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STUDIES ON THE CONVERSIONS OF AMINO ACIDS IN SOIL[1])

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CHAPTER 1

INTRODUCTION

Humus is a mixture of compounds formed in the soil by condensation and polymerisation of degradation products from animal and vegetable origin (SCHUFFELEN, 1950). It is a well-known fact that the composition of humus varies widely both in character and relative amounts of its components, depending on the nature of soil, climate, vegetation and moisture conditions. Many years ago nitrogen was already recognized as a constituent of humus (EGGERTZ, 1889), since all attempts to remove this element as an impurity had been without success. By degradation of the humus, followed by a fractionating procedure and identification of the products some insight was gained about its chemical structure and the structural bond of the nitrogen.

Humus formation occurs principally in the soil outside the living organisms. Undoubtedly the precursors are mainly derived from biological materials, and introductory processes of humification may occur in dying cells. The main processes, however, take place in the soil.

In spite of all attempts to elucidate the chemical processes leading to humus formation, this problem remained essentially unsolved, including the problem of nitrogen incorporation. In the soil a wealth of microbiological and chemical reactions occurs which can transform the degradation products of biological origin in many directions, but only a few of these involve the production of humus. The complex nature and the diversity of these processes are the main difficulties in solving this problem.

Many attempts were made to identify the precursors in the humification process, including nitrogenfree compounds, in particular lignin, carbohydrates and polyquinones, and several nitrogen containing substances, aromatic amino acids being the most important ones in this respect.

These results are partially based on a similarity in chemical structure between compounds isolated from the soil and humus degradation

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products. Partly they rest on investigations of substances which contribute to the formation of dark coloured material in cultures of special soil microorganisms. *Actinomycetes* proved to be most active in this respect. FLAIG c.s. (1952) stated that the aromatic amino acids contribute especially to the synthesis of these products. The other 23 amino acids he tested rarely caused an appreciable dark colouring, only small amounts of brown coloured compounds, at most one tenth of the quantity produced by aromatic amino acids.

The problem of the incorporation of nitrogen in humus compounds has often been considered. It is generally assumed that in nature this process can proceed along two different lines:

According to one view amino acids and related substances would be involved directly in the humification process, without any preceding N-mineralisation. This could happen either under the influence of the action of microorganisms in the soil or in a purely chemo-synthetic way, as has been revealed by laboratory experiments. So RAPER (1928) has been able to show that melanin could be formed out of tyrosine; ENDERS (1943) studied the reaction between glycine and methylglyoxal; WAKSMAN and IYER (1932) synthesized humus-like substances from lignin and protein. All the products formed were dark coloured, but at present it is not quite certain whether these processes contribute considerably to the production of humus, since the conditions in vitro may differ widely from those occurring in nature.

Considering the rapid deamination of amino acids by microorganisms in the soil, others are of the opinion that at first the N-containing organic substances are mineralised under liberation of ammonia. Only in the final stages of the humification process would the ammonia enter the N-free intermediates, formed either by action of microorganisms or synthesized by chemical reactions. In order to establish the importance of this view some investigators have tried to synthesize humus-like substances in this way. MATTSON c.s. (1943) was able to demonstrate the formation of such compounds by the action of ammonia on lignin under laboratory conditions and FLAIG (1950) by the reaction of ammonia with polyquinones. Both reactions were performed under conditions that might occur in soil.

In my opinion, the essential difference between these hypotheses is that, according to the view first mentioned, an important part of the carbon skeleton of the amino acid enters the humus fraction; according to the last mentioned hypothesis amino acid carbon atoms will take part in these syntheses only incidentally. When we started with this investigation little was known about this problem and only a few papers had appeared dealing with the subject. That is why we undertook this work.

CHAPTER 2

SURVEY OF LITERATURE

2.1. The presence of free amino acids in soil

Before discussing the conversion of amino acids in the soil, we will consider their occurrence.

Many attempts have been made to demonstrate the presence of free amino acids in soil (Bremner, 1952; Okuda ,1954; Sowden c.s., 1953; Sowden, 1957). In the early investigations these were found only in acid peat soils. Fowden, Dadd and Pearsall (1953) demonstrated paperchromatographically the presence of a few amino acids in extracts of this type of soil. These extracts were obtained by pressing out the soil sample and by concentrating the extract a hundred fold in vacuo at room temperature above sulfuric acid. The total quantity of amino acids present amounted to maximally 3 μg per gram of air dry soil. In nearly all samples serine, glycine, alanine, glutamic and aspartic acid were found. The presence of threonine, leucine-isoleucine, valine, β-alanine, γ-aminobutyric acid, asparagine, glutamine and some basic amino acids could occasionally be demonstrated. However the analytical methods for determining amino acids were improved considerably in recent years. With these highly sensitive methods it was possible to detect free amino acids in nearly every type of soil. Putnam and Schmidt (1959), Schmidt, Putnam and Paul (1960), Paul (1959), and Simonart (1954) investigated several neutral and alkaline soils. Instead of water they extracted the soil with 70–80 % ethanol, as this resulted in a higher yield. Paul (1959) extracted with 0.1 N $Ba(OH)_2$ and 0.5 N ammonium acetate; in this way an even larger number of amino acids could be detected. After concentrating to a 1000-fold they found 5–25 amino compounds in quantities of 0.05 μg–0.5 μg per gram of soil. This was much less than Fowden c.s. (1953) had found in acid peat soil.

In soils fertilized with glucose and sodium nitrate up to 40 different amino compounds could be detected in concentrations 10–50 fold as high as those in unfertilized soils.

Payne, Rouatt and Katznellson (1956) noticed a brownish colouring when the soil extracts were concentrated in vacuo at 40° C. With the procedure usually followed losses in amino content were reported. No decrease occurred when the extracts were concentrated by freeze-drying. The highest amount of free amino acids found in unfertilized soil has been 0.5 μg per gram of soil.

2.2. The combined amino acids in soil

Amino acids are the principal constituents of the soil nitrogenous material. This fraction gives on acid hydrolysis α-amino-N in a yield of about 25–50 % of the total soil nitrogen. As an example, Rendig (1951) hydrolysed a sample of virgin Miami silt loam with 3 N HCl

for 10 hours at 120° C. Afterwards he was able to isolate the following fractions, containing in terms of the nitrogen originally present: amino acids (including the basic amino acids) 34 %; ammonia released from amides during hydrolysis 20 %; non-basic-non-amino compounds 5 %; inorganic nitrogen compounds 1 %. The remaining fraction, with 40 % of the total N was not hydrolisable and could not be identified. The results of the various analyses depend to a considerable extent on the fractionating procedure used, but when carried out under standard conditions each procedure will give reproducible values.

Up to 1946–1947 the amino acids had to be identified by specific reactions; only a small number could be detected in soil hydrolysates (KOJIMA, 1947; WAKSMAN, 1938). Much better results were obtained both by paper-chromatography (BREMNER, 1946, 1949, 1951, 1955a; DAVIDSON c.s., 1951; PARKER c.s. 1952) and by column chromatography (SOWDEN, 1955; STEVENSON, 1954, 1956a, b); with these techniques all amino acids can be separated. SOWDEN (1956) found 12, BREMNER 21 (1950), STEVENSON 25 (1956a) and recently YOUNG and MORTENSEN (1958) 50 ninhydrin-positive substances in soil hydrolysates. These differences probably depend mainly on differences in the various analytical techniques used. However, with both chromatographic procedures a number of compounds, present in soil hydrolysates in low concentrations, remained unidentified.

The question then arises whether the combined amino acids fraction in the soil has a uniform composition. Indeed several authors have stated that the relative amounts of the various amino acids would be constant in many soil hydrolysates (BREMNER, 1946, 1949, 1951, 1952, 1954; SOWDEN, 1956). We must however remark that one meets these views only in older investigations in which less accurate analytical procedures were often applied. At present it is generally recognised that both the total and the relative amounts of the amino acids in the soil hydrolysates may vary. STEVENSON (1956b) found important differences depending on the vegetation. In a hydrolysate of soil from an experimental plot exclusively cropped with maize he found 30 % amino-N, expressed as percentages of total soil-N, against 40 % in a permanent grass border round about the same plot. In table 1 a survey is given of his results on the relative amino acid composition of these soil hydrolysates. CARLES (1959) also in many soil hydrolysates obtained varying amounts of amino acids.

From all these studies we may conclude that the amino acids in soil hydrolysate originate from divergent compounds. The problem arises: what is the nature of these compounds? It was generally assumed that a considerable percentage of the combined amino acids is derived from proteins.

However these proteins can scarcely be present in an unbound state. It appeared impossible to isolate proteins from soil; chemical identification of these compounds in soil or soil extracts gave negative results, e.g. the amino-end group reaction according to SANGER (BREMNER, 1957; SOWDEN c.s., 1953).

TABLE 1. AMINO ACIDS IN HYDROLYSATES FROM A SILT LOAM SOIL
(Data taken from STEVENSON (1957))

Soil sampled from the top soil. Since 1902 plot A had been cropped with maize, plot B with grass.
Hydrolysis: 16 hours with 6N HCl, at atmospheric pressure. After desalting the amino acids had been separated on a column of DOWEX 50.

Total N: A 0.138 %, B 0.323 % of dry matter
Amino-N-content: A 30.1 %, B 39.9 % of total N

Amino acid	A mg/100 g soil	B mg/100 g soil
glycine	21.8	73.7
alanine	31.7	76.4
β-alanine	5.0	17.3
serine	14.0	73.4
threonine	18.2	81.0
valine	21.3	62.0
leucine	19.5	63.4
isoleucine	16.1	47.7
proline	10.3	51.7
phenylalanine	7.4	23.6
tyrosine	8.0	23.3
aspartic acid	26.0	66.7
glutamic acid	20.0	94.4
ornithine	8.5	8.6
histidine	5.8	6.9
arginine	6.4	17.0
lysine	44.4	70.0
γ-aminobutyric acid	3.5	12.0
α-aminobutyric acid	1.0	3.5
α-ε-diaminopimelic acid	2.5	9.9
methionine	trace	trace
methioninesulfoxyd	11.3	22.7
methioninesulfone	trace	trace
cystine	trace	trace
cysteic acid	5.3	15.9

One could assume that most of the proteins present would be in the living or dead microorganisms; therefore the reactions just mentioned failed to reveal these compounds.

However according to the views of RUSSELL c.s. (1950) and SWABY (1959) the quantity of amino acids, formed by hydrolysis of the proteins of the intact microbes, could at best constitute only a minute fraction of the total amount of amino acids in the soil hydrolysate. They estimated that the countable microorganisms comprise only one percent of the soil organic matter.

More difficulties arise in determining which protein fraction is directly derived from dead microbes, as one cannot follow the estimation procedure of RUSSELL and SWABY in this particular case. One has to assume that this fraction is probably negligible.

In the top soil large quantities of proteins are supplied from plants and animals. These proteins are rapidly converted. There is no evidence of any great difference in the biochemical resistance of the proteins of microbes and those of other plants and animals. The reported occurrence of resistant proteins excreted by some fungi

(BOHONOS, c.s., 1942; MORTON c.s., 1955) seems to represent an exception. It seems therefore appropiate to assume that microbial proteins will also be rapidly converted.

Practically all the proteins in soil are present in a complex form. This prevented the extraction and isolation of these proteins. Several assumptions have been made about the character of these complexes. WAKSMAN and IYER (1932) stated that in soil lignin-protein complexes would be present, forming most of the resistant protein fraction. A second assumption is the adsorption of proteins on clay minerals, e.g. on bentonite (ENSMINGER c.s., 1942, 1944; LYNCH c.s., 1956, 1958; MCLAREN c.s. 1954a, b, 1956, 1958, 1959; PINCK c. s., 1951, 1954). In vitro both lignin- and bentonite-suspensions give rise to a rapid protein adsorption, about three fourths of the maximum adsorption occurring in the first few minutes. The protective action of lignin towards protein is probably due to physical absorption into a dense molecular gel network (ESTERMANN c.s., 1959). The clay-protein complex may derive its resistance to adsorption on the exterior surface or within their expanding lattice layers (PINCK c.s., 1951, 1954). Complexes subjected to in vitro digestion by proteolytic enzymes or by bacteria show a retardation in protein mineralisation, as compared with the conversion of unprotected protein (ESTERMANN c.s., 1959). Complexes with lignin are more resistant than those with bentonite. Added to the soil, both complexes may be decomposed however. The presence of lignin-protein complexes in actual samples of soil could not be demonstrated and up to the present there is no evidence for their existence (FORSYTH, 1946; MATTSON c.s., 1943; SOWDEN c.s., 1949, 1955; STEVENSON, 1956b).

ALLISON *et al* (1949) investigated the process of immobilisation of casein-carbon in a mixture of quartz sand, inoculated with a soil infusion, clay minerals having been added in part of the experiment. Adding kaolin to quartz sand brought about a 50 % higher carbon retention in a period of 1.5 to 4 months; after a 12 month period however no differences with quartz sand alone were observed. The clay mineral bentonite, unlike kaolin in having a large base exchange capacity, enhanced the carbon retention in a 1.5 to 4 month period about 100 %; 12 months later the increase was still 50 %. The role of clay protein complexes in the stabilisation of soil protein is therefore significant, although the practical importance is not known.

Complex formation may occur with other suitable materials, e.g. humus. BREMNER c.s. (1952, 1955a) isolated several humus fractions, hydrolysed these and found amino acids. After fractionating hydrolysis of a humic acid preparation he observed spots on the chromatogram of peptid-like substances that were not originally present (1955b). Presumably this result may be ascribed to the presence of a protein-humus complex.

It is obvious that part of the fraction of combined amino acids might consist of non-proteinous amino acids, either adsorbed to inorganic colloids, or as a constituent of organic matter. SOWDEN c.s. (1952) isolating various humus fractions found that 40–70 % of their nitrogen

was α-amino-N. This fraction was purified thoroughly by dissolving and precipitating repeatedly in order to reduce the content of minerals and of intact microbes to insignificant amounts.

The results of the experiments of BREMNER c.s. (1952, 1955a) mentioned on page 214, as well as those of SOWDEN can be partly interpreted by assuming the occurrence of humus-amino acid complexes in soil. It is not likely that the results of these hydrolysis experiments are due exclusively to the presence of humus protein complexes, according to the observations of DRAGUNOW (1958). He found that amino acids of a humic acid preparation were already released after $2^1/_2$ hours of refluxing with 1.5 N HCl. The complete hydrolysis of proteins of *Aspergillus Niger* could be accomplished only after 24 hours refluxing with 6 N HCl. DRAGUNOW ascribes this result to a difference in binding capacity between the amino acids and humus as compared with those in *Aspergillus* proteins. Although nothing was known about the nature of combination of the amino acids in his preparation, it may be assumed that he was engaged in examining humus-amino acid complexes.

SOWDEN (1956) noticed that the relative amounts of the amino acids in soil hydrolysates deviate from those in hydrolysates of proteins of several biological materials. In his soil hydrolysates the relative amount of glycine is higher and that of leucine and methionine less than in hydrolysates of microorganisms, herbage, yeast, vegetables and of grass. However, these results contribute very little to the solution of the problem.

STEVENSON (1956b) found in soil hydrolysates amino acids which were not yet detected in bacterial proteins. Secondary metabolic conversions may occur, yielding amino acids that were not present before. These amino acids would be bound to soil colloids.

In summary, the combined soil-amino acids are spread over the following fractions: a very small percentage is situated in the intact, living or dead microbes. Free resistant proteins are occasionally demonstrated, but only in minute quantities. Nearly all of the amino acids appear either as complex bound proteins, in clay-protein, or humus-protein complexes, or as amino acids bound in the organic soil polymers.

2.3. THE CONVERSIONS OF AMINO ACIDS IN SOIL

In this section we will consider what happens with the amino acids added to soil. Most experiments on the decomposition of these substances have been carried out during investigations of the nitrogen mineralisation, in which attention has mainly been confined to the conversions of the amino acid nitrogen.

The course of decomposition of nitrogeneous compounds in soil depends mainly on environmental conditions. Often the percolation method of LEES and QUASTEL (1946) is used. Although this procedure represents very unnatural conditions, it gives reliable results. A dilute solution of amino acids in water is continuously percolated, well

aerated, through a soil column and the decrease in its amino acid content is determined. Surprisingly the results obtained with the percolation method agrees rather well with those obtained in pot experiments (OWEN, WINSOR and LONG, 1950; SCHMIDT, PUTNAM and PAUL, 1960).

Under aerobic conditions an important part of the amino acid added to the soil is converted into CO_2, H_2O and NH_3 under uptake of an equivalent amount of oxygen (GREENWOOD and LEES, 1956, 1960) The ammonia can be oxidised to nitrite and nitrate, but under conditions of bad aeration and high pH to N_2O, N_2 and NO. These losses by denitrification can amount to one half of the nitrate-nitrogen present. Generally at least half of the amino acids is mineralised. The percentage of mineralised nitrogen depends on the C/N-ratio of the amino acid added, decreasing with an increase in ratio.

Most amino acids disappear completely in 2–9 days as appeared from the paperchromatographic analyses performed by GREENWOOD and LEES (1956), TOMBESI (1953), and SCHMIDT, PUTNAM and PAUL (1960). According to OWEN and WINSOR (1950), QUASTEL and SCHOLEFIELD (1950) and GREENWOOD and LEES (1956) the sulphur containing amino acids cysteïne and methionine were converted very slowly, whereas SCHMIDT, PUTNAM and PAUL (1960) recently reported a rapid decrease of methionine. They suppose that this may be due to different reaction conditions.

Adding a mixture of several amino acids, TOMBESI (1953) and SCHMIDT, PUTNAM and PAUL (1960), sometimes after a few days incubation, detected a new amino acid, probably β-alanine, which had not been present before. GREENWOOD and LEES (1960) however never observed the occurrence of a new amino acid.

The products formed from the amino acids which were not totally mineralised, have not yet been identified. Generally it is taken that these are used for the synthesis of microbial matter. GREENWOOD and LEES (1956) point out that the C/N-ratio of these substances varies between 1.2 and 3.9. They assume that these N-rich products may be considered to be bacterial proteins, but mostly bacterial matter has a C/N-ratio of 5–6.

Under anaerobic conditions the amino acid breakdown is different; leucine, methionine, phenylalanine, proline, hydroxyproline, threonine, tryptophan, tyrosine and valine were found to be fairly resistant. They were decomposed more slowly than alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, lysine and serine. A casein hydrolysate however was deaminated rapidly and completely. The anaerobic carbon mineralisation yield was very low; most of the carbon was recovered as volatile fatty acids (GREENWOOD and LEES, 1960).

2.4. DYNAMIC ASPECTS OF THE COMBINED AMINO ACID FRACTION IN SOIL

Two important problems concerning the stable amino acid complexes in soil are left: how were these amino acids immobilised, and what is

the rate of their release and breakdown? The second problem is particularly important.

SIMONART and MAYAUDON (1958) showed that not all amino acids in soil organic matter are derived from added proteins. A special humus fraction, incubated with C^{14}-labeled glucose for two months, gave on hydrolysis a considerable amount of labeled amino acids. Obviously the glucose carbon was used for synthesis of bacterial matter; the proteins and amino acids formed were afterwards partly incorporated in the humus fraction. WINSOR and POLLARD (1956) could demonstrate that the C/N-ratios of the immobilised residues, from various products rich in nitrogen, very often reach a value of about 6. This points too to a retention of proteins formed in microbes during the decomposition of the parent material.

It is not an easy matter to determine the rate of decomposition of the stable amino acids in soil. However, by their respiration processes the microbes will rapidly decompose the more unstable fractions of soil organic matter. A transient equilibrium is reached; the rate of production of carbon dioxide and the production of mineral nitrogen compounds are proportional to the rate of breakdown of the 'stable' organic matter. This process largely depends on environmental factors. It can be rapidly enhanced by air-drying and remoistening the soil, leading to a considerable increase in the content of phosphate, ammonia and free amino acids.

The data given do not allow us to determine what part of the supplied organic substances is converted into humus or into stable organic matter. KORTLEVEN c.s. (1960) investigated the relation between the formation and breakdown of humus, the humus content of the soil and the yearly supply of organic material. Depending on environmental and pedological conditions each soil type has an optimal humus content. He was able to demonstrate that, under favourable conditions, 25 % of the total supply of organic material was immobilised yearly. At present only a few factors have been investigated further.

In this paper an attempt has been made to increase the knowledge about these problems.

CHAPTER 3

METHODS

3.1. TREATMENT OF THE INVESTIGATED SOIL

A light sandy soil from the top layer of arable land in the vicinity of VENRAY was used in this investigation. This soil belongs to the diluvial deposits, typical for the southern provinces of the NETHERLANDS, and occurs especially on both sides of the valley of the river MEUSE. The results of an analysis of the soil were: 7 % silt and clay, 90 % sand, 3 % humus, no $CaCO_3$, 0,15 % total N, pH 5,6.

A stock of a hundred kilograms of this soil was stored in a glazed

stoneware open container, buried in the garden, separated from the soil underneath by a layer of washed gravel, and covered with fine-meshed nylon gauze to prevent contamination. The soil was subjected to the naturally occurring rainfall. All seedlings were removed. During storage a nitrification process gradually set in. The soil acidified and growth of moss was observed; at this stage the stock was discarded.

Originally the groundwater level was several meters below the surface of the soil, but under storage conditions this difference was only one meter. In the beginning this change in water level had a large influence on the microbial activity. Therefore the stock had to be left for some time under the new conditions before starting an experiment.

Samples of about one kilogram were taken by means of a soil core of 20 mm ϕ. Each sample was homogenised by rubbing it through a 1 mm sieve, and by mixing it thoroughly. Afterwards the soil was kept in darkness for a few days at a constant humidity and temperature of 23° C. During this period the microbial activity adjusted to the new conditions. In order to reduce changes in the bacterial population due to the treatment, the samples were kept under the same moisture conditions as the stock.

From these samples smaller ones of twenty gram each were taken for the measurements. For this purpose the soil was spread out in a reaction vessel (volume $\pm$ 100 ml.) in a layer of about 1 cm thickness, and 1 ml of water (23° C.) was sprinkled in tiny droplets onto the surface. If substrates had to be added they were dissolved previously in this water. In case of insufficient solubility, the compounds were mixed as a fine powder with the soil, after which 1 ml of water was added as just described. All experiments were performed at a constant temperature (23° $\pm$ 1 ° C.).

From preliminary experiments it appeared that no differences in O_2-uptake, CO_2-production or amino acid conversion could be demonstrated, whether the substrate was added as a solid or dissolved in water. Stirring of the soil sample in order to obtain a better distribution of the substrate gave no improvement in the results; in wet soil mixtures, water logging occurred with mixing and deviating figures resulted. In the method of addition described, mixing is not necessary, and is not recommended on account of the water logging.

3.2. Analytical procedures

The rate of O_2-uptake and of CO_2-production, the amounts of amino acids, keto acids, nitrate, and nitrite, and of ammonium present, the total nitrogen and the water content were determined.

Water content. The water content was calculated from the loss in weight of a sample of 10–20 g after drying in an oven at 105° C for 17 hours. Measurements were carried out in triplicate and the average loss is given in % dry weight. The standard deviation amounted to 1,3 % of the water content.

TOTAL NITROGEN. 5 g of soil was digested according to KJELDAHL with 4 g of selenium mixture (WIENINGER mixture, MERCK) and of 20 ml of concentrated sulfuric acid. The ammonia distilled off in a Parnas Wagner apparatus, was determined colorimetrically with NESSLER's reagent. If nitrate or nitrite was present in the soil, a reduction according to JODLBAUER had to precede the destruction. The standard deviation amounted to 3,5 % of the nitrogen content.

GAS EXCHANGE. The experiments involving O_2-consumption and CO_2-production were carried out in a manometric apparatus according to GORTER (1940). The apparatus consisted of 24 gas burettes, 25 ml each, graduated in 0.05 ml. The vessels had a volume of 100 ml. The temperature of the water bath like that of the room was 23° C $\pm$ 1° C. The vessels were mechanically shaken with 30 strokes per minute.

Each vessel was provided with an interchangeable inner vessel. 2 ml of 4 N NaOH was placed in it for CO_2-fixation. At the end of the experiment the inner vessel was connected to a second vessel, containing 10 ml of 25 % sulfuric acid in the main compartment, and allowed to equilibrate for 20 minutes in the water bath before mixing its content with the acid. The CO_2-evolution was measured.

All measurements were performed in triplicate, mean values are given in mMoles per sample of 20 g. The standard deviation of each triplicate, calculated from 30 measurements, was 2,4 % of the O_2-consumption and 1.4 % of the CO_2-production. Larger soil samples would have resulted in smaller dispersion, but technically this could hardly be achieved.

This method enabled us to determine the gas exchange of small soil samples under nearly natural conditions. The soil percolation method of LEES and QUASTEL (1946) also requires samples of 20–50 g but the experimental conditions differ extremely from those in nature. The bacterial processes, normally occurring in very thin water films on the soil particles (BARTHOLOMEV c.s., 1946) now proceed in a large volume of water. A more promising method for the determination of gas exchange makes use of the apparatus of SWABY and PASSEY (1953) modified by BIRCH and FRIEND (1956). Here the oxygen consumed is replenished automatically by means of electrolysis of acidified water; the hydrogen liberated is caught in a gas buret and serves as a measure for the oxygen uptake. Larger soil samples are needed. Although not carried out by the authors it would also be possible to estimate the amount of CO_2 produced at the end of the experiment.

The pH and inorganic nitrogen were determined in the extract obtained by shaking the sample with 5 volumes of 1 M NaCl and afterwards centrifuging at 1700 g for 15 minutes.

pH. The pH was determined in the extract by means of an Electrofact-pH-meter with a glass electrode.

AMMONIA. An aliquot of the extract containing 10–200 μg N was distilled at 100° C with 1 ml of a saturated solution of borax in a

Parnas Wagner apparatus. 30 ml of the distillate was collected in 1 ml 1 N sulfuric acid, made up to 50 ml; then the ammonia was determined colorimetrically at 450 mμ in 25 ml of the solution 1 ml of NESSLER's reagent having been added.

NITRITE and NITRATE. To the remaining solution, the ammonia having been distilled off, 50 mg DEVARDA-alloy and 1 ml 4 N NaOH were added and the nitrite and nitrate present reduced to ammonium. This was determined as described previously.

The measurements were performed in triplicate. The standard deviation of each triplicate determination, derived from 30 soil samples, was 3.3 % for the ammonia values and 4.2 % for the combined nitrite and nitrate values.

Known amounts of nitrate an ammonia added to the soil could be recovered quantitatively. No interference from labile N-compounds giving rise to the production of ammonia by the analytical procedures was demonstrated. Adding 200 times excess of amino acid-N did not disturb the results.

In soils with a high exchange capacity some of the ammonium ions remained bound to the adsorbtion complex after extraction with NaCl. Sufficient recovery could be brought about by extracting repeatedly or by percolation.

COMBINED AMINO ACIDS. To liberate the amino acids combined in the soil the sample was boiled under reflux with 25 volumes of 6 N HCl for 20–24 hours. The suspension was centrifuged and the sediment washed four times with 25 ml of water. The combined solutions were evaporated to dryness in vacuo at 40° C. Water was added and the extract again evaporated. This treatment was repeated until the excess of HCl had almost completely disappeared. The residue was taken up in 25 ml of water, 4 N NaOH was added to produce pH 5, and the mixture filtered and washed with water. The filtrate was diluted to 50 ml.

The desalting was brought about by adsorbtion of the amino acids on a cation exchanger in the H^+-form. (IMAC C 12, ACTIVIT, Amsterdam). The column (100 × 12 mm) was washed with distilled water till Cl^--free. The amino acids were eluted with 1 N ammonia (REDFIELD, 1953). The ammonia was distilled off in vacuo with a slight excess of 0.1 N $Ba(OH)_2$-solution; the barium ions were precipitated with an equivalent amount of sulfuric acid, and the amino acids determined as will be described further.

Hydrolysis of soil can give rise to considerable losses of several amino acids. STEVENSON (1956a) found losses of the aromatic amino acids, of methionine, cysteine and of arginine; LAATSCH c.s. (1953) reported similar losses. A solution containing known amounts of various amino acids was added to the soil and subjected to the same treatment. On the paper chromatogram compared with the control series sometimes a loss of basic amino acids could be demonstrated.

AMINO ACIDS. As NaCl interferes with the measurements water was used to extract the soil for determining the amino acids and keto acids. One extraction with 5 volumes of water sufficed. Unlike PUTNAM and SCHMIDT (1959), but in agreement with LEES and QUASTEL (1946), we did not get any appreciable adsorbtion of the amino acids added to the soil.

In nature and under storage conditions free amino acids were absent; even on a paper chromatogram no traces of amino acids could be detected.

QUALITATIVE DETERMINATION OF AMINO ACIDS. The amino acids were separated by two dimensional descending paper chromatography according to REDFIELD (1953), SCHLEICHER and SCHÜLL 2043 A paper (23 × 23 cm) was used. No special arrangements for temperature constancy were provided. The following solvents recommended by REDFIELD (1953) gave the best results: first direction: methanol, water, pyridine 80–20–4 (V/V), second direction: tertiairy butanol, n-butanone, water, diethylamine 40–40–20–4 (V/V). Only using fresh solvents the relative position of the spots was constant. Shifting and reversing of several spots was noticed using an aged solvent 2.

About 0.04μ mol N of amino acids was spotted on the chromatography paper. After development the paper was dried and steamed for 30–45 minutes to remove the diethylamine quantitatively, which otherwise interferes with the colour reaction. The amino acids were visualized by spraying with a 0.5 % solution of ninhydrin in 96 % ethanol. The paper was heated for two minutes at 105° C.; heating for a longer period interferes with the results by a strong background colouring. The spots appeared to be blue or yellow.

The following amino acids could be separated: arginine, ornithine, lysine, aspartic acid, glutamic acid, asparagine, glutamine, citrulline, glycine, histidine, α-alanine, hydroxyproline, β-alanine, γ-aminobutyric acid, proline, serine, tyrosine, methionine, valine, phenylalanine, threonine, leucine-isoleucine-norleucine and tryptophan. The spots of glucosamine and of α-aminobutyric acid coincided; those of leucine, isoleucine and norleucine partly coincided, likewise those of hydroxyproline and of β-alanine, but the latter varied in colour.

QUANTITATIVE DETERMINATION OF AMINO ACIDS. A non specific colour reagent for the quantitative determination is given by MOORE and STEIN (1948). The reagent consists of 10 mg of ninhydrin (B.D.H., recrystalized from water) dissolved in 0.5 ml methylethylene glycol, mixed with 0.8 mg of stannous chloride (MERCK) dissolved in 0.5 ml 0.4 M citrate buffer solution, pH 5.0. One ml of this reagent was added to a 0.2 ml amino acid solution, which contained 0.5–50 μg amino-N. It was heated for exactly 20 minutes in a boiling water bath, cooled, and diluted with 5 ml 50 % ethanol. The extinction is measured within an hour either at 570 mμ or at 440 mμ (for blue resp. yellow coloured solutions). Ammonia reacting with ninhydrin also, had to be removed beforehand. If only very small amounts were present, this could be omitted by correcting the results.

KETO ACIDS, QUALITATIVE DETERMINATION. Keto acids being unstable, they had to be converted into the stable phenyl hydrazones. Simple mixtures may be separated by paper chromatography, for the analysis of more complex mixtures of the hydrazones they must be reduced to the corresponding keto acids.

The determination of keto acids as hydrazones was carried out using the method of CAVALINI and FRONTALI (1954), in a simplified procedure as no proteins were present. To 30 ml of the water extract (page 221) 1 ml 0.2 % of 2-4-dinitrophenylhydrazine in 2 N HCl was added, and allowed to react for 20 minutes. When the hydrazones were formed, the solution was extracted with ethyl ether until all colouring substances were removed. The combined ether extracts were evaporated under vacuo to dryness, the residue taken up in 1 or 2 ml 1 N ammonia and extracted with an equal volume of chloroform to remove excess reagent and any neutral hydrazones present. An aliquot of this solution, containing 10–50 μg of the keto acid hydrazones was chromatographed descending with the solvent n-butanol, ethanol, water, 40–10–50 (v/v). The phenylhydrazones of the keto acids give yellow spots, brown-purple in ultra violet light. The reagent does not fluoresce. Spraying with alkali resulted in a better differentiation of the spots (TOWERS c.s., 1954).

CAVALINI and FRONTALI (1954) were able to determine keto acids quantitatively by eluting the spots and colorimetric determination.

As keto acids often form two isomers, preventing the analysis of complex mixtures by this method, TOWERS c.s. (1954) and ALFTHAN c.s. (1955) reduced the 2-4-dinitrophenylhydrazones into the corresponding amino acids. These can be identified easier quantitatively.

We prepared and isolated the hydrazones according to TOWERS, but for the reduction and purification the method of ALFTHAN was used. 20 g of soil, 120 ml ethanol, 50 mg 2–4–dinitrophenylhydrazine and 0.25 ml concentrated sulfuric acid were mixed in a blendor and allowed to react for one hour. The mixture was centrifuged and decanted, and the precipitate washed several times with ethyl acetate. The ethanol extract was evaporated to dryness at room temperature. The residue combined with the ethyl acetate extract, filtered, washed with a few ml of water, and extracted with a 1 % solution of sodium carbonate. The alkaline extract was acidified and extracted with ethyl acetate. The solution of the hydrazones was evaporated to dryness at room temperature, the residue taken up in absolute ethanol, and made to 50 ml.

25 ml of this solution was cooled at 0–5° C, 1.5 g tin powder added, after which dry hydrochloric acid gas was led through, under continously cooling, until all tin was dissolved. The reaction mixture was diluted with water, the Sn-ions were removed with H_2S and filtered off. The filtrate contained the amino acids formed out of the keto acids. After evaporating to dryness the procedures for desalting and two dimensional paper chromatography (page 220, 221) were carried out.

In order to establish the absence of amino acids in the original soil

extract several control measurements had to be done, omitting the reduction, or the addition of the reagent, or both.

QUANTITATIVE DETERMINATION OF KETO ACIDS. No method is available to isolate and to determine quantitatively all keto acids present in a complex mixture. Even the method in which the keto acids are reduced to the corresponding amino acids did not give reliable results. According to TOWERS c.s. (1954), considerable losses, up to 40 %, may arise.

In this investigation the total amount of keto acids present was determined as described by WOLF (1955) for plant material. Deproteinization could be omitted. To 3 ml of water extract (page 221) was added 1 ml of a 0.1 % solution of 2-4-dinitrophenylhydrazine in 2 N HCl, at a temperature of 25° C. After 25 minutes, the hydrazones were extracted with 8 ml ethyl acetate during 2 minutes mixing with an air current. The water layer was sucked off using a glass capillairy. The ethyl acetate solution was extracted with 6 ml 10 % Na_2CO_3-solution during two minutes mixing with an air current. It is essential to observe accurately the periods given. 5 ml of the water layer was mixed in a colorimeter tube with 5 ml 1 N NaOH, and colorimetred after exactly 5 minutes at a wave length of 430 mμ.

With this method 90–95 % of the amounts of keto acids added could be recovered. As it appeared that in a given set of experiments the deviation was practically constant, a control series with known amounts of a keto acid added was always measured. With these precautions correct results were obtained.

THE ACCURACY OF THE DETERMINATIONS. The accuracy with which the compounds in the soil could be estimated was given for each analytical method. All operations were performed threefold. The variability between the measurements depends largely on the macrohomogeneity of the soil sample. Technically we were able to obtain samples differing not more than a few percent. The only exception was the determination of the total nitrogen present, which may be attributed to the presence of inhomogeneities rich in nitrogen, such as plant material passing the sieves.

CHAPTER 4

CONVERSIONS OF AMINO ACIDS IN SOIL

It has been demonstrated in a number of studies that the soil is subjected to continuous alteration. Thus while investigating the conversions of substrates added to soil it is essential to take into account the reactions normally taking place.

4.1. THE SOIL INVESTIGATED UNDER THE EXPERIMENTAL CONDITIONS

Changes in the soil structure and in the ground water level, as affected by the pretreatment, alter the rate of the biochemical processes

such as nitrification, denitrification and ammonification. It appeared in the course of this investigation that under storage conditions the pH of the soil dropped and nitrate was accumulated. However the pH changes observed were small. It may be that they are the result of the higher moisture content. In situ this value was much lower than under storage conditions. BARTHOLOMEV c.s. (1951) indicates that nitrification increases with increasing moisture content, provided aeration is not the limiting factor.

It appeared that the variations depend on the season in which the soil samples were taken.

Changes in the nitrate content were also observed once more when the soil was kept under aerobic conditions at constant temperature and humidity. The soil respiration decreased gradually on storage. Previous to this decrease a temporary increase in the CO_2-production must have occurred, caused by the disturbance of the soil structure. In general the ratio $-\Delta O_2/\Delta CO_2$ varies between 0.84 and 1.35.

TABLE 2. GAS EXCHANGE IN COVERSAND SOIL WITH DIFFERENT MOISTURE CONTENTS

Soil sample from VENRAY. O_2-uptake and CO_2-production expressed in μMoles/day/20 g of soil (= 14.6 g of airdried soil). Incubation period 15 days. Temperature 23° C.

Moisture content, %	O_2-uptake, μMoles/day	CO_2-production, μMoles/day
27	5.5	5.4
72	5.6	5.3

Considerable changes in the moisture content of the soil under investigation scarcely influenced the rate of the gas exchange in a 15 day experiment. O_2-uptake and CO_2-production did not alter when the moisture content of the well aerated soil increased from 27 % to 72 %, and a layer of water was situated on the surface (Table 2).

This result simplified the experimental procedure, although this has to be verified with the other conversions studied.

4.2. MINERALISATION OF ALANINE IN SOIL

Amino acids added to the soil are mineralised in 2–9 days. A deamination takes place by which the ammonia splits off; the carbon moiety is disintegrated.

In Fig. 1 the course of the mineralisation of alanine in the soil is given. In all experiments the measurements were performed simultaneously on soil both with and without added substrates. The differences between these measurements were considered to be the result of conversion of substrate. In Fig. 1 and in all following experiments only these differences are given.

Within 3 days the alanine added had disappeared totally, the quantity of mineralised nitrogen remaining constant thereafter. The O_2-uptake increases throughout the whole experiment. This must be

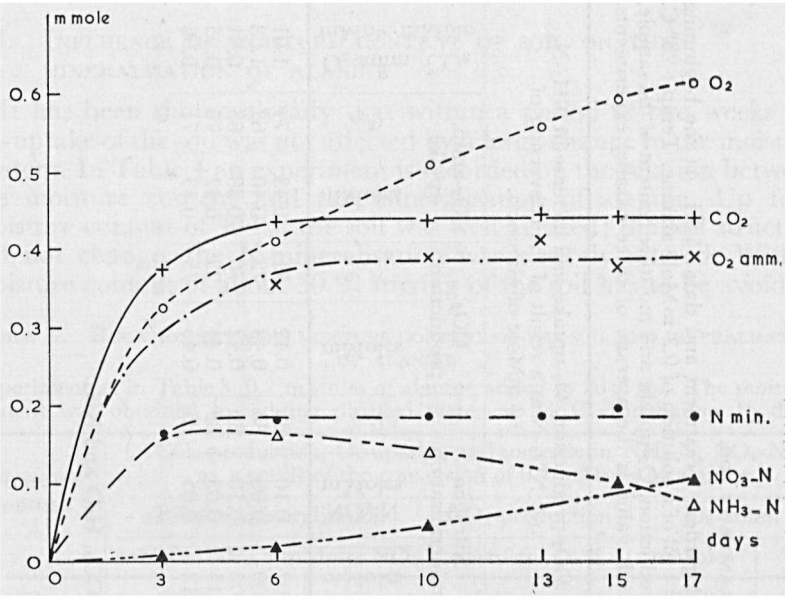

Fig. 1. Mineralisation of alanine in soil. 0.2 mMoles of alanine added to 20 g of coversand soil–Moisture content: 17 %– In this summation curve, the differences are given for soil without addition of alanine—The oxygen uptake is given with and without subtraction of the oxygen needed for the conversion of ammonia into nitrate. Each plot represents the mean of three determinations.

due to the conversion of ammonia into nitrate since the values of O_2-amm. (the O_2 consumed by the ammonification process) appeared to be constant. This value was obtained by diminishing the measured O_2-uptake by the O_2 equivalent of the conversion of NH_3 into NO_3^- calculated from the increase in NO_3-N.

From this experiment it appears that the mineralisation of alanine quickly comes to an end. At that time about 73 % of the carbon and 89 % of the nitrogen was mineralised, total oxidation being postulated. Part of the amino acid-C and N was immobilised, either by accumulation in microorganisms or by formation of "humic" substances.

The ratio between the oxygen used for the conversion of alanine and the carbon dioxide formed amounted to 0.91. By total oxidation of the alanine this value would have been 1.00. Thus the alanine-carbon not immobilised is oxidised for the greater part. In studying the formation of humus, our attention was directed mainly to the C and N that was not mineralised, since only these compounds might act as humus precursors.

The question arises whether the capacity of the soil to convert amino acids in the way described is equal for samples taken at any given time from the stock outside. In Table 3 some observations concerning this are summarised. In general the mineralisation yields are comparable wiht those reported in Fig. 1.

TABLE 3. MINERALISATION OF ALANINE IN SOIL

Soiltype: coversand from VENRAY.— 18–8–1956 brought in experimental garden. Treatment and data: § 3.1. At the beginning of the experiment to each soil sample of 20 g was added either 1 ml of water or 1 ml of a solution of 0.2 mMole alanine. Temp.: 23° C. At the end of the incubation all free amino acids were consumed. The CO_2-production, O_2-uptake and moisture content of the sample, and in the NaCl-extract nitrate-N and ammonium-N were determined. Each value is the average of three parallel experiments.

	storage time of soil before beginning of experiment (days)	month in which experiment started	period of incubation (days)	moisture content of soil (%)	Analysis of 20 g of soil after incubation in the absence of alanine					CO_2-production, O_2-uptake and increase in NH_3-N and NO_3-N as a result of the conversion of 0.2 mMole of alanine[4]).							
										Nitrogen mineralisation			CO_2 production		O_2-amm. uptake		
					pH, end exp.	NO_3-N μMoles	NH_3-N μMoles	CO_2 μMoles/day	O_2 μMoles/day	NH_3-N mMoles	NO_3-N mMoles	%[1])	mMoles	%[2])	mMoles	%[3])	O_2-amm./CO_2 mMole/mMole
A	0	11	20	14	5.0	18	0	8.9	12	0.07	0.10	85	0.42	71	0.43	71	1.01
B	21	1	12	23	5.1	—	—	—	—	0.09	0.08	85	0.43	72	0.40	67	1.08
C	41	2	19	22	5.1	36	0	7.8	7.0	0.08	0.10	89	0.42	70	0.38	63	0.91
D	64	3	8	23	5.1	32	0	8.2	6.9	0.15	0.03	89	0.44	73	0.42	70	0.95
E	27	4	10	21	4.9	36	0	10.7	10.5	0.12	0.05	85	0.45	75	0.43	71	0.96

[1]) Nitrogen mineralised in % of added alanine-N.
[2]) Carbon mineralised in % of added alanine-C.
[3]) O_2-amm. in % of the oxygen necessary for conversion of alanine into CO_2, H_2O and NH_3.
[4]) Corrected for the control series in absence of alanine.

4.3. Influence of moisture content of soil on the mineralisation of alanine

It has been shown already that within a period of two weeks the O_2-uptake of the soil was not affected by a large change in the moisture content. In Table 4 an experiment is recorded on the relation between the moisture content and the mineralisation of alanine. Up to a moisture content of 30 %, the soil was well aerated; the soil structure did not change, the N-mineralisation was scarcely altered. With a moisture content of about 30 % stirring of the soil has to be avoided,

Table 4. Relation between moisture content of the soil and mineralisation of alanine

Experiment as in Table 3. 0.2 mMoles of alanine added to 20 g. soil. The moisture content was obtained by adding distilled water at 23° C. Incubation 15 days.

Moisture content, %	CO_2-production, O_2-uptake, and increase in NH_3-N, NO_3-N as a result of the conversion of 0.2 mMoles of alanine.					
	nitrogen mineralisation		CO_2-production		O_2-amm.	
	mMoles NH_3	mMoles NO_3	mMoles	%	mMoles	%
22	0.095	0.075	0.43	72	0.40	66
27	0.076	0.086	0.45	75	0.45	75
42	0.104	0.029	0.45	75	0.42	70
72	0.111	0.036	0.44	74	0.41	70

as waterlogging may easily occur then. Above 30 % the structure could not be maintained and the aeration sharply decreased. The N-mineralisation yield increased somewhat, which agreed with the observations of Greenwood and Lees (1960) about the conversions of amino acids in soil under anaerobic conditions. Moreover, a sharp decline in the amount of nitrate formed was observed, the NH_3-content rose, doubtless by the decrease of the NH_3-oxidation rate, and by the decreasing immobilisation. The phenomenon of optimal nitrification by a moisture content approximating the field capacity of the soil has been observed by several investigators. As was expected, the total O_2-consumption decreased when the aeration was disturbed; the value O_2-amm. appeared to be constant.

These results facilitated the technical procedure, since slight changes in the moisture content of the soil gave rise to only small deviations of the total mineralisation. We assumed that a similar relation holds too for other amino acids.

When the soil is subjected to a change in moisture content lasting a relatively long period, the microbial population will change markedly, both quantitatively and qualitatively.

4.4. Influence of the storage period of soil on amino acid mineralisation

As previously mentioned, at the beginning of an experiment the activity of the soil gradually changed under the influence of new

conditions of constant temperature (23° C) and constant moisture content. Therefore the soil was kept for some time previously under conditions similar to those of the experiment. During winter, this acclimatisation had to be extended to several months. Thus we were able to reduce considerably the variability in results.

Beforehand we had to establish that this pretreatment of the soil did not seriously influence the mineralisation process of amino acids. The mineralisation of glutamic acid and of tyrosine were studied, both in concentrations of about equal amounts of carbon. Therefore the C-mineralisation, (but not the N-mineralisation) may be compared. At the end of the experiment the amino acids had totally disappeared and a state of dynamic equilibrium was obtained.

TABLE 5. INFLUENCE OF THE STORAGE OF SOIL ON THE MINERALISATION OF GLUTAMIC ACID AND TYROSINE

Experiment as described in Table 3. Storage of the soil during various periods at constant temperature (23° C) and moisture content (14 %). After these pretreatments amino acids were added. Incubation period: 18 days. Soil samples: 20 g.

Storage period, days	pH at end of experiment	CO_2-production, O_2-uptake and increase in NH_3-N, NO_3-N as a result of the amino acid conversion.						
		Nitrogen mineralisation			CO_2-production		O_2-amm. uptake	
		NH_3-N mMoles	NO_3-N mMoles	%[1]	mMoles	%[2]	mMoles	%[3]
0.12 mMoles of GLUTAMIC ACID added								
0	4.8	0.0018	0.086	73	0.41	69	0.35	64
25	4.8	0.0086	0.067	63	0.39	65	0.40	74
46	5.0	0.0352	0.058	78	0.41	68	0.33	60
0.06 mMoles of TYROSINE added								
0	5.1	0.0058	0.037	65	0.39	66	0.45	71
25	5.0	0.0	0.041	62	0.38	63	0.44	71
46	5.1	0.0049	0.039	67	0.38	63	0.44	71

1) N mineralised in % of added amino acid-N.
2) C mineralised in % of added amino acid-C.
3) O_2-amm. in % of the oxygen necessary for conversion of the amino acid in to CO_2, H_2O and NH_3.

The results are given in Table 5. It was found that for both amino acids the O_2-consumption and CO_2-production were within narrow variation limits independent of the period of the pretreatment of the soil up to 46 days. For the N-mineralisation also no large variations were observed, for tyrosine the values were very nearly constant.

About 71 % of the amino-N and 67 % of the C-atoms of the glutamic acid added were mineralised, the amount of O_2 taken up sufficed to oxidise 67 % of this compound. For tyrosine these results were 65, 64 and 71 % respectively. It is apparent that the degradation products of tyrosine remaining in the soil are partly oxidised.

The only difference brought about by the pretreatment of the soil that could be demonstrated was the decreasing rate of the nitrification processes with glutamic acid. After a pretreatment of three weeks practically all of the 0.12 mMole NH_3 produced was oxidised in 18 days, however after a six weeks pretreatment this was only 60 %.

It has been established in a variety of cases that the activity of the nitrifying microorganisms in soil samples decreases. Adding of ammonia to the sample stimulated the relevant microbial population which counteracts the fluctuations in nitrifying activity. The nitrate formed by this procedure was removed by percolating with water, after which the amino acid conversions could be carried out. In the last few years a similar stimulation of the organisms converting amino acids in soil has also been applied, but we did not follow this procedure as it would have created too unnatural a condition for this investigation and, in any event, the conversion of ammonia into nitrate was rather unimportant for the problem investigated.

4.5. The influence of concentration of amino acids on their mineralisation

Under natural conditions the amino acids are gradually released by hydrolysis of protein. For this reason we had to examine whether the mineralisation yield depends on amino acid concentration. Alanine, glutamic acid and tyrosine were added in various concentrations to soil samples. The results have been given in Table 6. During the incubation period the amino acids disappeared completely.

It will be noted that N-mineralisation increases the higher the amino acid concentration. This holds also for the percentage of N-mineralised. Degradation products of small amounts of amino acids are relatively more fixed than of larger quantities. Comparing equal molar concentrations it appears that the fixation is largest for tyrosine, least for alanine. As was expected the rate of the NO_3-production does not depend on the nature of the amino acid, but on the amount of NH_3 produced (Fig. 2).

The relative C-mineralisation, unlike the N-mineralisation, proceeds independently of the concentration of amino acids.

If an amino acid is immobilised in the soil without changing its C/N-ratio the amount of N in this fraction will be proportional to its amount of C. Graphically this would result in a point on the straight line OA (Fig. 3), where the abscissa represents the ratio of the quantity of carbon-immobilised (in m.atoms) and the number of C-atoms per N-atom in the amino acid molecule, and the ordinate represents the amount of N-immobilised (in m.atoms). The straight line OA is independent of the nature of the amino acid added.

A point deviating from OA would indicate the formation of a product differing in its C/N-ratio from that of the amino acid added.

In Fig. 3 no significant deviations were observed with small amounts of alanine and glutamic acid. When larger amounts of these amino acids were administered, compounds enriched in carbon were produced.

TABLE 6. INFLUENCE OF AMINO ACID CONCENTRATION ON MINERALISATION. Experiment as described in Table 3. Soil sample: 20 g. Moisture content: 21 %. Incubation period 9 days. In exp. A the soil sample was stored for 27 days; in exp. B: 47 days, and in exp. C: 51 days.

Amino acid added, mMoles	pH at end of exp.	CO_2-production, O_2-uptake and increase in NH_3-N+NO_3-N as a result of the amino acid conversion.						
		NH_3-N+NO_3-N		CO_2-production		O_2-amm. uptake		O_2-amm./CO_2
		mMoles	%[1]	mMoles	%[2]	mMoles	%[3]	mMole/mMole
		A ALANINE						
0.05	4.9	0.032	64	0.10	70	0.094	63	0.94
0.1	4.9	0.077	77	0.20	68	0.20	67	1.00
0.2	5.2	0.170	85	0.45	75	0.43	72	0.96
		B GLUTAMIC ACID						
0.05	4.9	0.033	66	0.17	69	0.13	58	0.75
0.1	5.0	0.073	73	0.27	72	0.28	62	0.77
0.2	5.2	0.164	82	0.36	75	0.67	74	0.89
		C TYROSINE						
0.025	4.8	0.010	40	1.16	70	0.088	37	0.47
0.05	4.8	0.024	48	0.29	64	0.28	59	0.96
0.1	4.8	0.064	64	0.60	67	0.66	69	1.10

[1]) N mineralised in % of added amino acid-N.
[2]) C mineralised in % of added amino acid-C.
[3]) O_2-amm. in % of the oxygen necessary for conversion of the amino acid into CO_2, H_2O and NH_3.

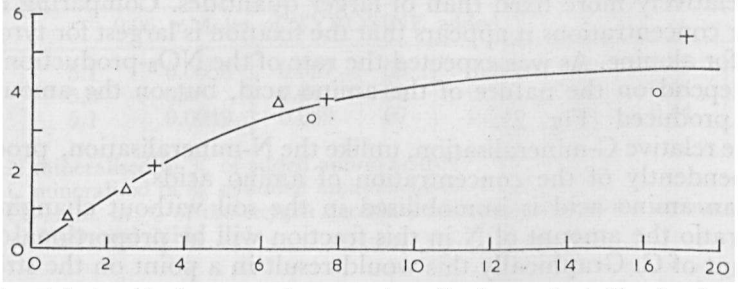

Fig. 2. Relationship between nitrogen mineralisation and nitrification in twenty days. Data in Table 6— Abscissa: NH_3–N + NO_3–N in milliatoms after an incubation period of twenty days— Ordinate: NO_3–N in milliatoms after the same period — + = alanine; o = glutamic acid; Δ = tyrosine.

The experiments with tyrosine resulted in a graph situated above the line OA, indicating a relatively high N content of the end-products formed.

The amino acids, particularly tyrosine, were not immobilised unaltered, as appeared from amino acid estimations of soil hydrolysates.

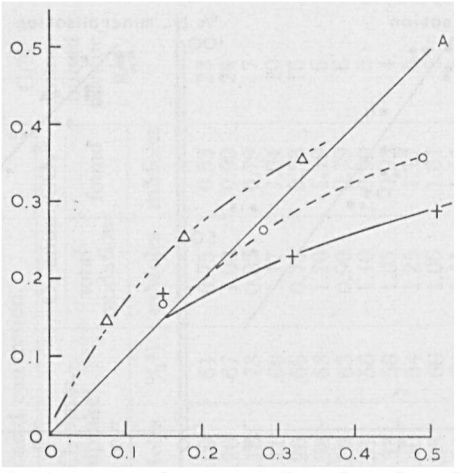

Fig. 3. Relationship between nitrogen and carbon immobilisation. Data in table 6—

Abscissa: $\frac{\text{milliatoms C-immobilised}}{\text{number of C-atoms added per N-atom}}$

—Ordinate: milliatoms N-immobilised— + = alanine; o = glutamic acid; Δ = tyrosine.

Shortly after the tyrosine had been added to the soil it could not be detected in the hydrolysates.

4.6. Comparative investigation on the mineralisation yields of amino acids

As was shown in Fig. 1, mineralisation of amino acids stopped in a few days, the percentages of carbon and nitrogen mineralised remained constant. These values were not affected by changing the storage and incubation periods of the soil, nor by altering its moisture content. Modifying the amounts amino acids added did not affect the percentage of the carbon mineralised.

However the mineralisation of nitrogen depended on the amounts of amino acid added. In order to eliminate this disturbing interference we had to add in each experiment equal amounts of amino acid-N. In this way we were able to compare the maximum mineralisation of various amino acids in each experiment. The results obtained might be disturbed if differences arose in the permeability of the cellwalls or in the biological activity of the relevant microorganisms for the various amino acids investigated. This will happen only in experiments too short to establish a dynamic equilibrium. For this reason we continued the experiments until this state of equilibrium had been reached.

The apparatus enabled us to determine simultaneously in triplicate the mineralisation of six amino acids; in the experiment to be described here twelve amino acids were investigated in two groups of six each. All environmental conditions remaining constant, except that the storage period differed two weeks, but this did not affect the results.

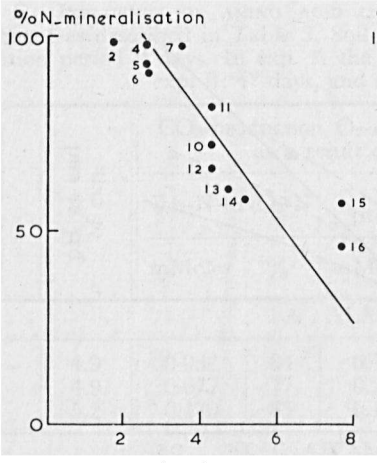

Fig. 4a

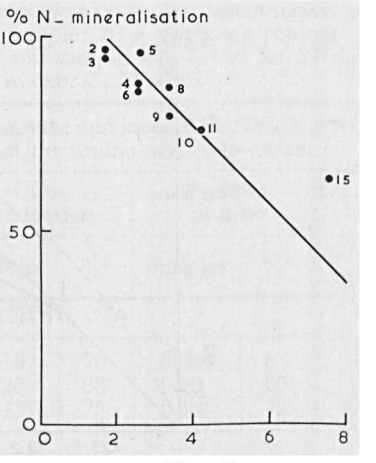

Fig. 4b

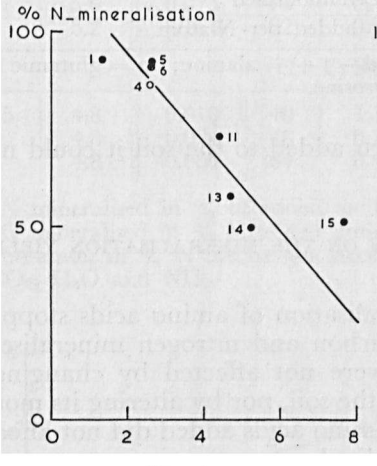

Fig. 4c

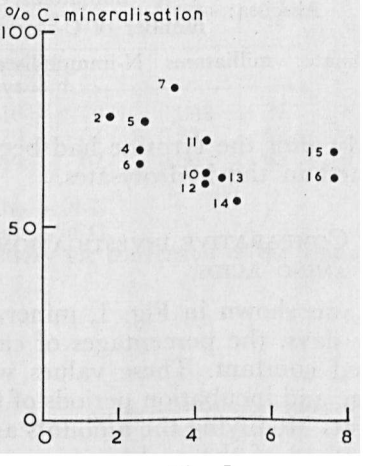

Fig. 5

Fig. 4. Relation between relative nitrogen mineralisation and the C/N-ratio of the added amino acid. Experiment A: data in Table 7 — Experiments A, B and C: Abscissa: C/N-ratio of the amino acid in g/g-

$$\text{Ordinate: } \frac{NH_3\text{-N} + NO_3\text{-N.}}{\text{animo acid-N.}} \cdot 100\ \%$$

1: arginine; 2: glycine: 3: histidine; 4: α-alanine; 5: serine; 6: lysine; 7: aspartic acid; 8: γ- aminobutyric acid; 9: threonine; 10: valine; 11: glutamic acid; 12: proline; 13: tryptophan; 14: isoleucine; 15: tyrosine; 16: phenylalanine.

Fig. 5. Relationship between relative carbon mineralisation and the C/N-ratio of the added amino acid. Data in Table 7 — Abscissa: C/N-ratio in g/g of the amino acid — Ordinate: CO_2-C · 100 %/amino acid-C.

TABLE 7. MINERALISATION OF SEVERAL AMINO ACIDS

Experiments as described in Table 3. Amino acid-N in all experiments: 0.1 milli-atoms. Moisture content: 18 %. Soil samples: 20 g. Incubation period: 10 days.

Amino acid added, mMoles		C/N-ratio, g/g	Storage period of soil before incubation, days	pH at end of exp.	CO_2-production, O_2-uptake and increase in NH_3-N+NO_3-N as a result of the amino acid conversion.								C/N immobilised residue, g/g
					NH_3-N+NO_3-N		CO_2-production		O_2-amm.-uptake		O_2-amm./CO_2		
											total oxidation	found	
					mMoles	%[1]	mMoles	%[2]	mMoles	%[3]	mMoles	mMoles	
glycine	0.1	1.71	12	5.5	0.098	98	0.15	74	0.09	61	0.75	0.53	23
alanine	0.1	2.57	12	5.1	0.097	97	0.21	70	0.20	67	1.00	0.90	24
serine	0.1	2.57	24	5.2	0.092	92	0.23	77	0.18	73	0.83	0.79	7
lysine	0.05	2.57	24	4.9	0.091	91	0.20	67	0.21	60	1.17	1.04	10
aspartic	0.1	3.42	12	5.1	0.097	97	0.35	86	0.20	66	0.75	0.58	16
valine	0.1	4.29	12	5.2	0.072	72	0.32	63	0.38	63	1.20	1.19	6
glutamic	0.1	4.29	12	5.0	0.081	81	0.36	72	0.28	63	0.90	0.79	6
proline	0.1	4.29	24	5.1	0.065	65	0.31	62	0.31	56	1.10	0.99	5
tryptoph.	0.05	4.71	24	5.0	0.060	60	0.35	64	0.33	58	1.05	0.95	4
ileuc.	0.1	5.14	12	5.3	0.058	58	0.34	57	0.40	54	1.25	1.17	5
tyrosine	0.1	7.71	24	5.1	0.056	56	0.62	69	0.63	66	1.06	1.01	5
phen.ala.	0.1	7.71	24	5.1	0.045	45	0.56	62	0.64	64	1.11	1.14	5

[1]) N mineralised in % of added amino acid-N.
[2]) C mineralised in % of added amino acid-C.
[3]) O_2-amm. in % of the oxygen necessary for conversion of the amino acid into CO_2, H_2O and NH_3.

The C/N-ratios of the amino acids tested ranged from 1.7 to 7.7.

In Fig. 4 (exp. A) and Table 7 the results of this experiment are summarised. Fig. 4 (exp. B and C) shows the results from further experiments. In Fig. 4 and 5 the ultimate values of the mineralisation of the nitrogen and of the carbon are plotted against the C/N ratios of the amino acids added. It appears that the relative nitrogen mineralisation decreases with increasing C/N-ratio. OWEN, WINSOR and LONG (1950) have already described this phenomenon.

The impression was gained that the results obtained with practically all amino acids could be fitted by a straight line. In all experiments performed tyrosine was an exception, being situated above this line.

When this straight line is extrapolated to the C/N-ratio zero it intersects the ordinate at a mineralisation value above 100 % in our experiments, whereas in the experiments of OWEN, WINSOR and LONG (1950) and of GREENWOOD and LEES (1956) this value is less than 100 %. We were not surprised. In our experiments, total nitrogen mineralisation has already been obtained with a C/N-ratio of 1.5–2.4. In this poor sandy soil only a minute quantity of carbon compounds is available for enzymatic disintegration. For a significant nitrogen fixation, carbon compounds have to be added. The authors cited used fertile garden soil for their experiments. Under these conditions relatively large amounts of carbon are available, which immobilise the ammonia formed.

In practically all the experiments the respiratory quotients obtained agreed fairly well with the expected values if the amino acids were completely oxidised (Table 7).

The C/N-ratio of the residue is the result of a complex system of reactions. The amino acid may be used to synthesize bacterial material

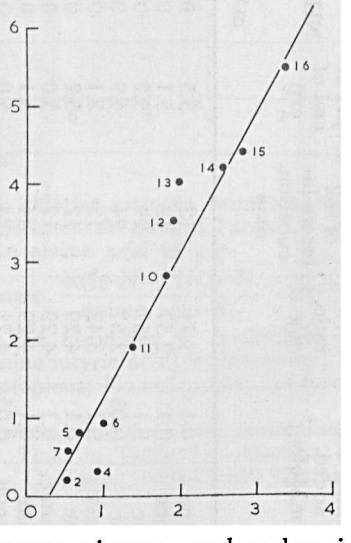

Fig. 6. Relationship between nitrogen- and carbon immobilisation. Data in Table 7 — Abscissa: mMoles C-immobilised; — Ordinate: mMolesN-immobilised.

or to produce resistant humus compounds. The values of the C/N-ratios calculated for the non-mineralised residues are, apart from those for glycine, alanine, lysine and aspartic acid remarkably constant (Fig. 6). The high values found for the amino acids just mentioned have less significance; because of the observed extremely low nitrogen immobilisation, the experimental error has a disproportionate influence.

CHAPTER 5

CONVERSIONS OF KETO ACIDS IN SOIL

5.1. Introductory remarks

The preceding chapter dealt with the decomposition of amino acids in soil. However the pathways of their decomposition were not discussed.

Research on microbiological conversions of amino acids has revealed several pathways of breakdown. For the most part, amino acids are converted by deamination or transamination into the relevant keto acids and ammonia, but other pathways have also been reported (Waksman, 1952). Lichstein (1951) has given a summary of the various pathways, of the intermediates that appear and of the enzyme systems taking an active part in the oxidative deamination.

We investigated whether keto acids are to be considered as intermediates during these microbiological reactions in the soil. This could be done by comparing the conversion rate of the keto acid and that of the relevant amino acid, or by identification of the keto acid as an intermediate in the breakdown of the amino acid.

5.2. The conversion rate of a keto acid and the relevant amino acid

The decomposition rates of alanine and of sodium-pyruvate, added in equimolar quantities to the soil, were determined by the methods described at pages 221 and 223.

Table 8 shows that pyruvate is broken down more rapidly than alanine. With α-ketoglutaric acid, phenylpyruvic acid and p-hydroxy-phenylpyruvic acid, corresponding results were obtained.

We also checked whether the conversion of sodiumpyruvate occurs chemically or microbiologically. For this purpose the test was repeated with sterilised soil samples, obtained by heating three times with intervals of 24 hours at 120° C. for 30 minutes, adding a sterile pyruvate solution, obtained by sterilising for 30 minutes at 120° C. The results have also been given in Table 8. The pyruvate was decomposed by the soil only very slowly, so that it is of no significance under natural conditions.

If keto acids were intermediates in amino acid breakdown one would expect that the carbon of a keto acid would be mineralised for a percentage corresponding to that of the relevant amino acid. This

TABLE 8. COMPARISON OF THE RATES OF BREAKDOWN OF ALANINE AND PYRUVIC ACID

Soil sample described in Table 3. Moisture content 17 %. Temperature 23° C.
Experiment A: 20 g of soil + 1 ml of water + 0.1 mMoles of alanine.
Experiment B: 20 g of soil + 1 ml of water + 0.1 mMoles of sodiumpyruvate.
Experiment C: 20 g of sterile soil (heated three times, at intervals of 24 hours, at 120° C for 30 minutes) + 1 ml of water (sterile) + 0.01 mMoles of sodium pyruvate (sterile). Control experiment: 20 g of soil + 1 ml of water. Each experiment was performed in triplicate.

Period of incubation, days	A alanine, mMoles	B Na-pyruvate, mMoles	C Na-pyruvate, mMoles
0	0.1	0.1	0.1
1	0.070	0	0.097
2	0.035	0	—
3	0	0	—
4	0	0	0.055
5	0	0	0.045
11	0	0	0.045

TABLE 9. COMPARISON OF THE BREAKDOWN OF AMINO ACIDS AND THE CORRESPONDING KETO ACIDS

Experiments as described in Table 3. Soil samples: 20 g. Moisture content: 22 %. Incubation period: 9 days.

	Substrate	mMoles	pH at end of exp.	NH_3-N + NO_3-N		CO_2-production		O_2-amm.-uptake	
				mMoles	%[1]	mMoles	%[2]	mMoles	%[3]
A	alanine	0.1	5.2	0.076	76	0.26	66	0.22	72
	Na-pyruvate+NH_4[4]	0.1	5.3	0.082	82	0.23	59	0.15	60
B	alanine	0.1	5.2	0.068	68	0.25	65	0.19	63
	Na-pyruvate+NH_4[4]	0.1	5.2	0.066	66	0.21	54	0.13	50
C	glutamic acid	0.1	4.6	0.071	71	0.32	64	0.26	58
	α-ketoglut.acid	0.1	4.4	—	—	0.30	60	0.18	45
	α-ketogl.+NH_4[4]	0.1	4.4	0.106	106	0.28	56	0.24	60
D	glutamic acid	0.1	4.6	0.071	71	0.32	64	0.26	58
	α-ketogl.+NH_4[4]	0.1	4.4	0.101	101	0.28	56	0.23	58
E	glutamic acid	0.1	5.1	0.070	70	0.34	68	0.30	67
	α-ketogl.+NH_4[4]	0.1	4.9	0.081	81	0.31	62	0.21	53
F	glutamic acid	0.1	4.9	0.069	69	0.36	72	0.26	58
	α-ketogl.+NH_4[4]	0.1	4.8	0.076	76	0.31	62	0.22	55
G	tyrosine	0.067	5.4	0.049	73	0.33	55	0.36	57
	pOHphen.pyruv.	0.067	5.5	—	—	0.06	10	0.06	10
	pOHph.pyr.+NH_4[4]	0.067	5.1	0.071	106	0.07	11	0.10	17
H	tyrosine	0.1	4.9	0.044	44	0.36	71	0.60	67
	pOHph.pyr.+NH_4[4]	0.1	4.9	0.099	99	0.18	21	0.15	17

[1] N mineralised in % of added substrate-N.
[2] C mineralised in % of added substrate-C.
[3] O_2-amm. in % of the oxygen necessary for conversion of the substrate into CO_2, H_2O and NH_3 (if present).
[4] keto acid + equivalent amount of NH_4Cl.

has been tested for alanine and pyruvate, glutamic acid and α-ketoglutaric acid and for tyrosine p-hydroxyphenylpyruvic acid (Table 9).

The result obtained was unexpected. Always with a keto acid the increase in CO_2-production was less than the increase with the corresponding amino acid. The differences were small for alanine—pyruvate and for glutamic acid-α-ketoglutarate, but very large for tyrosine-p-hydroxyphenylpyruvic acid.

This result can not be ascribed to a specific toxic action of the keto acids on the soil micro flora in the concentrations used. Otherwise one could not understand their rapid breakdown.

We could assume however that, by the rapid conversion of the keto acids, an intermediate is accumulated having a toxic action on the microorganisms in the soil. The breakdown of the corresponding amino acid would lead to the formation of the same compounds. But as this breakdown proceeds more slowly this substance would be present in a lower concentration. The toxic action would be less and

TABLE 10. RELATIONSHIP BETWEEN KETO ACID CONCENTRATION AND THE MINERALISATION PROCESS

Experiments as described in Table 3. Soil samples: 20 g. Moisture content: 21 %. Incubation period: 9 days. Data further to Table 6. These experiments were performed simultaneously with those in Table 6.

Substrate,[4] mMoles	pH at end of experiment	CO_2-production, O_2-uptake and increase in NH_3-N + NO_3-N as a result of the conversion of keto acid.					
		NH_3-N+NO_3-N		CO_2-production		O_2-amm.uptake	
		mMoles	%[1]	mMoles	%[2]	mMoles	%[3]
PYRUVIC ACID							
0.05	4.9	0.044	88	0.095	64	0.049	38
0.1	5.0	0.088	88	0.177	59	0.078	31
0.2	5.3	0.176	88	0.375	63	0.24	47
α-KETO GLUTARIC ACID							
0.05	4.8	0.035	70	0.14	57	0.069	34
0.1	4.8	0.075	75	0.30	60	0.18	45
0.2	4.7	0.174	87	0.58	58	0.42	53
P-HYDROXYPHENYLPYRUVIC ACID							
0.025	4.8	0.0193	77	0.08	35	0.056	25
0.05	4.7	0.048	96	0.107	24	0.078	17
0.1	4.6	0.103	103	0.14	16	0.15	16

[1] N 'mineralised' in % of added NH_4Cl.
[2] C mineralised in % of added keto acid-C.
[3] O_2-amm. in % of the oxygen necessary for conversion of the keto acid into CO_2 and H_2O.
[4] keto acid + equivalent amount of NH_4Cl.

one had to assume that the microorganisms would be able eventually to convert this compound into a non-toxic end-product.

This assumption was tested by investigating the effect of various concentrations of keto acids (Table 10). It appeared that within the range of the concentrations investigated neither pyruvic acid, nor α-ketoglutaric acid differ in their relative carbon mineralisation.

p-Hydroxypyruvic acid gave a different picture. Increasing concentrations of this keto acid inhibits the relative carbon mineralisation. This effect may be due to a toxic action of intermediate formed by the breakdown of this keto acid. The toxicity decreases by diminishing the amount of substrate added.

As the carbon immobilisation depended on the quantity of available nitrogen we have added an equivalent amount of ammonium-ions, in the form of NH_4Cl (Tables 9 and 10). It appears from Table 9 that this altered the carbon mineralisation very little. In several experiments some of the ammonium ions added were immobilised.

5.3. Keto acids in soil

From the preceding paragraph it may be concluded with fair certainty that keto acids act as intermediates during the amino acid breakdown in soil. The deviations found in the metabolism may be due to concentration effects, as has been shown for p-hydroxyphenylpyruvic acid. We then attempted to detect keto acids during the metabolic process.

5.3.1. The occurrence of free keto acids in soil. We had to investigate whether free keto acids are present in the untreated soil samples. By paper-chromatographic analyses of their extracts, treated with 2-4-dinitrophenylhydrazine (page 222) a spot with a high rF-value was observed, indicating the presence of a free keto acid.

As the reaction was scarcely significant we repeated the analysis with a soil extract concentrated tenfold. The spot was observed again and showed very clearly the brown-purple fluorescence characteristic for hydrazones of keto acids. The spot area is not influenced by the concentration of the reagent, which excludes the possibility that the keto acid was artificially formed by decomposition of the reagent by the soil.

However the assumption that the hydrazone of the unknown substance was derived from an acid carbonyl compound had to be proved. This was done by reducing the hydrazone. To this end a large amount of the hydrazone compound was prepared from the soil sample by the procedure described by Towers c.s. (1954). The extract obtained was concentrated tenfold and reduced according to Alfthan c.s. (1955) (page 222). The product obtained was afterwards isolated by paper-chromatography.

On the chromatogram a spot was visible, lacking on the control-chromatogram from an untreated soil sample. The spot gave all the characteristic reactions of an amino acid. The relative place and shape

of the spot were characteristic and reproducible (Fig. 7) but did not correspond to the amino acids mentioned on page 221. We did not isolate the compound in a pure state, so further analysis had to be omitted.

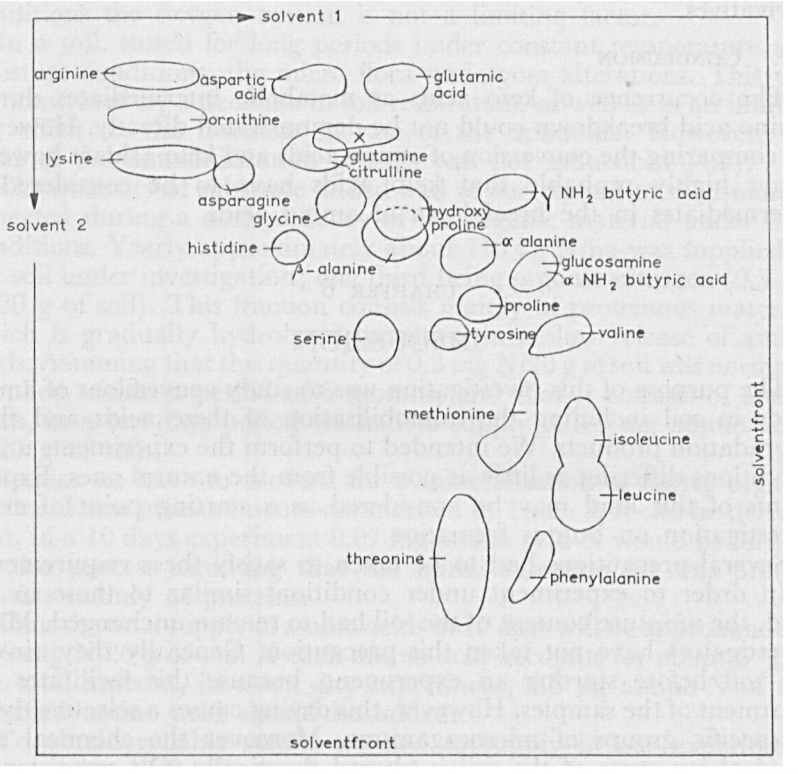

Fig. 7. Paperchromatogram of 23 known amino acids and the amino acid "X", prepared from the keto acid present in the untreated soil. Solvent I: methanol — water — pyridine 80–20–4 (V/V) Solvent II: butanone-2 — tertiairy butanol — water — diethylamine 40–40–20–4 (V/V).

5.3.2. THE OCCURRENCE OF KETO ACIDS DURING THE DECOMPOSITION OF AMINO ACIDS IN SOIL. During the experiments in which the decomposition of amino acids by soil was investigated, the presence of keto acids in the soil extract was regularly determined by paper-chromatography (page 222) and by colorimetry (page 223). The following amino acids had been tested: glycine, alanine, serine, valine, isoleucine, aspartic acid, glutamic acid, lysine, proline, phenylalanine, tyrosine and tryptophan. The soil extracts were sampled during the period in which the amino acid had not been completely converted, since this is the period in which one may expect that keto acids will be present.

In none of the soil extracts a keto acid could be demonstrated colorimetrically. Thus less than 0.5 % of the amino acid could be present in the form of a keto acid.

By the paperchromatographical test incidently several spots were observed. However since several spots of keto acids appeared as the result of the decomposition of only one amino acid one has to assume that these keto acids are secondary metabolic products, and not direct derivatives.

5.4. Conclusion

The occurrence of keto acids as metabolic intermediates during amino acid breakdown could not be demonstrated directly. However, by comparing the conversion of amino acids and keto acids it however seems highly probable that keto acids have to be considered as intermediates in the breakdown of amino acids.

CHAPTER 6

DISCUSSION

The purpose of this investigation was to study conversions of amino acids in soil including the immobilisation of these acids and their degradation products. We intended to perform the experiments under conditions differing as little as possible from the natural ones. Experiments of this kind may be considered as a starting point of every investigation on humus formation.

Several precautions had to be taken to satisfy these requirements.

In order to experiment under conditions similar to those in the field, the moisture content of the soil had to remain unchanged. Many investigators have not taken this precaution. Generally they air-dry the soil before starting an experiment, because this facilitates the treatment of the samples. However, this drying causes a selective dying of specific groups of microorganisms. Moreover the chemical and physical structure of the soil is altered drastically. On remoistening the soil, large amounts of easily decomposable substances such as amino acids and proteins become available. The activity of several groups of microorganisms is enhanced, resulting in a rush of decomposition, demonstrated by an increase in CO_2-production many times larger than the natural fluctuations.

In addition the soil structure preferably should remain undisturbed. This being impossible, we tried to pretreat the soil in such a way that in all experiments its structure was nearly the same. This structure did not differ much from those under field conditions in this arable soil.

One could claim that ideal similarity between experimental and natural conditions would demand that in our experiments the temperature should fluctuate. But since it was expected that this would complicate the interpretation of the results, we started our investigations working at a strictly constant temperature.

The experimental conditions also differed from the natural ones by substituting for the gasphase of the soil with air. Moreover, the carbon dioxide tension was zero and in closed reaction vessels the oxygen content decreased considerably. However, control experiments

have shown that the mineralisation yield of amino acids was equally high in the presence of CO_2 and in its absence. In our experiments the oxygen content never dropped below a value of 10 %. As may be seen from the literature (BARTHOLOMEV and AMER, 1951), under these conditions the oxygen tension is not a limiting factor.

In a soil, stored for long periods under constant temperature and moisture conditions, the micro flora undergoes alterations. This was demonstrated by a decrease in soil respiration, as well as in an inhibiting effect on the nitrification rate of the ammonia. However, the yield of the ammonification process was not influenced (4.4).

The quantity of substrate added had to correspond to the quantity expected during a normal decay of the organic material under field conditions. Yearly approximately about 110 kg N/ha was supplied to the soil under investigation; one third being organic nitrogen (0.3 mg N/20 g of soil). This fraction consists mainly of proteinous material, which is gradually hydrolysed, resulting in a slow release of amino acids. Assuming that this quantity of 0.3 mg N/20 g of soil was normally supplied within a period of 6 months, and that it consists of amino acids, in a ten days period we had to supply 0.016 mg amino acid-N/20 g of soil.

Besides we have to account for a mineralisation of native organic matter. From measurements summarised in Table 3, it can be derived that, in a 10 days experiment 0.07 mg amino acid-N would be formed by this process assuming that the initial substrate for this process consists mainly of proteins.

Thus the total supply of amino acids in 10 days will be approximately 0.09 mg N/20 g of soil. If each amino acid accounts for about 5 % of the total amount, in a ten day experiment, 4.5 μg amino-N of this specified amino acid should be added.

To obtain reliable results we had to supply in our experiments 45 μg amino-N (0.003 mmol), roughly ten times the amount produced in the soil under natural conditions.

In section 4.5 we studied the influence of the concentration of an amino acid on its conversion. With low concentrations a relatively small amount of the amino acid-N was mineralised, whereas the relative carbon mineralisation was not affected. Therefore in our experiments the immobilisation yield of nitrogen was estimated too low; natural conditions were more favourable for humus formation.

There was also the possibility that secondary reactions might occur, disturb the results. At a high pH-value, volatilisation of ammonia can occur (JEWITT, 1942); with a low pH-value, nitrite and ammonia can react to yield nitrogen (ALLISON c.s., 1948, 1951, 1952, WYLER and DELWICHE, 1954); depending on aeration and supply of organic material, denitrification processes of different character may occur (HAUCK c.s., 1956, JANSSON c.s., 1952, BREMNER c.s., 1954, BROADBENT, 1951, PINCK c.s., 1950, WHEELER c.s., 1958, WYLER c.s., 1954, GREENWOOD c.s., 1960): and finally ammonia can be bound tightly in the lattice layers of clay minerals (ALLISON c.s., 1953, STEVENSON, 1957). These losses can be observed only under specified conditions.

Often the presence of relevant microorganisms is essential. In several experiments we have drawn up the mineralisation balance: 98–100 % of the nitrogen added was recovered.

One could claim that this total recovery could be caused by an enhanced nitrogen mineralisation of the soil organic matter, balancing one of the losses mentioned. This can be verified in tracer experiments. As may be seen from Table 3, the N-mineralisation of the native organic matter in this type of soil proceeds very slowly. Therefore it is appropriate to assume that interferences of this kind did not occur.

PUTNAM and SCHMIDT (1959) investigated the adsorption of amino acids by soil adsorption complexes. Arginine and lysine remained adsorbed after percolating with water, whereas tryptophan dissolved. GREENWOOD and LEES (1960) could demonstrate the adsorption of arginine, histidine, lysine and tryptophan. According to the authors mentioned the adsorbed amino acids can be attacked by microbes.

Amino acids may also disappear by incorporation in bacterial matter. This activity may be promoted when mixtures of amino acids are added in the relative amounts present in proteins. We have investigated this point by incubating soil samples with single amino acids. After 1–5 days both a watery extract and an acid hydrolysate were prepared. The amount of an amino acid was estimated semi-quantitatively after paperchromatography. It appeared that the amino acid content of the extract equalled that of the hydrolysate, demonstrating that no amino acid incorporation had taken place. At most 5 % of the amino acids added (alanine or tyrosine) could have escaped our attention through variance in the samples and in the analytical procedure.

In section 5.2. we concluded that keto acids are converted more rapidly than the corresponding amino acids. The presence of keto acids in the soil during amino acid breakdown could not be demonstrated (5.3.2.) (GREENWOOD and LEES, 1960).

A comparison of the relative mineralisation yields of amino acids of keto acids administered in equivalent amounts (Table 9) revealed that the keto acids had been oxidised to a slightly lesser extent than the amino acids. Although these differences were small, they were significant. It is difficult to reconcile the assumption that amino acids in soil are broken down via keto acids with these results. However, one has to consider that during amino acid breakdown, keto acid will be produced at a relatively small rate, accumulating in very small amounts, less than one percent. In the experiments with keto acids their total amount was administered at once, resulting in a hundred fold higher concentration.

For this reason we investigated the effect of the concentration of a keto acid on its relative mineralisation. For pyruvic acid and α-ketogluric acid no concentration effect could be demonstrated, their mineralisation yield appeared to be constant 62 resp. 58 %. For the corresponding amino acids these values were 71 resp. 72 %, demonstrating the dissimilarity mentioned above. Only with p-hydroxyphenylpyruvic acid the mineralisation yield decreased when the

concentration was enhanced. 16 % of this acid was mineralised when 0.1 mmol had been administered, whereas this increased to 35 % with 0.025 mmol. The relevant amino acid tyrosine was mineralised for about 67 % (Table 6). We are willing to believe that the concentration effect observed supports the assumption that the dissimilarity between the mineralisation yield of a keto acid and its corresponding amino acid may be due to the totally different amounts of keto acids present in these experiments. One has to consider that the keto acid concentrations present in the soil are less than 4 % of the amounts investigated.

Table 9 shows that ammonia added together with a keto acid enhances the carbon immobilisation somewhat.

When we consider the relative N-mineralisation yield of an amino acid in relation to its C/N-ratio it appears that the yield decreases with increasing C/N-ratio (Fig. 4a, b, c, Table 7). The dependence may be seen from Fig. 6, in which the nitrogen immobilisation is plotted against the carbon immobilisation. The C/N-ratio of the immobilised fraction is practically constant.

These experiments demonstrated, that the N-mineralisation of tyrosine was relatively larger than that of the other amino acids, including phenylalanine. For the C-mineralisation, no distinct relation seems to exist. (Fig. 5). However the C/N-ratio of the immobilised fraction was nearly equal to that of the various amino acids.

In this respect it is remarkable that a similar value of the C/N-ratio was found by WINSOR and POLLARD (1956) and by JENSEN (1932) who incubated in soil substrates as sucrose and ammonium sulfate, dried fungi, bacteria and actinomycetes. The C/N-ratio of the immobilised fraction did not alter within 90 days.

From these experiments the conclusion may be drawn that the amino acids are altered by their immobilisation. The results of the hydrolysis experiments described on page 230 and 242 demonstrated however that no direct incorporation of amino acids occurred in significant amounts. The assumption that a complete mineralisation of amino acids preceeds, followed by a direct synthesis of humic substances from their degradation products, has to be excluded.

The equality of the C/N-ratios of the immobilised fractions suggested that the same endproduct is formed independent of the amino acids administered. This can best be understood by assuming that this is caused by a synthesis of microbial matter, particularly of proteins. However, since the total amount of microbial matter in the soil is very small as compared with the total soil organic matter, it has to be rapidly synthesised and their proteins and amino acids incorporated quickly into humous products.

The finding of KORTLEVEN c.s. (1960), that under favourable conditions up to 25 % of the total supplied organic material is converted into stable organic matter supports this view. It is also in accordance with the view of SWABY (1959) that the essential stage in the immobilisation of an amino acid falls in a period shortly after the death of the cells of an organism. In this situation both quinones and amino acids

are released and are then able to react with each other. Indeed these complexes can be formed in vitro; they are fairly resistent against microbial attack. Moreover, on acid hydrolysis the amino acids are liberated, as happens on hydrolysis of humic substances. If this reaction could be shown to account for the combined amino acid fraction in soil, then the variations and differences in amino acid content of different soil samples would be easily understood.

The results obtained may be of some value for further investigations into humus formation. Special attention has to be drawn to the interaction of various compounds in soil. It seems appropriate to extend these investigations to mixtures of amino acids, both without and in the presence of phenolic substances.

The formation of complexes between amino acids and soil organic matter would involve the production of compounds with a C/N-ratio higher than that of the parent amino acids, whereas the production of protein-clay complexes will lead to complexes with unaltered C/N-ratios. Moreover, in the humic substances, polymers of non-nitrogen-containing compounds are present. The important role of lignin, polyuronides and of polyphenols is well known. However, the processes are more complicated, since external conditions exert a considerable influence on the accumulation of metabolic products essential for humus synthesis (ENDERS, 1943).

SUMMARY

1. In studying the origin of nitrogen in humus formation it might be valuable to consider the conversions of amino acids in soil.

2. A survey was made of the literature on the presence and on the conversions of soil amino compounds. Speculations on the character of the resistant soil amino acid fraction have been given.

3. The soil was incubated with amino acids and conversions of amino acids were studied respirometrically. Oxygen consumption and carbon dioxide evolution were determined, and the content of amino acids, keto acids, ammonia and nitrate in aqueous or 1 N sodium chloride extracts.

4. The amino acids disappeared rapidly, mineralisation being completed within a few days. The ratio of the mineralisation products remained fairly constant during the period of investigation. When aeration was sufficient, small differences in moisture content gave no rise to alterations in the mineralisation yields. Under similar conditions the nitrification was enhanced by increasing moisture content. Storing the soil sample in the laboratory under constant temperature and moisture content did not give rise to differences in the mineralisation yield, but to a marked depression in the rate of nitrification. The relative carbon mineralisation yield was independent of the concentrations of amino acid administered; the relative nitrogen mineralisation yield increased with enhancing concentration. Amino acids with a high C/N-ratio give rise to lower nitrogen mineralisation percentages than those with low values.

5. During the decomposition of amino acids in soil no significant amounts of keto acids could be detected. The keto acids were broken down more rapidly than the corresponding amino acids. Their carbon mineralisation percentages were slightly lower than with amino acids. This might be due to a concentration effect, as the keto acids in the experiments mentioned had a 100 times higher concentration. It is quite reasonable to assume that keto acids are intermediates in amino acid breakdown in soil.

6. The differences between the laboratory conditions and those in nature, and the effect on the results of the experiments are discussed. No losses of substrates or

of reaction products occur by denitrification, volatilisation of ammonia, nor by irreversible fixation of ammonia and of amino acids to soil colloids. The amino acids are deaminated oxydatively; the nonmineralised fraction has a remarkably constant C/N-ratio. This accounts for most of the amino acids investigated. The assumption of SWABY that the amino acids in organisms that had just died would combine with quinones also becoming available may explain most of the properties of the resistant soil amino acid fraction.

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