Immobilization and mineralization of nitrogen in pasture soil

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Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus, prof. dr. ir. H. A. Leniger,
hoogleraar in de technologie,
in het openbaar te verdedigen op vrijdag 3 november 1972 te 16.00 uur
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Stellingen

I

De hypothese van Novák dat humificatie alleen plaats kan vinden door een complexe microflora is achterhaald.

B. Novák, Zentbl.Bakt.ParasitKde, Abt.II, 125: 566-577 (1970)

II

Een belangrijke bijdrage tot het verkrijgen van inzicht in de humussynthese in grond wordt geleverd door het bestuderen van humusachtige verbindingen verkregen met reincultures van micro-organismen in voedingsmedia van bekende samenstelling.

III

Het is voorbarig te veronderstellen, zoals Greenfield doet, dat de in een 6 N HCl hydrolysaat van de grond opgeloste stikstofverbindingen die niet geïdentificeerd kunnen worden als NH_4^+, hexosaminen of α-amino-N, grotendeels voorkomen in de vorm van aminozuren.

L. G. Greenfield, Pl.Soil 36: 191-198 (1972)

IV

Bij de omzetting van grasland in bouwland daalt het gehalte aan organische stof van de grond. De resultaten vermeld in dit proefschrift doen vermoeden, dat deze daling voor een belangrijk deel moet worden toegeschreven aan de tijdelijke afwezigheid van plantengroei en in mindere mate aan het mechanisch verstoren van de grond tijdens de bouwlandperiode.

V

Haenen stelt voor om in de zandgebieden de overschotten aan dierlijke mest te brengen op grasland, dat vervolgens enige jaren als bouwland wordt gebruikt. Daarop kan men dan verdere overschotten kwijt. Dit voorstel houdt geen rekening met de verontreiniging van het milieu.

Haenen suggests the disposal of animal manure on grassland in sandy areas. This land is used for some years as arable land on which further slurry can be spread. His suggestion does not consider environmental pollution.

J. A. H. Haenen, De Landbode: 372 (1972)

VI

Het nut van de algehele verwijdering van nitraat in installaties voor de biologische zuivering van afvalwater is twijfelachtig.

VII

Er zijn aanwijzingen dat de hoogst gelegen delen van het rivierklei-veen-inversielandschap oorspronkelijk ook het hoogst gelegen waren. Indien dit zo is, mag men niet meer van inversie spreken.

VIII

Massamedia vormen een uiterst geschikte voedingsbodem voor sensationele mededelingen.

Proefschrift van J. L. M. Huntjens
Wageningen, 3 november 1972

Abstract

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Also: Agric. Res. Rep. (Versl. landbouwk. Onderz.) 781.

The results obtained from turf samples indicate that growing plants are mainly responsible for the accumulation of soil organic nitrogenous compounds. Mixing of the soil of turf samples containing living plants did not stimulate the release of soil organic N.

Addition of unlabeled $(NH_4)_2SO_4$ did not promote the liberation of labeled nitrogen (N^{15}) recently immobilized in turf samples with living grass plants. This labeled part was mineralized more readily than the originally present soil organic matter upon killing of the grass plants.

The amino acid patterns of the hydrolysates of pasture soil, arable land and the humic acids of these soils were rather similar, resembling the amino acid composition of the hydrolysates of the 'humic acids' produces by streptomycetes in a glycerol-nitrate medium.

Soil organic matter was used as the only N source for the growth of a proteolytic *Pseudomonas* strain. The results obtained suggest that more protein-like material is incorporated in the soil organic matter of pasture than in that of arable land. The availability of N of 'humic acids', synthesized by a *Streptomyces* strain, to the *Pseudomonas* sp. was similar to that of humic acids extracted from grassland by NaOH.

Verantwoording

Dit onderzoek werd uitgevoerd op het Laboratorium voor Microbiologie van de Landbouwhogeschool te Wageningen.

Gaarne wil ik mijn dank betuigen aan mijn promotor prof. dr. ir. E. G. Mulder voor de vrijheid die hij me gaf om me in dit onderwerp te verdiepen. Ook ben ik hem zeer dankbaar voor het kritisch doornemen van dit proefschrift en voor de bijzondere hulp die hij me heeft geboden bij de samenstelling hiervan.

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Voor hun technische assistentie ben ik mej. A. G. M. Valckx, mevr. M. C. Hoogkamer-te Niet, de heren G. J. Rátz en G. J. K. Pesch dankbaar.

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Curriculum vitae

De schrijver van dit proefschrift behaalde in 1956 het diploma HBS-b aan het Bisschoppelijk College te Roermond. Na de vervulling van zijn militaire dienstplicht begon hij in 1958 zijn studie aan de Landbouwhogeschool te Wageningen. In 1963 behaalde hij het kandidaatsexamen en in 1965 het ingenieursexamen, richting bodemkunde en bemestingsleer. Daarna werd hij verbonden aan het Laboratorium voor Microbiologie van de Landbouwhogeschool te Wageningen als promotie-assistent. Op 1 mei 1968 werd hij in de wetenschappelijke staf opgenomen en is thans werkzaam als wetenschappelijk medewerker 1e klas.

Contents

1 Introduction

It is a well-known fact that the amount of organic nitrogen in grassland soil tends to increase with time. This accumulation of organic nitrogen continues for many years ('t Hart, 1950)[1]. An equilibrium is reached after several decades when the production of soil organic nitrogen is equal to its decomposition. In soil of arable land the amount of organic nitrogen generally remains constant or decreases.

Clement & Williams (1967) showed that organic nitrogen accumulated in the top 15 cm of soil under grass at an annual rate of 0.005 % of air-dried soil. When the weight of the soil layer amounts to 2.5×10^6 kg of dry soil, this percentage corresponds with an annual increment of 125 kg nitrogen per hectare over a depth of 15 cm. This phenomenon partly explains the fact that the recovery of fertilizer nitrogen in the shoots of the plants of grassland is often lower than 60 % (Mulder, 1949; van Burg 1962, 1970).

In the present investigation, an attempt has been made to study the mechanism responsible for the accumulation of organic nitrogen in grassland soil.

Pot experiments with soil of arable land and with turf samples of grassland have been performed. As the organic nitrogen content of soils generally is so high that small changes cannot be determined by conventional methods, labeled fertilizer nitrogen has been used. The effect of the presence of living plants and the effect of disturbing and mixing the turf samples on nitrogen transformations in the soil have been studied. For this reason balance-sheets of soil nitrogen and labeled fertilizer nitrogen have been determined.

Ploughing up of grassland is followed by a rapid decline of the soil organic matter content ('t Hart, 1950). It is likely that the chemical composition of soil organic matter of grassland differs from that of soil of arable land. This has been checked by estimating the amino acid pattern of hydrolysates of both types of soil. From the literature it is known that streptomycetes are able to synthesize humic acid-like compounds and that these organisms constitute a greater part of total cell count in soil of grassland than in soil of arable land. For these reasons the production of *Streptomyces* 'humic acid' has been investigated. The amino acid composition of the hydrolysed humic acid-like compounds has been compared with that of humic acids isolated from soil of arable land and grassland, respectively.

By analytical procedures used in soil chemistry it is impossible to determine the

1. For references Chapter 1: see Section 2.5, page 13.

fraction of soil organic matter which becomes easily decomposable after ploughing up of grassland. In the present study an attempt has been made to obtain more quantitative information about this fraction by determining the growth of a proteolytic *Pseudomonas* strain in a medium containing soil organic matter as the only nitrogen source.

2 Review of literature on the accumulation of soil organic nitrogen in pastures and on the biosynthesis of humic acid-like substances

2.1 The fate of fertilizer nitrogen in grassland soils

Many investigations have shown that the recovery of fertilizer nitrogen in the shoots of grass plants of permanent pastures is low. Mulder (1949), Cunningham & Cooke (1958), van Burg (1962, 1970) and Woldendorp (1963) recorded that this recovery seldom exceeds 60 %, it usually being less than 50 %.

Losses by leaching of fertilizer nitrogen from grassland were recorded by Woldendorp et al. (1966). In lysimeters filled with sandy soil and sown with grass, scarcely any losses were found to occur when ammonium nitrate was applied during the growth season, except when heavy rainfall followed application. Therefore, losses of available fertilizer nitrogen in grassland soil must be due to volatilization or to incorporation in the roots or in soil organic matter. Woldendorp (1963) determined nitrogen balance-sheets in turf samples of permanent grassland using labeled fertilizer nitrogen. Under varying experimental conditions where leaching was impossible, 45–65 % of a nitrate dressing was found in the shoots, 10–25 % in the roots, and 7–22 % in the soil, while 10–40 % had been lost by denitrification. When ammonium nitrogen had been supplied, about 10 % was lost by denitrification after its transformation to nitrate by nitrifying bacteria.

Grassland soils are characterized by the rapid disappearance of fertilizer nitrogen when applied during the growth season. Richardson (1938) observed that the added fertilizer was entirely absorbed within at most a fortnight. Without added fertilizer, the inorganic nitrogen content of grassland soil is low throughout the whole year. Richardson found values for ammonium nitrogen between 2 and 8 and for nitrate nitrogen between zero and 2 mg per kg dry soil. The low inorganic nitrogen content of soil of permanent pasture has most commonly been attributed to a high nitrogen absorption rate due to microorganisms which are stimulated by the large quantities of organic materials added to the soil by the grass roots. A different explanation has been given by Theron (1951, 1963). He found that a very considerable mineralization of nitrogen took place as soon as the grass cover was removed, though large quantities of root residues still remained in the soil. He suggested, therefore, an inhibition of the mineralization, due to a toxic effect of an exudate of the living grass roots on the bacteria mineralizing nitrogen.

2.2 Accumulation of soil organic nitrogen in grassland

It is a well-established fact that large increases in organic nitrogen occur in grassland soil. Richardson (1938) estimated that the soil organic nitrogen can increase for more than 150 years. After such a long period of time, the production and decomposition of soil organic matter will be equal and the content of soil organic nitrogen will remain constant. Determinations of soil nitrogen changes under pure grass swards (Parker, 1957; Clarke, 1970) showed annual increases of soil nitrogen of the order of 20 to 40 kg per hectare, although no fertilizer nitrogen was applied. Recent investigations of Barrow (1969) showed an annual increase of 38 kg N per hectare in the top 12.5 cm of sandy soils under pastures, which were only fertilized with superphosphate. The accumulated nitrogen in these pastures was mainly derived from symbiontic nitrogen fixation due to the presence of clover. Clement & Williams (1967) analysed all the organic materials – roots and other underground plant organs were included in the soil samples – and observed that under a ryegrass/white clover sward, organic nitrogen accumulated in the top 15 cm at an annual rate of 100 to 110 kg N per hectare. Application of 45 kg fertilizer nitrogen per hectare resulted in an annual increment of 125 kg N per hectare. With an annual dressing of 314 kg N, increases under grazed leys averaged up to 180 kg N per year.

The mechanism of nitrogen accumulation under grass is imperfectly understood. In the presence of living plants, carbonaceous material is added to the soil by dead root hairs, dead root cells and root excretions. These plant-derived compounds may be used by microorganisms as energy source and as carbon source to build up their cell material. The formation of microbial cells includes immobilization of nitrogen in their cell organic matter. This immobilized nitrogen may be derived from nitrogenous fertilizers and from mineralization of soil organic nitrogen, of nitrogenous plant materials and of microbial cells. In grassland, the production of carbonaceous material may be so high that more nitrogen is immobilized than mineralized, resulting in accumulation of soil organic nitrogen.

The soil organic matter is stable as long as the soil is under grass. Ploughing up of grassland soils is followed by a rapid decline of the organic matter content ('t Hart, 1950). Due to this treatment, the plants are killed and the environmental conditions in the soil are changed. Killing the grass plants by ploughing will arrest the continuous flow of root-derived carbonaceous material resulting in a decrease of soil organic nitrogen and an increase of inorganic nitrogen. In the following, the changes caused in the soil of grassland by ploughing will be discussed in order to clarify the circumstances which are leading to accumulation of soil organic nitrogen in grassland.

2.2.1 Influence of living grass plants on the supply of carbonaceous material to the soil

It is rather difficult to determine the amount of carbonaceous material which is delivered by living plants to the soil. Goedewaagen & Schuurman (1950a) estimated that the annual production of roots, produced by grass crops, amounts to approxi-

mately 5000 kg per hectare. According to Shamoot et al. (1968) the quantities of organic debris (excluding roots) remaining in the soil per 100 units of root weight ranged between 20 and 50 units. For grassland these figures correspond with an annual production of 1000 to 2500 kg per hectare. Due to the fact that part of these organic debris has already been decomposed to CO_2 during the experimental period, the total production will be even higher.

Goring & Clark (1949) showed that the mineralization of nitrogen was depressed by the presence of crops in comparison with the fallow soil. They ascribed this to the re-immobilization of mineralized nitrogen, caused by the microbial decomposition of root compounds with a low nitrogen content.

2.2.2 Influence of living grass plants on the moisture content of the soil

The accumulation of soil organic nitrogen in grassland may be caused by the continuous production of carbonaceous material by plants, but according to Harmsen & van Schreven (1955) other factors may also be responsible. They wrote, 'the accumulation of humus in grassland cannot entirely be explained by the generally higher amounts of plant residues produced in grassland and forest than in arable land, because even the scantiest vegetation on grasslands, prairies, forests, etc., is proved to be able to build up a certain level of humus, while even luxuriously growing crops with high applications of farm manure, compost, or green manure can never entirely avert the depletion of humus and total N in arable soil'. Harmsen (1951) applies the theory of Enders (1942) on humus formation to the situation occurring in grassland. This theory is based on an accelerated synthesis of humus by microbes under unfavourable conditions. These conditions, i.e., frequent and abrupt changes in humidity, temperature and irradiation, occur in the extreme surface of the soil and Harmsen (1951) therefore proposed to regard this soil layer as the main site of humus formation. The accumulated humus is expected to be transported to lower layers by biological activity.

The major part of the root system of grassland soils is concentrated in the surface soil. Goedewaagen & Schuurman (1950b) showed that 62 per cent of the total root mass was generally present in the top layer 0–5 cm and 87 per cent in the layer 0–20 cm This may have very important consequences as far as the moisture content of the surface soil of grassland is concerned. The grass plants will take up their water mainly from the layer 0–20 cm, causing a rapid decrease of the moisture content of the soil during periods of the growth season without rainfall. Although the fluctuation of the moisture content in the layer 0–20 cm may be less frequent than in the extreme surface of the soil, the theory of Enders about humus formation may also be valid for this deeper soil layer.

In contrast to the suggestions of Harmsen about humus synthesis, the experiments of Birch (1958) showed that intermittent drying and wetting of soil during incubation enhanced the amount of mineralized nitrogen. With plant residues added, it appeared that the more frequently the soil went through the drying and remoistening cycle the

greater was the amount of mineralized plant nitrogen (Birch, 1964). The results of these experiments suggest that accumulation of soil organic nitrogen would be prevented by cyclic drying and wetting of the soil. However, this suggestion is not in agreement with the observed accumulation of organic matter in grassland soils. Two reasons for this deviation may be given.

1. In the soil samples without added plant residues tested by Birch (1958), carbonaceous material apparently did not come available at a sufficient rate to re-immobilize the mineralized nitrogen and to bring about accumulation of humus. The latter process can only occur when carbon and nitrogen are supplied by other sources than the original humus stock. In grassland the supply of available carbonaceous material may be so large that the mineralized nitrogen – eventually appearing in the soil after remoistening by rainfall following a dry period – is readily incorporated in proliferating microbial cells, as far as this nitrogen is not taken up by the grass plants. When the conditions change and become unfavourable for microbial growth (dry soil), humus formation might be favoured by the death of microbial cells.

2. In the soil samples containing plant residues tested by Birch (1964), it is possible that besides nitrogen mineralization of plant residues, the synthesis of humus had also been favoured by intermittent drying and wetting during the incubation period.

When it does not rain for a long period of time, the soil may become so dry that microbial decomposition of humus compounds and of plant residues will stop. The mineralization of soil organic matter increases with increasing moisture content, it being optimal at 50–60 % of the waterholding capacity (Jansson, 1958). Experiments of Grootenhuis (1961) indicate that more nitrogen is mineralized in ploughed pasture soils during a wet summer than during a dry summer. Robinson (1957) noted that at moisture levels between the wilting point and field capacity, the percentage mineralization increased with increasing moisture content. A small decrease in the moisture content below the wilting point was sufficient to prevent the substantial formation of mineral nitrogen. No mineralization occurred in air-dry soil.

In summary, it may be stated that the course of the moisture content in pasture is an important factor in the accumulation of soil organic nitrogen. Frequent changes of the moisture content may stimulate humus synthesis. A prolonged period of dryness may retard mineralization. This effect of moisture content on humus metabolism is expected to be less important in arable land where the plant roots may penetrate in deeper soil layers to absorb water. Moreover, this land is occupied by the crop only during part of the year, resulting in a more constant and higher level of the moisture content of the soil.

2.2.3 Influence of disturbing the soil

Woldendorp (1963) found that the mineralization rate in turf samples or permanent grassland with killed root systems was promoted by mixing the soil. An explanation of this effect may be that the poorly aerated soil aggregates are destroyed so that oxygen may penetrate more readily in all parts of the soil.

2.2.4 Influence of aeration

The observation of Woldendorp (1963) that denitrification is stimulated by the presence of living plants indicates that the oxygen concentration is grassland soil is low. The level of oxygen concentration which reduces nitrogen mineralization is not known. Experiments of Greenwood (1961) indicate that the respiration rates of simple organic compounds in water-saturated crumbs (mean radius 1.55 mm) of a loam soil were only slightly affected by reduction of the oxygen partial pressure in the atmosphere from 15 to 1.7 cm of mercury. Due to the inability of detecting any products of anaerobic decomposition at an oxygen partial pressure at which more than 80 % of the soil provided conditions for anaerobic decomposition, Greenwood suggested that these products diffuse into regions where sufficient oxygen is available to maintain aerobic metabolism. Novák (1971) investigated mineralization and humification under aerobic and anaerobic conditions. Greater amounts of humic substances were formed from straw under aerobic conditions. The products of anaerobic metabolism of straw and glucose were more readily mineralized and humified by the aerobic soil microflora than glucose.

These results may indicate that in grassland soils both the rate of mineralization and the rate of humification are stimulated by the occurrence of aerobic and anaerobic regions at the same time. It is likely that the level of oxygen concentration is dependent on the moisture content of the soil. As the moisture content of grassland is frequently changed, the same may be valid for the oxygen concentration.

2.3 Building units of nitrogenous soil organic matter

A large number of compounds of plant, animal, and microbial origin in varying stages of decay may be involved in humus production. Due to the fact that lignin is relatively resistent to biological attack, Wacksman & Iyer (1932) postulated that humic acids are formed by reaction between lignin and proteinaceous material in soils. Isolation of a small amount of a lignin-protein complex has been reported by Tinsley & Zin (1954) and by Jenkinson & Tinsley (1960). Mattson & Koutler-Andersson (1943) suggested that part of the soil nitrogen is present as complexes of oxidized lignin and ammonia. Studies by Sørensen (1962) showed that reactions between oxidized lignins and amino acids may occur during the biological decomposition of plant materials in soils.

However, there is no evidence that lignin accounts for a significant proportion of the organic matter of the soil. Although lignin yields appreciate amounts of phenolic aldehydes when oxidized with nitrobenzene in alkaline solution, only trace amounts are obtained from humic acids of soil organic matter (Bremner, 1955a; Morrison, 1958, 1963).

Hydrolysis of soils (Keeney & Bremner, 1964) and of humic acid preparations with 6 N HCl (Bremner, 1955b) showed that from 20–50 % of the total nitrogen in most soils and in humic acids is in the form of bound amino acids, and from 5–10 % is in the

form of combined hexosamines. Ammonium nitrogen liberated by hydrolysis was found to account for 15–25 % of the total soil organic nitrogen.

There are several theories as to how the amino acids of soil organic matter are combined. Polymer formation of amino compounds (amino acids, peptides and proteins) with carbohydrates or their decomposition products (sugar, methyl glyoxal, etc.) has been suggested to occur by the so-called Maillard reaction (Maillard, 1912, 1917; Enders, 1942; Schuffelen & Bolt, 1950).

Swaby & Ladd (1962) proposed that amino acids are incorporated as single units during the oxidative polymerization of phenols. It is unlikely, however, that amino acids occur only as separate units, because Sowden (1966a, 1966b) showed the presence of peptides in hydrolysates upon partial hydrolysis of soil with concentrated acid at room temperature. Simonart et al. (1967) isolated a protein fraction from soil humic acid. Evidence for its occurrence in soil humic acids has also been obtained by showing that the proteolytic enzyme pronase releases α-amino acids from humic acids (Ladd & Brisbane, 1967).

Kononova & Aleksandrova (1959) suggested that organic residues of plant and animal origin are first decomposed to simpler compounds through the activity of microorganisms. Some of the relatively simple products may be utilized by microorganisms to synthesize humic substances.

Some authors (Laatsch et al., 1952; Flaig, 1950, 1964) suppose the occurrence of a reaction of ammonia, amino acids, peptides or proteins with oxidized phenolic compounds derived from lignin degradation or from microbial metabolism.

Much information about substances involved in humus formation has been obtained from studies with artificial humic acids. Haider et al. (1965) showed that in the presence of phenol oxidases only such phenols which have no methylated hydroxyl groups reacted with amino acid compounds. The polymeric reaction products of oxidized phenols with amino acids were stable against hydrolysis. It was also found that after hydrolysis only the N-terminal amino acid of incorporated peptides, which is bound to oxidized phenols, could not be recovered. Using serum albumin, it was found that there is a reaction of the N-terminal amino acid and also of the ε-amino group of lysine residues with phenols during oxidation. Ladd & Butler (1966) prepared phenolic polymers, either nitrogen-free or incorporating amino acids, peptides, or proteins, from *p*-benzoquinone and catechol under mild oxidative conditions. Results showed that polymers, in which peptides and proteins are incorporated, resemble humic acids more closely than those incorporating separate amino acids. Experiments with polymers incorporating peptides showed that the bond between the carbon atom of an aromatic ring and the nitrogen atom of an α-amino acid is far more stable to acid hydrolysis than peptide bonds.

2.4 Formation of humic acid-like substances by pure cultures of microorganisms

Under laboratory conditions pure cultures can give insight into the processes taking place in the natural environment. Many experiments about humus formation by pure cultures of fungi and actinomycetes have been described in the literature. An advantage of such model experiments is that the influence of different carbon sources (such as lignin, phenols and non-aromatic compounds) and different nitrogen sources (such as inorganic nitrogen, amino acids, peptides and proteins) on humic acid formation can easily be investigated.

Wieringa (1958) and Woldendorp (1963) pointed out that actinomycetes constitute a greater part of the total cell count in grassland than in non-grassland soil. Flaig & Kutzner (1960) found the number of peptone-browning streptomycetes isolated from grassland to be generally higher than that from arable land of the same soil type. The formation of humic acid-like compounds by cultures of such streptomycetes was shown by Scheffer et al. (1950), Laatsch et al. (1950), Flaig et al. (1952). Glycerol was generally used as the carbon source and amino acids or peptone as the nitrogen source. Von Plotho (1950) found a fall of pH of the nutrient medium after inoculation with actinomycetes, which was followed by a rise of pH when the culture solution darkened, indicating autolysis of the cells. No humic acids were formed below a pH value of 7.5. Küster (1952) concluded from his experiments with streptomycetes that the aromatic building units of humic acids are not only supplied by plants (lignin), but may also be derived from the metabolic products of microorganisms. Küster (1958) demonstrated that the peptone-browning reaction depends on the presence and the activity of phenol oxidases. Two types of these oxidases can be distinguished, viz. tyrosinase (catechol oxidase) oxidizing monophenols and *o*-dephenols, and laccase (*p*-diphenol oxidase) oxidizing *o*- and *p*-diphenols (Küster, 1955; Kutzner, 1968). When the phenols have been oxidized, they can polymerize, incorporating not only phenols but also nitrogenous substances. Matschke (1970) showed that many types of nitrogenous substances may be involved in these condensation reactions. He found 15 different amino acids in hydrolysates of humic acids isolated from cultures of *Streptomyces aureus*.

Kang & Felbeck (1965) extracted humic acid-like substances from spores and mycelium of *Aspergillus niger* with alkali. Elementary analysis, methoxyl content and other characteristics showed that the 'humic acids' isolated from spores more closely resembled soil humic acids than those isolated from the mycelium. The yields of humic acid-like compounds, expressed as percentage of ash-free tissue weight, amounted to 24 and 16 % respectively, for spores and mycelium.

Martin et al. (1967) isolated humic acid-like substances from *Epicoccum nigrum*, grown in a glucose-asparagine medium. The yield amounted to about 10 % of the organic material synthesized by this fungus. The authors concluded that certain microbes, even when provided with relatively simple organic carbon and nitrogen sources, are able to produce substances which are similar to naturally occurring humic materials. Addition of certain phenols increased yields of 'humic acid' by 70–200 %.

Haider & Martin (1967) isolated two phenols from the nutrient solution of *E.nigrum* during the first week of growth of this organism. It appeared that during later stages of growth the fungus altered these phenols by introduction of additional OH groups, by decarboxylation and by oxidation of methyl to carboxyl groups, forming a large number of different polyphenols. The synthesis of humic acid-like compounds coincided with the disappearance of the phenols, increase of ammonia concentration and rise of pH of the culture medium. The results obtained with C^{14}-labeled phenols indicate that most of the side chain and methoxyl carbon atoms were released as CO_2 or were utilized for cell synthesis, while most of the ring carbon was incorporated in 'humic acid', or remained in solution. Phenolases were not found in the nutrient solution of *E.nigrum;* a weak phenolase activity was detected in the mycelium. However, polyphenols identified in the nutrient solution before 'humic acid' formation began, were shown to be strongly autoxidizable at pH values of 6 or above. The results obtained suggest a mechanism of synthesis of humic acid-like substances in two steps, viz. (a) alteration by soil fungi of phenols synthesized by these fungi or derived from other sources (e.g. lignin decomposition) to autoxidizable phenolic compounds and (b) autoxidation of these compounds. During the latter process the phenols will react with other phenols and with amino acids or peptides to form polymers.

Martin & Haider (1969) reported that up to 33 % or more of the substances synthesized by *Stachybotrys* cultures consisted of humic acid-like compounds. The polymer yields were even greater when the fungi were cultivated on plant residues. During the growth of *Strachybotrys atra* and *Epicoccum nigrum* in bean straw or corn stalks media, most of the phenols detected were similar to those produced in synthetic glucose media; in some instances even higher concentrations were found in the straw and in the corn stalks media. In additions to the phenols of fungal origin, other phenolic compounds were found in the latter material which had probably been derived from plant residues. The results of this investigation suggested a partial degradation of the lignin and the incorporation of some of the degradation products in the 'humic acid'.

The investigations with pure cultures suggest that a large number of phenolic radicals take an active part in the formation of soil humic acids. These radicals may react with other phenols and amino compounds. The latter substances are released in soil during the breakdown of plant and microbial cells and due to excretion by living roots. The possibilities for reactions or combinations are unlimited. Dubach & Metha (1963) remarked that probably no two molecules of humic acid are exactly the same. Swaby & Ladd (1962) suggested that humic acids are three-dimensional polymers consisting of many different amino acid and phenol units without ordered sequence. According to these authors the resistance of humic acid to microbial decomposition could be explained by its occurrence as a large spherical molecule, consisting of many heterogeneous units, so that many extra-cellular enzymes from many different microorganisms would be needed to decompose it piece by piece from the outer surface. The concept of this theory is still important, although the occurrence of peptide bonds in humic acids has been proved.

2.5 References

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INFLUENCE OF LIVING PLANTS ON IMMOBILIZATION OF NITROGEN IN PERMANENT PASTURES

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SUMMARY

The presence of plants has a negative effect on the net nitrogen mineralization rate in samples of turf from permanent grassland. This effect is probably caused by root excretions and dead roots which lead to the immobilization of mineralized nitrogen. When the plants of a turf sample have been killed by repeated cutting and removal of the shoots the mineral nitrogen immobilized due to the presence of plants is subsequently more readily mobilized than that immobilized in the greater part of the soil organic matter. The constant presence of plants is responsible for the accumulation of nitrogen in the organic matter of the soil of permanent grassland.

INTRODUCTION

Accumulation of organic nitrogen in permanent pastures in temperate regions is well-known [10 7 2 3]. The amount of organic nitrogen in the soil of arable land generally remains constant or decreases. The purpose of the present work was to investigate the mechanism responsible for the accumulation of organic nitrogen in permanent grassland.

The quantity of organic nitrogen in soil is usually too high for the estimation, by the conventional Kjeldahl method, of changes in the organic nitrogen content resulting from a particular treatment. For this reason a technique involving labeled fertilizer nitrogen was used. After application, the labeled nitrogen becomes distributed among the tops and roots of plants and the soil organic matter. Consequently, it is possible to estimate the quantity of nitrogen present in the plant tissue and the soil organic matter which has been derived from the fertilizer.

In most of the experiments using labeled nitrogen described in the literature the soil was obtained from arable land. In addition, the fertilizer is usually added at the same time as the seed is sown. However, in permanent grassland the soil is always occupied by plants and in this case the labeled fertilizer nitrogen has to be applied to a soil which is already occupied by a vegetation. One of the few authors who has carried out such experiments is Woldendorp [15].

In attempting to determine the causes of the difference in organic matter content between permanent pasture and arable soil, the principal differences between both environments should be taken into consideration. In the case of grassland, plants are present throughout the year, whereas on arable land they are often present for no more than four to five months. A second point of difference is that arable land, in contrast with grassland, is disturbed by ploughing each year.

Influence of plants

In grassland soil the amount of inorganic nitrogen is generally low, viz. 0–5 ppm NO_3–N and approximately 5 ppm NH_4–N [10 12]. In the case of some fields, Simpson [12] found 40 ppm NO_3–N during the summer; this was ascribed to dryness which damaged the plants, and consequently NO_3–N accumulated in the soil. Theron [13] observed that the presence of living plants reduced net mineralization of nitrogen; he ascribed this to the presence of compounds which inhibit mineralization [14]. This hypothesis was based on the fact that mineralization during incubation experiments was favoured by predrying the soil at room temperature for one day. It was postulated that as a result of predrying the ammonification inhibitor would be destroyed. A more feasible explanation would seem to be that predrying of the soil causes the death of soil organisms which would result in increased mineralization of nitrogen on rewetting the soil. According to Barrow [1] the living grass would absorb all inorganic nitrogen from the soil, which would result in a deficiency of available nitrogen for the soil microorganisms, with consequent reduction in the rate of decomposition of the soil organic matter.

A more acceptable explanation for the reduced mineralization of nitrogen in pastures is that under the influence of living grass the mineralized nitrogen is subsequently re-immobilized into microbial cell tissue. The organic matter necessary for this immobilization

would be provided by dying roots and root excretions. In this connection it is desirable to know how much plant derived organic matter is added to the soil annually. Goedewaagen and Schuurman [4] estimated the total yearly production of root tissue in a pasture to be 4500 kg dry matter per hectare. According to Shamoot *et al.*[11] deposition of organic debris (excluding roots) from plant roots would amount to 1000–200 kg dry matter a hectare during seven months of active growth (Rhizo-deposition). This could mean that 5 to 6 tonnes of dry matter would be produced in the form of roots and organic debris depositions. On the assumption that the carbon of approximately 35 kg plant dry matter is necessary for immobilization of 1 kg nitrogen into microbial cell tissue in soil, it would be possible for 140 to 170 kg nitrogen per hectare to be immobilized into microbial cell tissue each year.

On arable soils after the harvest of the crop no more than 1000 to 2000 kg of dry matter would remain in the form of stubble and roots [8]. These residues together with the organic debris depositions from plant roots amount to a yearly dry matter supply to the soil of 2000–4000 kg per hectare, immobilizing 60 to 110 kg nitrogen according to the foregoing assumption.

Influence of disturbing the soil

Woldendorp [15] found that turf samples obtained from permanent pastures, and in which the grass roots had been subsequently killed, had a higher nitrogen mineralization rate when the soil of the turf was mixed than when it remained undisturbed. A possible explanation for this is that the turf is better aerated following mixing of the soil. However, ammonification is not necessarily retarded under anaerobic conditions [6].

MATERIALS AND EXPERIMENTAL PROCEDURE

Soil and experimental conditions

Turf samples, 9.4 cm in diameter and 7.5 cm thick, were obtained from a grass sward, eight years old established on sandy soil. The turf samples were placed in pots without drainage holes, and the pots were sunk so that their rims were level with the surface of the sward. The pots were covered with plastic caps during rainy weather to prevent waterlogging. The pots were weighed twice a day and the water content was kept at 60 per cent of W.H.C. by addition of distilled water. Each pot contained approximately 620 g of dry

soil, which had a pH of 6.5, containing 4.8 per cent organic matter and 2100 ppm of total nitrogen.

To compare the nitrogen transformations in pastures with those in arable soil, one experiment was carried out by filling some pots in spring with fertile sandy soil from an arable field. This field had been fallowed since the previous autumn. The soil had a pH of 6.3 and contained 4.4 per cent organic matter and 1400 ppm of total nitrogen. Each pot contained 580 g of dry soil. Before starting the experiments all the pots received 0.33 g KH_2PO_4 and 0.30 g $MgSO_4.7\,H_2O$.

Design of experiments

Experiment 1. The objective of this experiment was to study the transformation of mineralized N into organic N and vice versa in permanent pastures in which the roots had been killed and in arable soil. The root systems of the turf samples in the pots were killed by clipping the grass several times [15] during the period from 13 to 23 May. The soil surface was covered with glass-wool to prevent evaporation and the growth of algae. When the roots had died, 47.6 mg N as $(NH_4)_2SO_4$, containing 21.0 mg excess N^{15}, was added to 8 pots containing turf samples with killed roots and to 8 pots filled with arable soil. The labeled fertilizer was added on 23 May. Disappearance of the NH_4–N from the soil was estimated by analysis of the soil from two pots in each treatment, 6 hours after addition of the labeled fertilizer (to estimate the chemical fixation of NH_4–N) and on 31 May, 21 June and 22 July.

Experiment 2. The purpose of this experiment was to study the effect of the presence of living plants and the effect of disturbing and mixing of the soil of the turf samples containing the killed plant roots on nitrogen transformations in the soil. The turf samples in 18 pots were enriched with 38.3 mg N asKNO_3, containing 20.4 mg excess N^{15}, on 24 May 1966. In additional turf samples, which had received the same quantity of nitrogen but in the unlabeled form, the mineral nitrogen was estimated periodically in order to determine when the inorganic nitrogen had disappeared from the soil. This was found to have occurred by 8 June. On this date the 18 pots containing turf samples were divided into three series, I, II and III, each consisting of 6 pots. The grass shoots of the pots of series I were cut 3 cm above the soil surface on 8 June and the tops discarded. The root systems of the turf samples in the pots of series II and II were killed by clipping the grass shoots very close to soil level several times between 8 June and 20 June the tops being discarded on each occasion. The soil of the turf samples in series III was mixed on 23 June. Nitrogen balance-sheets of two turf samples in each series were determined on 23 June, 22 July and 22 August. The grass shoots of the turf samples of series I were clipped very close to soil level when balance-sheets had to be determined. The grass of the turf samples of series I analysed on 22 August had been clipped 3 cm above the soil surface on 22 July before being clipped close to the soil level on 22 August.

The average maximum and minimum temperatures during the first 14 days after addition of the fertilizer were 19.3°C and 10.4°C, respectively, at 3 cm below the surface of the soil.

Experiment 3. In this experiment a study was made of the effect on mineralization of soil organic nitrogen of clipping the shoots of the grass plants and mixing the soil without damaging the plant roots. The turf samples in 14 pots received 98.5 mg N as $(NH_4)_2SO_4$, containing 24.4 mg excess N^{15}, on 11 June 1966. The grass shoots of all the turf samples were cut on 16 June and analysed. The inorganic nitrogen had disappeared from the soil on 27 June. On this date the 14 turf samples were divided into 7 series, each containing two pots, which were treated as follows:

Series I: used for establishing nitrogen balance-sheets on 27 June including the nitrogen contained in the clipped grass shoots which had been removed. In all the other series the grass shoots were clipped 3 cm above soil level on 27 June and the clippings analysed.

Series II: used for determining nitrogen balance-sheets on 22 July. The grass shoots in the remaining series were clipped on this date and the clippings analysed.

Series III: used for nitrogen balance-sheets on 16 August. The soil of the turf samples in series V and VII was mixed on this date without damaging the plant roots.

Series IV and V were analysed on 12 September and those of series VI and VII on 5 October.

The average maximum and minimum temperatures during the first 14 days after addition of the fertilizer were 24°C and 16°C, respectively, at 3 cm below the surface of the soil.

Analysis

The methods used for establishing nitrogen balance-sheets of the turf samples are described by Woldendorp [15]. Determinations were made of total nitrogen content of the soil (including the nitrogen of the roots and inorganic nitrogen), inorganic nitrogen (NH_4^+ and NO_3^-) in the soil, and the nitrogen content of the roots and shoots of the grass plants. Soil nitrogen was obtained by subtracting the inorganic and root nitrogen from total nitrogen. The N^{15}-content was estimated by means of a Metropolitan Vickers mass spectrometer.

RESULTS AND DISCUSSION

Nitrogen transformation in turf samples containing killed roots and in arable soil samples (Experiment 1)

The mineralization of nitrogen in turf samples with killed roots was considerably greater than in arable soil (Table 1, 6th column). The mineralized nitrogen has probably been derived from a particular fraction of the organic matter, viz. the easily decomposable fraction. In the case of the arable soil this part of the organic nitrogen had already been mineralized and the products leached out from the soil before it was placed in the pots. Two months after the addition

TABLE 1

Nitrogen mineralization and distribution (mg N per pot) of added fertilizer nitrogen* in turf samples containing killed roots and in arable soil (Experiment 1)

Date of analysis	Pot no	Inorganic nitrogen				Fertilizer-N (N^{15}) in the fraction org. + fixed N**
		$N^{14} + N^{15}$			Fertilizer-N (N^{15})	
		NH_4^+	NO_3^-	$NH_4^+ + NO_3^-$	$NH_4^+ + NO_3^-$	
Grassland (plants dead on 23 May)						
23 May	189	51	3	54	35.0	7.7
at 6 p.m.	186	48	3	51	41.8	5.0
31 May	185	48	12	60	36.0	
	188	43	15	58	35.6	11.8
21 June	200	33	43	76	33.8	11.8
	183	36	48	84	29.7	19.3
22 July	196	11	91	102	33.4	14.3
	195	15	77	92	31.6	13.2
Arable soil						
23 May	1	42	3	45	40.3	7.5
at 6 p.m.	6	42	5	47	41.1	8.4
31 May	2	41	7	48	43.1	4.5
	8	41	8	49	41.5	5.9
21 June	18	28	24	52	38.8	9.1
	3	25	24	49	36.1	8.9
22 July	4	4	49	53	37.3	9.1
	7	3	49	52	35.2	8.2

* 47.6 mg N in the form of labeled $(NH_4)_2SO_4$ containing 21.0 mg excess N^{15} added on 23 May at noon.

** The amount of fertilizer-N found in this fraction 6 hours after the addition of the fertilizer-N is assumed to be fixed. The remaining part is assumed to be immobilized by biological processes in the soil.

TABLE 2

Net mineralization of soil organic nitrogen (mg $N^{14} + N^{15}$ per pot) during the period 23 June–22 August in turf samples with living plants (Experiment 2, Series I)

Date of analysis*	Inorg. N ($NH_4^+ + NO_3^-$) in soil	N in roots	N in cut shoots				Inorg. + plant N	Net mineralization of soil org. N after 23 June	Dry matter of shoots
			23 June	22 July	22 Aug.	Total			
23 June	7	86	34			34	127	—	1.44
22 July	7	81		28**		28	116	−11	2.07
22 Aug.	6	78		11**	23	34	118	− 9	2.28

* 38.3 mg N, as labeled KNO_3 containing 20.4 mg excess N^{15} added to each pot on 24 May; mineral nitrogen used up by 8 June when the shoots were cut at 3 cm above soil level and discarded.

** Difference in nitrogen yield is due to the difference in cutting height.

of the nitrogenous fertilizer practically all the NH_4^+ had been converted to NO_3^- in both soils indicating deficiency of organic material whereby the NH_4-N could be utilized and immobilized. Woldendorp [15] found mineralization of nitrogen in undisturbed turf with killed roots to be less pronounced than was the case in the present investigation. Probably these different results depend on the use of turf of differing origin.

The amount of residual fertilizer nitrogen decreased more rapidly in turf than in arable soil. This decrease coincided with an increase of fertilizer nitrogen in the organic fraction. The greater immobilization of fertilizer nitrogen in the turf as compared with soil from arable land indicates the presence of larger amounts of metabolizable organic material in the turf.

Influence of living plants and of mixing of the soil of turf samples containing killed grass roots on nitrogen transformations in grassland soil (Experiment 2)

The total nitrogen yield of the shoots of the living grass plants, removed at different lengths of time after all the fertilizer N had been absorbed by the plants (Series I), was nearly the same at the different harvest dates (Table 2). Moreover, there was no change in inorganic soil nitrogen, while the root nitrogen decreased slightly during the experimental period. Consequently the net mineralization of organic nitrogen was slightly negative. In spite of this, the dry weight of tops increased considerably during the experimental period

In turf containing killed plant roots, the content of inorganic nitrogen increased by approximately 45 mg between 23 June and 22 August (Table 3). This result is in agreement with the finding from Experiment 1 (Table 1). Disturbing and mixing the soil of the turf did not promote mineralization, an observation which is in contrast with the results of Woldendorp [15]. This difference may have been due to the dependence of mineralization on the type of turf and on the incubation temperature. Woldendorp incubated the turf samples at a constant temperature of 28°C.

In turf containing killed plant roots, the roots lost approximately 20 mg N during two months (Table 3) If complete mineralization of this nitrogen had occurred the amount of inorganic nitrogen would have increased by the same amount during this period. Since the increase in inorganic nitrogen in the soil was considerably larger, it

TABLE 3

Net mineralization of soil organic nitrogen (mg N^{14} + N^{15} per pot) during the period 23 June–22 August in turf samples containing killed roots (Experiment 2)

Date of analysis *	Inorg. N ($NH_4^+ + NO_3^-$) in soil	N in roots	Inorg. + root N	Net mineralization of soil organic nitrogen after 23 June
Turf samples, the soil of which remained undisturbed (Series II)				
23 June	10	75	85	—
22 July	31	59	90	5
22 Aug.	57	50	107	22
Turf samples, the soil of which was disturbed and mixed (Series III)				
23 June	14	75	89	—
22 July	26	67	93	4
22 Aug.	57	56	113	24

* 38.3 mg N, as labeled KNO_3, added to each pot of turf on 24 May; mineral nitrogen completely absorbed by 8 June when the plant shoots were removed. Plants killed by clipping the shoots very close to the soil surface during the period 8–20 June, the shoots being discarded. Soil of turf samples of series III disturbed and mixed on 23 June.

TABLE 4

Distribution of previously immobilized fertilizer nitrogen (mg labeled N per pot) between the soil, plant roots and shoots in turf samples with living plants (Experiment 2, Series I)*

Date of analysis	Soil org. N	Inorg. N ($NH_4^+ + NO_3^-$) in soil	N in roots	N in shoots			
				23 June	22 July	22 Aug.	Total
23 June	6.4	0.3	4.3	7.7			7.7
	7.9	0.4	4.1	6.2			6.2
22 July	6.9	0.4	4.9		5.8**		5.8
	4.9	0.4	4.1		5.2		5.2
22 Aug.	6.4	0.3	2.8		2.3**	4.0	6.3
	6.1	0.3	3.4		2.6	2.8	5.4

* mg fertilizer N not accounted for was partly removed when the shoots of the grass plants were cut on 8 June and discarded, and probably partly lost by denitrification.
** Difference in nitrogen yield is due to the difference in cutting height.

must be assumed that the difference was derived from soil organic matter (Table 3, net mineralization).

This effect of the presence of living plants in reducing the net mineralization of nitrogen in soil (Tables 2 and 3) is in accordance with the results of Goring and Clark [5].

The distribution of fertilizer nitrogen between plant shoots and roots and the soil was approximately the same on 23 June, 22 July and 22 August in the case of turf samples with living plants (Table 4).

Killing of the plants associated with the turf samples reduced the amount of fertilizer nitrogen in the roots; the amount of fertilizer

TABLE 5

Mineralization of fertilizer nitrogen (mg labeled N per pot) incorporated in the roots and soil organic matter in turf samples containing killed roots. The fertilizer nitrogen applied on 24 May had been completely absorbed by 8 June, when the plant shoots were removed and the roots began to die. All the roots were dead by 23 June (Experiment 2)

Date of analysis	Soil of turf sample undisturbed			Soil of turf sample disturbed		
	Soil org. N	N in roots	Inorg. N ($NH_4^+ + NO_3^-$) in soil	Soil org. N	N in roots	Inorg. N ($NH_4^+ + NO_3^-$) in soil
23 June	9.0	3.5	0.8	7.5	3.1	0.7
	6.4	2.8	0.5			
22 July	6.3	2.0	2.3	3.0	2.6	1.8
	5.5	2.0	1.8	6.2	2.0	1.7
22 Aug.	4.4	1.8	2.6	7.8	1.9	3.0
	7.8	1.8	3.8	3.1	1.8	2.8

TABLE 6

Comparison of mineralization of organic nitrogen compounds of the following sources in soil: (1) total soil organic nitrogen ($N^{14} + N^{15}$), (2) fertilizer nitrogen (N^{15}) recently incorporated in soil organic matter, (3) total nitrogen in root tissue ($N^{14} + N^{15}$), (4) fertilizer nitrogen (N^{15}) recently incorporated in root tissue, following killing of the roots of the plants of the turf samples. The figures for $N^{14} + N^{15}$ are derived from Table 3, those for N^{15} from Table 5 (Experiment 2)

Date	Soil organic N (mg per pot)						Root N (mg per pot)					
	Total N ($N^{14} + N^{15}$)			Fertilizer-N (N^{15})			Total N ($N^{14} + N^{15}$)			Fertilizer-N (N^{15})		
	mg N	Loss		mg N	Loss		mg N	Loss		mg N	Loss	
		mg N	%		mg N	%		mg N	%		mg N	%
23 June	1200			7.6			75			3.1		
22 Aug.		23	2	5.8	1.8	24	53	22	30	1.8	1.3	42

nitrogen in the inorganic nitrogen of the soil increased by an amount greater than that derived from the decomposing roots during the experimental period (Table 5). Although the amount of fertilizer nitrogen in the organic matter did not change significantly, it must be assumed that part of the mineralized nitrogen had been derived from the soil organic matter. The nitrogen of the soil organic matter was less easily mineralized than nitrogen recently immobilized in soil organic matter, the comparable proportions being approximately 2 and 24 per cent, respectively (Table 6).

Thirty per cent of the total nitrogen was lost from the killed root material during the experimental period; this is only a slightly lower proportion than the proportion of fertilizer nitrogen lost from the roots during the same period (42 per cent; Table 6).

Influence of date of removal of plant shoots and mixing of the soil of turf samples containing living plant roots on mineralization of soil organic nitrogen (Experiment 3)

During the experimental period (from 27 June till 5 October) there was practically no uptake of nitrogen derived from the organic fraction of the soil (Table 7, 11th column, and Table 8, 3rd and 11th

TABLE 7

Influence of date of removal of the grass shoots and disturbance and mixing of the soil of the turf samples, on the uptake of nitrogen by grass plants (mg $N^{14} + N^{15}$ per pot) and dry-matter production (g per pot). (Experiment 3)

Series no	Date of analysis*	Inorg. N ($NH_4^+ + NO_3^-$) in soil	N in roots	N in grass shoots							Total dry matter in grass shoots
				Date of removal of grass shoots							
				16/6	27/6	22/7	16/8	12/9	5/10	Total N	
I	27 June	7	111	52	93					145	3.8
II	22 July	6	92	60	35	57				152	4.7
III	16 Aug.	5	98	59	35	20	37			151	5.1
IV	12 Sept.	5	116	57	43	27		36		163	5.3
V**	12 Sept.	6	93	61	34	26		41		162	5.7
VI	5 Oct.	6	97	53	41	30			37	161	5.3
VII**	5 Oct.	4	102	61	36	21			32	150	5.1

* 98.5 mg N added as labeled $(NH_4)_2SO_4$ to each pot of turf on 11 June 1966; mineral nitrogen completely absorbed by 27 June. The grass shoots of all the pots were cut on 16 June and on subsequent dates as indicated.

** Mixed on 16 August.

TABLE 8

Influence of date of removal of grass shoots and disturbance and mixing of the soil on the distribution (mg labeled N per pot) of 98.5 mg fertilizer nitrogen. The nitrogen was added as labeled $(NH_4)_2SO_4$ on 11 June (Experiment 3)

Series no	Date of analysis	Soil org. N	N in roots	N in grass shoots						
				Date of removal of grass shoots						
				16/6	27/6	22/7	16/8	12/9	5/10	Total N
I	27 June	12.0	10.4	27.5	42.7					70.2
				35.8	40.9					76.7
II	22 July	10.3	5.8	37.5	17.3	21.0				75.8
		8.5	4.8	40.1	21.3	17.5				78.9
III	16 Aug.	10.9	6.2	31.1	18.8	10.1	11.0			71.0
		11.5	4.4	36.1	21.6	10.4	8.7			76.8
IV	12 Sept.	10.0	6.2	35.4	22.7	11.1		10.0		79.2
		11.6	7.6	34.6	24.4	9.0		7.4		75.4
V**	12 Sept.	18.9*	8.6*	47.9*	39.9*	19.9*		12.5*		120.2*
		12.1	5.5	39.4	19.3	10.8		9.4		78.9
VI	5 Oct.	11.8	5.5	34.0	24.4	11.5			7.9	77.8
		10.2	6.1	30.2	23.2	13.5			10.0	76.9
VII**	5 Oct.	14.3	4.2	38.9	20.2	8.3			6.1	73.5
				39.8	20.5	9.2			7.4	76.9

* Erroneously, 153.9 mg fertilizer nitrogen applied.
** Mixed on 16 August.

column). Clipping and removal of the shoots at different dates and even mixing of the soil of the turf samples on 16 August did not stimulate the mineralization of soil organic nitrogen. This means that the very low mineralization rate in turf and consequently the accumulation of organic matter in permanent grassland is mainly due to the presence of living plants. Theoretically, two processes may be responsible for this phenomenon. (a) The presence of living plants may inhibit mineralization [14]. This theory is not acceptable (see Introduction). (b) Plant growth results in accretions to the soil of organic material in the form of root excretions and dead roots which are associated with the immobilization of part of the mineral nitrogen [5 9]. In experiments 2 and 3 the C–N ratio of this organic material was apparently so high that all of the mineralized nitrogen was immobilized again as soon as it was released. As a result of killing the plants the accretion of organic material to the soil presumably decreases, whereupon a net mineralization (Table 3) of soil organic nitrogen occurs.

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THE INFLUENCE OF LIVING PLANTS ON MINERALIZATION AND IMMOBILIZATION OF NITROGEN

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SUMMARY

Changes in the pattern of distribution of the nitrogen of the soil and seedling grass plants have been investigated when the grass plants were grown in pots of sandy soil, from a pasture, at pH 5.7. Net mineralization of soil nitrogen was not observed during an experimental period of one month in the absence of added nitrogenous fertilizer (Table 2).

Addition of labeled nitrogen (as ammonium sulphate) to the soil at the beginning of the experimental period resulted in a negative net mineralization during this period (Table 4b). When none of the fertilizer nitrogen remained in its original form in the soil it was found that approximately 12 per cent of the labeled nitrogen had been immobilized in soil organic compounds.

Clipping of the grass at this date was followed by a decrease in the amount of labeled soil organic nitrogen, indicating that mineralization was not depressed by living plants.

The application of unlabeled ammonium sulphate subsequent to the utilization of the labeled nitrogen did not decrease the amount of immobilized labeled nitrogen in the soil organic matter, as would be expected if the organic nitrogen compounds of the soil had been decomposed to ammonia. This was thought to be due to the fact that decomposition of organic nitrogen compounds in permanent grassland results in the production of peptides, amino acids etc. which are utilized by microorganisms without deamination taking place.

In pots with ageing grass plants, labeled organic nitrogen compounds were found to be translocated from the grass shoots to the soil (Table 7).

Net mineralization of soil organic nitrogen was positive in the contents of pots containing killed root systems (Table 3b). About 8 per cent of the labeled nitrogen added to the contents of such pots, in the form of ammonium sulphate, was found to be present in soil organic nitrogen compounds approximately 4 weeks after application, while a total of about twice this amount of soil organic nitrogen was mineralized during that period.

From the results obtained in this investigation, it is concluded that the constant presence of living plants is responsible for the accumulation of nitrogen in organic compounds in permanent grassland. No evidence was obtained that the decomposition of such compounds in the soil is inhibited by living plants.

INTRODUCTION

In previous experiments [10], accumulation of nitrogen in organic form in permanent grassland was shown to be associated with the presence of living plants. In seeking on explanation of the mechanism of this accumulation, two possibilities were considered. Firstly, mineralization of organic nitrogen compounds might be inhibited by the presence of plants. Secondly, mineralized nitrogen might be immobilized by the activity of microorganisms utilizing carbonaceous material such as root excretions and dying roots.

The production of carbonaceous material depends on photosynthesis which is eliminated by killing the plants and reduced by clipping the shoots. Therefore, a study was made of mineralization and immobilization of nitrogen in the contents of pots containing root systems which had been killed, and also in the contents of pots containing living plants, the shoots of which were or were not clipped during the experiment. Labeled fertilizer nitrogen was used in the investigation.

In arable soil, a continuous mineralization-immobilization cycle of nitrogen occurs [11]. If this is also true for permanent grassland, the addition of labeled fertilizer nitrogen should increase the release of previously immobilized labeled nitrogen. In the present investigation this hypothesis has been tested.

EXPERIMENTAL MATERIALS AND METHODS

Soil and experimental conditions

Soil, obtained from the 1–7 cm layer of a nine years old pasture on sandy soil*, was sieved to remove the greater part of the roots (December 1967). Immediately thereafter, 60 pots without drainage holes were filled with 665 g of moist soil (moisture content 25 per cent; organic-matter content 5.6 per cent) and sown with perennial ryegrass. Each pot contained 950 mg soil nitrogen in organic form and 30 mg nitrogen in root residues. The water content of the soil was kept at 60 per cent of the waterholding capacity. Subsequently N, P, K, Mg and minor-elements were added to the soil and the grass shoots were clipped several times. The plants were grown in a phytotron under controlled conditions. The temperature was maintained at 20°C during the day (between 8.00 a.m. and 8.00 p.m.) and at 15°C during the night (between 8.00 p.m. and 8.00 a.m.). The relative humidities during these periods were 70 and 75 per cent, respectively.

* The same pasture as used in previous experiments [10].

TABLE 1

Experimental programme

Series	Number of pots	Fertilizer treatment		Date when grass shoots were clipped off	Date of removal of grass shoots to kill the root systems	Date of sampling for analysis
		mg N added per pot	Date added			
I	5	—	—	—	—	14 May
II	3	—	—	—	—	31 May
III	3	—	—	—	—	12 June
IV	2	175.6 N^{15}	14 May	—	14 May	14 May
Va	2	175.6 N^{15}	14 May	—	14 May	12 June
Vb	1	175.6 N^{15}	14 May	—	14 May	12 August
VI	3	175.6 N^{15}	14 May	—	—	31 May
VII	3	175.6 N^{15}	14 May	31 May	—	12 June
VIII	4	175.6 N^{15}	14 May	—	—	12 June
IX	4	175.6 N^{15}	14 May	31 May	—	12 June
		100 N^{14}	31 May			
X	4	175.6 N^{15}	14 May	—	—	12 June
		100 N^{14}	31 May			
XI	3	175.6 N^{15}	14 May	31 May	—	12 August
XII	3	175.6 N^{15}	14 May	—	31 May	12 August

On 14 May 1968, the grass shoots in all the pots were clipped at a height of 5 cm above the soil level. On this date the pH of the soil was 5.7. The grass was kept in an erect position by surrounding the pots with transparent perforated plastic.

Design of experiments

After clipping the grass shoots on 14 May the pots were divided into the following 12 series (Table 1).

Series I, II and III were used for determining the distribution of nitrogen as organic soil N, root N, inorganic N and N in the grass shoots on 14 May, 31 May and 12 June, respectively.

Series IV and V were used for experiments in which the grass plants were killed. The root systems of the grass plants were killed by clipping the grass shoots as close to the soil surface as possible on 14 May, so that no further growth occurred. Immediately after this treatment 175.6 mg labeled N as $(NH_4)_2SO_4$ (175.6 mg N contained 52.1 mg excess N^{15}) were added to each pot. In the pots of series IV the amounts of the various forms of N were ascertained 3 hours after the addition of the fertilizer nitrogen. Of series V two pots were analysed on 12 June (series Va) and one on 12 August (series Vb).

Series VI and up to and including XII (containing living grass plants) were fertilized with labeled nitrogen (similar to series IV and V) on 14 May.

Series VI was analysed on 31 May when the inorganic N had practically disappeared from the soil. This was also the case with similar pots where the same amount of unlabeled fertilizer had been added.

The shoots of the grass plants in series VII and IX were clipped on 31 May and the shoot material analysed. Series IX and X received an additional amount of 100 mg N as unlabeled $(NH_4)_2SO_4$ on 31 May. Series VII, VIII, IX and X were analysed on 12 June.

The shoots of the grass plants of series XI were clipped and the root systems of the grass plants of series XII were killed by clipping of the shoots on 31 May. The amounts of the different forms of nitrogen were determined in both series on 12 August.

Analytical methods

The methods used for determination of the amounts of the different forms of nitrogen were the same as those used by Woldendorp [22]. At the end of the experiments the contents of the pots were packed in plastic bags and stored at −18°C until analysed. At the time of analysis the contents of the pots were weighed after thawing, then thoroughly mixed and weighed again to determine moisture loss during the mixing procedure.

Total nitrogen in soils. The Kjeldahl method was used for the estimation of the total nitrogen content of the soil (including that of the roots and inorganic nitrogen). Five samples, each of 15 g, were taken from the contents of each pot for analysis. Each sample was then digested with 30 ml conc. H_2SO_4 and 10 g of a mixture of 950 g K_2SO_4, 15 g $CuSO_4.5\ H_2O$ and 20 g Se. When nitrate was present, reduction with reduced iron powder was carried out prior to digestion [5]. After digestion, 75 ml water were added and the ammonia was then steam-distilled and collected in 20 ml of a 2 per cent boric acid solution followed by titration with 0.1 *N* H_2SO_4 using methylredbromocresolgreen as indicator.

Total nitrogen in roots. Three to five samples, each of 100 g, were taken from the contents of each pot and washed with tapwater. The washing water was strained through a 32-mesh sieve in order to recover small roots. The latter and the soil particles remaining on the sieve were resuspended in water and after separation the small roots were collected on the sieve. This was repeated twice whereupon the small roots were added to the bulk of the roots. The combined root material was then dried, ground and suspended in carbon tetrachloride to separate it from the heavier soil particles. Finally the powdered root material was dried and analysed by digestion with 20 ml conc. H_2SO_4 and 4 g of the Se, $CuSO_4$, K_2SO_4 mixture. Ammonia was estimated by distillation into 10 ml of a 2 per cent boric acid solution followed by titration with 0.1 *N* H_2SO_4.

Ammonia and nitrate in soils. Two samples, each of 100 g and known moisture content, were taken from the contents of each pot. Extraction was carried out by shaking the soil mechanically with 250 ml 1 *N* K_2SO_4-solution for 1.5 hours. After removal of the soil by filtration, a 100-ml sample of the filtrate was pipetted into a 500-ml Kjeldahl flask and the pH adjusted to 6.3 by addition of 0.2 *N* NaOH using methylred as an indicator; 150 ml distilled

water were then added followed by 50 ml borate buffer containing 8 g boric acid + 40 g $Na_2B_4O_7 . 10 H_2O$ per liter [4]. Ammonia was distilled off, collected in boric acid and titrated with 0.02 *N* H_2SO_4. After distilling off the ammonia the samples were concentrated to a volume of 100 ml in the same Kjeldahl flask. Ten ml of a 50 per cent NaOH-solution were than added, and the samples further concentrated to 40 ml. During the latter procedure ammonia, derived from the breakdown of soil organic matter, which interferes with the nitrate estimation was driven off. The nitrate in the concentrated solutions was reduced by $FeSO_4$ and $AgSO_4$ and the resulting ammonia distilled off as in the method of Cotte and Kahane [8].

Organic soil nitrogen. The amount of organic soil nitrogen was calculated by subtracting the inorganic and root nitrogen fron the total nitrogen.

Total nitrogen in the grass shoots. Immediately after being clipped off, the grass shoots were dried under forced ventilation. The dried material was weighed and ground and duplicate samples analysed by digestion with conc. H_2SO_4 and a mixture of Se, $CuSO_4$ and K_2SO_4. Ammonia was estimated by distillation into 10 ml of a 2 per cent boric acid solution followed by titration with 0.02 *N* H_2SO_4. The moisture content of the samples was determined at the time of the nitrogen analyses.

Estimation of isotopic composition. In the case of samples containing N^{15} the ammonia obtained in the various distillations was distilled off again and collected in 0.1 *N* HCl. After conversion of the ammonia into N_2 in a Rittenberg vessel using NaOH and NaOBr (sodium hypobromite), the N^{15}-abundance was estimated by means of a Metropolitan Vickers mass spectrometer.

Example of calculation

In order to facilitate understanding of the data presented in the tables, an example of the calculations made for the contents of a pot containing living plants is given below (pot no 3, Tables 4a and 4b).

In the fraction 'plant-N + inorganic N', 278 mg N were present on 12 June. This fraction contained 129 mg N on 14 May, *i.e.* there had been an increase of 149 mg N of which 141 mg was labeled and had consequently been derived from the fertilizer added. In Table 4b this is indicated by the symbol P15. The remaining increase in N, 149 — 141 = 8 mg N, had been derived from the soil organic nitrogen (P14). Of the fertilizer N, 11 mg had been immobilized (I15). Thus the net mineralization of nitrogen, P14–I15, was 8 — 11 = —3 mg N. In calculating the amount of mineralized soil nitrogen which had been re-immobilized (I14), the following equation was used:

$\frac{I14}{I15} = \frac{P14}{P15}$; this is based on the fact that both the immobilized nitrogen and the nitrogen taken up by the plants had been derived from the same mixture of mineralized soil nitrogen and fertilizer nitrogen.

Consequently,

$$I14 = \frac{P14 \times I15}{P15}$$

TABLE 2

Distribution of nitrogen on three different dates (mg N per pot) in the contents of pots containing living grass plants and to which fertilizer nitrogen was not added

Series	Date of analysis	Shoots	Roots	Inorganic N	Plant-N + inorganic N
I*	14 May	58	58	13	129
II**	31 May	62	63	3	128
III**	12 June	61	64	3	128

* Mean values of 5 pots.
** Mean values of 3 pots.

In this example I14 was equal to $8 \times 11/141 = 0.6$ mg N. The gross mineralization (P14 + I14) therefore amounted to $8 + 0.6 = 8.6$ mg N and the net mineralization (P14 − I15) to -3 mg N.

RESULTS

The distribution of nitrogen in the contents of the pots containing living plants, and to which fertilizer N was not added (Table 2), did not change between 14 May and 12 June, indicating that net mineralization of nitrogen did not occur during this period.

Contents of the pots with killed grass roots

The addition of 175.6 mg labeled fertilizer-N to the contents of the pots containing killed plant roots was followed, 3 hours after the addition of the fertilizer, by an increase in the unlabeled N in the root material and in the inorganic soil nitrogen (Table 3a, series IV). As far as the inorganic soil nitrogen is concerned, the increase can be explained by exchange with the labeled nitrogen (I15). The remaining increase in the unlabeled nitrogen (calculated as net mineralization in Table 3b) cannot be the result of microbial activity during these 3 hours and consequently it is difficult to explain.

During the period 14 May to 12 June a clear mineralization of organic N-compounds occurred in the contents of these pots (Tables 3a and 3b). The resulting unlabeled inorganic nitrogen was derived partly from the roots and partly from the soil organic matter (M14). The amount of immobilized fertilizer nitrogen (I15) increased during the experimental period, but this increase was smaller than the in-

TABLE 3a

Distribution of nitrogen (mg N per pot) following the addition of labeled ammonium nitrogen on 14 May, in the contents of pots containing killed grass roots

Series	Pot no	Amount of labeled fertilizer nitrogen added	Date of analysis	Root-N			Inorganic N			Root-N + inorg. N			Immobilized + fixed N^{15} (I15)
				N^{15}	N^{14}	Total	N^{15}	N^{14}	Total	N^{15}	N^{14}	Total	
I	*	0	14 May	—	58	58	—	13	13	—	71	71	—
IV	45	175.6	14 May + 3 h	2	67	69	154	18	172	156	85	241	6
	50	175.6	14 May + 3 h	2	62	64	154	19	173	156	81	237	4
Va	18	175.6	12 June	4	44	48	139	56	195	143	100	243	11
	56	175.6	12 June	3	46	49	139	60	199	142	106	248	17
Vb	27	175.6	12 August	4	41	45	123	59	182	127	100	227	18

* Average values of 5 pots.

TABLE 3b

Mineralization of soil organic matter nitrogen (M14) and immobilization + fixation of fertilizer nitrogen (I15), at different periods of time after addition of fertilizer N, in the contents of pots containing killed grass roots (mg N per pot)

Series	Pot no	Labeled N added on 14 May	Date of analysis	Increase in root-N + inorg. N after 14 May				Net mineralization
				F15*	M14**	Total N	I15	M14–I15
IV	45	175.6	14 May + 3 h	156	14	170	6	8
	50	175.6	14 May + 3 h	156	10	166	4	6
Va	18	175.6	12 June	143	29	172	11	18
	56	175.6	12 June	142	35	177	17	18
Vb	27	175.6	12 August	127	29	156	18	11

* F15 = fertilizer nitrogen (N^{15}) in root tissue and in inorganic form in the soil.
** M14 = soil nitrogen mineralized after 14 May (= increase in root-N + inorganic soil-N after 14 May).

crease in M14, so that a net mineralization occurred. The immobilization of fertilizer nitrogen was brought about by available carbonaceous material, which enabled the micro-organisms to utilize this nitrogen. However, the C–N ratio of the decomposable material was so low that more nitrogen was mineralized than was immobilized. After 12 June the amount of inorganic nitrogen present decreased (series Vb), probably due to denitrification. This is suggested by the amount of fertilizer nitrogen recovered (127 + 18 = 145) which is considerably lower than in the case of the contents of the pots analysed previously. No explanation can be given for the loss of N^{15} three hours after the addition of the labeled fertilizer.

Contents of the pots containing living plants

In the case of the contents of the pots containing living plants (Tables 4a and 4b) more labeled nitrogen had been immobilized (I15) on 31 May than was the case with the contents of the pots containing killed grass roots on 12 June. In comparing the results for the contents of these two sets of pots, it should be borne in mind that the total amount of fertilizer nitrogen remained in the soil during the entire experimental period in the case of the contents of the pots containing dead grass roots, whereas in the case of the pots containing living plants the greater part of the fertilizer N was removed by the crop. This might mean that where there was a low concentration of fertilizer nitrogen the value for I15 in the case of the contents of the pots containing killed grass roots would have been condersiably lower still.

Effect of clipping. Clipping of the grass shoots on 31 May caused a decrease in I15 as indicated by the result of the analyses, on the 12 June samples. This is a strong indication that the presence of living plants did not inhibit nitrogen mineralization. In the case of the pots in which the grass shoots were not clipped on 31 May (series VIII) the amount of fertilizer nitrogen in the soil organic matter was approximately the same on 12 June as that found on 31 May. If it is assumed that mineralization of immobilized N^{15} in the contents of the pots containing plants clipped on 31 May has proceeded in the same way as in the contents of the pots containing plants which were not clipped on 31 May, the higher values for I15 in the contents of the latter pots have been due to a larger quantity

of carbonaceous matter excreted by the roots resulting in re-immobilization of the mineralized N^{15}.

The mineralization of unlabeled soil nitrogen in the contents of pots containing living plants (P14 in series VI, VII and VIII) was less than M14 in the contents of the pots containing killed grass roots. Even the gross mineralization of N in the contents of the pots containing living plants was lower than M14 in the contents of the pots containing killed grass roots (Table 3b). This might indicate that, in contrast to the conclusion reached in the foregoing paragraph, the presence of living plants does inhibit the mineralization of soil organic nitrogen compounds.

The decrease in I15 (mineralization of previously immobilized N^{15}), due to clipping of the grass shoots on 31 May, was attended by increased mineralization of unlabeled soil nitrogen (P14 in Table 4b, series VII). Since the pool of labeled soil organic nitrogen amounted to no more than 20 mg N for the contents of a pot and the pool of unlabeled nitrogen to approximately 950 mg N, the relatively rapid release of N^{15} (from I15) as contrasted with the slow release of N^{14} (from soil organic compounds) points to a clear difference in availability of the two types of soil organic nitrogen.

Addition of unlabeled $(NH_4)_2SO_4$. The application of an additional amount of ammonium nitrogen (100 mg N^{14} per pot) on 31 May did not result in a decrease in I15 – the labeled nitrogen immobilized from 14 May to 31 May (Table 5). These results are in conflict with the concept of a continuous mineralization-immobilization cycle (see Introduction), according to which dilution of mineralized, labeled soil nitrogen with N^{14} would have decreased I15. To explain this inconsistency, it might be suggested that the decomposition of immobilized N^{15}-compounds, and probably that of N-compounds in general, in permanent grassland does not proceed as far as ammonia, but only to such compounds as peptides, amino acids etc, which are utilized by microorganisms without being deaminated. Soil microorganisms immobilizing nitrogen in their cells probably utilize these sources of nitrogen in preference.

If this hypothesis is correct the calculations of I14 will be affected. This calculation (see example of calculation) is based on the fact that both the immobilized nitrogen and the nitrogen taken up by the plant are derived from the same mixture of mineralized soil nitrogen

TABLE 4a

Effect of clipping the grass shoots on the distribution of labeled and unlabeled nitrogen in the contents of pots containing living grass plants (mg N per pot)

Series	Pot no	Labeled fert. N added on 14 May	Clipping date	Date of analysis	Grass shoots			Roots			Inorg. N total	Plant-N + inorg. N			Imm. + fixed N^{15} (115)
					N^{15}	N^{14}	Total N	N^{15}	N^{14}	Total N	$N^{14}+N^{15}$	N^{15}	N^{14}	Total N	
I		0		14 May	—	58	58	—	58	58	13	—	129	129	—
VI	1	175.6		31 May	117	60	177	18	59	77	8	135	127	262	27
	7	175.6		31 May	108	75	183	19	60	79	8	127	143	270	19
	11	175.6		31 May	114	71	185	20	55	75	8	134	134	268	17
VII	3	175.6	31 May*	12 June	124**	80**	204**	17	54	71	3	141	137	278	11
	13	175.6	31 May*	12 June	119**	87**	206**	17	58	75	3	136	148	284	15
	47	175.6	31 May*	12 June	121**	90**	211**	15	54	69	3	136	147	283	10
VIII	24	175.6	Shoots	12 June	111	79	190	25	63	88	3	136	145	281	22
	12	175.6	not clip-	12 June	104	75	179	26	65	91	3	130	143	273	27
	37	175.6	ped on	12 June	112	71	183	23	58	81	3	135	132	267	19
	66	175.6	31 May	12 June	116	70	186	25	64	89	3	141	137	278	18

* Grass clipped at 5 cm above soil surface; at final analysis, grass clipped at soil level.

** Grass shoots of 31 May + those of 12 June.

TABLE 4b

Effect of clipping of grass shoots on gross mineralization and net mineralization of N in the contents of pots containing living grass plants (mg N per pot)

Series	Pot no	Labeled fertilizer N added on 14 May	Date of clipping grass shoots	Date of analysis	Increase of plant-N + inorg. N after 14 May			Immobilized N^{15} N^{14}		Gross mineralization	Net mineralization
					P15*	P14*	Total N	I15**	I14**	P14+I14	P14−I15
VI	1	175.6		31 May	135	−2	133	27	−0.4	−2.4	−29
	7	175.6		31 May	127	14	141	19	2.1	16.1	− 5
	11	175.6		31 May	134	5	139	17	0.7	5.7	−12
VII	3	175.6	31 May	12 June	141	8	149	11	0.6	8.6	− 3
	13	175.6	31 May	12 June	136	19	155	15	2.0	21.0	4
	47	175.6	31 May	12 June	136	18	154	10	1.3	19.3	8
VIII	24	175.6	no	12 June	136	16	152	22	2.6	18.6	− 6
	12	175.6	cutting	12 June	130	14	144	27	2.9	16.9	−13
	37	175.6	on	12 June	135	3	138	19	0.4	3.4	−16
	66	175.6	31 May	12 June	141	8	149	18	1.0	9.0	−10

* P15 = labeled fertilizer nitrogen in the fraction 'plant-N + inorganic N'.
P14 = mineralized soil nitrogen in the fraction 'plant-N + inorganic N'.
** I15 = immobilized labeled fertilizer nitrogen.
I14 = re-immobilized mineralized soil nitrogen.

and fertilizer nitrogen. If the microorganisms preferentially utilize the organic nitrogenous decomposition products from soil organic matter rather than the ammonia of the added labeled fertilizer, then the value for P14 will be too low and, consequently, the calculated value for I14 has been underestimated (Tables 4a and b).

Such underestimation of P14 and I14 may also explain why the gross mineralization (P14 + I14) in the contents of pots containing living plants was lower than M14 in the contents of pots containing killed roots. The effect of living plants depends on the production of carbon energy sources which enable the microorganisms to utilize the organic N-compounds without the production of ammonia. From the results obtained it may be concluded that the mineralization of soil organic nitrogen compounds to ammonia is reduced by the presence of living plants. There is no evidence that the decomposition of soil organic compounds to products like amino acids is effected.

TABLE 5

Effect of 100 mg unlabeled N, added as $(NH_4)_2SO_4$ on 31 May, on nitrogen transformations in the contents of pots containing either grass with unclipped shoots or grass with shoots clipped on 31 May (mg labeled N per pot)

Series	Pot no	Labeled N added on 14 May*	Unlabeled N added on 31 May	Date of clipping of grass shoots	Date of analysis	Grass shoots N^{15}	Roots N^{15}	Immobilized + fixed N^{15} (115)
VII	3	175.6	—	31 May**	12 June	124***	17	11
	13	175.6	—	31 May**	12 June	119***	17	15
	47	175.6	—	31 May**	12 June	121***	15	10
IX	8	175.6	100.0	31 May**	12 June	127***	10	13
	32	175.6	100.0	31 May**	12 June	128***	12	20
	20	175.6	100.0	31 May**	12 June	130***	12	14
	67	175.6	100.0	31 May**	12 June	124***	10	23
VIII	24	175.6	—	no	12 June	111	25	22
	12	175.6	—	clipping	12 June	104	26	27
	37	175.6	—	of shoots	12 June	112	23	19
	66	175.6	—	on 31 May	12 June	116	25	18
X	9	175.6	100.0	no	12 June	121	21	18
	38	175.6	100.0	clipping	12 June	110	20	28
	40	175.6	100.0	of shoots	12 June	116	18	24
	57	175.6	100.0	on 31 May	12 June	114	18	21

* The inorganic N had almost disappeared on 31 May (as a consequence of uptake by the plants, immobilization and loss by denitrification).

** Grass shoots clipped at 5 cm above soil surface on 31 May and cut off at soil surface on 12 June.

*** Grass shoots of 31 May + those of 12 June.

Nitrogen translocation. From the data of Table 4 it will be seen that some labeled nitrogen was translocated from the grass shoots to the roots of intact plants (shoots not clipped on 31 May) during the period 31 May to 12 June. Clipping of the grass shoots on 31 May resulted in upward transport of labeled nitrogen from the soil and from the roots to the grass shoots (series VII). This is in accordance with the results from previous investigations [22]. The supply of an additional 100 mg of unlabeled N on 31 May (Table 5) promoted the upward movement of labeled nitrogen from the roots to the shoots. This translocation was more pronounced where the

TABLE 6

Effect of living grass plants and killed grass roots, respectively, on the distribution of labeled N, previously incorporated into the grass plant tissues, in the contents of the pots (mg labeled N per pot)

Series	Pot no	Labeled N added on 14 May	Treatment	Date of analysis	Grass shoots N^{15}	Roots N^{15}	Inorg. N^{15}	Immobilized + fixed N^{15} (I15)
VI	1	175.6		31 May	117	18	—	27
	7	175.6		31 May	108	19	—	19
	11	175.6		31 May	114	20	—	17
XI	31	175.6	Grass shoots	12 August	115**	17	—	24
	41	175.6	clipped	12 August	112**	17	—	28
	68	175.6	on 31 May*	12 August	114**	18	—	26
XII	6	175.6	Grass roots	12 August	115***	6	15	14
	22	175.6	killed	12 August	116***	8	18	12
	54	175.6	on 31 May	12 August	114***	8	18	14

* Grass clipped at 5 cm above soil surface and cut off at soil surface on 12 August
** Grass shoots of 31 May + those of 12 August.
*** Grass shoots of 31 May.

grass shoots were clipped on 31 May than where they remained unclipped.

In the case of the contents of the pots containing roots killed by cutting off the grass shoots at soil level on 31 May (Table 6) one third of the labeled nitrogen previously immobilized in soil organic matter and more than half of the labeled nitrogen in the roots was mineralized during the period between 31 May and 12 August. Clipping of the grass shoots on 31 May had not changed the N^{15}-distribution pattern when this was determined on 12 August (series XI) in contrast to that of the contents of the pots analysed on 12 June (series VII Tables 4a and 7).

The shoots of the grass plants of the pots of series XI had not been clipped between 31 May and 12 August. When harvested on 12 August 49 mg labeled nitrogen were present in the grass shoots and roots of this series, while 58 mg were present in the plants of series VII which were harvested on 12 June (Table 7). From these findings, the amount of immobilized labeled nitrogen had increased by 14 mg

TABLE 7

Effect of ageing of plants on the re-distribution of previously incorporated labeled nitrogen (mg N per pot)

Series	Pot no	Labeled N added on 14 May	Date of clipping grass shoots	Data of analysis	N^{15} contents of grass shoots			Grass roots N^{15}	Immobilized + fixed N^{15}	Plant tissue-N $N^{14}+N^{15}$
					Cutting date					
					31 May	12 June	Total			
VII	3	175.6	31 May	12 June	80	44	124	17	11	275
	13	175.6	31 May	12 June	78	41	119	17	15	281
	47	175.6	31 May	12 June	81	40	121	15	10	280
					31 May	12 August				
XI	31	175.6	31 May	12 August	83	32	115	17	24	276
	41	175.6	31 May	12 August	82	30	112	17	28	280
	68	175.6	31 May	12 August	79	35	114	18	26	281

between 12 June and 12 August. Thus during this period labeled nitrogen had been transferred from the grass plants to the soil. Such a transfer has also been observed by Woldendorp *et al.*[23] in the case of ageing perennial ryegrass plants. Since no change in total nitrogen content of the plants (N^{14} + N^{15}) had taken place between 12 June and 12 August, labeled nitrogen in the plants must have been replaced by unlabeled soil nitrogen, which became available during the experimental period. This indicates that the decomposition of organic matter was not inhibited by the presence of living plants. Consequently, when the net mineralization of N in the contents of pots to which fertilizer N was not added (*e.g.* series I, II and III) is nil during a certain period, this might be the net result of immobilization of nitrogen which has been transferred from the plants to the soil and of the uptake of nitrogen derived from decomposition of organic nitrogen compounds in the soil.

DISCUSSION

A net mineralization of nitrogen was not observed, during the period 14 May to 12 June, in the contents of pots to which fertilizer N had not been added and containing living grass plants. In those cases where fertilizer nitrogen (as labeled $(NH_4)_2SO_4$) had been added, the net mineralization of N during this period was negative

(Table 4b, series VI and VIII). In spite of this, part of the nitrogen in the plants had been derived from unlabeled organic nitrogen compounds of the soil (P14). In contrast to this result, unlateled ammonium sulphate added during the above-mentioned period, did not promote the liberation of previously immobilized labeled soil nitrogen (Table V). Tyler and Broadbent [19] arrived at the same conclusion in the case of soil in which Sudan grass had grown, but without presenting convincing evidence. Their statement was based only on a comparison of the percentage of labeled nitrogen in the shoots (dry matter = 100) before and after the addition of unlabeled fertilizer N, the percentages being lower after the addition of unlabeled fertilizer N.

In a previous experiment in which samples of turf were used [10], two dosages of labeled nitrogenous fertilizer were applied, *viz* 58.5 and 153.9 mg N as $(NH_4)_2SO_4$. At the end of the experiment the amount of unlabeled soil nitrogen which had been taken up by the grass plants was the same in both cases, indicating that the higher dosage of fertilizer nitrogen had not resulted in increased uptake of soil nitrogen by the plants.

The results from the experiments with turf samples do not agree with those obtained when arable soil was used since in the latter case increased levels of added fertilizer nitrogen were accompanied by an increased uptake of soil nitrogen [1 2 6 7 13 15 16 17 18 20 21]. To explain the results of the experiments obtained when arable soil was used, three hypotheses have been put forward in the literature:

1. That the increased mineralization of soil organic matter is due to the stimulation of the soil-microflora by the added nitrogenous fertilizer.

2. That the organic compounds utilized by the microorganisms of the rhizosphere are derived from roots with C–N-ratios reduced as a result of the addition of fertilizer nitrogen [13 14]. Under these conditions the micro-organisms can obtain adequate amounts of nitrogen from decaying root hairs, sloughed off root cells and root excretions and do not utilize the mineralized soil nitrogen which would therefore be available for uptake by the plants.

3. That there is interchange of fertilizer nitrogen for soil nitrogen during the immobilization-mineralization cycle [17]. This hypothesis could largely explain the results from many experiments. Almost all the investigations recorded in the literature are performed with

seedling plants supplied with labeled fertilizer nitrogen before or at the time of sowing. At the start of the experiments the plants have little influence on the nitrogen transformations so that mineralization of soil nitrogen will proceed to ammonia and nitrate and such mineralized soil nitrogen will be diluted with the labeled fertilizer N. During the growth of soil microorganisms labeled fertilizer nitrogen will be utilized as well as mineralized soil nitrogen, thus allowing more soil nitrogen to be available for uptake by plants than in the absence of fertilizer N.

In samples of turf with living grass plants the conditions obtaining are quite different and it is thought that these are the cause of the differing results. Plant density is high in turf and, in contrast with arable land, living roots occupy a considerable proportion of the soil. This leads to a rapid uptake of added fertilizer nitrogen by the plants, so that its dilution effect (hypothesis 3) is of little importance.

A further result of the high root density in turf is the presence of relatively large amounts of plant residues and root exudates. This brings about a high level of microbial activity and probably a high demand for nitrogen compounds. Under these conditions the N mineralization-immobilization cycle apparently proceeds only as far as compounds such as amino-acids (*cf* the data of Table 5) due to the fact that microorganisms utilize amino acids more readily than inorganic nitrogen as a source of nitrogen.

Concerning the immobilization of mineral nitrogen two possible mechanisms may be considered (Woldendorp [22]). Firstly, mineral nitrogen may be immobilized in soil organic matter by way of higher plants. The results recorded in Table 7 show that labeled nitrogen was transferred from the grass plants to the soil. This results from the excretion of nitrogenous compounds by roots and the decay of dead roots. Secondly, fertilizer nitrogen may be immobilized directly in microbial cells. The results of the experiments with soil containing dead grass roots showed a net mineralization of soil nitrogen. Obviously, under these conditions carbonaceous material did not become available sufficiently rapidly to re-immobilize the mineralized soil nitrogen. However, when living plants were present the net mineralization of N was negative (series VI and VIII). This indicates that in the rhizosphere of grass plants microorganisms immobilize fertilizer nitrogen, using root excretions, dead root hairs and root cells as the carbon source [3 13]. Apparantly there is a continuous supply of

carbonaceous material from the living plants. These conditions occur in permanent grassland; they are probably a very important cause of the accumulation of nitrogen in the form of organic compounds in these soils.

According to Haider *et al.*[9] and Ladd *et al.*[12] a biochemical process may be involved in nitrogen accumulation in permanent grassland, viz. the reaction of fertilizer nitrogen (NH_4–N), soil organic nitrogen compounds (*e.g.* amino acids or proteins) or metabolized plant constituents with polyphenols.

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AMINO ACID COMPOSITION OF HUMIC ACID-LIKE POLYMERS PRODUCED BY STREPTOMYCETES AND OF HUMIC ACIDS FROM PASTURE AND ARABLE LAND

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Summary—Strains of streptomycetes, cultivated in a glycerol-nitrate medium, synthesized humic acid-like substances. With one strain 13·7 per cent of the NO_3^-–N in the medium was converted into humic acid-like substances after 59 days. Hydrolysis with 6 N HCl released 40 per cent of the 'humic acid' N as NH_4^+– and amino acid–N.

With humic acids isolated from soils of arable land and 10-yr-old pasture about 60 per cent of the humic acid N was found to be present as NH_4^+– and amino acid–N in the hydrolysate.

The amino acid patterns of the hydrolysates of pasture soil, arable soil and the humic acids of these soils were rather similar, resembling the amino acid composition of the hydrolysates of the 'humic acids' of two *Streptomyces* strains.

INTRODUCTION

IN PREVIOUS studies (Huntjens, 1971a, 1971b) the accumulation of soil organic nitrogen in permanent pastures was explained by the continuous accretion of degradation products of dead roots and root exudates from the living plants in the soil. The C–N ratio of these root compounds is apparently so high that all the nitrogen from decomposing soil organic compounds is used as a nitrogen source by microorganisms and immobilized in cell material as soon as it is released. The exudates of wheat roots contain organic acids, sugars and amino acids; these compounds can be expected to occur also in root exudates of grasses (Clark and Paul, 1970).

Several investigators have established that fungi and streptomycetes synthesize phenolic compounds from non-aromatic carbon and nitrogen sources. These compounds give rise to the formation of dark-coloured, humic substances (Martin and Haider, 1971).

Wieringa (1958) and Woldendorp (1963) pointed out that the actinomycetes constitute a greater part of the total cell count in grassland than in non-grassland soil.

In the present study the production of humic acid-like polymers by several streptomycetes in a glycerol–nitrate medium was investigated. In order to obtain more information about the contribution of streptomycetes to the accumulation of soil organic nitrogen in pastures, a comparison of the amino acid composition of hydrolysates of the humic acid-like substances from streptomycetes and of hydrolysates of soil organic matter from arable land and from pasture was made.

MATERIALS AND METHODS

Microorganisms, media and cultivation

Four strains of *Streptomyces* from a collection of actinomycetes, isolated from soil beneath pasture, were used. They were kept on slants containing 1·0% glucose, 0·7%

yeast extract and 1·2% agar. With two strains the slants turned dark brown (strain A) and light brown (strain B), respectively, but strains C and D produced no pigment on this medium. The four strains grew very well on a glycerol–nitrate medium.

For production of humic acid-like substances the cultures were grown in a medium containing 10 ml glycerol, 0·5 g $MgSO_4.7\ H_2O$, 1·25 g K_2HPO_4, 2·16 g KNO_3, 1 g $CaCO_3$ and 0·1 g yeast extract/l. of tap water. The following trace elements were added: 10 mg $FeCl_3.6\ H_2O$ and 1 ml of a mixture containing 0·1 g $CuSO_4.5\ H_2O$, 1·0 g $ZnSO_4\ .7\ H_2O$, 1·0 g $MnSO_4.7\ H_2O$, 10 mg Na_2MoO_4, 10 mg H_3BO_3 and 10 mg $CoCl_2$/l. The pH of the nutrient solution was adjusted to 7·0 with 10% HCl.

The spores of 7-day-old slants of the *Streptomyces* strains were inoculated into 1 l. of sterile nutrient solution contained in 5 l. Erlenmeyer flasks and the flasks were incubated on a shaker at 30°C for several weeks.

Isolation of humic acid-like polymers from Streptomyces *culture medium*

To isolate humic acid-like polymers, the cultures were centrifuged at 10,000 *g* and the pelleted cells were extracted twice with 0·1 N NaOH solution and centrifuged. The supernatants were added to the culture filtrate and filtered to remove suspended material. The humic acid-like substances were precipitated by adjusting the pH of the solution to 1·0 with conc. HCl. After standing overnight, precipitates were collected by centrifugation at 600 *g*, washed twice with 0·1 N HCl and redissolved in 0·5 N NaOH. Total nitrogen was determined by the Kjeldahl procedure (Bremner, 1965a).

Soils and extraction of soil humic acid

Soils from 10-yr-old pasture and arable land, both established on the same sandy soil were compared. The pH of these soils was 6·0. The soil organic nitrogen contents were 1·37 and 0·82 g/kg, respectively, on a dry weight basis. The soil samples were air-dried, sieved (1·0 mm), and soil humic acids isolated (Stevenson, 1965).

Hydrolysis of soils and humic acids

A sample of soil or humic acid, containing about 10 mg N, was placed in a 250-ml round-bottom flask and hydrolysed (Bremner, 1965b). After washing the hydrolysis residue with small portions of water, the hydrolysate was evaporated to about 15 ml at 40°C in a rotary-evaporator. The distillation procedure was repeated twice, each time adding about 50 ml of distilled water to the flask before distillation. Water was added and the extract evaporated to dryness. This last treatment was repeated until the HCl had almost completely disappeared. The residue was taken up in 20 ml citrate buffer pH 2·20 and further purified by centrifugation for 10 min at 10,000 *g*.

Analysis of hydrolysates

The amino acid composition of 0·5-ml aliquots of each purified hydrolysate was determined (Moore and Stein, 1954) with the aid of a Biocal 200 Amino Acid Analyser. Acid and neutral amino acids were eluted from a column containing 52 × 0·9 cm Biorad A6 spherical ion exchange resin, and the basic amino acids were separated on a 22 × 0·9 cm Biorad A5 ion exchange resin column (Biocal instruction sheets).

A sample of casein was hydrolysed and analysed in the same way as described above for comparative purposes.

RESULTS

Production of humic acid-like substances in the Streptomyces *cultures*

The culture solutions of the *Streptomyces* strains A, B, C and D had turned brownish-red, yellow, yellow and colourless, respectively, after 3 days of incubation (Table 1). After 2 weeks, these culture solutions were black, reddish-brown, yellow and light yellow, respectively.

TABLE 1. 'HUMIC ACID' PRODUCTION BY DIFFERENT STRAINS OF *Streptomyces* AFTER DIFFERENT PERIODS OF INCUBATION, N CONTENT EXPRESSED AS PERCENTAGE OF NO_3–N ORIGINALLY PRESENT IN THE NUTRIENT MEDIUM

Incubation time (days)	Strain A			Strain B		
	Colour* of nutrient medium	Final pH	% of NO_3^-–N	Colour of nutrient medium	Final pH	% of NO_3^-–N
16	B	8·9	6·0	RB	8·9	2·1
21	B	8·9	8·5	RB	9·0	1·9
50	B	8·9	6·9	RB	9·3	1·3
64	B	8·9	7·2	RB	ND	ND
	Strain C			Strain D		
16	Y	8·4	0·5	LY	8·2	0
21	Y	ND	ND	LY	ND	ND
50	Y	ND	ND	LY	ND	ND
64	Y	ND	ND	LY	ND	ND

* B, black; RB, reddish-brown; Y, yellow; LY, light yellow.
ND—Not determined.

The initial pH of the medium was 7·0. After inoculation it increased quickly in the medium with $CaCO_3$ (Table 1) but slowly in the medium without $CaCO_3$ (Table 2).

The different strains produced different amounts of humic acid-like substances. A higher production of these substances was accompanied by the formation of more dark pigment in the glycerol–nitrate medium (Table 1). Strain D which had formed no black pigment did

TABLE 2. 'HUMIC ACID' PRODUCTION BY *Streptomyces* STRAIN A, N CONTENT EXPRESSED AS PERCENTAGE OF NO_3^-–N ORIGINALLY PRESENT IN THE NUTRIENT MEDIUM*

Incubation time (days)	Colour of nutrient medium	Final pH	Humic acid N (% of NO_3^-–N)
6	YB†	7·5	3·1
13	B	7·2	8·5
19	B	7·2	8·5
38	B	8·5	10·8
59	B	8·9	13·7

* The nutrient medium contained 0·125 g/l $CaCl_2.2\,H_2O$ instead of 1 g/l $CaCO_3$.
† YB, yellowish-brown; B, black.

not produce 'humic acid'. Increasing amounts of 'humic acid' were formed with increasing age of the culture of strain A, which had received no $CaCO_3$ in the medium (Table 2). After 59 days 13·7 per cent of the original amount of NO_3^-–N had been converted into humic acid-like substances.

Nitrogenous compounds in soils and in humic acids of arable land and pasture

The occurrence of various nitrogenous compounds in soil of arable land and pasture was estimated after hydrolysis with HCl, and expressed as a percentage of total soil nitrogen (Table 3). Hydrolysis of the pasture soil liberated higher percentages of the different types of amino acids but a slightly lower percentage of NH_4^+ than was the case with arable soil. In hydrolysed soil samples from arable land 53·5 per cent of total soil nitrogen was amino acids and NH_4^+. In the pasture soil this figure amounted to 59·7 per cent.

TABLE 3. NITROGENOUS COMPOUNDS FROM HYDROLYSED SOILS AND FROM HYDROLYSED HUMIC ACID PREPARATIONS OBTAINED FROM SOIL AND FROM CULTURES OF STREPTOMYCETES

	Amino acid N as % of total N of soil or of humic acid									
					'Humic acid'					
	Soil		Humic acid		Age of culture (days) Strain A			Strain B		
N compounds	Arable land	Pasture	Arable land	Pasture	21	50	64	21	50	Casein
Neutral amino acids	22·7	26·8	25·3	27·8	22·7	20·0	17·1	36·8	30·2	48·3
Acidic amino acids	7·9	9·2	8·8	8·9	7·0	5·6	4·9	9·2	7·5	20·7
Basic amino acids	12·0	14·3	15·2	16·4	8·7	10·4	9·5	12·4	7·1	23·0
Total amino acid N	42·6	50·3	49·3	53·1	38·4	36·0	31·5	58·4	44·8	92·0
Ammonium N	10·9	9·4	10·2	9·3	3·7	5·2	4·6	6·9	8·4	7·3
Total amino acid N + ammonium N	53·5	59·7	59·5	62·4	42·1	41·2	36·1	65·3	53·2	99·3

The nitrogen of humic acids isolated from soils of arable land and pasture represented 27·4 and 32·5 per cent, respectively, of the total soil organic nitrogen. Upon hydrolysis with HCl approximately 60 per cent of the nitrogen present in the humic acids was accounted for as amino acid- and NH_4^+–N (Table 3). This value was slightly higher in humic acids derived from grassland than in those from arable land. The same was true of the different types of amino acids. However, NH_4^+–N (in % of total humic acid nitrogen) released by hydrolysis of humic acids from arable land was slightly higher. These results agree with those obtained from acid hydrolysis of whole soil.

Nitrogenous compounds in humic acid-like substances from cultures of streptomycetes

The amount of nitrogen present in the form of amino acids and NH_4^+ in the hydrolysates of the humic acid-like substances from strains A and B (calculated as percentages of the total nitrogen in the 'humic acid') decreased with increasing age of the cultures (Table 3). These amounts were lower in the humic acids from strain A (42·1–36·1 per cent) than in those from strain B (53·2–65·3 per cent). The percentages of NH_4^+–N in the hydrolysed 'humic acids' tended to increase with age of the culture, in contrast to most of the different groups of amino acids which decreased.

When a comparison is made between the various groups of amino acids contained in humic acids from soil and from streptomycetes, it will be seen that the humic acid-like material from strain B resembled the soil material more closely than that of strain A. This is true of the acidic and basic amino acids and of NH_4^+. Neutral amino acid contents were higher in the humic acid of strain B than in that of the soil. In the humic acid-like material from strain A all of the nitrogenous fraction contents measured were lower than those of soil.

Comparison of the amino acid composition of hydrolysable fractions of soils and humic acids from arable land, pasture and Streptomyces *strains*

To compare the amino acid composition of the hydrolysable fractions of the different samples tested, the amount of nitrogen of each estimated amino acid has been calculated as percentage of the total amino acid nitrogen of each sample. These calculations (Table 4) show that the amino acid patterns of both soils tested (from arable land and pasture, respectively) were very similar. The same was true of the two humic acid hydrolysates of both soils. However, there were small differences between the soil samples and the humic

TABLE 4. AMINO ACID COMPOSITION OF ACID HYDROLYSATES OF SOILS, HUMIC ACID PREPARATIONS FROM SOILS AND 'HUMIC ACIDS' ISOLATED FROM CULTURES OF STREPTOMYCETES

	N in amino acid as % of total amino acid N									
					'Humic acid'					
	Soil		Humic acid		Age of cultures (days) Strain A			Strain B		
Amino acid	Arable land	Pasture	Arable land	Pasture	21	50	64	21	50	Casein
Neutral	53·3	53·3	51·4	52·3	59·0	55·6	54·2	62·9	67·3	52·7
Cysteic acid	1·2	0·8	0·6	0·6	0·1	0·3	0·3	1·7	0·2	0·1
Threonine	4·7	4·8	4·9	5·6	4·2	3·9	3·8	5·0	4·9	3·5
Serine	5·6	5·0	5·7	5·6	3·9	4·2	3·5	5·1	5·8	5·8
Proline	5·4	5·4	3·9	4·3	6·0	3·9	4·1	5·8	4·2	9·9
Glycine	12·2	11·7	10·6	10·0	9·6	10·6	10·8	11·1	12·5	2·7
Alanine	8·4	8·6	7·5	7·5	11·2	10·3	10·1	10·8	11·6	3·5
Valine	4·5	4·8	5·1	5·1	5·7	5·0	5·1	7·0	7·4	5·8
Methionine	1·4	1·2	1·2	1·1	2·9	3·3	3·2	1·5	2·0	2·3
Isoleucine	3·1	2·8	3·0	3·0	3·6	3·3	3·2	3·4	3·6	4·0
Leucine	4·5	4·6	5·1	5·1	7·0	6·7	6·0	7·5	9·4	8·0
Tyrosine	0·7	1·0	1·2	1·5	2·1	1·7	1·6	2·4	2·5	3·6
Phenylalanine	1·7	2·8	2·6	2·8	2·9	2·5	2·5	3·1	3·4	3·5
Acidic	18·6	18·3	17·9	16·7	18·2	15·6	15·5	15·7	16·7	22·6
Glutamic acid	8·7	8·0	7·7	7·1	9·4	8·6	8·5	7·5	8·4	16·8
Aspartic acid	9·9	10·3	10·2	9·6	8·8	7·0	7·0	8·2	8·3	5·8
Basic	28·2	28·5	30·9	30·8	22·6	28·9	30·1	21·2	15·8	25·1
(a)*	6·4	7·2	4·5	3·9	1·0	2·0	2·2	0·3	0·5	0
(b)*	4·9	4·2	4·1	3·6	1·6	2·5	3·2	0·3	0·5	0
Lysine	5·6	5·6	7·7	7·5	5·2	6·1	6·3	4·1	3·6	10·0
Histidine	2·8	3·6	4·9	4·5	5·5	5·0	5·7	4·1	3·3	5·9
Arginine	8·5	8·0	9·7	11·3	9·4	13·3	12·7	12·3	8·0	9·2

* With the assumption that each molecule of (a) and (b) contains one atom of nitrogen (these compounds were not identified).

acid samples. Valine, leucine, tyrosine, lysine, histidine and arginine contents were slightly lower and proline, glycine, alanine, glutamic acid and the unidentified amino acid (a) contents slightly higher in the soil hydrolysates than in the hydrolysates of the humic acids derived from the soils.

Although the amino acid composition of the hydrolysed *Streptomyces* 'humic acids' differed from those of the soil humic acids, much more pronounced differences existed between the humic acid hydrolysates and the casein hydrolysate. This was particularly true of proline, glutamic acid, lysine and histidine which were about twice as high in casein than in the humic acids and of glycine, alanine and aspartic acid which were much lower in casein. The unknown ninhydrin positive peaks (a) and (b) did not occur in casein. Small differences in amino acid patterns were also observed between both strains of *Streptomyces* and between *Streptomyces* cultures after 21, 50 and 64 days incubation. In addition to the amino acids recorded in Table 4, trace amounts of other compounds were observed on the chromatograms obtained from hydrolysates of soil and soil humic acids, viz. ethanolamine, one small peak between lysine and histidine and another small peak between ammonium and arginine.

DISCUSSION

Certain microorganisms on ageing are able to synthesize humic acid-like compounds. Since streptomycetes are among the dominant microorganisms in pasture soil, the capacity of some strains of these organisms to synthesize humic acids has been tested. The yield of such compounds was rather high with *Streptomyces* strain A grown in the medium without $CaCO_3$; 13·7 per cent of the applied NO_3^-–N was converted into 'humic acid' nitrogen. From the data of Von Plotho (1950) and Scheffer *et al.* (1950) it may be calculated that 6–10 per cent of glutamic acid nitrogen, which was used as the nitrogen source for streptomycetes in their experiments, was converted into 'humic acid' nitrogen after 40 days. The occurrence of many amino acids in the hydrolysates of humic acid-like substances, isolated from cultures of streptomycetes in the present study, agrees with the qualitative results of Matschke (1970).

The humic acid-like substances collected from the cultures of *Streptomyces* strain A released less nitrogen in the form of amino acids and NH_4^+ upon hydrolysis with 6 N HCl than the soil humic acid or soil organic matter. Ladd and Butler (1966) demonstrated that synthetic phenolic polymers, incorporating individual amino acids, release less polymer nitrogen upon acid hydrolysis than phenolic polymers incorporating peptides and proteins. The latter polymers resemble the natural humic acids more closely than the former ones. The results of Ladd and Butler (1966) might suggest that humic acids obtained from soil might have incorporated more peptides and proteins than those isolated from the *Streptomyces* cultures of strain A. According to many contemporary investigators, the formation of humic materials is not enzymatically controlled, but is the result of the spontaneous condensation of microbial degradation products (Flaig, 1966). The occurrence of proteins in an environment is partly dependent on the presence and activity of proteolytic enzymes. Streptomycetes are known to be proteolytic and the proteolytic activity may have been higher in the *Streptomyces* culture than in the soil during the formation of humic substances. Therefore, less protein and more separate amino acids may have been incorporated in the *Streptomyces* 'humic acids' than in those of the soil.

Quantitative comparison of the amino acids released after acid hydrolysis show that *Streptomyces* 'humic acids' resemble natural humic acids and suggests that streptomycetes may contribute to the humus formation in soil. Since the amino acid distribution in hydro-

lysates of arable and pasture soil is rather similar, the results obtained in the present study do not allow the conclusion that streptomycetes contribute to a larger extent to humus formation in pasture than in arable land. The similarity in amino acid pattern of hydrolysed arable and pasture soil agrees with the results of Keeney and Bremner (1964), who found that cultivation of virgin soils led to a marked decrease in all fractions of nitrogen, but, on the average, had little effect on the relative distribution of different fractions of nitrogen upon acid hydrolysis.

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AVAILABILITY OF MICROBIAL AND SOIL ORGANIC NITROGEN TO A *PSEUDOMONAS* STRAIN AND THE EFFECT OF SOIL ORGANIC MATTER ON THE AVAILABILITY OF CASEIN NITROGEN

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Summary—A microbiological method has been devised to determine the availability of microbial and soil organic N to a proteolytic *Pseudomonas* strain. The growth of the *Pseudomonas* sp. on soil fractions or on microbial material as the sole N source was compared with the growth on casein-N.

The nitrogenous constituents of 3-day-old *Arthrobacter* cells were found to be more resistent to degradation than those of 6-day-old cells during an incubation period of 4 days.

The percentage of available soil organic N and available humic acid N increased with the pH of the medium in which the availability was determined. The percentage of available organic N in grassland soil was twice that in arable soil.

The proportion of N utilized by the proteolytic *Pseudomonas* sp. from humic acids extracted from the soil with sodium pyrophosphate (pH 7) was four times higher than that utilized from the total soil organic matter. When NaOH had been the extractant of the humic acid fraction, the relative availability of the N of the humic acid fraction was twice as high as that of the entire soil organic matter.

The availability of N of humic acid-like compounds, synthesized by a *Streptomyces* strain, to the *Pseudomonas* sp. was similar to that of humic acids extracted from grassland soil by NaOH, viz. 11–25 per cent (depending upon the pH of the culture solution) of the total N contained in the humic acid fraction.

The availability of casein-N decreased in the presence of soil or soil humic acids. This reduction of casein assimilation was partly offset by the addition of $CaCO_3$ to the test medium; it appeared to be a pH effect.

The results obtained suggest that more protein-like compounds are incorporated in the soil organic matter of grassland than in that of arable land.

INTRODUCTION

SOILS developed under grass generally have a higher organic nitrogen content than soils of arable land. Ploughing up of permanent grassland is followed by a rapid decline of the organic matter and organic nitrogen content ('T Hart, 1950). The conditions in soils of grassland probably favour the retention of soil organic nitrogen as humic substances. The literature on this subject has been discussed by Stevenson (1965) and Harmsen and Kolenbrander (1965). The mechanism responsible for the differences in effect between the soil uses is imperfectly understood. It seems probable ('T Hart, 1950) that a large proportion of the organic matter occurring in permanent grassland is stable under the conditions of grassland and unstable under the conditions of arable land. However, analytical procedures used in soil chemistry are unable to separate these two fractions. Analyses of hydrochloric acid hydrolysates have shown that the hydrolysability of the organic matter of both types of soil is rather similar (Keeney and Bremner, 1964). The same was true of the amino acid

composition of these hydrolysates (Huntjens, 1972). Studies on the hydrolysis of soil organic matter have shown that 20–50 per cent of total nitrogen in most soils occurs in the form of bound amino acids (Flaig, 1971). Several investigators have suggested that this amino acid nitrogen is incorporated as peptides or proteins in the humus molecules (Simonart *et al.*, 1967; Ladd and Butler, 1966). Treatment of soil humic acids with the proteolytic enzyme pronase liberated about one third of the amino acids released by acid hydrolysis, demonstrating the presence of peptide or protein components in soil humic acids (Ladd and Brisbane, 1967). This relatively low availability of humic acid nitrogen may be due to the fact that soil humic acids may inhibit proteolytic activity (Ladd *et al.*, 1968; Ladd and Butler, 1969a, 1969b).

In the present study a method was developed to determine the availability of organic nitrogen to a protein-decomposing *Pseudomonas* sp. The sources of organic nitrogen investigated were cells of an *Arthrobacter* sp., humus-like substances isolated from filtrates of *Streptomyces* cultures, and organic matter from arable and grassland soil. In addition the effect of humic acids and soil organic matter on the availability of casein nitrogen to the *Pseudomonas* sp. was investigated.

MATERIALS AND METHODS

Preparation of the nitrogen-containing samples

A soil *Arthrobacter* sp. was grown in a medium (pH 7·0) containing 5 g glucose, 0·5 g $MgSO_4.7\,H_2O$, 1·25 g K_2HPO_4, 0·56 g $(NH_4)_2SO_4$, 1 g $CaCO_3$ and 0·1 g yeast extract, and trace elements (Huntjens, 1972) in 1 l. of tap water to obtain microbial cell nitrogen. The glucose was sterilized separately and added to 50 ml portions of sterile medium contained in 8 conical flasks of 250 ml capacity. They were inoculated with cells from 2-day old cultures on yeast extract-glucose agar and incubated on a rotary shaker at 25°C for 3 days. The cells of four flasks were then harvested by centrifugation and washed twice with distilled water. The pellets of two of these flasks (R3) were used to determine the availability of the cell nitrogen using the *Pseudomonas* sp. as test organism. The pellets of the other two flasks were resuspended in 10 ml of water, cooled with ice and exposed to ultrasonic vibrations in a MSE ultrasonic disintegrator for 30 min at 20 kc/s. The availability of the nitrogen of the disintegrated cells (UR3) was also determined. The remaining four flask cultures received 5 ml of sterile glucose solution (50 g/l) and after incubation for a further 3 days the cells were treated in the same way as recorded above (R6 and UR6).

Samples of soil from arable land and grassland (Huntjens, 1972) were treated with 0·5 N NaOH to isolate humic acids (Stevenson, 1965) or with 0·1 M neutral sodium pyrophosphate (Bremner and Lees, 1949). The isolation of *Streptomyces* 'humic acid' from filtrates of strain A has been described (Huntjens, 1972). The washed precipitates of humic acids were redissolved in 0·5 N NaOH or dried by lyophilization.

Determination of biologically-available nitrogen

Twenty Gram-negative bacteria, isolated from grassland soil, were tested for their ability to hydrolyse casein. The organism surrounded by the largest transparent halo when grown for 2 days on agar containing casein was selected to test the microbial degradation of nitrogenous compounds in soil samples. This organism was called *Pseudomonas* strain 31.

Duplicate samples containing about 6 mg N were transferred to 250 ml conical flasks and mixed with 50 ml of the culture solution used for the growth of *Arthrobacter* cells, except that no $(NH_4)_2SO_4$ was added. To study the effect of pH during the incubation period on

the availability of organic nitrogen, $CaCO_3$ was replaced by 0·12 g $CaCl_2.2\ H_2O$/l in a number of control vessels. The pH of the contents of all the flasks was adjusted to 7·0. Glucose was sterilized separately. The flasks were inoculated with *ca.* 10^8 cells from a 2-day-old culture of *Pseudomonas* grown on yeast extract-glucose agar, incubated on a rotary shaker at 25°C for 4 days, unless otherwise stated, and then serial dilutions were made in test tubes containing 0·9% NaCl. Ten 5-μl portions of suitable dilutions were pipetted on to agar plates (10 g glucose, 7 g yeast extract and 12 g agar, in 1 l. of tap water) using an Agla-micrometer syringe outfit (syringe sterilized in alcohol, rinsed with sterile water and thereafter with the culture dilution, see Hartman, 1968). The agar plates were dried by storage at 60°C for 2 h and condensation water removed before use. The colonies in the drops were counted under a magnification of 12× after 20 h incubation at 25°C. The total number of organisms per conical flask was then calculated. The amount of available nitrogen in each sample was calculated by comparing the total number of *Pseudomonas* cells of flasks with the soil sample as the only nitrogen source with a standard curve obtained by plotting *Pseudomonas* numbers against known amounts of added casein. Such a standard curve was set up for every series of determinations.

Ethylene oxide sterilization

Soils and humic acids were sterilized with ethylene oxide by a modification of the method of Gilbert *et al.* (1964). A vacuum vessel was filled with water to a depth of 1 cm and conical flasks containing soil or lyophilized humic acids were placed in it. The air was removed (pressure reduced to 10 mm of Hg) to allow the ethylene oxide to vaporize. The vessel was kept at room temperature for 2 days, then opened and evacuated several times to remove the poisonous vapour.

Preliminary experiments had shown that simultaneous sterilization of nutrient solution and soil with ethylene oxide was not satisfactory, as no growth occurred after inoculation with *Pseudomonas*. Therefore soil samples and freeze-dried humic acids were sterilized separately with ethylene oxide vapour at room temperature, after which the heat-sterilized (120°C) nutrient solution was added. Care was taken that the mixture had a pH of about 7. Casein and the *Arthrobacter* cells were always sterilized at 120°C.

Analytical methods

Glucose was detected with Luff–Schoorl reagent (25 g $CuSO_4.5\ H_2O$, 50 g citric acid, and 388 g $Na_2CO_3.10\ H_2O$ are dissolved in water and made up with water to 1 l.). Two ml of this reagent and 1 ml of culture solution were heated to the boiling-point in a test tube and the formation of a red colour showed the presence of sugar.

Total nitrogen was determined by the Kjeldahl procedure (Bremner, 1965). Carbon and hydrogen were determined by a dry-combustion method (Hösli-apparatus).

RESULTS

The standard curve

A representative standard curve of numbers of *Pseudomonas* cells (Fig. 1) shows a linear relationship between number of cells and amount of casein, if the latter is present in the range of 0–3·5 mg N/flask. A standard curve covering the range from 0 to 2·5 mg casein-N was used in all experiments except for the determination of the availability of the nitrogen of the *Arthrobacter* cells for which up to 5 mg casein-N was used. The standard curve (0-2·5 mg casein-N] always showed a linear relationship. The pH of the culture solution

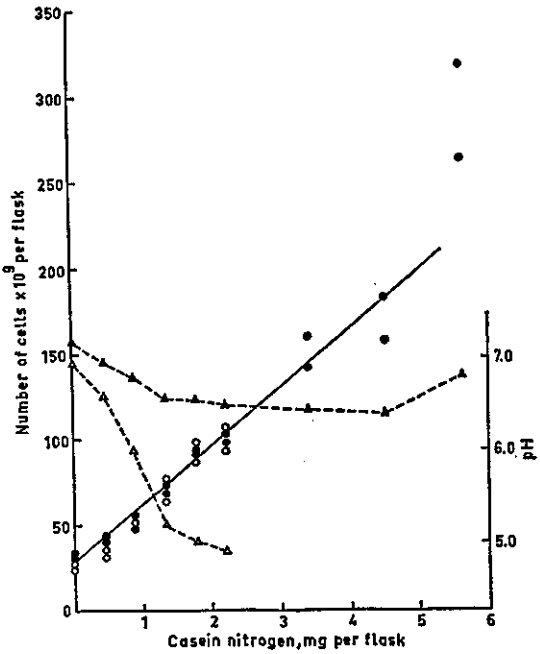

FIG. 1. Relationship between casein-N, number of cells and pH of the culture solution 4 days after inoculation with *Pseudomonas* strain 31. Number of cells: in the absence of $CaCO_3$ (○); in the presence of $CaCO_3$ (●); pH in the absence of $CaCO_3$ (△); in the presence of $CaCO_3$ (▲).

decreased slightly with increasing amounts of casein. This decrease was less when $CaCO_3$ was present. The number of cells was independent of the presence of $CaCO_3$ in the medium and of the final pH (Fig. 1). Glucose was detected at the end of the incubation period at all casein concentrations, except with 4·5 and 5·5 mg casein-N. The rise in pH at the highest aasein concentration was due to exhaustion of glucose which was probably accompanied by cmmonification or respiration of acid products.

Availability of nitrogen of Arthrobacter *cells*

The availability of nitrogen of heat-sterilized *Arthrobacter* cells was determined 1, 2, 3 and 4 days after inoculation with *Pseudomonas* strain 31. Figure 2 shows that the nitrogen of the 3-day-old *Arthrobacter* cells (R3) was less available than that of the 6-day-old cells (R6) during an incubation period of 4 days. Disintegration of the cells promoted the availability of the nitrogen of both types of *Arthrobacter* cells. The nitrogen of the 6-day-old disintegrated cells (UR6) was more readily available than that of the 3-day-old disintegrated cells (UR3). Glucose was present in all of the four series at the end of the incubation period, although it was reduced to a very low concentration with the UR6-cells as the nitrogen source. The magnitudes of the standard errors of the availability percentages decreased during the incubation period and were small at 4 days after inoculation with the *Pseudomonas* sp.

Chemical data of soils and humic acids

Table 1 gives the percentages of total soil nitrogen extracted from both soils by different

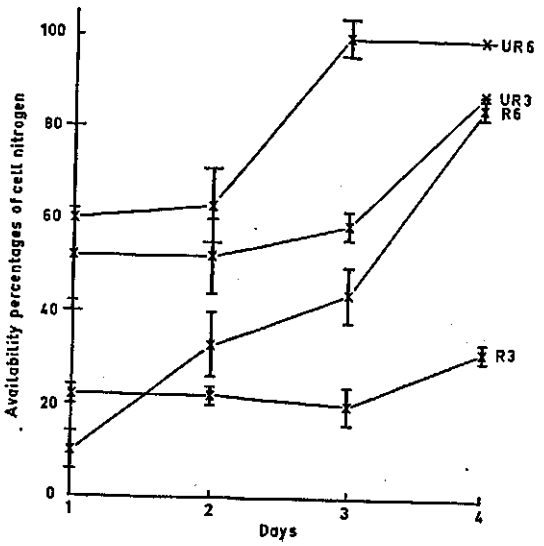

FIG. 2. Influence of time of incubation with *Pseudomonas* strain 31 on the availability of 5 mg N of *Arthrobacter* cells. The availability percentages after 1, 2, 3 and 4 days were obtained from their respective standard curves after 1, 2, 3 and 4 days. Three-days-old *Arthrobacter* cells, R3; the same, disintegrated, UR3; 6-days-old *Arthrobacter* cells, R6; the same, disintegrated, UR6. The vertical lines show the magnitudes of the standard errors of each value.

extractants. The results are rather similar for both soils, although soil of arable land contained less organic nitrogen than that of grassland. The only striking difference is that more humic acid nitrogen was extracted with 0·5 N NaOH from the grassland soil. The nitrogen contents of humic acid preparations were higher in those prepared from soil of grassland than in those prepared from soil of arable land causing a reverse effect on the C–N ratio (Table 2). The 'humic acids' isolated from *Streptomyces* contained 8·4% N and the C–N ratio was therefore very low (5·2).

TABLE 1. NITROGEN EXTRACTED FROM SOIL BY SODIUM PYROPHOSPHATE (NaPP) AND SODIUM HYDROXIDE

Soil	Total N (% of dry matter)	Extractant	N extracted (% of total soil N)		
			Fulvic acids*	Humic acids†	Total N extracted
Arable land	0·08	0·1 M—NaPP (pH 7·0)	8·1	15·2	23·3
		0·1 M—NaPP (pH 9·9)	11·3	27·2	38·5
		0·5 M—NaOH	32·0	27·4	49·4
Grassland	0·14	0·1 M—NaPP (pH 7·0)	9·9	14·2	24·1
		0·1 M—NaPP (pH 9·9)	13·2	24·3	37·5
		0·5 M—NaOH	33·6	32·5	66·1

* Fraction which is soluble in alkali and in mineral acids.
† Fraction which is soluble in alkali and insoluble in mineral acids.

TABLE 2. ELEMENTARY ANALYSIS OF HUMIC ACIDS ISOLATED FROM SOILS AND FROM *Streptomyces* CULTURES (ASH-FREE BASIS)

	Extractant	C	H	N	C/N
Arable land	0·1 M—NaPP (pH 7·0)	42·5	4·7	2·9	14·7
	0·5 M—NaOH	40·6	5·4	3·0	13·5
Grassland	0·1 M—NaPP (pH 7·0)	42·8	5·1	3·2	13·4
	0·5 M—NaOH	43·7	5·7	3·7	11·8
Streptomyces		44·0	4·9	8·4	5·2

Biologically-available nitrogen in soil and in humic acids

The nitrogen uptake by *Pseudomonas* strain 31 from grassland soil was higher than that from soil of arable land (Table 3). The addition of $CaCO_3$ to the test medium caused a higher pH at the end of the incubation period (final pH) and a greater availability of soil nitrogen than without this addition.

TABLE 3. EFFECT OF ADDITION OF 1 g $CaCO_3$/l CULTURE SOLUTION ON THE AVAILABILITY OF NITROGEN OF HEAT-STERILIZED SOILS (120°C) TO *Pseudomonas* STRAIN 31

Soil	Addition of $CaCO_3$	Final pH*	Availability of nitrogen (% of total soil N)
Arable land	−	6·4	0
	+	6·9	1
	+	7·0	5
Grassland	−	6·2	8
	+	6·8	14
	+	6·9	19

* pH value of the medium at 4 days after inoculation with the *Pseudomonas* strain.

Sterilization of soil by heating at 120°C may have changed the properties of the soil organic matter and increased the availability of the organic nitrogen. To demonstrate such an effect, the availability of the nitrogen of heat-sterilized soil was compared with that of soil which was sterilized by ethylene oxide at room temperature. The results (Table 4) show that available nitrogen of the ethylene oxide-treated soils was slightly higher than that of heat-sterilized soil.

TABLE 4. EFFECT OF METHOD OF STERILIZATION ON THE AVAILABILITY OF SOIL ORGANIC NITROGEN TO *Pseudomonas* STRAIN 31

Soil	Sterilization method	Addition of $CaCO_3$	Final pH	Availability of nitrogen (% of total soil N)
Arable land	120°C	+	6·9	1
	Ethylene oxide	−	6·6	2
	Ethylene oxide	+	7·1	4
Grassland	120°C	+	6·8	14
	Ethylene oxide	−	6·5	17
	Ethylene oxide	+	6·8	18

The availability of the nitrogen of humic acids isolated from both soils by 0·5 N NaOH was tested with *Pseudomonas* strain 31. The humic acids were sterilized at 120°C in conical flasks containing culture solution. The results of this experiment show (Table 5) that humic acid nitrogen of soil from arable land was less available than that of grassland soil, while the availability tended to increase with higher pH. In humic acids from soil of arable land the percentage of nitrogen taken up by the *Pseudomonas* strain was higher than that taken up from the soil at the same pH (cf. Tables 5 and 3). From the results of Table 5 it is probable that the higher availability of nitrogen in the presence of $CaCO_3$ is a pH effect.

TABLE 5. EFFECT OF ADDED $CaCO_3$ AND pH OF THE MEDIUM ON THE AVAILABILITY TO *Pseudomonas* STRAIN 31 OF NITROGEN OF HEAT-STERILIZED HUMIC ACIDS ISOLATED FROM THE SOIL BY 0·5 N NaOH

Origin of humic acids					
Arable land			Grassland		
Addition of $CaCO_3$	Final pH	Availability of N (% of total humic acid N)	Addition of $CaCO_3$	Final pH	Availability of N (% of total humic acid N)
–	5·8	8	–	6·3	13
–	6·0	6	–	6·6	11
–	7·0	7	–	6·9	16
–	7·1	12	–	7·0	17
+*	6·7	8	+	6·7	19
+	7·0	7	+	7·0	14
+	7·1	11	+	7·1	19
+	7·4	11	+	7·3	24

* 1 g $CaCO_3$/l.

Table 6 shows the effects of the extractant used for the isolation of humic acids from soil on the availability of the humic acid nitrogen. The uptake of nitrogen from humic acid extracted with pyrophosphate was twice as high as that from humic acid extracted with NaOH. However, it should be noted that the former type of humic acid represented only 15 and the latter type about 30 per cent of the total soil organic nitrogen (Table 1).

TABLE 6. AVAILABILITY TO *Pseudomonas* STRAIN 31 OF NITROGEN OF HEAT-STERILIZED HUMIC ACIDS EXTRACTED FROM SOIL BY 0·1 M SODIUM PYROPHOSPHATE (pH 7) AND 0·5 M SODIUM HYDROXIDE, RESPECTIVELY

Soil	Extractant	Addition of $CaCO_3$	Final pH	Availability of nitrogen (% of total humic acid N)
Arable land	NaPP	–	6·7	16
	NaPP	+	6·8	15
	NaOH	+	6·7	8
Grassland	NaPP	–	6·7	34
	NaPP	+	6·7	32
	NaOH	+	6·7	19

The humic acid nitrogen extracted by pyrophosphate and sterilized by ethylene oxide treatment was much less available than that sterilized at 120°C (Table 7). This is in contrast with the results obtained with soil (Table 4); it may indicate that the fraction of humic acids extracted by pyrophosphate is very sensitive to high temperature which increases its availability. The results of Table 7 can also be explained by incomplete removal of the ethylene oxide.

TABLE 7. EFFECT OF METHOD OF STERILIZATION ON THE AVAILABILITY TO *Pseudomonas* STRAIN 31 OF NITROGEN OF HUMIC ACIDS EXTRACTED FROM SOILS BY 0·1 M SODIUM PYROPHOSPHATE (pH 7)

Soil	Sterilization method	Addition of $CaCO_3$	Final pH	Availability of nitrogen (% of total humic acid N)
Arable land	120°C	−	6·7	16
	Ethylene oxide	−	7·7	0
	Ethylene oxide	+	7·3	0
Grassland	120°C	−	6·7	34
	Ethylene oxide	−	7·8	5
	Ethylene oxide	+	7·3	17

The biological availability of 'humic acid' nitrogen isolated from *Streptomyces* strain A and sterilized at 120°C ranged from 11 to 25 per cent dependent on the final pH. With *Streptomyces* 'humic acid' sterilized by ethylene oxide no growth of *Pseudomonas* was observed, apparently due to the incomplete removal of the ethylene oxide from the lyophilized 'humic acid'. This is remarkable because the 'humic acid' of *Streptomyces* and the soil humic acid fractions were sterilized in the same container at the same time; in the former no growth occurred after inoculation with *Pseudomonas*, whereas normal growth occurred on media supplied with the soil humic acids.

The effect of soil organic matter on the availability of casein nitrogen

The amount of casein nitrogen taken up by the *Pseudomonas* strain in the presence of soil organic matter was obtained by subtracting the biologically estimated amount of nitrogen (mg N) taken up from a culture medium with soil organic matter as the only nitrogen source from the amount of nitrogen taken up from a medium containing both soil organic matter and casein. The availability of casein to *Pseudomonas* strain 31 in the presence of soil organic matter was obtained by expressing this difference (mg N) as percentage of the amount of casein nitrogen applied to the culture solution.

In order to ensure that the presence of soil organic matter did not interfere with the uptake of hydrolysed casein by *Pseudomonas* strain 31, an experiment was performed in which the effect of humic acids on the growth of *Pseudomonas* strain 31 with casamino acids as the nitrogen source was studied. The growth of the test organism and as a consequence the uptake of the casamino acids were not reduced by the soil humic acid (Table 8).

However, the availability of casein nitrogen to the test organism was reduced by the presence of soil from both arable land and grassland (Table 9). The reduction was very pronounced in the medium without $CaCO_3$. In the presence of $CaCO_3$ (1 g/l.) the availability of casein nitrogen to *Pseudomonas* strain 31 was reduced to about 50 per cent by the presence of soil.

TABLE 8. EFFECT OF HUMIC ACIDS ON THE AVAILABILITY OF NITROGEN OF CASAMINO ACIDS TO *Pseudomonas* STRAIN 31

Soil	Humic acid N (mg)	Casamino acid N (mg)	Addition of $CaCO_3$	Final pH	Available to *Pseudomonas* (% of total casamino acid N)
	0	0·92	−	5·7	100
	0	0·92	+	6·3	100
Arable land	4·78	0·92	−	5·8	100
	4·78	0·92	+	6·4	143
Grassland	3·75	0·92	−	6·1	117
	3·75	0·92	+	6·4	120

TABLE 9. EFFECT OF HEAT-STERILIZED SOILS ON THE AVAILABILITY OF CASEIN NITROGEN TO *Pseudomonas* STRAIN 31

Soil	Soil (mg N)	Casein (mg N)	Addition of $CaCO_3$	Final pH	Available to *Pseudomonas* (% of total casein N)
	0	2·2	−	5·0	100
	0	2·2	+	6·5	100
Arable land	5·9	2·2	−	5·4	16
	5·9	2·2	+	6·5	61
Grassland	6·3	2·2	−	5·2	7
	6·3	2·2	+	6·3	53

Soil samples sterilized by ethylene oxide had the same effect as the heat-sterilized soil samples on the availability of casein nitrogen (Table 10).

TABLE 10. EFFECT OF SOIL (STERILIZED WITH ETHYLENE OXIDE) ON THE AVAILABILITY OF CASEIN NITROGEN TO *Pseudomonas* STRAIN 31

Soil	Soil (mg N)	Casein (mg N)	Addition of $CaCO_3$	Final pH	Available to *Pseudomonas* (% of total casein N)
Arable land	6·3	1·7	−	6·0	34
	6·3	1·7	+	6·7	53
Grassland	6·7	1·7	−	5·4	7
	6·7	1·7	+	6·3	59

The same experiments as recorded in Tables 9 and 10 were repeated with humic acids extracted from both soils by 0·5 N NaOH and 0·1 M sodium pyrophosphate (pH 7), respectively. The results were very similar to those obtained with soil. To demonstrate the effect of pH on the availability percentages of casein nitrogen in the presence of soil or humic acids, all the data available have been plotted against the final pH (Fig. 3). The results show that the final pH, controlled to some extent by the presence or absence of $CaCO_3$, was an important factor as to the influence of soil organic matter on the availability of casein nitrogen to *Pseudomonas* strain 31.

Streptomyces 'humic acid' which was sterilized at 120°C reduced the availability of casein to about 40 per cent at a final pH of 6·7.

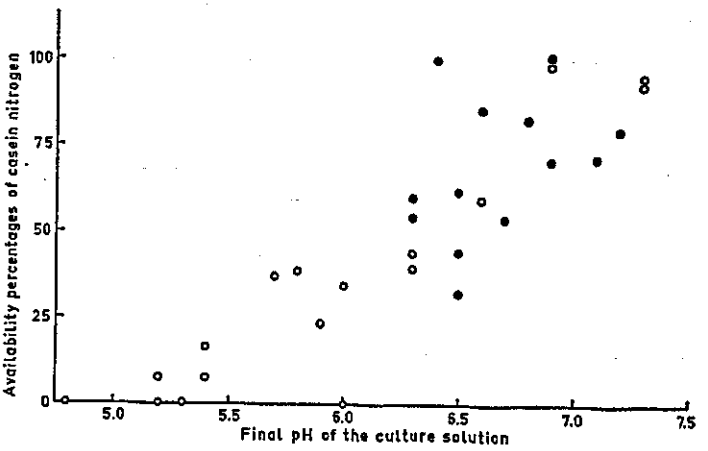

FIG. 3. Effect of final pH of the culture solution on the availability to *Pseudomonas* strain 31 of 2 mg casein-N in the presence of 6 mg soil organic-N. In the absence of $CaCO_3$ (○); presence of $CaCO_3$ (●).

DISCUSSION

The availability of soil nitrogen to *Pseudomonas* strain 31 was slightly enhanced by sterilization with ethylene oxide in comparison with sterilization at 120°C (Table 4) demonstrating that soil organic matter as a whole is stable to high temperature treatment. Organic nitrogen of grassland soil was available to a considerably higher percentage than that of soil of arable land (Tables 3 and 4). This large difference was probably partly due to the easily-decomposable nitrogenous compounds present in small roots and root hairs in the grassland soil.

The availability to the test organism of humic acid nitrogen, extracted from soil or arable land by 0·5 N NaOH (Table 5), was approximately twice as high as that of total soil organic nitrogen (Table 3) showing that humic acids are not representative for soil organic matter. With humic acids from grassland soil the same availability percentages were found as with the whole soil. This similarity was probably due to the presence of easily-decomposable root residues in the grassland soil, increasing the availability percentages of soil nitrogen. These root residues were absent from the humic acid preparations.

The proteolytic *Pseudomonas* sp. utilized similar percentages of nitrogen from humic acids, extracted from soils with sodium hydroxide, as Ladd and Brisbane (1967) found with the proteolytic enzyme pronase. The humic acids extracted from soil by 0·1 M sodium pyrophosphate (pH 7) were more easily decomposable than those extracted by 0·5 N NaOH. This is in accordance with the mineralization experiments of Stanford (1968).

Reduced availability of soil nitrogen to soil microorganisms as compared with the availability of proteins like casein might be due to (a) the occurrence of the soil organic nitrogen in non-protein compounds, (b) protection of proteins to microbial degradation by certain soil constituents (chemical fixation or perhaps adsorption), (c) fixation or adsorption of the released amino acids by soil constituents, (d) fixation or adsorption of microbial proteolytic enzymes by soil constituents, reducing the enzyme activity.

Reduction of the activity of proteolytic enzymes by humic acids has been recorded by Ladd and Butler (1969a,b). By using dipeptides it was shown that this reduction was relieved by methylation of the carboxyl groups of the humic acids (Butler and Ladd, 1969),

addition of inorganic cations (Ladd and Butler, 1970), or acetylation of the proteolytic enzymes (Ladd and Butler, 1971). The latter investigation indicates that the inhibition is due to adsorption of the proteolytic enzymes by the humic acid molecules. Ladd and Butler (1970) suggested that humic acids reversibly bind enzymes by a cation-exchange mechanism causing changes of enzyme activity. Inorganic cations in sufficiently high concentration are able to displace the enzymes from the complexes. The concentrations of divalent and monovalent cations required to desorb the enzymes from the humic acids amounted to 10^{-2} and 10^{-1} M, respectively. In the present investigation the reduction by humic acids in release of available nitrogen compounds from casein by *Pseudomonas* cells was partly relieved by the addition of 1 g $CaCO_3$/l which resulted in an increase of Ca^{2+} concentration of at the most 10^{-2} M and an increase in the final pH. The availability of casein nitrogen to *Pseudomonas* strain 31 in the presence of humic acids correlated with the final pH of the culture solution (Fig. 3). This agrees with the results of Hoffman and Teicher (1957), who observed that addition of $CaCO_3$ increased the proteolytic activity of the soil. The stimulation of release of available nitrogen, observed in the present investigation, may also have been due to the beneficial effect of liming on bacterial growth (Mulder, 1950). Such an effect has apparently not been involved in the present study where the growth and response of the test organism to casein in the absence of soil were independent of the variation in pH (Fig. 1). An explanation of the pH effect on the release of available nitrogen, as shown in the present paper, cannot be given without further study. At a lower pH, casein may be protected against microbial degradation by the formation of humic acid-casein complexes or by partial inactivation of the proteolytic enzymes by the formation of humic acid–enzyme complexes, or by both of these processes.

In a previous investigation (Huntjens, 1971) no evidence was obtained of any reduction in the rate of decomposition of soil organic compounds in grassland soil to available nitrogenous compounds like amino acids. Therefore, the accumulation of unavailable soil organic nitrogen in such soils was ascribed to the immobilization of decomposed soil organic compounds. e.g. amino acids, by the presence of an excess of carbon compounds. The results of the present study suggest that reduction of protein decomposition may be partly responsible for the low availability of soil organic nitrogen in grassland soil. However, the accumulation of soil organic nitrogen in these soils cannot be explained by reduced protein decomposition, as this reduction also occurs in soil or arable land where no accumulation of soil organic nitrogen takes place.

A pH rise not only enhanced the availability of soil organic nitrogen to the *Pseudomonas* strain but also enhanced the availability of casein in the presence of humic acids. This is further evidence that soil organic matter contains protein-like compounds. The higher availability of humic acid nitrogen from grassland soil as compared with that of humic acids from soil of arable land indicates that more protein-like material is incorporated in the soil organic matter of grassland.

Hydrolysis with 6 N HCl released about the same percentages of amino acid nitrogen of total N from both soils and their humic acids (Huntjens, 1972). Whereas proteolysis by a growing *Pseudomonas* culture, as was studied in the present investigation, released more nitrogen (calculated as % of total soil nitrogen) from soil organic matter of grassland than from that of arable land, showing different characteristics of both soils.

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7 General discussion

The purpose of the present work was to investigate the mechanism responsible for the accumulation of soil organic nitrogen in permanent grassland. The low recovery of fertilizer nitrogen in the grass shoots is probable partly associated with this accumulation, which implies the immobilization of nitrogen in microbial cells or soil organic matter.

The soil organic nitrogen of turf samples of which the grass roots had been killed was found to be mineralizable to a higher degree than that of arable land (the content of mineral nitrogen in the pots containing approximately 600 g of dry soil increased by 45 mg and 6 mg, respectively, during two months, Table 3.1). The immobilization of labeled fertilizer nitrogen in these turf samples was greater than that in soil of arable land (from 47.6 mg labeled fertilizer nitrogen which was applied as $(NH_4)_2SO_4$ 7.3 and 0.7 mg N, respectively, were immobilized, Table 3.1). This points to the presence of larger amounts of metabolizable carbonaceous material in the turf. Disturbing and mixing the soil of the turf samples with killed roots did not promote mineralization (Table 3.3).

In the case of turf samples containing living plants, the net mineralization of soil organic nitrogen was slightly negative (approximately – 10 mg N per pot, Table 3.2). Mixing of the soil of turf samples containing living plants did not stimulate the release of soil organic nitrogen (Tables 3.7 and 3.8). Comparison of these results with the positive net mineralization rates of the turf samples with killed roots (22–24 mg N during two months, Table 3.3) indicates that growing plants are mainly responsible for the accumulation of soil organic nitrogen in grassland. In spite of this, experiments with labeled fertilizer nitrogen showed that part of the nitrogen taken up by the plants (P14) had been derived from unlabeled soil organic nitrogen (Table 4.4b). The amount of immobilized (labeled) fertilizer nitrogen (I15) was greater than P14, resulting in a negative net mineralization (P14–I15) and consequently in an accumulation of soil organic nitrogen (6–16 mg N per pot containing 532 g of dry soil). When the photosynthesis of the grass plants was reduced by clipping the shoots after the fertilizer nitrogen had been taken up, the net mineralization of soil organic nitrogen increased due to a reduced immobilization (Table 4.4b) The increased mineralization was attributed to the excretion of less carbonaceous matter by the roots of the clipped grass plants, resulting in a reduced immobilization of fertilizer nitrogen. Removal of the grass shoots by clipping may cause the death of part of the root system of the turf and may enrich the soil with more decomposable lignin-containing material. Apparently this lignin did not promote the incorporation of available nitrogen in the soil

organic matter as was the case with the excretion products of roots of plants which were not clipped.

Mineralization of soil organic nitrogen was expected to proceed as far as ammonia. However, addition of unlabeled ammonium sulphate did not promote the liberation of previously immobilized labeled fertilizer nitrogen (Table 4.5). These results suggest that the decomposition of organic nitrogen compounds in pasture proceeds to such compounds as peptides, amino acids etc,. which are utilized by microorganisms without being deaminated.

Nitrogen recently immobilized in turf samples with living grass plants was mineralized more readily than soil organic matter upon killing of the grass plants. This was demonstrated in a pot experiment with turf samples dressed with labeled nitrate (Table 3.6). The plants were killed when the nitrate had been used up. Two months later 2 % of the originally present soil organic nitrogen had been liberated (N^{14}) as contrasted with 24 % of the more recently immobilized nitrogen (N^{15}).

The water content of the soil in the pot experiments was kept at 60 per cent of the waterholding capacity by addition of water twice a day. This treatment is favourable for microorganisms, but, according to Harmsen (Section 2.2.2) less favourable for humus formation. Therefore, more labeled fertilizer nitrogen might have been incorporated in soil organic matter under natural conditions where the changes in soil humidity are more extreme than those of pot experiments.

High numbers of chromogenic streptomycetes occur in pasture soil. Several strains of such organisms were tested for the ability to produce humic acid-like substances in a glycerol-nitrate medium. All the strains tested were found to produce humic substances in this medium (Table 5.1). These results suggest that microorganisms may contribute to the synthesis of soil humic acids.

Hydrolysis with 6 N HCl released less nitrogen in the form of amino acids and ammonia from humic acid-like substances synthesized by *Streptomyces* strain A (40 %) than from soil of pasture (60 %) or arable land (54 %) and from humic acids obtained from these soils (60 %), see Table 5.3. From these results it might be concluded that less protein is incorporated in the humic acid-like substances produced by streptomycetes than in the soil organic matter. Due to the fact that streptomycetes are proteolytic organisms, the concentration of protein may have been lower in the *Streptomyces* culture than in the soil during the spontaneous condensation of the building units leading to the synthesis of humic substances. No evidence was obtained that the chromogenic streptomycetes contribute more extensively to the humus formation in pasture soil than in arable soil, because the amino acid composition of the hydrolysates of both soils were rather similar (Table 5.4).

A more marked difference between the soil organic matter of pasture and that of arable land was when soil or fractions of soil organic matter were used as the only nitrogen source for the growth of a proteolytic *Pseudomonas* strain. It appeared that a larger percentage of the organic nitrogen in grassland soil was available to the *Pseudomonas* than it was the case with arable soil (Tables 6.3, 6.4, 6.5 and 6.6).

The availability to the proteolytic *Pseudomonas* sp. of nitrogen of humic acids

extracted from the soil with sodium pyrophosphate (pH7) was twice as high as that of humic acids extracted with NaOH (Table 6.6). The availability of the nitrogen of the latter humic acid fraction was generally higher than that of the entire soil nitrogen (Tables 6.3 and 6.5). These results show that humic acids are not representative for soil organic matter.

The addition of $CaCO_3$ to test medium promoted the availability of soil nitrogen, apparently as a result of the higher pH value of the medium (Tables 6.3 and 6.5).

The availability to the *Pseudomonas* sp. of nitrogen of humic acid-like compounds, synthesized by a *Streptomyces* strain, varied between 11 and 25 % (depending upon the pH of the culture solution) of the total nitrogen contained in this fraction. It was similar to that of humic acids extracted from grassland soil by NaOH.

The availability of casein nitrogen was independent of the pH of the culture solution (Fig. 6.1) as contrasted with that of soil organic nitrogen which was dependent on the pH (Table 6.3). The availability of casein nitrogen was reduced in the presence of soil organic matter (Tables 6.8, 6.9 and Fig. 6.3), probably owing to the presumed inhibition of the proteolytic activity of the *Pseudomonas* strain. The reduction of the availability of casein due to the presence of soil organic matter was partly eliminated by the addition of $CaCO_3$ to the test medium, demonstrating that it was a pH effect (Fig. 6.3).

This response to low pH of the microbial decomposition of soil organic nitrogenous compounds and that of casein in the presence of soil organic matter, is a further indication that the latter contains protein-like compounds. The higher availability to the *Pseudomonas* strain of soil organic nitrogen from pasture as compared with that from arable land indicates that more protein-like material is incorporated in the soil organic matter of pasture. It is likely that these protein-like residues are decomposed after ploughing up of grassland resulting in a release of available nitrogen.

8 Samenvatting

Immobilisatie en mineralisatie van stikstof in grasland

Een onderzoek werd ingesteld naar de ophoping van stikstof in organische verbindingen in de zode van grasland. Deze ophoping is een gevolg van het feit dat de vastlegging (immobilisatie) van stikstof in organische stof de afbraak (mineralisatie) overtreft.

Voor dit onderzoek werden potproeven met zoden van grasland uitgevoerd. De zoden werden bemest met gemerkte ammoniak- of nitraatstikstof. Op verschillende tijdstippen na de stikstofgift werden stikstofbalansen opgesteld door de bovengrondse delen, wortels en grond te analyseren.

De netto-mineralisatie van de bodemstikstof in organische vorm in graszoden waarin de plantenwortels waren gedood was positief en werd niet bevorderd door het homogeniseren van de grond (tabel 3.3). Bij een poging de opbouw van de grond van graszoden te verstoren doch de planten intakt te laten, bleek dat dit geen invloed had op de opneming van stikstof door de grasplanten (tabellen 3.7 en 3.8).

De aanwezigheid van groeiende planten verlaagde de netto-mineralisatie van de organische stof van de grond tot nul (tabel 3.7); soms werden zelfs negatieve waarden verkregen (tabel 3.2). Onder deze omstandigheden bleef het gedeelte van de gemerkte kunstmeststikstof dat tijdens de groei van planten in de organische stof van de grond werd vastgelegd daarin aanwezig zolang de planten intakt werden gelaten (tabellen 3.4 en 3.8). Dit geldt uiteraard alleen voor de daar vermelde proefomstandigheden. Deze waarnemingen wijzen erop dat het vrijkomen van stikstof uit organische bodembestanddelen wordt tegengegaan door de aanwezigheid van groeiende planten.

Uit proeven met graszoden die bemest waren met gemerkte stikstof in de vorm van KNO_3 en waarvan de planten gedood waren op het moment dat geen anorganische stikstofverbindingen in de grond meer waren aan te tonen, bleek gedurende de hierna volgende periode van twee maanden de ongemerkte bodemstikstof in organische vorm minder gemakkelijk te mineraliseren dan de kort van tevoren in de organische stof vastgelegde (gemerkte) stikstof; de afneming van stikstof in organische vorm voor beide fracties bedroeg respektievelijk 2 en 24 % (tabel 3.6).

Om de spreiding tussen de resultaten van verschillende potten met dezelfde behandelingen te reduceren, werden potproeven met ingezaaid gras uitgevoerd. De grond die hiervoor werd gebruikt was afkomstig van grasland. Nadat de grond in de potten goed doorworteld was, werd bemest met gemerkte stikstof in de vorm van ammoniumsulfaat. Na ongeveer twee weken werd geen stikstof in deze vorm teruggevonden. Op dit tijdstip bleek dat minder stikstof uit de organische stof van de grond ter beschikking van de plant was gekomen (P14) dan ammoniumstikstof in de organische

fractie van de grond was vastgelegd (I 15), zie tabel 4.4b. Dit betekent dat een toename van de hoeveelheid organische stikstofverbindingen in de grond had plaats gevonden. Gemiddeld werd 12 % van een stikstofgift in de organische fractie van een grond vastgelegd. Dezelfde resultaten werden een maand na toevoeging van de kunstmeststikstof verkregen. Indien twee weken na de toediening van de anorganische stikstofverbinding de bovengrondse delen van de grasplanten werden geknipt en verwijderd, bleek na nogmaals twee weken geen toename van de bodemstikstof in organische verbindingen te hebben plaats gevonden.

Toediening van ongemerkte ammoniumsultaat, nadat de gemerkte stikstof door de planten was opgenomen en in de organische stof van de grond was vastgelegd, had geen afname van de hoeveelheid gemerkte organische bodemstikstof (I15) tot gevolg (tabel 4.5). Een dergelijke afname had kunnen worden verwacht indien de afbraak van de organische stikstofverbindingen tot ammoniak zou verlopen. De verkregen resultaten doen vermoeden dat de afbraak van organische stikstofverbindingen in grasland verloopt tot produkten zoals aminozuren en peptiden die door micro-organismen weer worden geassimileerd en in celbestanddelen worden vastgelegd zonder dat ammonifikatie heeft plaats gevonden.

Tijdens het ouder worden van het ingezaaide gras werd gemerkte stikstof van de bovengrondse delen naar de grond getransporteerd (tabel 4.7). Deze verrijking van de grond met gemerkte stikstof had echter geen ophoping van stikstof in organische verbindingen tot gevolg, omdat evenveel ongemerkte bodemstikstof door de grasplanten werd opgenomen.

De positieve invloed van groeiende planten op de ophoping van organische stikstofverbindingen in de grond wordt waarschijnlijk ten dele veroorzaakt door de uitscheiding van produkten door wortels die kunnen fungeren als koolstof- en energiebron voor micro-organismen. Tijdens het afsterven van bepaalde micro-organismen kan een deel van hun stikstofverbindingen worden omgezet in humusachtige verbindingen. Aangezien veel *Streptomyces*-soorten in staat zijn tot de produktie van deze verbindingen en streptomyceten in grond van grasland een groter percentage van de totale microflora uitmaken dan in grond van bouwland, werden deze organismen onderzocht op het vermogen om humusachtige stoffen te produceren (zie hoofdstuk 5). Dit werd nagegaan in een glycerol-nitraat medium. Een stam zette 13,7 % van de nitraatstikstof om in humuszuurachtige verbindingen (tabel 5.2). Hydrolyse met 6 N HCl maakte 40 % van de stikstof van dit 'humuszuur' vrij als ammonium- en aminozuurstikstof (tabel 5.3). Bij humuszuren geïsoleerd uit grond van bouwland en van tien jaar oud grasland kwam door een dergelijke hydrolyse in beide gevallen 60 % van de humuszuurstikstof vrij als ammonium- en aminozuurstikstof.

De aminozuursamenstelling van de hydrolysaten van grond van grasland, grond van bouwland en van de humuszuren van deze gronden bleken vrijwel gelijk te zijn (tabel 5.4). Deze samenstelling toonde veel overeenkomst met die van de hydrolysaten van 'humuszuren' van twee *Streptomyces* stammen.

In hoofdstuk 6 werd een micro-biologische methode geïntroduceerd om de beschikbaarheid van stikstof in micro-organismen en die in organische bodembestanddelen

te bepalen. Dit werd gedaan door de groei van een eiwitsplitsende *Pseudomonas*-soort in een vloeibaar medium met het te bestuderen materiaal als enige stikstofbron te vergelijken met de groei in een medium met caseine als stikstofbron.

De stikstofhoudende verbindingen van drie dagen oude *Arthrobacter*-cellen bleken resistenter te zijn tegen afbraak dan die van zes dagen oude cellen (fig. 6.2).

De beschikbaarheid van de in organische vorm voorkomende bodemstikstof voor de *Pseudomonas*-stam nam toe met stijgende pH van het medium waarin de beschikbaarheid werd bepaald; deze beschikbaarheid was bij grasland hoger dan bij bouwland (tabellen 6.3 en 6.5).

De eiwitsplitsende *Pseudomonas*-stam maakte procentueel tweemaal zoveel stikstof vrij uit humuszuren die geëxtraheerd waren met natriumpyrofosfaat (pH7) dan uit humuszuren die geëxtraheerd waren met natriumhydroxide (tabel 6.6). De uit de grond geëxtraheerde humuszuren waren in het algemeen beter beschikbaar dan de stikstof van de grond als geheel.

De beschikbaarheid van stikstof van humuszuurachtige verbindingen, gesynthetiseerd door een *Streptomyces*- stam, was gelijk aan die van humuszuren die met natriumhydroxide uit grond van grasland waren geëxtraheerd doch beter dan die van humuszuren uit bouwland. Afhankelijk van de pH van de voedingsoplossing werd 11 tot 25 % van de aanwezige stikstof door de *Pseudomonas* opgenomen.

De beschikbaarheid van caseinestikstof voor de *Pseudomonas*-stam was slechter zowel in aanwezigheid van grond (tabellen 6.8 en 6.9) als in aanwezigheid van humuszuren. Deze verminderde caseine-assimilatie werd gedeeltelijk opgeheven door de toedinening van $CaCO_3$ aan het testmedium; het bleek een pH-effect te zijn (fig. 6.3).

De verkregen resultaten doen vermoeden dat de organische stof van de grond eiwitachtige verbindingen bevat en dat deze verbindingen in de organische stof van grasland in grotere hoeveelheden voorkomen dan in die van bouwland.