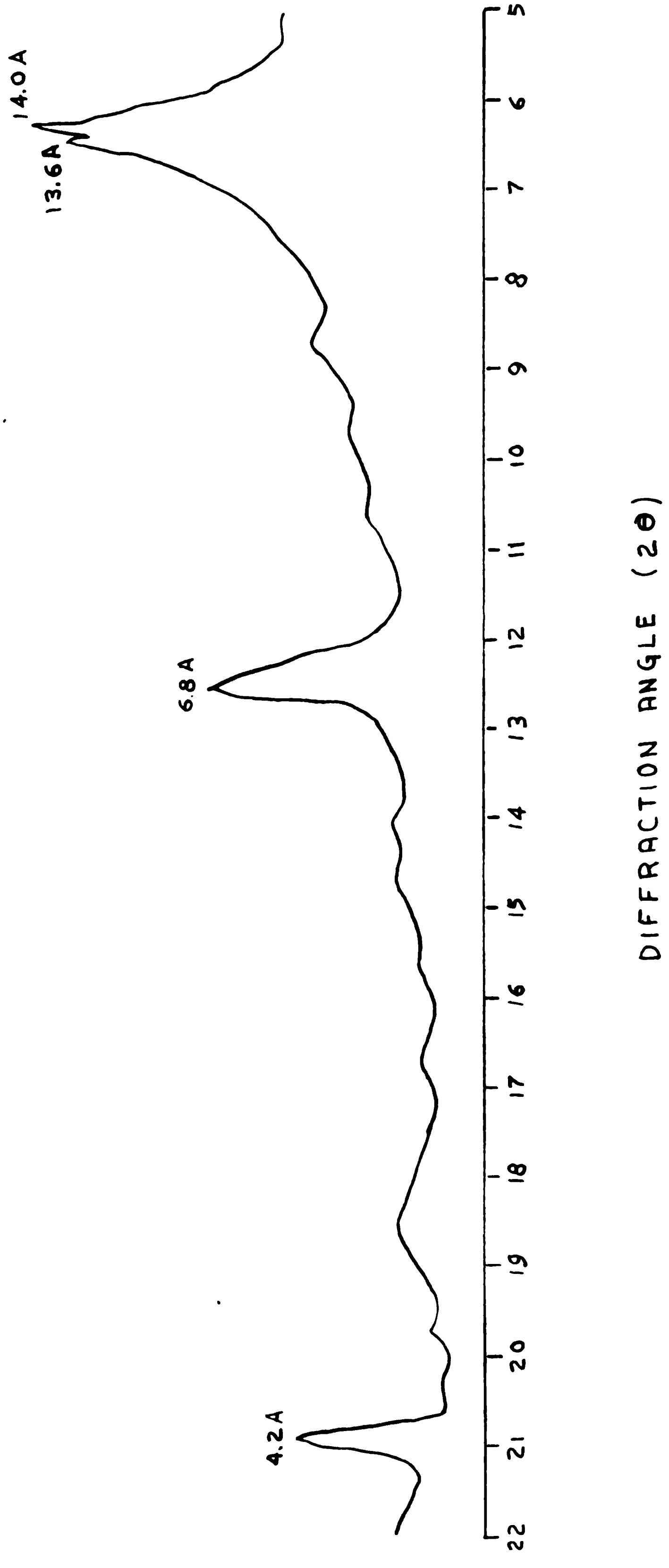

14.0A
13.6A
6.8A
4.2A
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
DIFFRACTION ANGLE (2θ)

FIGURE 4.

X-RAY DIFFRACTION PATTERNS OF DIALYZED CLAY SUSPENSIONS AND WYOMING BENTONITE AFTER HEATING TO 110°C

Pattern A - Corresponds to the check soil

Pattern B - Corresponds to the 6.25 tons of clay/acre treatment

Pattern C - Corresponds to the 50 tons of clay/acre treatment

Pattern D - Corresponds to Wyoming Bentonite

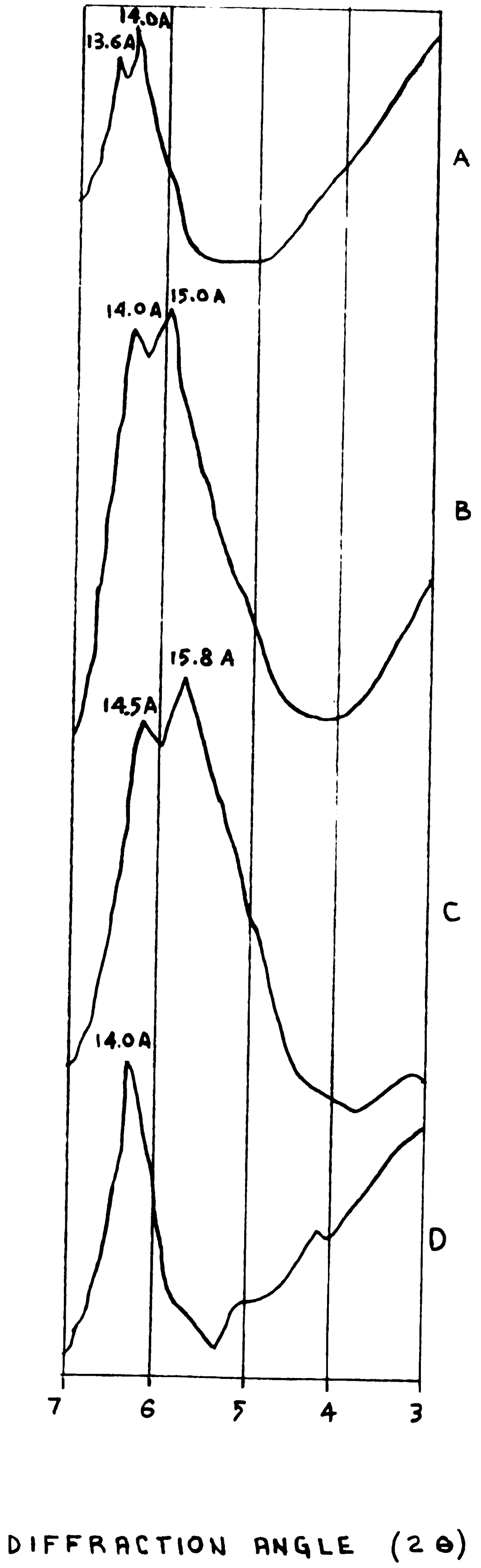

14.0A
13.6A
A
14.0A
15.0A
B
15.8 A
14.5A
C
14.0A
D
7
6
5
4
3
DIFFRACTION ANGLE (2θ)

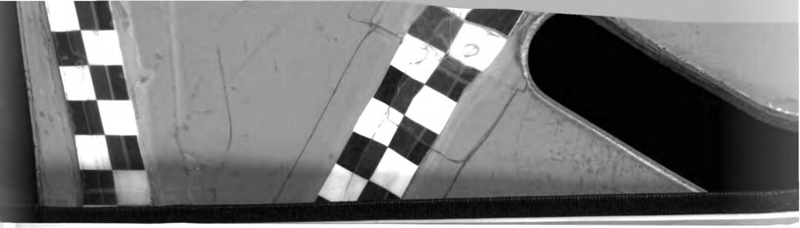
FIGURE 5.

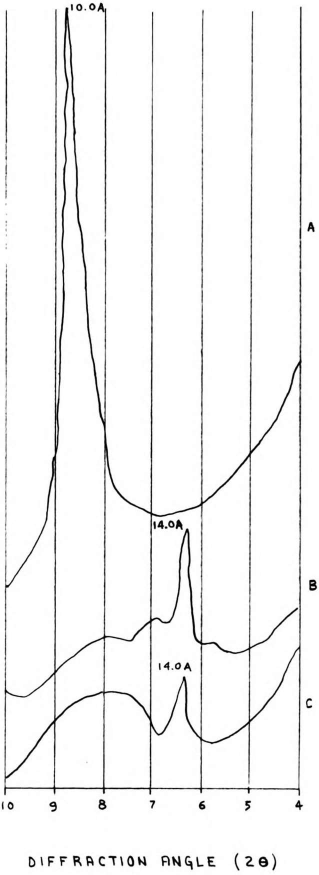
X-RAY DIFFRACTION PATTERNS OF DIALYZED CLAY SUSPENSIONS AND WYOMING BENTONITE AFTER TREATMENT WITH POTASSIUM CHLORIDE AND HEATING TO 500°C

Pattern A - Corresponds to Wyoming Bentonite

Pattern B - Corresponds to the check soil

Pattern C - Corresponds to the 6.25, 12.5, 25, and 50 tons of clay/acre treatments




10.0A
14.0A
14.0A
A
B
C
10
9
8
7
6
5
4
DIFFRACTION ANGLE (2θ)

suggesting disorientation of the crystal lattice, probably due to an incomplete burning of entrapped organic molecules between the clay lattice.

The high nitrogen content of the clay suspensions, along with the results of X-ray and electrophoresis studies, made us suspect that some nitrogenous organic constituents were interacting with the Wyoming Bentonite applied to the sandy soil. The low C:N ratios of the colloidal suspensions indicated that not all of the nitrogen associated with the clays was organic. However, as will be shown in the next chapter, there was no relation between clay treatment and the hydrolyzable ammonia fraction, where inorganic nitrogen would appear. The close inverse relationship between cataphoretic mobility and carbon content suggested that organic, rather than inorganic, nitrogen compounds were principally effective in altering the charge of the clay.

NITROGEN FRACTIONATION

The results of fractionating the total soil nitrogen of the different soil treatments into acid and non-acid hydrolyzable forms are presented in Table 7. As pointed out before, there was a definite trend for total soil nitrogen to increase with level of clay application. There was, essentially, no difference in the non-acid hydrolyzable nitrogen, especially in the case of the clay-treated soils; the check soil showed a somewhat lower absolute amount of acid resistant nitrogen, but this represented a larger proportion of the total soil nitrogen than in the soils treated with clay. The proportions of nitrogen liberated during hydrolysis are in agreement with those reported in the literature (26, 79, 97, 106). The data indicates that there was a distinct trend for the acid-hydrolyzable nitrogen to increase absolutely and percentagewise with increasing clay treatments.

It appears that the clay-associated organic nitrogen may have been protected from enzymatic transformation in the soil (100), but yet, it was susceptible to release by strong acid hydrolysis. On the other hand, the non-acid hydrolyzable nitrogen appears to have been independent of the amount of clay and of the acid hydrolyzable or total nitrogen. This suggests that this acid resistant soil nitrogenous fraction was associated with the original soil organic matter or humus, rather than with any nitrogenous organic fractions formed during the three-year period since clay treatment. Mattson and Koutler-Andersson (91) regard this acid-resistant fraction to be the product of chemical fixation

Table 7.

Recovery of Soil Nitrogen in Hydrolyzable and Non-Hydrolyzable Forms as Related to Clay Treatment

N fraction	Clay treatment None	6.25T	12.5T	25T	50T
	N, - mg. per 100 g. soil				
Total soil	24.5	29.5	29.0	30.5	31.0
Non-hydrolyzable	6.8	7.2	7.2	7.4	7.4
Hydrolyzable (Fraction A)	17.4	21.4	21.8	22.6	24.3
Not accounted for	-0.3	-0.9	0	-0.5	+0.7
	N, - percent of total soil N				
Non-hydrolyzable	27.8	24.4	24.8	24.3	23.9
Hydrolyzable (Fraction A)	71.0	72.5	75.5	74.1	78.4
Not accounted for	-1.2	-3.0	0	-1.6	+2.3

of ammonia which occurs simultaneously with the autoxidation of lignin or other aromatic constituents or decomposition products of plant materials. Stevenson (137) has suggested that amino acids or peptides may be similarly complexed by oxidized humic materials. Broadbent (29) has expressed the opinion that a sizable part of the organic nitrogen in soils may be in forms not closely related to compounds produced by plants or microorganisms, but may, instead, be produced in the soil. The data in Table 7 suggest that the non-hydrolyzable nitrogen fraction may have been more directly related to the degree of oxidation of such soil-formed nitrogen complexes than to the quantity or quality of plant residues returned or to the quantity of clay associated with their decomposition during the three-year period since treatment.

A further fractionation of the acid-hydrolyzable nitrogen was achieved by making it alkaline with calcium hydroxide and subjecting it to distillation in vacuo at 40°C. This treatment results in the release of hydrolyzable ammonia and the precipitation of an alkali insoluble fraction, called humin. There is left an acid-and alkali-soluble supernatant (Fraction B). Table 8 shows the results of this fractionation. The proportion of humin nitrogen decreased about half as the result of clay treatment, but there was no apparent significance in the differences between the several clay treatments. It should be noted that the absolute amount of nitrogen as well as the percent of the total nitrogen present in the soil hydrolysates after removal of ammonia and humin increased with successive clay treatments. This trend was less evident

Table 8.

Recovery of Nitrogen in Soil Hydrolysate (Fraction A) as Related to Clay Treatment

	Clay treatment				
N fraction	None	6.25T	12.5T	25T	50T
	N, - mg. per 100 g. soil				
Fraction A	17.4	21.4	21.8	22.6	24.3
NH_3-N	1.8*	4.5	3.8	4.2	3.5
"Humin" N	3.7	2.2	2.6	2.5	1.9
Fraction B	11.9	14.2	14.2	16.2	19.5
Not accounted for	--	-0.5	-1.2	+0.3	-0.4
	N, - percent of total soil N				
Fraction A	71.0	72.5	75.5	74.1	78.4
NH_3-N	11.4	15.2	13.1	13.8	11.3
"Humin" N	15.1	7.4	9.0	8.2	6.1
Fraction B	48.6	48.1	49.0	53.1	62.9
Not accounted for	--	-1.7	-4.1	+1.0	-1.3
	N, - percent of total N in Fraction A				
NH_3-N	16.1	21.0	17.4	18.6	14.4
"Humin" N	21.3	10.3	11.9	11.1	7.8
Fraction B	68.3	66.3	65.1	71.7	80.2
Not accounted for	--	-2.3	-5.5	+4.4	-1.6

*Calculated by difference rather than analyzed in this one sample only.

when the nitrogen in Fraction B was compared with the total nitrogen in the acid hydrolyzable fraction (Fraction A). It is possible that this lack of consistent trend in the proportion of hydrolyzable nitrogen recovered in Fraction B may have been related to the trends in hydrolyzable nitrogen not accounted for. It is known that Kjeldahl procedures do not give as complete recovery of nitrogen from organic materials as can be achieved with the Dumas catalytic ignition procedure (23, 29).

The origin of the hydrolyzable ammonia has been regarded as speculative (136). It may come from decomposition during hydrolysis of acid amides (124) or amino sugars (132) or organic complexes (91). Without doubt, exchangeable ammonia is included, as well as some of the ammonia fixed irreversibly by clay minerals (122).

According to Shorey (124), secondary products formed by polymerization and condensation during the laboratory procedures of some of the primary splitting products can account for the presence of humin nitrogen.

Stevenson (132) considers that the acid hydrolyzable nitrogen left after removing the ammonia and humin is essentially amino-acid nitrogen. By this criterion the values of amino acid nitrogen reported here were observed to be higher than those reported by some workers (26, 79), but similar to those of others (53, 132).

The results of the nitrogen fractionation so far presented showed that the preferential accumulation of nitrogen over carbon with increasing clay treatments observed in Table 1 was principally due to an in-

crease in acid hydrolyzable nitrogen. Since the increase was largely in the acid-and alkali soluble Fraction B, it was inferred that clay treatment had resulted in an accumulation of amino nitrogen. To find out how this amino nitrogen was distributed in the soil with respect to the clay treatments was the next objective in the present work. The first step in this direction was to fractionate the acid and alkali soluble nitrogen (Fraction B) into its basic (Fraction D) and non-basic (Fraction C) constituents. This was achieved by precipitating the basic nitrogen compounds with phosphotungstic acid, as the Hausmann protein fractionating procedure (65). The results of this fractionation are presented in Table 9.

The values in Table 9 are quantitatively similar to those reported by Kojima (79). The results of this step revealed that both basic and non-basic forms of hydrolyzable nitrogen increased following applications of Wyoming Bentonite to the soil. In the case of the non-basic Fraction C, there was no consistently direct relationship with the amounts of clay which had been applied. However, the nitrogen in the basic Fraction D increased quite consistently with clay application up to 25 tons per acre. Although there was no additional increase where 50 tons of clay was applied, the basic nitrogen recovered was still three times that found in the check or the soil which received the 6.26 tons per acre application. This finding appears to be in agreement with Stevenson's proposal (135) that the ability of basic amino acids to resist decomposition in soils may depend upon the kind and amount of

Table 9.

Recovery of Nitrogen in Fraction B as Related to Clay Treatment

N fraction	Clay treatment None	6.25T	12.5T	25T	50T
	N, - mg. per 100 g. soil				
Fraction B	11.9	14.2	14.2	16.2	19.5
Fraction C (non-basic)	9.3	13.6	12.7	11.8	16.5
Fraction D (basic)	1.3	1.3	2.1	4.4	4.0
Not accounted for	-1.3	+0.7	+0.6	0	+1.0
	N, - percent of total soil N				
Fraction B	48.6	48.1	49.0	53.1	62.9
Fraction C (non-basic)	38.0	46.8	43.8	38.7	53.2
Fraction D (basic)	5.3	4.4	7.2	14.4	12.9
Not accounted for	-5.3	+2.4	+2.1	0	+3.2
	N, - percent of total N in Fraction B				
Fraction C (non-basic)	78.1	95.8	89.4	72.8	84.6
Fraction D (basic)	10.9	9.2	14.8	27.2	20.5
Not accounted for	-10.9	+4.9	+4.2	0	+5.1

clay mineral present. Other workers (3, 53) have, previously, suggested a similar interaction.

In order to determine how much of the nitrogen in the basic and non-basic fractions could be accounted by the Van Slyke nitrous acid procedure, these two separates were subjected to this treatment following the recommendations of Peters and Van Slyke (109). The elemental nitrogen evolved was measured manometrically. A reaction time of three minutes was allowed. Alpha-amino nitrogen, if present, is deaminated by this procedure. However, Kojima (79) and Bremner (20) have observed that Van Slyke's nitrous acid method for α-amino nitrogen determination of soil hydrolysates yields results higher than expected. The results of this step are shown on Table 10. There appeared to be no relation between the Van Slyke-nitrogen and clay treatment in either fraction. We were, therefore, lead to attempt a more detailed fractionation of the acid hydrolyzable nitrogen left after removal of ammonia and humin.

The acid hydrolyzable supernatant was then fractionated into functional constituents by the process of column separation recommended for protein hydrolysates by Fromageot (59). Here, basic materials, such as diamino acids, are retained in a silica column, the acidic or dicarboxylic in an alumina column, the aromatic in a charcoal column, and the final perfusate contains neutral non-aromatic materials. The procedure was tested using pure amino acid mixtures belonging to the basic, acidic, neutral non-aromatic, and aromatic groups. These

Table 10.

Van Slyke Nitrogen in Hydrolyzable Fractions from Soils with Varying Clay Treatments

N fraction	Clay treatment				
	None	6.25T	12.5T	25T	50T
	Amino N,* - mg. per 100 g. soil				
C (non-basic)	6.10	7.46	9.02	8.50	5.46
D (basic)	0.77	0.75	0.81	3.09	0.10
C + D	6.87	8.21	9.83	11.59	5.56
	Amino N, - percent of N in Fraction B				
C (non-basic)	65.59	54.85	71.02	72.03	33.09
D (basic)	59.23	57.69	38.57	70.23	2.50
C + D	64.81	55.10	66.42	71.54	27.12
	Amino N, - percent of hydrolyzable N (Fraction A)				
C (non-basic)	35.06	34.86	41.38	37.61	22.46
D (basic)	4.42	3.50	3.72	13.72	0.41
C + D	39.48	38.36	45.09	51.28	22.88
	Amino N, percent of total soil N				
C (non-basic)	24.89	25.28	31.10	27.87	17.61
D (basic)	3.41	2.54	2.79	10.13	0.32
C + D	28.04	27.83	33.90	38.00	17.94

* Nitrous acid releases other -NH_2 groups besides α -amino.

were simultaneously fractionated by Fromageot's procedure singly, in mixtures, and in mixtures added to soil fractions. Micro Kjeldahl analysis for total N showed reliable recoveries and the procedure was, therefore, adopted.

The results of applying Fromageot and Lederer fractionation procedure to the soils are shown in Tables 11 and 12. They are considered highly informative and suggest that this procedure can be utilized in studying soil nitrogenous fractions. It is significant to note in Table 12 that the results of this fractionation of the acid hydrolyzable nitrogen (ammonia and humin free) served to check our previous findings and revealed further details about active functional groups. The basic and non-basic nitrogen recovered by this new method, expressed as percentage of the total soil nitrogen or as percentage of total nitrogen in Fraction B, was found to be very close to the recovery of the same nitrogenous groups obtained by phosphotungstic acid separation as shown in Table 9. Functional group separation by columns is a more detailed separation and, therefore, more informative. Moreover, it is quicker and less laborious. No similar agreement for these two methods of fractionation was detected for Fraction A (Table 11). This is taken to imply that humin present in Fraction A interfered in the further separation of the soil hydrolyzable nitrogen into functional group constituents by this method.

A considerable proportion of the hydrolyzable nitrogen in these soils was in the form of neutral non-aromatic constituents (Tables 11 and 12).

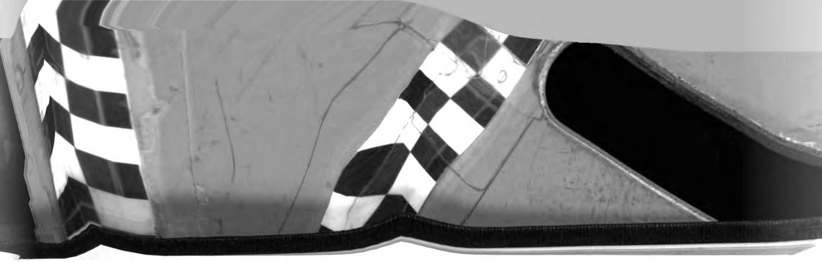


Table 11.

Distribution of Nitrogen Among Column Separates from Total Hydrolysate (Fraction A), as Related to Clay Treatment

Column separate	Clay treatment None	 6.25T	 12.5T	 25T	 50T
	Total N, - as percent of total soil N				
Basic	1.80	9.05	1.72	2.43	5.81
Acidic	7.26	9.05	14.51	2.62	6.00
Aromatic	5.80	14.03	3.66	0	0
Neutral, non-aromatic	45.06	41.39	59.86	60.72	41.87
Total of separates	59.92	73.52	79.76	65.77	53.67
Fraction A	71.02	72.54	75.52	74.09	78.39
	Total N, - as percent of total N in Fraction A				
Basic	2.53	12.48	2.29	3.27	7.41
Acidic	10.22	12.48	19.31	3.54	7.65
Aromatic	8.16	19.35	4.86	0.00	0.00
Neutral, non-aromatic	63.45	57.06	79.63	81.95	53.41
Total of separates	84.36	101.36	106.10	91.16	68.48
Not accounted for	-15.64	+1.36	+6.10	-8.84	-31.52

Table 12.

Distribution of Nitrogen Among Column Separates from Fraction B, as Related to Clay Treatment

	Clay treatment				
Column separate	None	6.25T	12.5T	25T	50T
	Total N, - as percent of total soil N				
Basic	4.69	4.75	7.14	14.10	13.23
Acidic	13.42	10.51	10.86	9.38	6.39
Aromatic	5.14	4.75	4.90	6.23	6.84
Neutral, non-aromatic	23.88	24.14	25.86	26.23	36.38
Total of separates	47.15	44.15	48.76	55.94	62.84
Fraction B	48.50	48.13	48.90	53.11	62.90
	Total N, - as percent of total N in Fraction B				
Basic	9.66	9.86	14.57	26.54	21.02
Acidic	27.65	21.83	22.18	17.65	10.15
Aromatic	10.59	9.86	10.00	11.73	10.87
Neutral, non-aromatic	49.16	50.14	52.82	49.38	57.84
Total of separates	97.06	91.69	99.56	105.31	99.90
Not accounted for	-2.94	-8.31	-0.44	+5.31	-0.10

These increased with clay treatments up to one and a half times the check soil, for the highest level. An outstanding feature of the results of this fractionation is that while the basic constituents increased with clay treatments, the acidic decreased. Their range of variation (Table 12) was of a similar magnitude, since they gave rise to not very divergent maxima and minima. The maximum for the basic amino separates was about 13 to 14 mg. of nitrogen per 100 g. of soil for the higher clay treatments and a minimum of 5 mg. for the check. These changes corresponded to a maximum of about 13 mg. of nitrogen in the acidic separate from the check soil and about 6 mg. minimum for the highest clay treatment.

The latter results point toward some state of inverse equilibrium between the basic and the acidic nitrogenous constituents in their relationship to increasing clay treatment. It is recalled that Bremner (23) is of the opinion that basic nitrogenous compounds are associated with humic acid. Stevenson (136) has suggested that diamino molecules could form points of attachment of humic colloids to clays. Our results suggest that acidic and basic nitrogenous constituents are dependently interrelated in the associative interactions which occur in soils between organic materials and clay minerals.

It thus appears that equilibrium states achieved in the humification process reflect some sort of chemical balance between clay minerals and the acidic and basic constituents of humus. The total charge of the mineral-organic colloidal complexes in soil apparently modifies the course of decomposition of fresh plant residues in such a way as to maintain the electrical stability of the system. The negatively charged clay minerals were found here to promote a preferential retention of hydrolyzable basic nitrogen compounds. It is conceivable that these were held by cation exchange forces, as was inferred by Birch and Friend (17) when they suggested that the so called "priming" action of fresh organic matter on the breakdown of soil humus is a displacement reaction caused by exchange between products of decomposition of the fresh organic matter and similar organic compounds previously protected by the clay.

The observed reduction in hydrolyzable acidic nitrogen compounds (Table 12) might reflect an enhanced microbial dissipation of these materials. However, the increase in neutral non-aromatic nitrogen was of the order of 3 times greater than the decrease in acidic nitrogen. This would indicate that, under the influence of clay, an increasing quantity of both basic and acidic nitrogen compounds were incorporated into neutral organic combinations chemically very resistant in nature. As will be shown later, the chromatographic behavior of the neutral non-aromatic fractions on paper was such as to conclude that they were indeed incompletely hydrolyzed complexes, rather than mixtures of ultimate splitting products. Their chemically resistant nature may be inferred from their apparent resistance to hydrolysis. However, the possibility must yet be entertained that the observed complexes may have arisen as artifacts by oxidative condensation and polymerization following the neutralization of the hydrolysates with LiOH (Figure 2).

With the purpose of obtaining some idea as to the distribution of the ninhydrin reacting compounds in the different soil nitrogenous fractions, the modified photometric ninhydrin procedure of Troll and Cannan (140) was applied. Several workers (41, 66, 95, 127) have expressed the results of this procedure in terms of the leucine equivalent nitrogen. This is done by comparing color development by unknown solutions after reaction with ninhydrin with that for a series of leucine standards. The 570 mμ filter is used. The procedure was originally elaborated by Harding (70). This is just an empirical estimation of amino groups since the different amino acids give not only differences in the shade of color, but differences in the depth of color per unit of the amino radical (65). Gortner and Gortner (65) have regarded the ninhydrin reaction as the most delicate reagent for detecting the presence of an α - amino acid. The reaction is not specific.

Tables 13 and 14 contain the results of the photometric ninhydrin reaction applied to Fractions A and B and their column separates. The leucine equivalent nitrogen of the total hydrolysate (Fraction A) was less than the total for the column separates (Table 13). This was not expected, since Fraction A contains ammonia and it is known that ammonia gives the purple color with ninhydrin. Apparently, passing the whole acid hydrolyzable fraction through the columns promoted some kind of liberation of the amino and carboxylic groups required to develop the purple color (65). This is an indication that the ammonia in

Table 13.

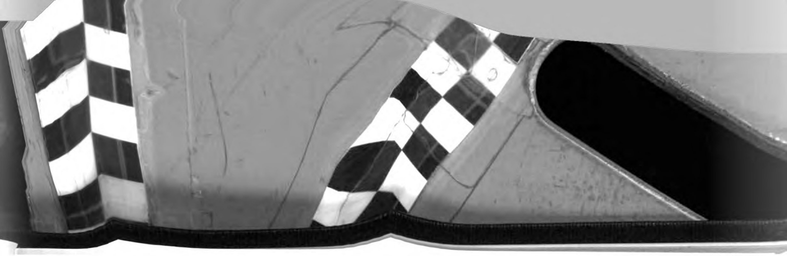
Occurrence of Leucine-Equivalent Ninhydrin-Reactive Compounds in Fraction A and its Column Separates, as Related to Clay Treatment

	Clay treatment				
Column separate	None	6.25T	12.5T	25T	50T
	Leucine-equivalent amino - N, as percent of total soil N.				
Basic	2.00	2.54	1.00	1.15	2.84
Acidic	4.24	1.12	2.62	1.84	1.55
Aromatic	3.59	3.05	2.66	2.95	2.61
Neutral, non-aromatic	3.06	2.58	3.34	2.88	2.03
Total of separates	12.90	9.28	9.62	8.82	9.03
Fraction A	2.41	3.80	4.34	3.34	3.35
Total N in Fraction A	71.02	72.54	75.52	74.10	78.40
	Leucine-equivalent amino - N, as percent of total N in Fraction A				
Basic	2.82	3.50	1.33	1.55	3.62
Acidic	5.98	1.54	3.49	2.48	1.90
Aromatic	5.06	4.20	3.60	3.98	3.33
Neutral, non-aromatic	4.31	3.55	4.45	3.89	2.59
Total of separates	18.16	12.80	12.80	11.90	11.52
Fraction A	3.39	5.23	5.78	4.51	4.28

Table 14.

Occurrence of Leucine-Equivalent Ninhydrin-Reactive Compounds in Fraction B and its Column Separates, as Related to Clay Treatment

	Clay treatment				
Column separate	None	6.25T	12.5T	25T	50T
	Leucine-equivalent amino - N, as percent of total soil N				
Basic	1.84	0.71	0.75	1.11	3.16
Acidic	4.20	2.61	2.48	2.10	1.94
Aromatic	3.26	1.97	2.76	1.97	3.61
Neutral, non-aromatic	5.95	2.74	1.38	1.61	1.45
Total of separates	15.26	8.00	7.38	6.79	10.16
Fraction B	2.45	8.61	4.14	6.56	6.90
Total N in Fraction B	48.60	48.10	49.00	53.10	62.90
	Leucine-equivalent amino - N, as percent of total N in Fraction B				
Basic	3.78	1.41	1.55	2.09	5.02
Acidic	8.66	5.42	5.07	3.95	3.08
Aromatic	6.72	4.08	5.62	3.70	5.74
Neutral, non-aromatic	12.26	5.70	2.82	3.02	2.30
Total of separates	31.42	16.62	15.07	12.77	16.15
Fraction B	5.04	17.88	8.45	12.34	10.97



Fraction A is not present as such but rather as an interfering compound or complex.

The recovery of ninhydrin reactive compounds from the acid hydrolyzable fractions after removing ammonia and humin (Fraction B, Table 14) was similar to the summation of the corresponding column separates. This emphasizes the necessity for getting rid of ammonia and humin during the process of soil amino nitrogen fractionation.

With the reservations already pointed out above, the quantities of ninhydrin reactive nitrogen are considered to be low when compared with the total nitrogen in the fractions in Tables 11 and 12.

Leucine-equivalent nitrogen found in the phosphotungstic acid separates in Fraction B is shown in Table 15. It is interesting to note that the values for basic leucine-equivalent nitrogen presented here are roughly of the same order as those presented in Table 10 for the Van Slyke-nitrogen of the same basic separate.

The nitrogen content for the non-basic phosphotungstic acid separate obtained by the Van Slyke method is much higher than by the colorimetric ninhydrin procedure. The lack of uniformity in the behavior of these two fractions makes it impossible to draw any definite conclusions as to their amino nitrogen content. It does appear that the Van Slyke nitrogen of the basic phosphotungstic acid separate (Fraction D) is of a similar chemical nature to the leucine-equivalent nitrogen of the same separate. The same cannot be said for these forms of nitrogen in the non-basic separate (Fraction C).

Table 15.

Occurrence of Leucine-Equivalent Ninhydrin-Reactive Compounds in Fractions C and D as Related to Clay Treatment

N fraction	Clay treatment None	6.25T	12.5T	25T	50T
	Leucine-equivalent amino - N, as percent of total soil N				
C (non-basic)	9.42	12.92	4.38	12.06	10.35
D (basic)	1.71	1.76	2.62	8.79	4.42
C + D	11.15	14.68	9.00	20.85	14.77
	Leucine-equivalent amino - N, as percent of total N in fraction				
C (non-basic)	24.84	28.02	10.00	31.19	19.45
D (basic)	32.31	40.00	34.28	60.91	34.25
C + D	25.75	29.06	15.07	41.73	22.34

PAPER CHROMATOGRAPHY

It was hoped that column separates would be adequately purified for identification of constituent amino acids by paper chromatography. Accordingly, two dimensional paper chromatograms were run for each fraction and column separate of each of the five soils. The technique utilized has been described under Methods of Analyses. The results are presented in Figures 6 to 23. In these figures we have shown generalized chromatographic patterns for the different column separates. The spots represent material which gave a positive test when the papers were sprayed with ninhydrin. These figures were pantographed from the original papers.

A good separation of standard amino acids was achieved with this procedure (Figure 6). However, no comparable separation was obtained with any fraction or separate from any of the soils. There were differences in the paper patterns for different clay treatments, but these were not interpretable. Nevertheless, the results are considered valid, in that they were reproducible and certain patterns of migration were associated with given fractions or separates. Definite trends in mobility of ninhydrin reactive materials were also associated with increasing clay treatment.

It is considered worth mentioning that the basic group of ninhydrin reactive compounds separated with the phosphotungstic acid were analyzed before and after desalting. The intensity and the purple area on the paper was considerably greater before desalting. Moreover,

FIGURE 6.

CHROMATOGRAM OF STANDARD AMINO ACIDS

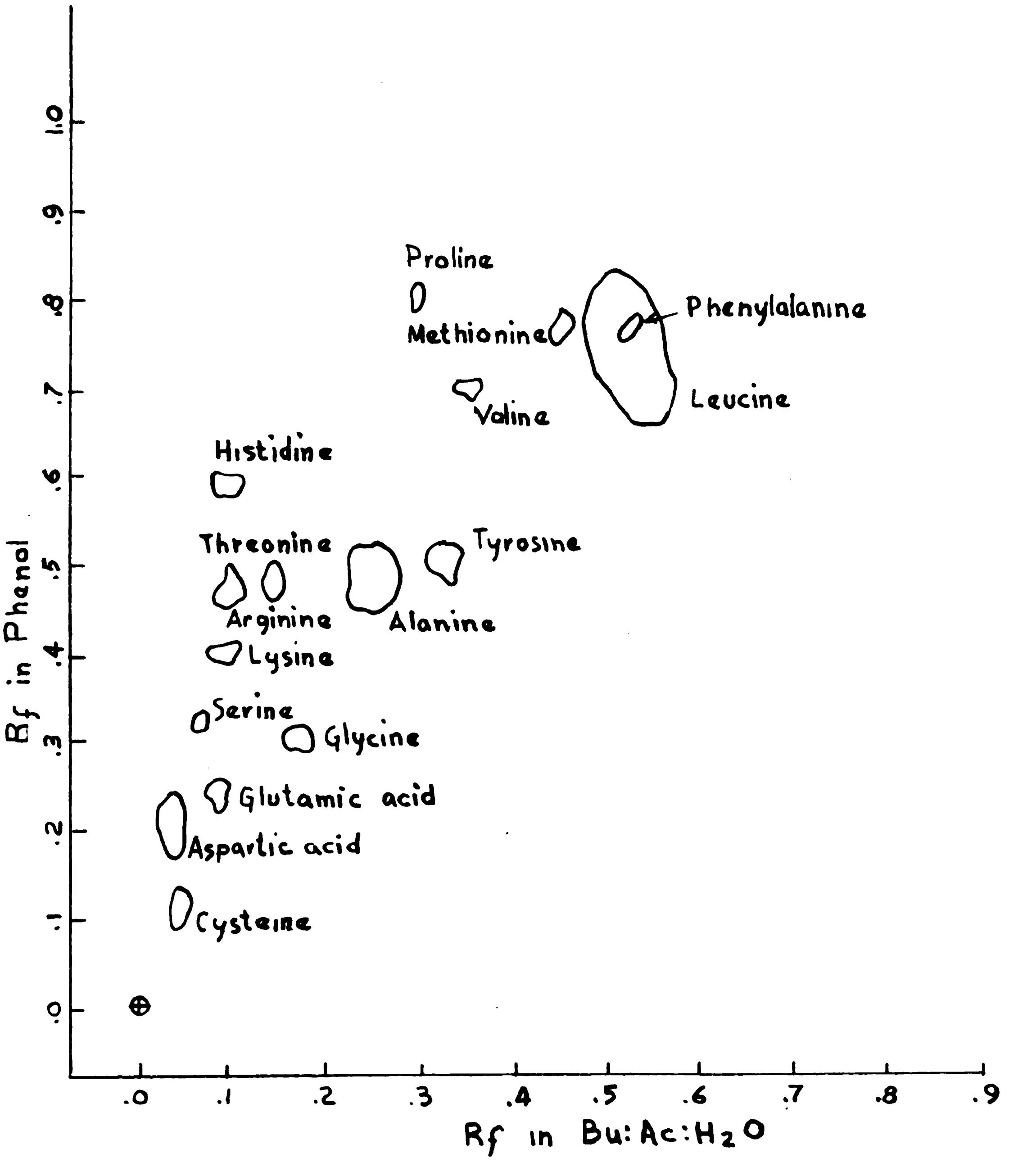

Proline
Methionine
Phenylalanine
Leucine
Valine
Histidine
Threonine
Tyrosine
Arginine
Alanine
Lysine
Serine
Glycine
Glutamic acid
Aspartic acid
Cysteine
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
.0
Rf in Phenol
.0
.1
.2
.3
.4
.5
.6
.7
.8
.9
Rf in Bu:Ac:H2O

Fig. 6

FIGURES 7 TO 12.

CHROMATOGRAPHIC PATTERNS OF THE BASIC-NINHYDRIN COMPONENTS

Figure 7 - Phosphotungstic acid-precipitated fraction of the check soil.

Figure 8 - Phosphotungstic acid-precipitated fraction of the 6.25 tons of clay/acre treatment.

Figure 9 - Phosphotungstic acid-precipitated fraction of the 12.5 tons of clay/acre treatment.

Figure 10 - Phosphotungstic acid-precipitated fraction of the 50 tons of clay/acre treatment.

Figure 11 - Generalized pattern for the components of all the soils retained by the silica columns before removing ammonia and humin from the hydrolysate.

Figure 12 - Generalized pattern for the components of all the soils retained by the silica columns after removing ammonia and humin from the hydrolysate.

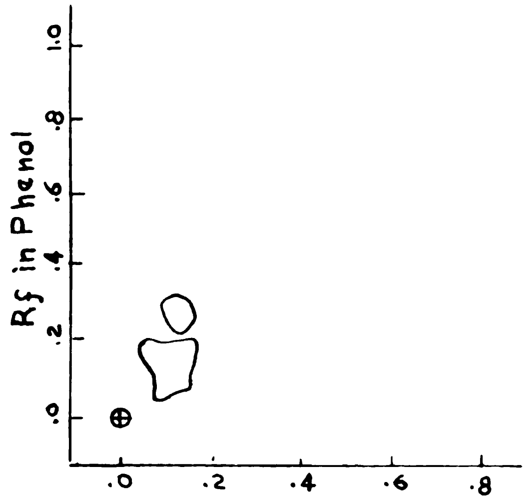


Fig. 7

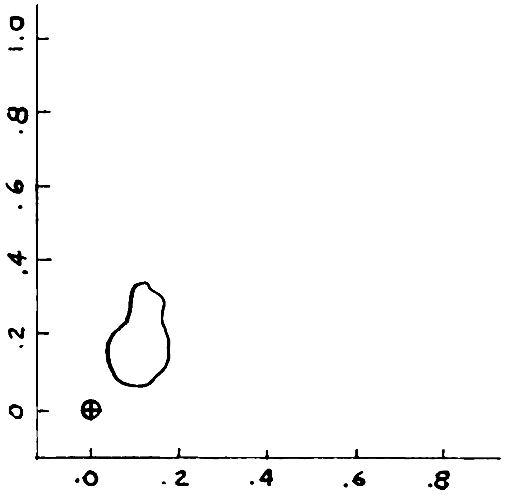


Fig. 8

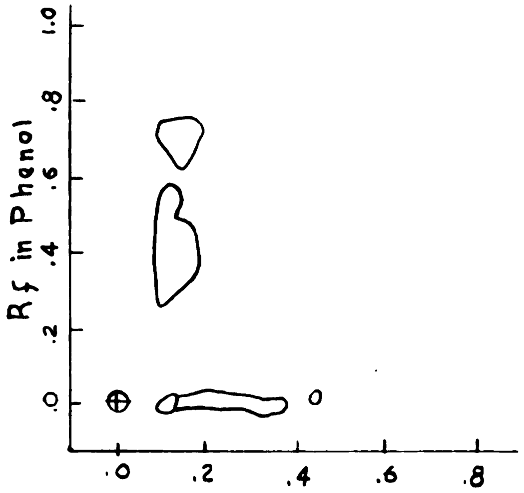


Fig. 9

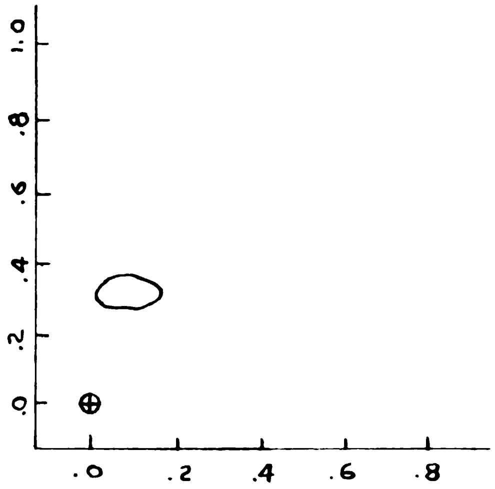


Fig. 10

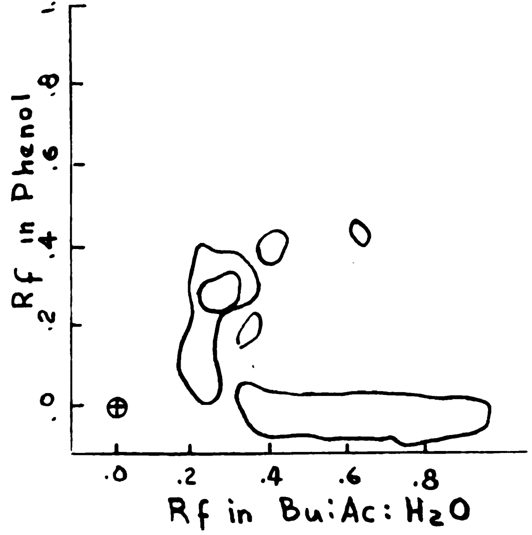


Fig. 11

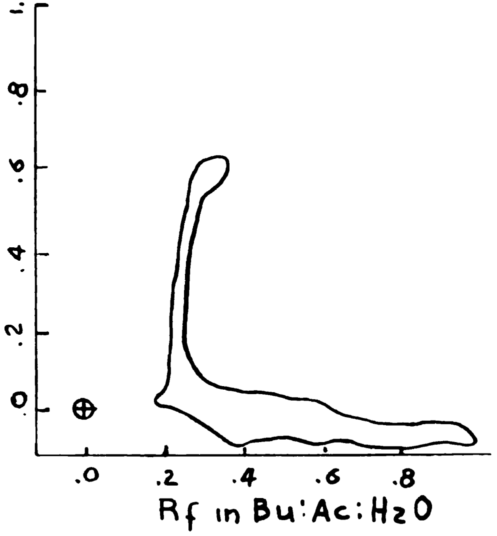


Fig. 12

FIGURES 13 TO 18.

CHROMATOGRAPHIC PATTERNS OF THE ACIDIC NINHYDRIN COMPONENTS

Figure 13 - Generalized pattern of the phosphotungstic acid supernatant of all soils.

Figure 14 - Components of the check soil retained in the alumina columns before removing ammonia and humin from the hydrolysate.

Figure 15 - Components of the 6.25 tons of clay/acre treatment retained in the alumina column before removing ammonia and humin from the hydrolysate.

Figure 16 - Components of the 25.5 tons of clay/acre treatment retained in the alumina column before removing ammonia and humin from the hydrolysate.

Figure 17 - Components of the check soil retained in the alumina column after removing ammonia and humin from the hydrolysate.

Figure 18 - Generalized pattern of the components of all soils, except the check, retained in the alumina columns after removing ammonia and humin from the hydrolysate.

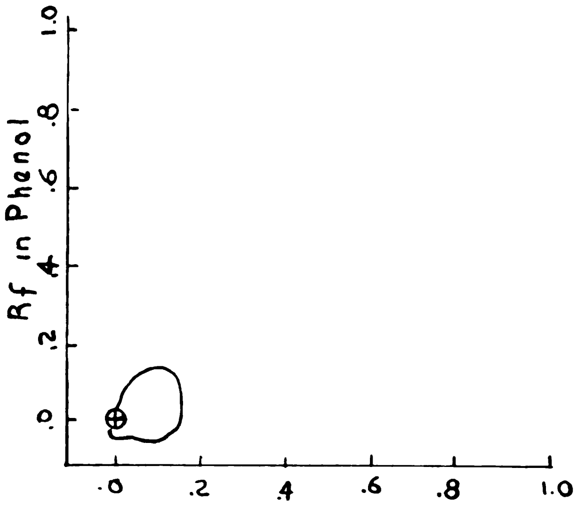


Fig. 13

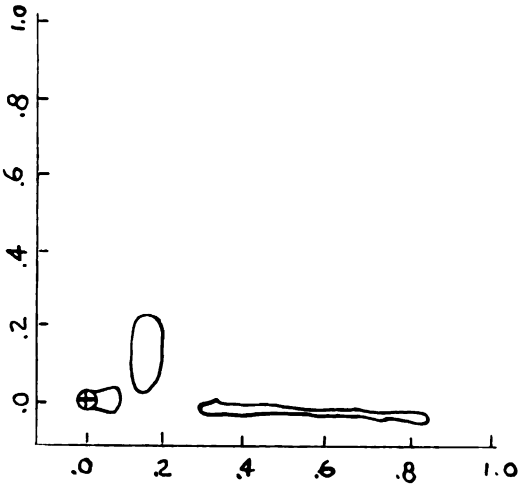


Fig. 14

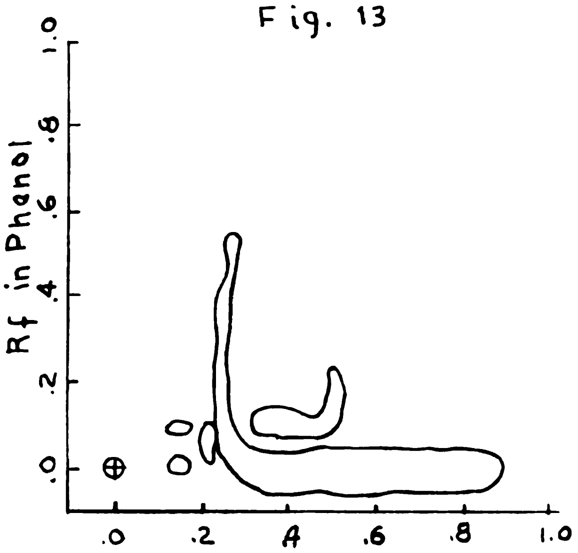


Fig. 15

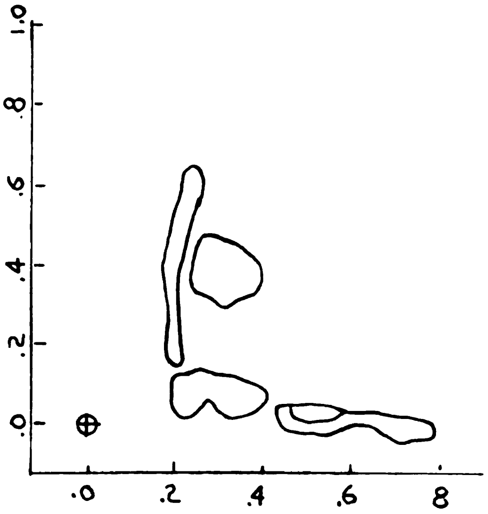


Fig. 16

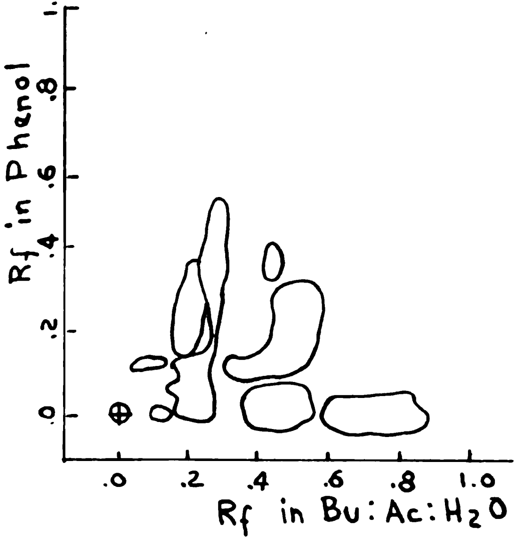


Fig. 17

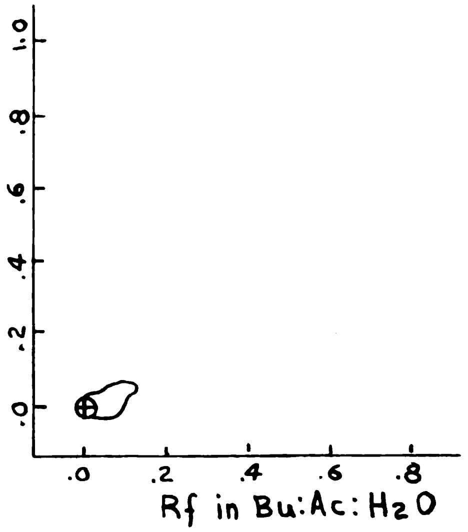


Fig. 18

FIGURES 19 TO 23.

CHROMATOGRAPHIC PATTERNS FOR THE AROMATIC AND THE NEUTRAL NON-AROMATIC NINHYDRIN COMPONENTS

Figure 19 - Generalized pattern for the aromatic components of all the soils retained in the charcoal columns before removing ammonia and humin from the hydrolysate.

Figure 20 - Generalized pattern for the aromatic components of the check soil and the 6.25 and 12.5 tons of clay/acre treatment retained in the charcoal columns after removing ammonia and humin from the hydrolysate.

Figure 21 - Generalized pattern for the aromatic components of the 24.4 and 50 tons of clay/acre treatment retained in the charcoal columns after removing ammonia and humin from the hydrolysate.

Figure 22 - Generalized pattern for the neutral non-aromatic components of all the soils before removing ammonia and humin from the hydrolysate.

Figure 23 - Generalized pattern for the neutral non-aromatic components of all the soils after removing ammonia and humin from the hydrolysate.

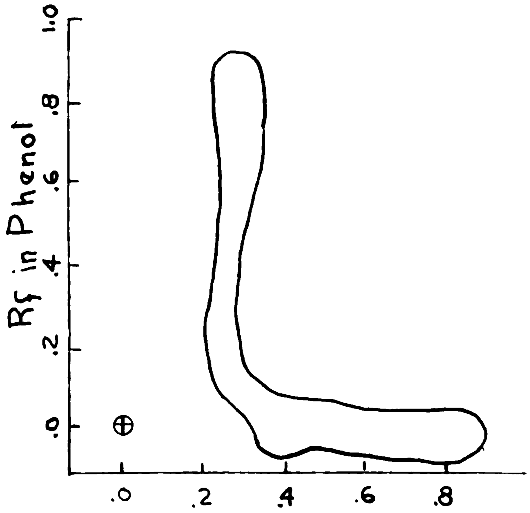


Fig. 19

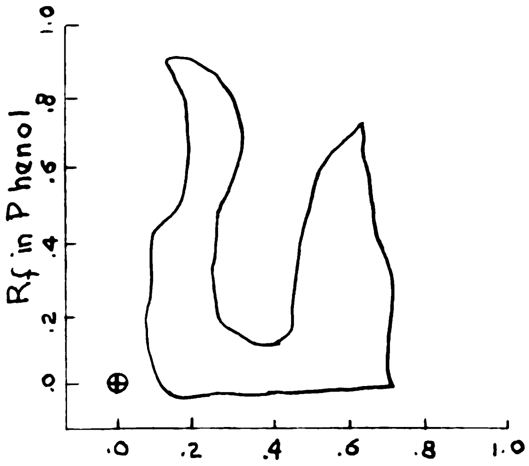


Fig. 20

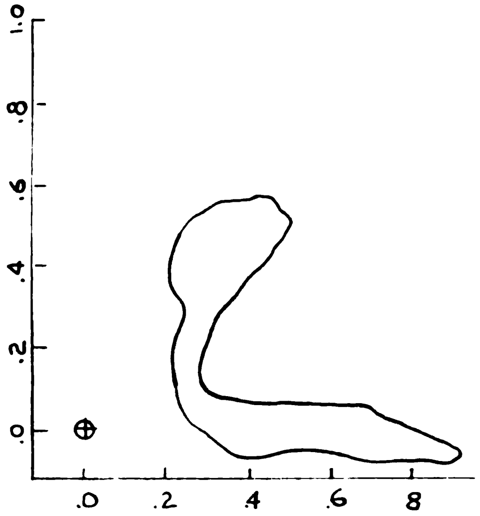


Fig. 21

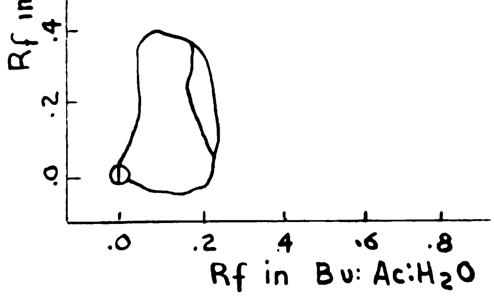


Fig. 22

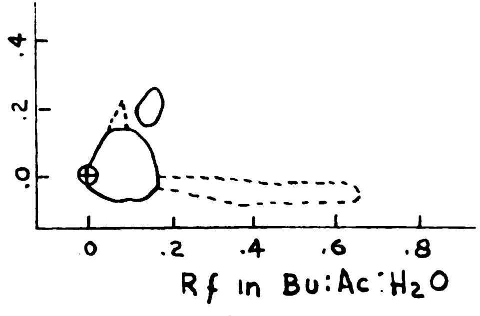


Fig. 23

the ninhydrin photometric procedure (140) applied to this group before and after desalting definitely showed a loss of ninhydrin reactivity with desalting.

Before desalting, the phosphotungstic acid-precipitated fractions showed considerable "trailing", especially toward the phenol front. It was, therefore, considered important to desalt the samples following recommendations of Zweig and Hood (157) and also to find out by X-ray if there was clay present in the fractions. The results of the X-ray studies are presented in the Appendix.

The chromatograms of the desalted basic amino fraction precipitated with phosphotungstic acid are presented in Figures 7 to 10. All soils showed components with low mobility in butanol-acetic acid (Rf = 0.1 - 0.2), but with variable mobility in 80% phenol. Maximum mobility in phenol (Rf = 0.5 - 0.7) occurred with the soils treated with 12.5 and 25 tons of clay per acre. With these two treatments rather distinct spots were obtained which correspond to lysine, arginine, and histidine in Figure 6. Both of these treatments showed a very similar chromatographic pattern. Figure 9 represents the pattern for both soils. In these two soils only, there appeared ninhydrin-reactive components immobile in phenol but with varying mobility in butanol-acetic acid. These correspond to none of the standard amino acids. If it could be assumed that these phenol immobile components were present in organic complexes with basic amino acids in the other soils, it would account for the reduced mobility and indistinct separation of components with phenol affinity in the soils of lower clay treatment (Figures 7 and 8). The effect of intermediate clay treatments in this case would appear to be due to absorption of basic amino acids by clays, preventing their reaction with other organic molecules to form strictly organic complexes. The disappearance of the phenol immobile constituent at the highest level of clay treatment (Figure 10) may have been related to the depressed yield of crops and reduced level of residue return, or to progressively greater breakdown of these constituents during desalting. Gas evolution was more vigorous with desalting at higher clay contents.

The latter explanation appears more likely, considering the results depicted in Figure 11. The generalized chromatogram in Figure 11 represents basic constituents retained on the silica column in the Fromageot procedure. They were eluted with 0.1 N hydrochloric acid and concentrated in vacuo at 40°C. However, the eluate was not desalted prior to spotting on paper. The distribution of ninhydrin-reactive materials was essentially the same for all soils. An intense purple spot in the check soil with Rf = 0.28 in phenol and 0.27 in butanol-acetic acid-water was found to become progressively smaller and less intense with increasing clay treatment. Coincident with the disappearance of this spot, three other spots were observed to increase in intensity. These three spots, all exhibited greater mobility in butanol-acetic acid-water than the first spot. Two exhibited greater mobility in phenol, the other a lower mobility than the first. None of these spots corresponded to any of the standard amino acids shown on Figure 6 or with

phosphotungstate-precipitated basic constituents which were fairly well resolved in Figure 9. The three well defined spots in Figure 11 may be amino acids with substituted organic groups which have altered their mobility in the solvents used. While phosphotungstic precipitation and careful desalting may, more effectively, separate constituent basic ninhydrin-reactive compounds, the less drastic separation achieved by silica absorption may serve to isolate multimolecular groupings or complexes which represent significant units in the larger humic complex. The relation to clay treatment which was observed among the silica absorbed constituents suggest that these materials were formed in the soil rather than as artifacts arising during hydrolysis.

Attention is called to the distribution of the ninhydrin-reactive compounds in Figure 12. These also represent basic constituents retained in the silica column, but are free of ammonia and humin. The elimination of ammonia and humin did not help to separate ninhydrin- reactive constituents for identificational purposes. In fact, the distinct spots observed in Figure 11 were lost completely. There was an increase in materials with a low mobility in both solvents which resulted in the closing of the "L" shaped pattern at its vertex. This behavior appears to have resulted from the complexing of compounds with differential mobility in phenol and in butanol-acetic acid. This complex formation must have occurred during the distillation following addition of $Ca(OH)_2$ to remove ammonia and humin.

When paper chromatograms of the phosphotungstate-precipitated

basic constituents (Figures 7 to 10) are compared with the corresponding fractions separated from the silica column by Fromageot's procedure (Figures 11 and 12), it is noted that by the latter procedure ninhydrin-reactive constituents were separated which had a greater range of mobility in butanol-acetic acid than those obtained by the precipitation procedure. The elimination of ammonia and humin (Figure 12) brought about a smaller resolution of the ninhydrin-reactive components in this basic fraction.

The foregoing suggests that Fromageot's procedure permits a better separation of the ninhydrin-reactive components of the soil hydrolysates. This is further suggested when Figure 13, showing the non-basic constituents from the phosphotungstate supernatant, is compared with the acidic constituents retained in the alumina column (Figures 14 to 16). The former (Figure 13) showed practically no migration of the ninhydrin-reactive components. There appeared a single very intense pink spot. Figures 14 to 16 show a greater resolution with increasing clay treatments.

There was a distinct difference in the degree of resolution between the acidic column fractions before and after removing the ammonia and humin. As was observed in Figures 11 and 12, there was better resolution of ninhydrin-reactive constituents in the column separates from clay-treated soils before removal of ammonia and humin (Figures 15, 16 and 18). On the other hand, removal of ammonia and humin improved the resolution of acidic ninhydrin-reactive constituents in the check

soil (Figures 14 and 17). This interaction between clay content of the sample and the alkaline distillation employed to remove ammonia and humin from the hydrolysate is difficult to interpret. It is apparent, however, that clay treatment in the field markedly altered the proportions of complexable constituents in the hydrolysate.

The general chromatograms of the aromatic fractions are shown in Figures 19 to 21. These showed a distinct tendency for the "L" shaped migration patterns found in the case of the basic fractions after removal (Figure 12), and of the acidic fractions before removal, of ammonia and humin (Figures 15 and 16).

The chromatographic behavior of the neutral non-aromatic fractions is shown in Figures 22 and 23. These did not show much migration in either solvent. It is recalled that these fractions were quickly desalted. The neutral non-aromatic fraction before removing humin and ammonia is shown in Figure 22. This showed a rather larger spot containing an amalgam of color which varied from a deep purple, grading through pink into a yellow border at the farmost butanol front. The colors appeared uniformly distributed along the phenol front. Figure 23 represents this same fraction after removing ammonia and humin. There appears to be not much difference in resolution in the neutral non-aromatic fractions before and after removing ammonia and humin.

The total area and intensity of the spots for the neutral non-aromatic fractions clearly increased with increasing clay treatments, except for the 12.5 tons of clay per acre treatment, which showed the

least area and color intensity. This is consistent with the fact that total nitrogen in this separate increased with clay treatment (see Table 12). The low mobility of these materials in both solvents suggest that they were relatively complex, large molecules.

From their behavior during paper chromatography it is clear that the various hydrolytic fractions and separates studied did not contain any significant quantities of free amino acids but, rather, amino acids complexed with other organic compounds. It would appear that the mixture of colors observed in the neutral non-aromatic fractions represent undissociated amino acids complexed to an organo-phyllic counterportion. The resulting complexes exhibited a neutral, non-migratory behavior, due to internal electrochemical compensation of their functional groups. Most of these complexes are not definite compounds. If they were, they would give distinct spots instead of trailing across the paper or producing spots with a mixture of colors. The fact that they were neutral to silica and alumina columns suggests that both the amino and carboxylic groups were tied up.

The organic compounds which are tied up with the amino acids appear to be aromatic in nature. This is suggested by the fact that the "L" shaped pattern of the aromatic group is to be observed also in the basic group after, and in the acidic group before, alkaline distillation to remove ammonia and humin. This is compatible with the concept that amino acids in soils are held in complexes with humic acids.

It was found that the ninhydrin color reaction did not reach maximum intensity for several days after the papers were sprayed. This, too, would suggest that amino acid groups were tied up in some way so that they could react only slowly with ninhydrin. Most workers (25, 29, 48, 73, 106, 130, 136) infer from the high amount of amino derivatives obtained from soil humate hydrolysates, and the high amount of non-acid hydrolyzable nitrogen in humus, that the soil amino acids are essentially associated with humic acid rather than being present as free amino acids or as normal proteins.

Sowden and Parker (130) and Bremner (23, 27) have shown that soil amino acids are very strongly associated with humus. Their work also showed that humus is not uniform since the dinitrophenyl amino acid derivatives of humic acid preparations showed striking differences in susceptibility to hydrolysis and solubility. Sowden has reported (129) the presence of an unknown interfering material in his chromatographic elution columns. According to his own description, "it did not seem likely that it would be one of the more common amino acids". It reacted readily with the Moore & Stein reagent* but very slowly with a ninhydrin-in-butanol spray. Full color intensity did not develop for more than 24 hours after spraying and was pink rather than blue or reddish blue as is true for most amino acids. Because of the interference of this material they were unable to properly separate tyrosine and phenylalanine, two aromatic amino acids.

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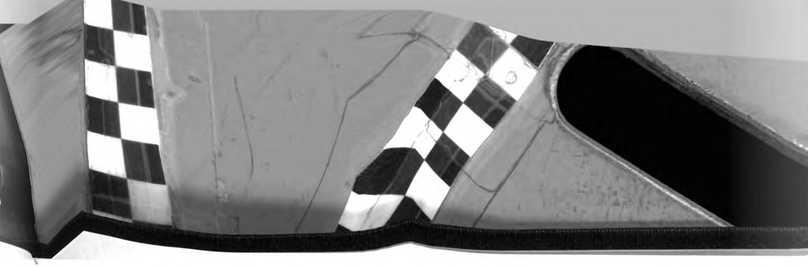
* Sequence of pyridine, 80% phenol, and ninhydrin.

The possibility that clay could have been present in the soil preparations was rejected on the basis of X-ray analyses, since it was found that glycerated soil hydrolysates proved to be X-ray amorphous. Diffraction patterns of unglycerated preparations showed no maxima which could be attributed to the native soil clay or Wyoming Bentonite. The details of this part of the work is fully discussed in the Appendix where a mechanism is tentatively suggested to explain the results.



SUMMARY

Chemical and physical studies with a clay-amended Plainfield sand three years after field applications of Wyoming Bentonite gave the following results:

1. Increased rate of treatment with Wyoming Bentonite resulted in an increased positive electrophoretic mobility at pH 6.0 to 6.1 of the colloidal fraction of the soil. Calculated Ƶ potentials were inversely related to the carbon content of the colloidal fraction.
2. The C:N ratio of the soil decreased with increasing levels of clay application. This was due, principally, to an increase in total nitrogen and more specifically to an increase in nitrogenous materials which were solubilized by hydrolysis in 6N HCl.
3. Fractionation of the hydrolyzable nitrogen showed an increase with increasing level of clay application in basic and non-basic components soluble in the presence of excess $Ca(OH)_2$, while ammonia nitrogen was essentially unaltered and humin nitrogen decreased.
4. The recovery of basic and non-basic nitrogen by phosphotungstate precipitation was in close agreement with the recovery by column separation. The results with column separation revealed that the observed tendency for non-basic forms of nitrogen to increase with clay treatment was very largely due to an increase in neutral non-aromatic constituents.
5. Aromatic forms of nitrogen were essentially unrelated to treatment, whereas nitrogen in acidic constituents decreased and basic forms of

nitrogen increased with increasing amounts of clay.

6. Paper chromatograms showed that the ninhydrin-reactive nitrogen in the various hydrolyzable fractions and separates was not present in the form of free amino acids. The amino acids appeared to be complexed with other organic compounds. These complexing compounds appeared to be aromatic in nature.

7. It was not clear whether the amino nitrogen complexes observed had been formed in the soil or whether they had arisen as artifacts during chemical treatment. However, their distribution and their mobilities in phenol and in butanol-acetic acid-water on paper chromatograms were clearly influenced by the level of clay application.

8. The release of CO_2 and nitrate during incubation indicated that the added clay had a greater stabilizing influence on residual soil nitrogen than carbon.

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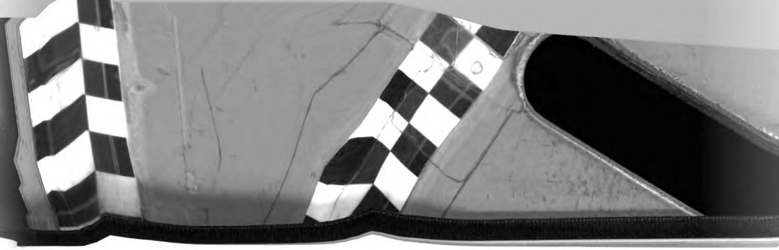

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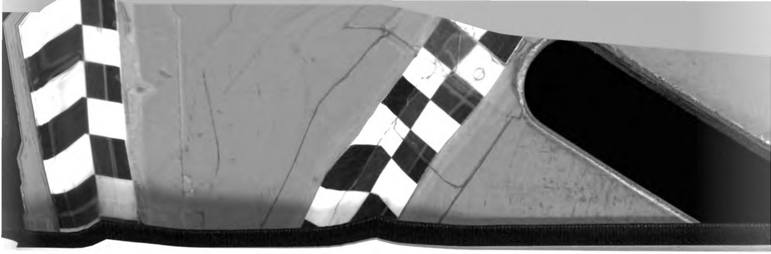

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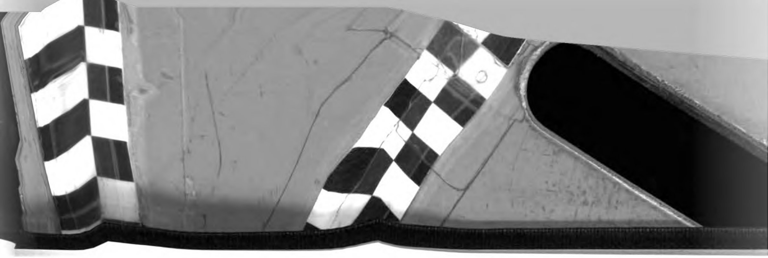

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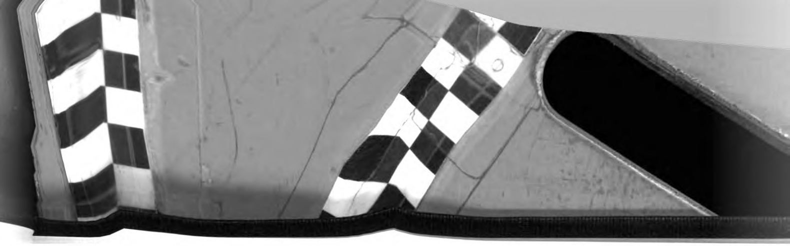

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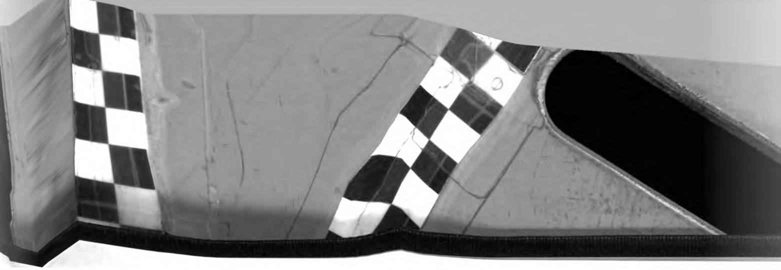
APPENDIX: GLYCERATION EXPERIMENT

INTRODUCTION

X-rays were used in an effort to detect clay in the hydrolyzable fractions used in paper chromatography. The so called 'grauhuminsaure', which are high molecular weight substances from which amino acids are isolated, have been reported (48, 119) to form complexes with clay minerals. Preliminary results with X-rays showed evidence that clay was not present in the hydrolyzable fractions used in paper chromatography. Nevertheless, glyceration was adopted in order to achieve greater discrimination by expanding the lattice of the clay mineral. Glyceration caused the disappearance of the X-ray patterns. As far as the author is aware, this is the first time such a reaction has been reported in the literature. Its significance in identifying amino acid derivatives is not understood. Moreover, it is suspected that a similar mechanism could mask soil amino acids. In fact, Scheffer et al. (119), were able to prepare synthetic humic acids which proved to be X-ray amorphous.



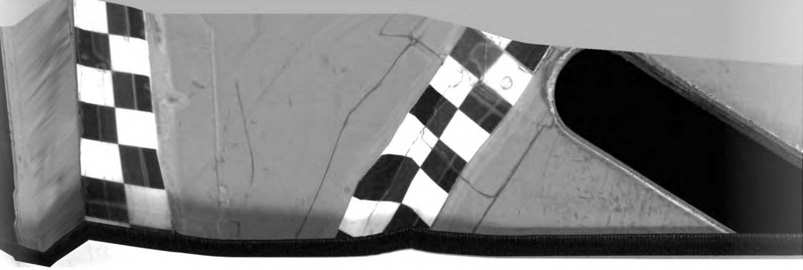
REVIEW OF THE LITERATURE

Glycerol is a triatomic alcohol having the formula CH_2OH - $CHOH$ - CH_2OH. It shows the characteristic reactions of a primary and a secondary alcohol together in a single molecule. Oxidation of a primary alcohol yields an acid, while a secondary alcohol yields a ketone. Alcohols react with many acids to form esters. They can also undergo dehydration reactions to olefins in the presence of strong acids and other strongly electrophilic reagents. Glycerol is a polyfunctional molecule, as are amino acids. The interaction of polyfunctional molecules may lead to condensation reactions giving rise to polymers of multiple inter-and intra-molecular linkages, as in vulcanized rubber (35). Thus, a dibasic acid, for instance, and glycerol, give esters of high molecular weight in which there are cross links joining the chain together (35).

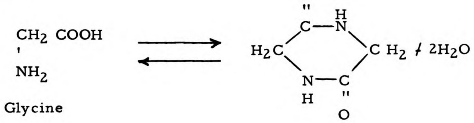
Alpha and δ-amino acids tend, like the corresponding hydroxy acids, to form cyclic derivatives on heating.

Alpha and δ-hydroxy acids form cyclic inner esters which are called lactones. These heterocyclic ring systems are most easily formed when the ring contains five or six atoms in all (35, 49, 54). The imides are similar compounds where the substituting group is not an OH group like in the hydroxy acids, but an NH_2. These cyclic imides are called lactams, and their stability is also dependent upon configurations involving five or six atom members (54, 104).

When heated, α-amino acids and their esters form a type of cyclic



amide known as diketopiperazine, according to a reaction of the following type:



Glycine

Diketopiperazine

As they are readily formed when α-amino acids or esters are subjected to high temperature, they are found among the products of protein hydrolysis which involves long heating (49, 65, 75).

Glycerol and amino-dicarboxylic acid additions, leading to the formation of polyamides, are known (35). When glycerol is heated in a solution of α-amino acids, water is lost and diketopiperazine cyclic amides are formed (75, 104). In 1900, Balbiano and Trasciatti, as cited by Katchalski (75) described the preparation of polyglycine by heating glycine in glycerol. The polymer formed is insoluble in water and the usual organic solvents. Its properties are described as resembling those of a horny protein and yield glycine methyl ester and diglycine methyl ester, respectively. When Balbiano's polymerization reaction was studied by Maillard (75, 83) he was able to isolate, in addition to the horny, water-insoluble polymeric product, the water-soluble intermediate triglycyl-glycine and glycine anhydride. The ratio between the final horny product and the stable six-membered diketopiperazine varied depending on the conditions used.

Because diketopiperazines appear to be present as an artifact in protein hydrolysates and because it was suspected to be present in the present work, it is considered appropriate to discuss some of the literature describing its behavior. The following remarks are cited from Katchalski (75):

> "No data on the mechanism of polymerization of glycine in glycerol are available. Carothers suggests that the condensation reaction, probably, involves the formation of glycerol esters as transitory intermediates. Maillard made the curious observation that an acqueous solution of triglycyl-glycine and diketopiperazine deposits an insoluble material on standing. Analytical evidence indicated that the insoluble material is a hexapeptide. Except for the solubility effect in acqueous solution, there is no obvious reason why this coupling of diketopiperazine with the polypeptide should stop at the hexapeptide stage. The glycine anhydride may, therefore, play its part as an intermediate in the polymerization process. Esters of amino acids and peptides, generally, undergo condensation more readily than the free amino acids and free peptides. The linear product of polycondensation of esters of amino acids are often accompanied by diketopiperazines in varying quantities. Frequently, a diketopiperazine is the only compound which can be isolated

from the condensation mixture".

Diketopiperazines have been reported to react readily with aniline, ammonia, and primary and secondary amines (75). They may, also, undergo polymerization in the solid or liquid state, as well as in solution (75). Water acts as a catalyst in this polymerization reaction.

The following factors were considered (56) to favor the polymerization reaction:

a) elevated temperature

b) use of solvents

c) the passage of gases through solutions of the esters, -CO_2 was found to promote condensation

d) reduced pressure

The polycondensation of peptide derivatives, as studied by Magee and Hofmann (82), deserves our attention in connection with Bremner's (20) finding that some constituent of humic acid preparations interfered with the estimation of free amino groups by the Van Slyke manometric technique (109). The former observed that the rate of interaction of nitrous acid with the hydrazide group of diglycyl-glycine hydrazide greatly exceeded its rate of interaction with the free amino group of the peptide. The hydrazide can be formed when the $NaNO_2$ is added to the peptide.

Diketopiperazine has been studied extensively by itself and in its connection with protein hydrolysates (4, 38, 47, 76, 78, 118). Klarman (78) found that glycine-alanine-tyrosine piperazine did not give a pos-

itive Millon's test despite the fact that it contained tyrosine. He concluded that the piperazine nucleus interferes with this test. Katchalsky (75) has emphasized that the presence of functional groups in addition to the normal α-amino and α-carboxyl groups may tend to produce unexpected complications in these condensation reactions of amino acids leading to diketopiperazines formation. Klarman (78) has described the color reactions of diketopiperazines. Aromatic dinitro compounds (carbonyl reagents) were found to give a characteristic reaction with diketopiperazines when heated for a short time. Precipitated moist copper oxide which reacts readily with amino acids in proteins, and in ninhydrin and biuret tests, does not give any reaction with diketopiperazines.

Ambrose and Ellis (4, 47, 76) have described the infra red properties of diketopiperazine. Kellner (76) assigned a C = O vibration of 2.9μ(3,448 cm.$^{-1}$) for the first overtone of diketopiperazine and 3.0μ (3,333 cm.$^{-1}$) for the fundamental valence vibration of N - H groups for diketopiperazine. The CH_3 vibration was found at 3.4 to 3.5 μ (2,941 to 2,857 cm.$^{-1}$).

No information about X-ray studies with glycerated amino acids was found in the literature. The information presented in this work appears to be the first. Katchalsky (75) has presented and discussed some of the work which has been done in X-ray of polyamino acids polymerized in ethyl acetate and benzene. Brown et al. (32), has reported interplanar spacings of 5.2 and 11.7 $\overset{o}{A}$ as the two principal diffraction rings observed from the X-ray powder diagrams of (1:1 mole) copolymers of DL-β-phenylalanine and L-leucine, and DL-phenylalanine and α-amino isobutyric acid. Shifting of the 11.7 Å diffraction peak was observed according to the X-ray technique employed. Bernal (14) has prepared an extensive report on a preliminary X-ray study of fifteen amino acids and related compounds, including diketopiperazine.

As far as the infra red spectra of amino acids is concerned, Bellamy (12) has presented a detailed review of the literature on this subject. He points out that no absorption in the usual NH stretching region of 3,500 to 3,300 cm.$^{-1}$ (2.9 to 3.0μ) is shown by any amino acid or hydrochloride. This is supported by the work of many investigators cited (12). According to Bellamy, all amino acids capable of possessing the NH_3^+ structure, and their hydrochloride, show two characteristic absorption bands at 1,600 to 1,500 cm.$^{-1}$ (6.3 to 6.7μ), in addition to an ionic carbonyl absorption which also takes place in this region. The first of these, at 1,660 to 1,610 cm.$^{-1}$ (6.0 to 6.2μ), is often weak, while the second, at 1,550 to 1,485 cm.$^{-1}$ (6.5 to 6.7μ), is usually more intense.

The NH vibration for amido acids (NH_2 present in an amide, instead of an amine) falls in the range 3,390 to 3,260 cm.$^{-1}$ (3.0 to 3.1 μ). The location of the amide I absorption peak (carbon-carbon and C-H links) was found by Randall et al. (113), to fall between 1,600 to 1,620 cm.$^{-1}$ (6.3 to 6.2μ) in twelve out of fifteen cases. Other investigators have observed the same specific absorption (12, 60)for the amide II

(carbon-nitrogen and nitrogen-hydrogen) links in 25 compounds studied.

No infra red absorption bands characteristic of amides, proteins, polypeptides, and amino acids are reported in the literature (12) for frequencies greater than 4,825 cm.$^{-1}$ (2.1 μ) or less than 880 cm.$^{-1}$ (11.4 μ). There is, nevertheless, information on infra red absorption by aromatic compounds, including aromatic amines, in the region about 4,000 cm.$^{-1}$ (2.5 μ). Bell (11), for instance, studying the absorption spectra of organic derivatives of ammonia, particularly anilines, found that the band occurring in the region 2.8 μ (3,571 cm.$^{-1}$) may be regarded as due to amino groups. Alkyl and aryl introductions on the amino group of aniline caused the band to become shallower and further additions (yielding tertiary amines) caused the 2.8 μ (3,571 cm.$^{-1}$) band to disappear. A 2.3 μ (4,348 cm.$^{-1}$) band is present in aniline, as well as in substituted anilines. Ellis (46) observed a band in toluene, xylene, and mesitylene in the region 2.3 to 2.4 μ (4,348 to 4,167 cm.$^{-1}$) while Glatt and Ellis (63) reported absorption bands at 4,216, 4,322, 5,671 cm.$^{-1}$ (2.4, 2.3, 1.8 μ) and higher frequencies for polythene and Parowax. They also found a 4,291 cm.$^{-1}$ (2.3 μ) band in nylon, a polyamide.

Colthup (34) reported that the band within the range 1,625 to 1,575 cm.$^{-1}$ (6.2 to 6.3 μ) was characteristic for most aromatic materials. Again, according to Bellamy (12), these bands are highly characteristic of the aromatic ring itself and taken in conjunction with the C-H stretching band near 3,030 cm.$^{-1}$ (3.3 μ), they afford a ready means of recognition for this structure. The following remarks are cited from Bellamy (12):

> "On theoretical grounds the main 1,600 and 1,500 cm.$^{-1}$ (6.3 and 6.7μ) bands of the phenyl ring can be expected to occur also in polycyclic materials where there should be expected some broadening of the over all ranges in which they can occur."

The 1,600 to 1,500 cm.$^{-1}$ (6.3 to 6.7μ) aromatic absorption bands are notorious for the very wide fluctuations in intensity which are encountered in this region. Frequently, the bands are weak in conjugated structures, and are often shown only as shoulders on other bands (12).

However, heterocyclic aromatics such as pyridine and pyrimidines also give a similar pair of bands in the 1,600 cm.$^{-1}$ (6.3μ) region from the C=C and C=N links, although in this case the third skeletal vibration is usually at appreciably lower frequencies (12). In certain cases, where the carbonyl absorption is capable of shifting toward the 1,600 cm.$^{-1}$ (6.3μ) region under the influence of strong hydrogen bonds, it is difficult to differentiate between the two (12).

Bellamy has presented a detailed discussion of the infra red absorption of aromatic molecules and their derivatives in the region about 1,000 cm.$^{-1}$ (10μ). But, as he pointed out (12), the lower intensity of these bands renders them less generally useful than the higher-frequency absorptions already described. "No information is available in the literature on these absorptions, and the correlations depend, almost entirely, on the unpublished work of those workers who have drawn up correlation charts (12)".

EXPERIMENTAL

The samples used in the X-ray and infra red analyses were the same ones used to spot the paper chromatograms. Diffraction patterns of the soil fractions were obtained with a Philips Norelco X-ray spectrometer (1950 model) equipped with a copper target. One mililiter of the sample was served over a microscope slide and completely dried under an infra red lamp, except when otherwise indicated.

The infra red absorption analyses were done with a model-21, double beam, self recording spectrophotometer, using NaCl plates, and Nujol or hexachlorobutadiene as mounting medium whenever the sample was not thick enough to smear it directly on the salt plates.

Glyceration was done by mixing the sample with C.P. glycerine (4:1 sample to glycerine ratio). For the infra red studies, the samples were dried inside a vaccum oven at 70°C for four days after glyceration.

For both analyses pure amino acid systems were prepared in aqueous solution and half of them were glycerated as indicated above.

DISCUSSION OF RESULTS

X-Rays

The X-ray diffraction peaks for the dicarboxylic fraction retained by the alumina column from the $Ca(OH)_2$ treated hydrolysate of the check soil are shown in Table 16. Three outstanding diffraction peaks are readily recognized: 11.3 Å, 5.6 Å, and 3.8 Å. The last peak is about 1.5 times stronger than the other two. The 11.3 Å and 3.8 Å diffraction peaks are considered complex in the sense that they showed a compact zig-zagging at their tips. The 11.3 Å peak has an accompanying 11.8 Å peak close to its base. Several other small peaks are also present. The similarity of those peaks with those reported by Brown et al. (32) for their amino acid copolymers is noticeable.

The glycerated counter portion of the above mentioned fraction showed no diffraction peaks at all. Heating the glycerated samples was then considered worthwhile. Heating for 4 hours at 200°C did not alter the results. But when the sample was heated for 2 hours at 500°C, the material over the plate turned, from a dark brown, to a whitish color and two small clear peaks appeared at 4.22 Å and 3.50 Å and a strong peak at 3.35 Å. These peaks could be interpreted as being due to salts which may have been able to show up when the rest of the material was "burned". Salt metal chelation by organic matter is, therefore, indicated. It should be pointed out that these samples were all desalted by the procedure described in the paper chromatography section of this work, so that salt protection by chelation seems to be plausible during

Table 16.

X-Ray Diffraction Data for Some of the Soil Fractions Before and After Glyceration

Soil fraction	Treatment	Most prominent peaks observed Å	Remarks
Alumina column sorbate of the check soil after removing ammonia and humin	Unglycerated	11.3, 5.6 and 3.8	Small peaks also present at 11.8, 3.6, 3.5, 3.3, and 3.2
	Glycerated	No diffraction	
Alumina column sorbate of the 50 tons of clay/acre before removing ammonia and humin	Unglycerated	7.6, 4.3, 3.8, 3.1, 2.5, and 1.9	Band around 17.4 and small peaks at 8.4, 5.9, and 2.9
	Glycerated	No diffraction	
Alumina column sorbate of the 6.25 tons of clay/acre after removing ammonia and humin	Unglycerated	No diffraction	
	Glycerated	No diffraction	
Alumina column sorbate of the 50 tons of clay/acre after removing ammonia and humin	Unglycerated	13. Å	One single sharp peak
	Glycerated	No diffraction	
Silica column sorbate of the 50 tons of clay/acre before removing ammonia and humin	Unglycerated	7.6, 4.3, 3.8, and 3.0	Band around 17.4 and small peaks at 8.4, 5.9, and 2.5
	Glycerated	No diffraction	

amino acid isolation from soils.

Table 16 also shows the diffraction pattern of the unglycerated portion of the separate retained by the alumina column from the soil hydrolysate (Fraction A) for the treatment which received 50 tons of clay per acre. Sharp peaks are observed at 7.6 Å, 4.3 Å, 3.78 Å, 3.1 Å, 2.5 Å, and 1.9 Å. Small, but distinct, peaks are present also at 17.4, 8.4, 5.9, and 2.9 Å. Except for the 3.8 Å peak, which was very prominent here again, the rest do not correspond to those for the dicarboxylic acid separate from the soil hydrolysate after ammonia and humin separation (Fraction B). It might be inferred that the removal of the humin precipitate or of ammonia caused the elimination of peaks for organic compounds capable of reacting with glycerol. Since all the peaks disappeared after glyceration of this fraction, too, the possibility must also be entertained that crystal orientation of organic molecules over the plate was altered by the presence of glycerol.

The behavior of glycerated soil amino fractions is emphasized here for the reason that similar reactions with other organic soil constituents may, seriously, interfere with the isolation of amino acids from soils. Furthermore, the possibility that such reactions may go on in the soil itself should not be overlooked.

That mechanisms similar to the one just described may take place without glyceration, is suggested by the results observed in the dicarboxylic acid fraction of the soil hydrolysate after removing the humin precipitate and ammonia from the soil hydrolysate of the 6.25 tons of clay per acre treatment. No reflections were shown by this sample even before glyceration. Still, this sample showed a positive ninhydrin reaction. It is appropriate to point out that a careful visual inspection of the ninhydrin color test run on other of the glycerated dicarboxylic acid fractions after they were concentrated in vacuo to a gummy syrup, demonstrated that they had lost their sensitivity to the ninhydrin reaction. These samples showed a gradual blue-violet coloration when heated, not with ninhydrin itself, but with the phenol-pyridine reagent of Troll and Cannan (140) so that, when ninhydrin was actually added to the sample, there was little increase in purple coloration. It should be recalled that Sowden (129) recently reported that some peculiar compound eluted from the Moore and Stein column between phenylalanine and tyrosine showed an abnormal behavior toward ninhydrin. "It reacted readily with Moore and Stein reagent but very slowly with ninhydrin in butanol spray. Full color intensity did not develop in the paper chromatograms for more than 24 hours after spraying." Attention is brought to the fact that a similar retardation in color over the paper was also observed, in many instances, in the present work (see paper chromatographic section). A color change similar to that described above when the glycerated dicarboxylic acid concentrates were treated with the phenol-pyridine reagent, has also been observed for diketopiperazines by Klarman (56, 78). The former observed that aromatic nitro compounds (carbonyl reagents) were found to give characteristic color reactions with diketopiperazines. It was observed, in the present work, that most of the gummy concentrates, prepared as indicated above, were capable of decolorizing an alcoholic solution (0.1%) of p-nitrophenol with the consequent precipitation of a white substance which is believed to be of the same salty nature as the one described above when one of the dicarboxylic fractions used in X-ray analysis was ignited at 500°C. This belief is partially supported by the fact that a similar white precipitate separated out of the brown gummy concentrate after boiling in 95% alcohol, but without the apparent disappearance of the brown gummy compounds.

The X-ray pattern for the unglycerated dicarboxylic acid fraction of the soil hydrolysate after humin and ammonia separation for the 50 tons per acre clay treatment showed only one peak at 13.0 Å, of very high intensity. Here again, glyceration caused the peak to disappear.

The behavior toward glyceration of other samples was similar to that described above. The basic amino fraction of the hydrolysate from the 50 tons per acre clay treatment showed very intense peaks at 7.56, 4.29, 3.8, and 3.5 Å, before glyceration. Glyceration caused the disappearance of those peaks. Other basic fractions showed no peaks before or after glyceration.

As a matter of comparison, some pure amino acids were run (see Table 17). In the particular cases of lysine and glutamic acid, glyceration caused the disappearance of peaks characteristic for the pure amino acids.

It is recalled that Scheffer et al. (119) found that synthetic humic

Table 17.

X-Ray Diffraction Data for Some Amino Acids Before and After Glyceration

Amino acids	Treatment	Most prominent peaks observed Å	Remarks
Glutamic acid	--	8.7, 4.9, 4.4, 2.7, 2.5	Smaller peaks were present including one at 2.33 Å
Glutamic acid	Glycerated	4.4, 2.7, 2.3	
Lysine	--	19.6, 10.3, 3.4	Bands rather than peaks were found at about 5.0 to 4.6 and 3.9 Å
Lysine	Glycerated	No peaks	No peaks
Cystine	--	4.8 and 3.13	Other small peaks at 9.4, 4.2, 4.0, 3.3, 2.8, 2.7 Å
Histidine	--	7.7, 6.2, 4.3, 3.9, 3.6, 3.5, 3.4, 3.1, 2.6	Other smaller peaks at 8.7, 4.4, 3.3, 3.2, 2.9, 2.8, 2.7, 2.4 Å
Arginine	--	5.0, 4.4, 3.5, 3.4, 2.7, 2.4, 2.3	Small peaks at 10.2, 4.5, 4.3, 3.1, 2.5 Å
Phenylalanine	--	16.4, 5.2, 5.0, 4.9, 4.8, 4.3, 4.0, 3.9, 3.4	Other peaks at 3.6, 3.1, and 2.6 Å

acids, produced on the basis of multivalent phenol, were X-ray amorphous and formed difficultly insoluble humates with polyvalent cations. The fact that glycerol is capable of "erasing" the X-ray diffraction pattern of soil amino hydrolysates and two pure amino acids tested, is considered very interesting.

The results of X-ray analyses, together with the heating procedure, demonstrated that clay was not present in the soil nitrogenous fractions.

Infra Red

The infra red spectra of some of the fractions discussed above are shown on Plate 1. The broken line portion of these spectrograms represent the portions which can be accounted for, either by glycerol or by the solvent used in mounting the preparation. Except for curves 17, 18, 19, and 20 on Plate 1, the rest show in successive pairs the infra red spectrograms of glycerated soil fractions or pure amino acids and their unglycerated counterparts. Curves 18, 19, and 20 show the infra red spectra of the solvents employed for smearing the samples on the salt plates.

The glyceration behavior of the acidic separate from Fraction B for the 12.5 tons of clay per acre treatment is shown in Curves 1 and 2. Glyceration suppressed absorption at 3,840 cm.$^{-1}$ (2.6μ), enhanced the absorption which appears as a shoulder at 3,257 cm.$^{-1}$ (3.1μ), and left intact the shoulder at 2,941 cm.$^{-1}$ (3.4 μ). The glyceration of this fraction brought about a pronnounced absorption band at 1,198 cm.$^{-1}$ (8.4μ) and broadened the band at 1,053 cm.$^{-1}$ (9.5μ).

PLATE I

INFRA RED ABSORPTION SPECTRA OF SOIL NITROGENOUS CONSTITUENTS AND AMINO ACIDS BEFORE AND AFTER GLYCERATION

Curve 1 - Unglycerated constituents of Fraction B retained in the alumina column from the 12.5 tons of clay per acre treatment. Nujol was used as mounting medium.

Curve 2 - Glycerated constituents of Fraction B retained in the alumina column from the 12.5 tons of clay per acre treatment. Glycerol served as mounting medium.

Curve 3 - Unglycerated constituents of Fraction A retained in the silica column from the 6.25 tons of clay per acre treatment. Nujol was used as mounting medium.

Curve 4 - Glycerated constituents of Fraction A retained in the silica column from the 6.25 tons of clay per acre treatment. Glycerol served as mounting medium.

Curve 5 - Unglycerated neutral non-aromatic, final perfusate of Fraction B from the check soil. Nujol was used as mounting medium.

Curve 6 - Glycerated neutral non-aromatic, final perfusate of Fraction B from the check soil. Nujol was used as mounting medium.

Curve 7 - Unglycerated constituents of Fraction A retained in the alumina column from the 12.5 tons of clay per acre treatment. Nujol was used as mounting medium.

Curve 8 - Glycerated constituents of Fraction A retained in the alumina column from the 12.5 tons of clay per acre treatment. Glycerol served as mounting medium.

Curve 9 - Unglycerated constituents of Fraction B retained in the alumina column from the 25 tons of clay per acre treatment. Nujol was used as mounting medium.

Curve 10- Glycerated constituents of Fraction B retained in the alumina column from the 25 tons of clay per acre treatment. Nujol was used as mounting medium.

Curve 11 - Unglycerated asparagine. Nujol was used as mounting medium.

Curve 12 - Glycerated asparagine. Glycerol served as mounting medium.

Curve 13 - Unglycerated glutamic acid. Nujol was used as mounting medium.

Curve 14 - Glycerated glutamic acid. Glycerol served as mounting medium.

Curve 15 - Unglycerated lysine. Nujol was used as mounting medium.

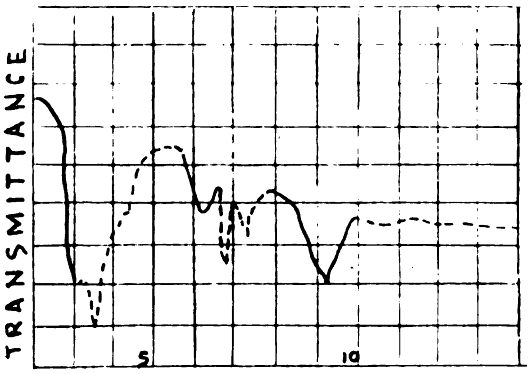
Curve 16 - Glycerated lysine. Glycerol served as mounting medium.

Curve 17 - Glycerated constituents of Fraction B retained in the alumina column from the check soil. Hexachlorobutadiene was used as mounting medium.

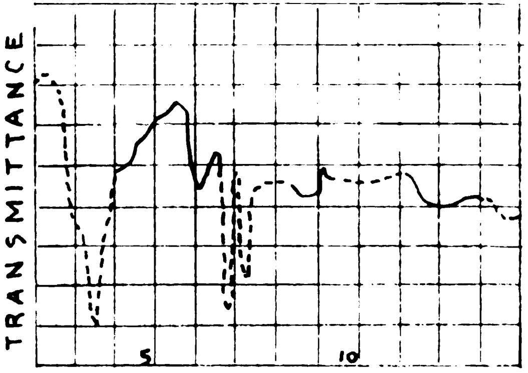
Curve 18 - Glycerol.

Curve 19 - Nujol.

Curve 20 - Hexachlorobutadiene.


Curve 1


Curve 2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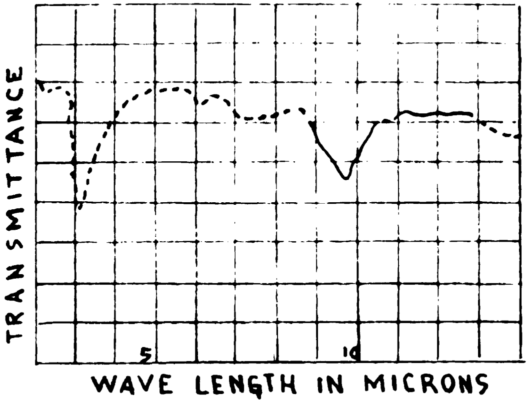
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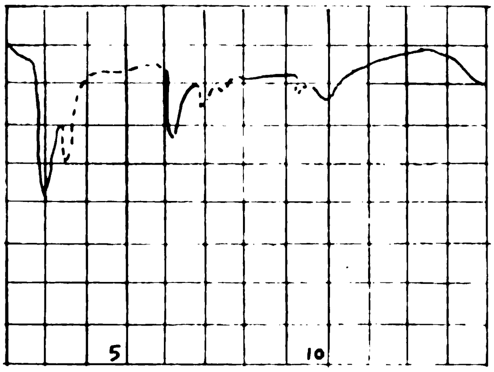
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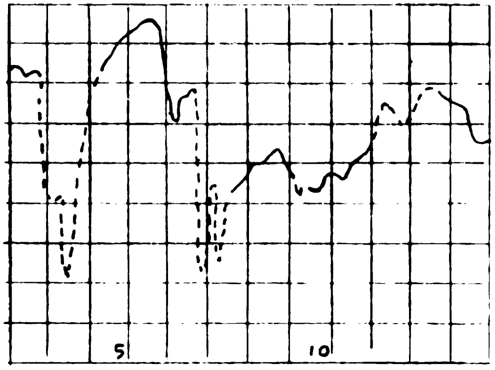
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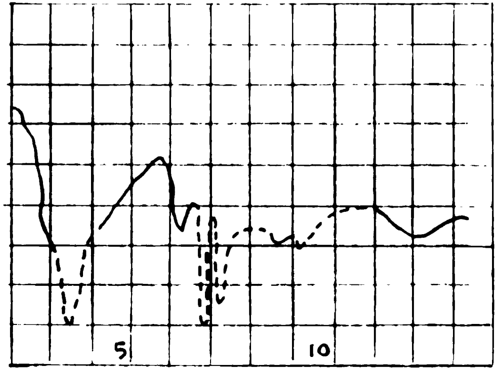
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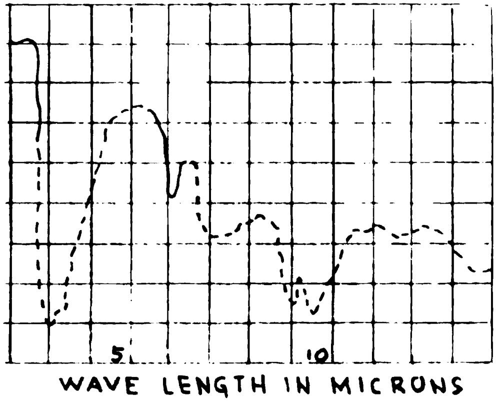
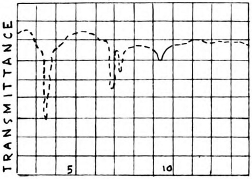

Curve 8

PLATE 1

Curve 9



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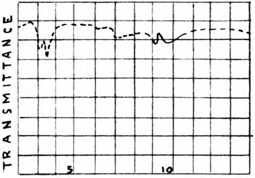
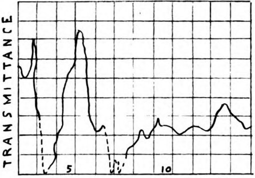
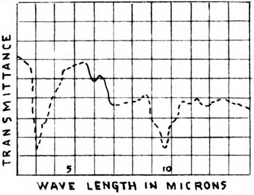


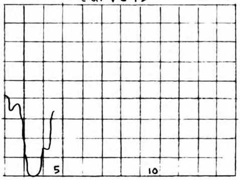
Curve 11



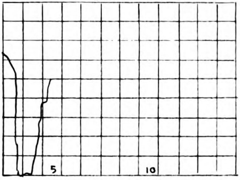
Curve 12



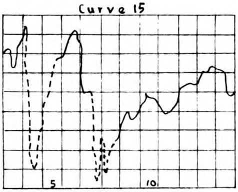
Curve 13



Curve 14



Curve 15



Curve 16

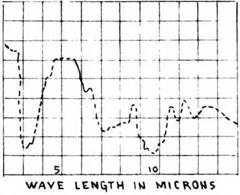


PLATE 1 (continued)

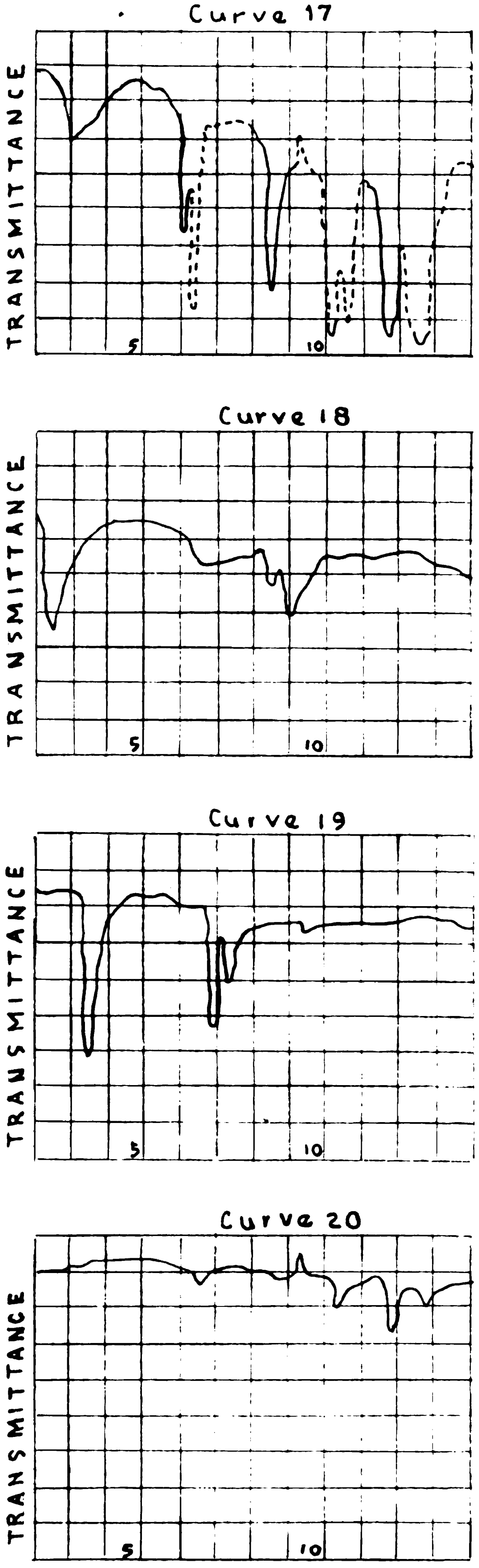


PLATE 1 (continued)

It should be pointed out that in all cases of glyceration the absorption band at 1,613 cm.$^{-1}$ (6.2 μ) was found to be narrower and in most cases very suppressed and shifted to higher frequencies.

The glyceration contrast of the basic separate from Fraction A of the hydrolysate for the 6.25 tons clay per acre treatment is shown in Curves 3 and 4. In this case, the absorption which appears as a shoulder at 2,353 cm.$^{-1}$ (4.3 μ) was considerably suppressed by glyceration. The 1,176 to 1,143 cm.$^{-1}$ (8.5 to 8.8 μ) absorption band disappeared after glyceration, while a new absorption band appeared at 1,026 cm.$^{-1}$ (9.7 μ). Despite the fact that both the untreated sample and glycerol showed absorption at 830 to 815 cm.$^{-1}$ (12.0 to 12.3 μ), the glycerated sample did not show any absorption in this region.

Changes in the infra red absorption were very pronounced for the neutral non-aromatic separate from Fraction B of the check soil as the result of glyceration (Curves 5 and 6). First, a new band at 4,000 cm.$^{-1}$ (2.5 μ) appeared after glyceration. The band at 3,333 cm.$^{-1}$ (3.0 μ) was shifted to about 3,125 cm.$^{-1}$ (3.2 μ), and two weak shoulders appeared, one at 2,353 cm.$^{-1}$ (4.3 μ) and the other at 2,222 cm.$^{-1}$ (4.5 μ). A distinct absorption band appeared in the glycerated sample at 1,545 cm.$^{-1}$ (6.5 μ), a less pronounced one at 1,205 cm.$^{-1}$ (8.3 μ), and a shoulder at 1,117 cm.$^{-1}$ (8.9 μ). Changes in the absorption spectrum also occurred at higher frequencies. An absorption band appeared after glyceration at 1,035 cm.$^{-1}$ (9.7 μ), with a shoulder at the edge of this band at 943 cm.$^{-1}$ (10.6 μ). A pronounced peak appeared at

719 cm.$^{-1}$ (13.9μ) after glyceration.

The acidic separate of Fraction A from the 12.5 tons treatment showed distinctive changes in its infra red spectrogram as a consequence of glyceration, (Curves 7 and 8). The 1,626 cm.$^{-1}$ (6.2μ) band was either displaced to 1,468 cm.$^{-1}$ (6.8μ) or it was eliminated and that at 1,468 cm.$^{-1}$ (6.8μ) represents a new site or type of vibration. Three bands were eliminated by glyceration, one at 1,961 cm.$^{-1}$ (5.1μ), one at 1,136 cm.$^{-1}$ (8.8μ), and another at 816 cm.$^{-1}$ (12.3μ).

It is interesting to observe that the infra red absorption spectrum of the acidic separate from Fraction B of the 25 tons clay treatment was not altered above the 1,111 cm.$^{-1}$ (9.0μ) region when glycerated (Curves 9 and 10). However, a pronounced change of the 1,080 cm.$^{-1}$ (9.3μ) band was very evident after glyceration. This was manifested by a distinct broadening of the 1,080 cm.$^{-1}$ (9.3μ) absorption band and the appearance of a new band at 1,015 cm.$^{-1}$ (9.8μ).

Infra red spectrograms were prepared for pure amino acids before and after glyceration. The infra red spectra of asparagine before and after glyceration, (Curves 11 and 12), show considerable alterations due to glyceration. It should be noted that the sharp infra red absorption band appearing at a frequency of 4,082 cm.$^{-1}$ (2.4μ) was eliminated by glyceration. All amino acids studied showed the same behavior. This absorption band was more pronounced for the unglycerated diamino acids (lysine and asparagine) than for glutamic acid. It is, therefore, suspected that this band is associated with the amino group rather than with the carboxylic or acid amide group and that glycerol is capable of interacting with this particular group.

Other outstanding effects of glycerol on asparagine are the complete elimination of the 2,083 cm.$^{-1}$ (4.8 μ) absorption band and the shifting to higher frequencies with the simultaneous narrowing of the 1,667 to 1,538 cm.$^{-1}$ (6.0 to 6.5 μ) band. Furthermore, glyceration caused the disappearance of the following bands of asparagine: The 1,247 cm.$^{-1}$ (8.0 μ), the 1,143 cm.$^{-1}$ (8.8 μ), the 1,000 cm.$^{-1}$ to 939 cm.$^{-1}$ (10.0 to 10.7 μ), the 749 cm.$^{-1}$ (13.4 μ), and the 717 cm.$^{-1}$ (13.9 μ) bands. Glyceration of this amino acid brought about an absorption band at 1,081 cm.$^{-1}$ (9.3 μ), despite the fact that the unglycerated asparagine and the glycerol itself are transparent in this region.

Curves 13 and 14 show the infra red spectrogram of glutamic acid for the overtone region only. The near infra red region was run to a very low transmittance and, therefore, considered inadequate for discussion. As observed above, glyceration eliminated the 4,082 cm.$^{-1}$ (2.4 μ) absorption band. In this particular amino acid this band appeared much less prominent than for asparagine and lysine.

Lysine infra red absorption spectrograms show interesting variations due to the action of glycerol (Curves 15 and 16). Here again, glyceration eliminated the 4,082 cm.$^{-1}$ (2.4 μ) absorption band. A noticeable effect associated with glycerated lysine is the higher infra red transmittance in the region 2,841 cm.$^{-1}$ (3.5 μ). This can also be observed in the case of glutamic acid, but is not quite so apparent with asparagine. The appearance of an absorption band at 1,739 cm.$^{-1}$ (5.8 μ) and the disappearance of the 1,608 and 1,567 cm.$^{-1}$ (6.2 and 6.4 μ) absorption peaks to give instead a single peak at 1,613 cm.$^{-1}$ (6.2 μ), are also attributed to the effects of glycerol in lysine. A band appeared at 1,183 cm.$^{-1}$ (8.5 μ) because of glyceration and the 1,149 cm.$^{-1}$ (8.7 μ) band disappeared. As in the case of asparagine, the glycerated lysine showed an absorption band at 1,075 cm.$^{-1}$ (9.3 μ). The 971 to 930 cm.$^{-1}$ (10.3 to 10.8 μ) band of the unglycerated lysine disappeared by glyceration; so did the 801 cm.$^{-1}$ (12.5 μ) band and the prominent 719 cm.$^{-1}$ (13.9 μ) band.

It should be noted that all the glycerated pure amino acids, as well as glycerol itself and some of the unglycerated soil hydrolysate fractions, show a high absorption of infra red at a frequency just above 3,500 cm.$^{-1}$ (2.9 μ). Bellamy (12) has associated this frequency with free OH stretching vibration.

The glycerated dicarboxylic fraction of the check soil hydrolysate after treatment with $Ca(OH)_2$ was completely dried and a crystalline substance was obtained. Curve 17 shows its infra red spectrogram. This particular fraction was mulled in hexachlorobutadiene. Free glycerol is considered to be totally absent. The broken line represents the portion of the spectrum accounted for by hexachlorobutadiene. Three absorption regions are clearly observed in this spectrum; the 4,000 to 2,173 cm.$^{-1}$ (2.5 to 4.6 μ) broad band, the 1,612 cm.$^{-1}$ (6.2 μ), and the 1,163 cm.$^{-1}$ (8.6 μ). It should be pointed out here that these crystals showed no X-ray diffraction pattern; they showed optical activity as determined* with polarized light in the microscope and showed parallel extintion. The crystals were heated to 250°C without melting. Their refractive index, as compared with oils of known refractive indices, was found to be 1,530 ± .002. The substance showed pleochroism, probably dichroism, as shown by a green and yellow-brown color when rotated under polarized light in the microscope.

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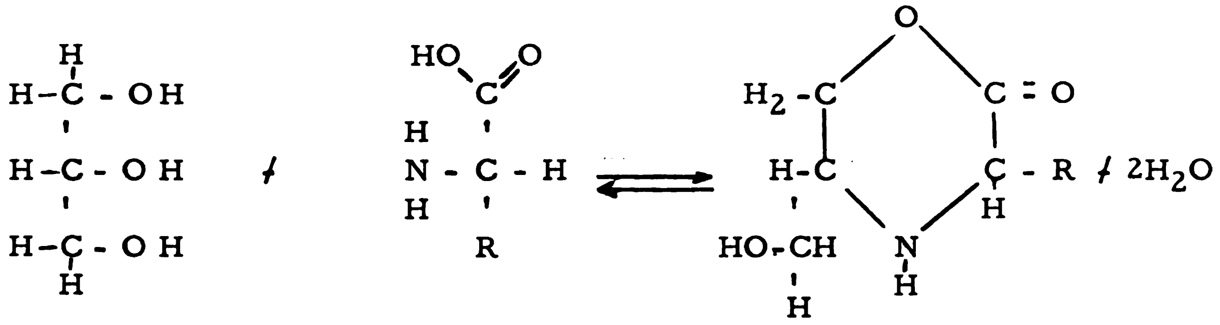
* The author is endebted to Mr. N. Yassoglou, Graduate Student in Soil Science, Michigan State University, for the microscopical analyses.

CONCLUSIONS

From the results of the infra red absorption experiment it seems safe to infer that the absence of X-ray diffraction in the glycerated soil amino fractions, as well as in the glycerated pure amino acids, is associated with definite chemical changes.

The 4,082 cm.$^{-1}$ (2.4 μ) absorption band is attributed to the NH_2 groups, although no confirmatory evidence was found in the literature, except for the skeletal vibration of aromatic systems or their derivatives. The fact that the 4,082 cm.$^{-1}$ (2.4 μ) band disappears by glyceration, added to the fact that the free OH stretching vibration (3,600 to 3,700 cm.$^{-1}$, equivalent to 2.8 to 2.7 μ) then becomes evident, suggests that glycerol reacts with the amino acids. The following reaction is tentatively suggested as the most plausible:



Esterification of the glycerol OH and the carboxylic group of the amino acid followed by dehydration could give rise to the cyclic amino lactone. It should be observed that such a cyclic condensation could be repeated to form polycyclic amino lactones, the properties of which would be expected to be similar to some of the fibrous peptides, as far as molecular stretching, dichroism, resonnance, and spatial configuration are concerned (8).