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CHANGES IN ENZYMIC ACTIVITY AND DISTRIBUTION OF ACID-SOLUBLE, AMINO ACID–NITROGEN IN SOIL DURING NITROGEN IMMOBILIZATION AND MINERALIZATION

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Summary—During a period of immobilization of nitrate-^{15}N and mineralization of organic N in a sandy-loam, changes were recorded in: (a) the concentration of an added carbon source, glucose-^{14}C; (b) evolution of $^{14}CO_2$; (c) bacterial populations; (d) distribution and concentration of newly-synthesized, acid-soluble, amino acid-^{15}N; and (e) distribution and activities of several oxidative and hydrolytic enzyme systems.

Added glucose-^{14}C was rapidly metabolized by the soil microflora. After 1·5 day's incubation, when only 3·6 per cent of the added glucose was present, 68 per cent of the ^{14}C remained in the soil-microbial system. During this period there was a marked increase in viable bacterial numbers and an almost complete immobilization of nitrate-^{15}N. On continued incubation, microbial metabolites were oxidized at decreasing rates, the more rapid phase corresponding to a period of net decline in the viable bacterial population.

Soil was fractionated by a relatively mild procedure into components containing: (a) extractable proteins; (b) extractable amino acids and peptides; (c) particulate material containing microbial cells, cell debris and material bound to larger soil particles; and (d) microbial metabolites mainly bound to soil colloids. Although the total, acid-soluble, amino acid-^{15}N remained relatively constant for about 50 days, there were marked changes in their concentration in different fractions, especially in the extracts and in the fraction containing fine colloidal material. However, the relatively large decline in labelled, acid-soluble, amino acid-^{15}N occurred during the active phase of oxidation of microbial metabolites when little net mineralization of labelled compounds occurred.

Increases in enzymic activities generally coincided with increased viable bacterial populations although there were some exceptions, notably casein and benzoyl arginine amide-hydrolysing enzymes. The stabilities of the newly-formed enzymes varied markedly. The greatest relative changes in activity occurred with the casein-hydrolysing enzymes. Their activity reached a maximal value after the main flush of bacterial growth, was short-lived and was to a large extent extractable. The formation and disappearance of this extracellular proteolytic activity coincided approximately with that of a secondary peak of extractable, newly-synthesized, protein-^{15}N. In general however, changes in enzymic activity could not be identified with changes of protein-^{15}N concentrations of the different fractions.

INTRODUCTION

OF THE various organic nitrogen compounds formed in soil during immobilization of mineral nitrogen, those comprising the alkali non-distillable, acid-soluble fraction appear quantitatively to be the most important (Cheng and Kurtz, 1963; Stewart, Porter and Johnson, 1963; Simpson and Freney, 1967; Broadbent, 1968; Danneburg, Haunold and Kaindl, 1968; McGill, 1971). A significant proportion of the nitrogen of this fraction is accounted for as amino acid-N (Cheng and Kurtz, 1963; Chu and Knowles, 1966; Broadbent, 1968; McGill, 1971) derived presumably from the hydrolysis of peptides and proteins. Subsequent remineralization leads to decreased amounts of all forms of organic-N, the

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acid-soluble amino acid-N again being a major contributor (Keeney and Bremner, 1964, 1966; Freney and Simpson, 1969; Moore and Russell, 1970; McGill, 1971). Changes in the concentration of the total, newly-synthesized, acid-soluble, amino acid-N during a period of immobilization-mineralization reflect the differences in the net rates of synthesis and degradation of many proteins and peptides. These also vary in their nature, function and distribution. For example, Wagner and Mutatker (1968) have indicated that microbial cell wall proteins decompose less readily than other proteins in soil. Sorensen (1969, 1972) has suggested that extracellular enzymes may contribute to the amino acid-N fraction, the persistence of the enzyme protein being related to the clay content of the soil. Thus it may be anticipated that if soil nitrogenous compounds were fractionated according to their biological properties (Jansson, 1967; Persson, 1968), the proportion of nitrogen immobilized within a given fraction would change with time.

In this paper, we have examined the distribution of ^{15}N-labelled, acid-soluble, amino acid-N, formed during immobilization and subsequent remineralization of nitrate-^{15}N. Changes in the concentrations of amino acid-^{15}N of different fractions and in the activities of several enzymes during the incubation period have been compared. The procedure used for soil fractionation is relatively mild to minimize microbial cell destruction and loss of enzyme activity, and has been adapted from that of McGill (1971), who has investigated the dynamics of immobilized nitrogen transformations through microbial biomass and extracellular metabolites.

MATERIALS AND METHODS

Soil

A Chernozemic soil, Bradwell fine sandy loam (Ellis, Acton and Moss, 1970) was sampled to a depth of 25 cm from a summer-fallow site, air dried for 2 days and, after removing coarse plant material, ground and sieved (0·5 mm). The soil, stored dry at about 22°C, contained 50 μg/g of nitrate-N.

Soil incubations

Several incubation experiments were set up to run concurrently. Samples of air dried soil were moistened (16·7 per cent final) with distilled water or solutions of glucose–sodium nitrate and, after thorough mixing in Erlenmeyer flasks, were placed in moist, CO_2-free, aeration trains and incubated at 22°C for up to 105 days. Initially, the concentrations of glucose-C and nitrate-N in soils receiving these nutrients, were 3 mg and 0·17 mg/g dry wt of soil, respectively. During the incubation period, flask contents were briefly mixed immediately before subsampling.

(a) *Large scale incubation.* In this, the main experiment, 1 kg air dried soil was mixed with 200 ml of a solution of glucose and ^{15}N-labelled sodium nitrate (7·68234 $^{15}N\%$ excess final, assuming equilibration with nitrate already present in the soil). At appropriate time intervals, 65 g of moist soil were removed, 25 g of which were assayed for enzyme activity within 2 h of subsampling, and the remainder stored frozen until analysed for residual glucose, microbial numbers, ammonium-N, nitrate-N and acid-soluble amino acid-N.

In a parallel experiment, 600 g soil were moistened with 120 ml distilled water and subsamples were assayed for enzyme activity and inorganic-N only.

(b) *Medium scale incubation.* Soil (100 g) was mixed with 20 ml of glucose–sodium nitrate solution and incubated for 5 and 50 days, at which times subsamples were immediately fractionated and assayed for their enzyme activities.

(c) *Small scale incubation.* Soil (50 g) was mixed with 10 ml of a solution of ^{14}C-labelled glucose (3μCi) and sodium nitrate. Evolved carbon dioxide was trapped in glass towers containing 40 ml 0·4 N NaOH. Total CO_2 evolved was calculated after titrating residual alkali with standard HCl; CO_2 originating from labelled substrate was determined from the radioactivity of 0·5 ml aliquots of trapping solution added to 10 ml of a toluene-Triton X-100 scintillant (Turner, 1968). Control flasks, containing soil moistened with distilled water only, and blanks, without soil and substrate, were also included. All small scale experiments were duplicated.

Soil fractionation

Soil samples were fractionated to determine the distribution of: (a) enzyme activities; and (b) acid-soluble amino acid-N. The procedure adopted prior to the amino acid-N analyses is outlined in Fig. 1 and is based upon the methods of Anderson (1972) and McGill (1971), but modified to further reduce destruction of microbial cells and loss of enzymic

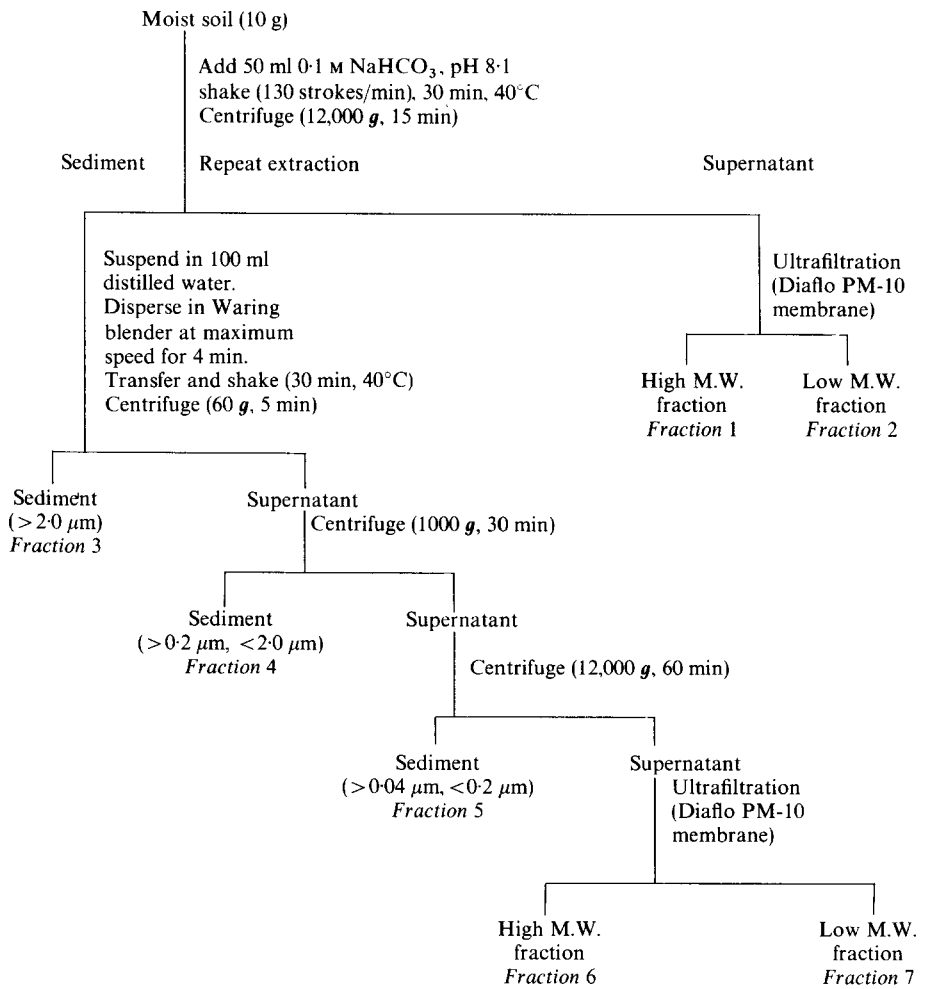

FIG. 1. Fractionation of Bradwell soil.

activity. Duplicate samples of moist soil were each briefly extracted twice with 0·1 M $NaHCO_3$ by shaking in 250 ml capacity, polycarbonate centrifuge bottles. The pooled extracts (about 200 ml) were concentrated to 0·1 volumes at 1° C by using a Diaflo ultrafiltration assembly, fitted with a PM-10 membrane (dia. 7·6 cm, 275·8 kPa). Material retained by the membrane was successively washed three times by adding 1 vol cold distilled water then concentrating to 0·1 vol between additions. Supernatants obtained after the final centrifugation were treated similarly. All fractions were freeze-dried before chemical analysis.

Preparation of soil fractions for enzymic activity assays followed a similar procedure except that: (1) soils were extracted and fractionated immediately after sampling and fractions 3, 4 and 5 were assayed on the day of their preparation; and (2) the initial bicarbonate extracts were dialysed against distilled water for 16 h at 1°C before fractionating and concentrating by membrane filtration. Fractions 1 and 6, i.e. material not passing through the membrane were stored frozen until assayed. Fractions 2 and 7, representing extracted material of lower molecular weight were not tested for enzyme activity.

Enzyme assays

Table 1 summarizes the conditions employed. Assays of hydrolytic enzymes involved shaking (130 oscillations/min.) reactants in capped tissue culture tubes (capacity 20 ml) (Ladd and Butler, 1972). The enzymic reactions were terminated by rapidly cooling the tubes in water at about 5°C, then adding acid or alkali. Hydrolyses of benzyloxycarbonyl-phenylalanyl leucine (ZPL) and of benzoylarginine amide (BAA) were determined after reacting aliquots of acidified supernatants with a ninhydrin reagent and relating the absorbances at 570 nm with those of similarly-treated leucine and ammonium chloride standards (0·3 μmoles), respectively (Ladd and Butler, 1972). Casein hydrolysis was determined after reacting trichloracetic acid (TCA)–soluble components with the Folin reagent and comparing the absorbance at 700 nm with that of a tyrosine standard (0·12 μmoles) (Ladd and Butler, 1972). *p*-Nitrophenylphosphate (PNPP) hydrolysis by soil phosphatases was determined by relating absorbances at 410 nm of the alkaline supernatants to that of a *p*-nitrophenol standard (Tabatabai and Bremner, 1969).

The dehydrogenase assay was based upon the reduction of triphenyltetrazolium chloride in the presence of endogenous soil substrates (Ross, 1971) and added succinate. All reactants were added to tissue culture tubes fitted with serum caps, through which were inserted syringe needles connected to a vacuum line. After evacuating for 3 min, the tubes were incubated without shaking at 40°C for 120 min, when the reaction was terminated by the addition of methanol. The reaction product, formazan, was extracted by shaking the tubes, now fitted with screw caps, at 40°C for 60 min and, after centrifugation, absorbances were determined at 485 nm.

Oxidation of various ^{14}C-labelled substrates by soil was assayed using Erlenmeyer flasks (25 ml capacity), sealed with rubber serum caps (Kontes Glass Co., Vineland, New Jersey, U.S.A.) fitted with centre wells, in which were placed pieces of fluted Whatman no. 1 filter paper (Hobbie and Crawford, 1969). Suspensions of soil and substrate were incubated for 90 min at 22°C on a rotary action shaker, then mixed with 0·2 ml 2*N* H_2SO_4, injected through the serum cap. Then 0·2 ml phenethylamine were injected carefully on to the filter paper and the flasks shaken for a further 60 min. The filter paper with the absorbed CO_2 was transferred to a counting vial containing 10 ml of scintillation fluid and the radioactivity determined.

TABLE 1. CONDITIONS EMPLOYED IN ENZYME ASSAYS

Enzyme	Substrate	Buffer (μmoles)	Moist soil (g)	Volume (ml)	Temp (°C)	Time (min)	Reagent to terminate reaction	Reaction products	Reference to modified assay
Protease	Casein (30 mg)	Tris-HCl pH 8·1 (300)	0·5	3·0	50	120	1·0 ml 17·5% TCA	Folin reagent reactive compounds	Ladd and Butler, 1972
	ZPL (2 μmoles)	Tris-HCl, pH 8·1 (100)	0·3	3·0	40	20	1·0 ml 0·5 *N* HCl	leucine	Ladd and Butler, 1972
	BAA (2 μmoles)	Tris-HCl, pH 8·1 (100)	0·3	3·0	40	20	1·0 ml 0·5 *N* HCl	ammonium chloride	Ladd and Butler, 1972
Phosphatase	PNPP (10 μmoles)	sodium maleate pH 6·5 (400)	0·3	5·0	40	10	1·0 ml 0·5 *M* $CaCl_2$, 4·0 ml 0·5 *N* NaOH	*p*-nitrophenol	Tabatabai and Bremner, 1969
Dehydrogenase	Succinate (100 μmoles)	Tris-HCl, pH 8·1 (1000)	1·0	2·0	40	120	10 ml methanol	formazan	Ross, 1971
Oxidase	Endogenous ^{14}C-labelled glucose, acetate, succinate or alanine (each 4 μmoles, 0·04 μCi)		0·5	4·0	25	90	0·2 ml 2 *N* H_2SO_4	$^{14}CO_2$	Hobbie and Crawford, 1969

In all assays, treatments were at least duplicated. Net enzyme activity, with one exception, was obtained after correction for two controls viz. without soil and without substrate. Because of non-enzymic interaction between casein and soil, it was preferable to calculate casein hydrolysis from the amounts of product released within the reaction time interval, 5–120 min. Under the conditions described, net enzymic activity was directly related to the weight of soil used.

Bacterial counts

Total bacterial numbers were determined by a direct count technique in which micro-organisms were stained with fluorescein isothiocyanate; and viable bacterial numbers were determined by a dilution plate count technique (Babiuk and Paul, 1970).

Chemical analyses

Residual glucose was extracted from previously frozen soil samples by shaking suspensions in 0·5 N K_2SO_4 for 10 min, and measured by reacting aliquots of the extract with an anthrone reagent then relating absorbances at 630 nm to those obtained with glucose standards (Oades, 1967).

Soil mineral nitrogen was extracted with 2 M KCl and ammonium-N and nitrate-N determined by distillation with MgO and Devarda's alloy–MgO mixture, respectively (Bremner, 1965). Amino acid-N was estimated after reacting hydrolysates of freeze-dried soil fractions with ninhydrin (Bremner, 1965). All N analyses were duplicated. The two distillates from each treatment were pooled, acidified with 0·2 ml 1 N H_2SO_4, "spiked" with 2 mg non-enriched ammonium-N and concentrated to 2–3 ml at 45–50°C under reduced pressure (Bremner, 1965). An M.A.T. GD 150 mass spectrometer was used to determine ^{15}N per cent abundance of aliquots of the distillates (Bremner, 1965; Johns, 1971).

RESULTS

Glucose oxidation

Figure 2 shows that glucose was rapidly metabolized after a lag period of approx 0·5 days. After incubating for 1·5 days, 96·4 per cent of the added glucose was metabolized and 31·2 per cent of the original glucose carbon was evolved as CO_2, thus demonstrating that 65 per cent of the added glucose-C still remained in the soil as microbial biomass or metabolites at the

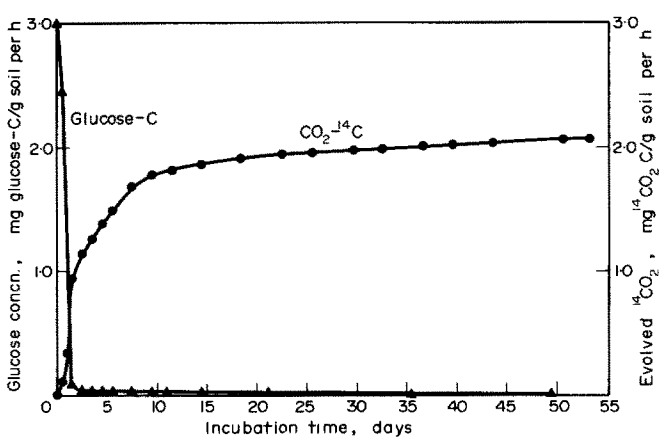

FIG. 2. Oxidation of ^{14}C-glucose by Bradwell soil.

cessation of the very active growth and decomposition phase. The rate of disappearance of glucose during the active oxidation phase was calculated to be equivalent to a half life of 0·22 days. ^{14}C-labelled microbial metabolites formed from glucose were oxidized less readily, exhibiting (from semi-log plots) half lives of approx 4 days during the 1·5–20 day incubation period, and of 190 days upon entering a more stabilized phase, (after 30 days) when 33 per cent of the glucose-derived carbon still remained in the soil.

During the first 5 days' incubation, positive priming of soil carbon oxidation occurred. The maximal priming effect took place after 1·5 days and was equivalent to the release of 0·23 mg soil–C/g dry weight soil/day. After 2·5 days, no priming was evident, but after 3·5 days, a second, lesser priming effect (equivalent to 0·10 mg soil–C/g dry weight soil per day) was apparent. The double prime was also observed by Keefer and Mortensen (1963) and by Shields (1972), who suggested that this pattern may reflect qualitative changes in the enzymic and microbial constitution of the soil following metabolism of the added substrate.

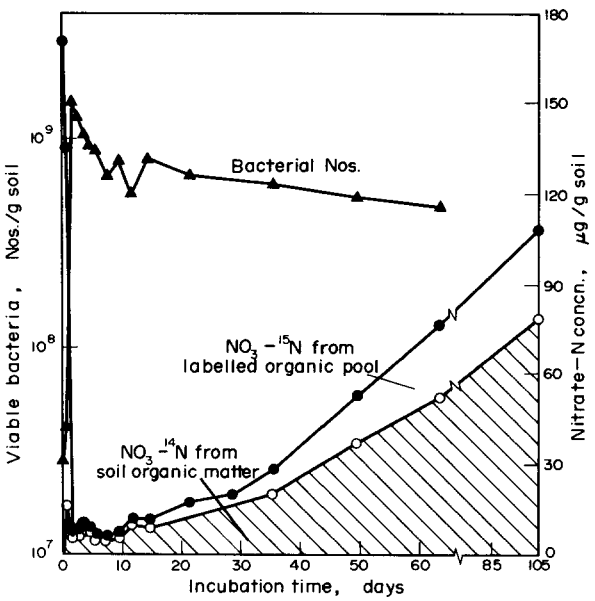

FIG. 3. Effect of glucose oxidation on viable bacterial numbers and nitrate immobilization.

Bacterial growth and nitrogen immobilization

Figure 3 shows that after a lag period of 0·5 days, viable bacterial numbers increased rapidly due to utilization of the added glucose. Following exhaustion of this substrate after 1·5 days, bacterial numbers decreased, rapidly at first but less so after approx 10 days' incubation.

This pattern of decline in bacterial numbers corresponded to that of the oxidation of ^{14}C-labelled microbial metabolites (Fig. 2), suggesting that the latter were derived, at least in part, from products released from the dead bacterial cells. Nevertheless, after 63 days' incubation, viable bacterial counts were still an order of magnitude greater than those observed before substrate addition. The viable bacterial counts may be taken as an indication of microbial activity but cannot be used to calculate the incorporation of labelled substrate into identifiable microbial cells.

Total bacterial numbers ranged from 10^9 cells/g dry weight of soil in the initial sample to $1{\cdot}8 \times 10^9$ cells/g dry weight of soil after 4·5 days' incubation, but showed no obvious patterns of population changes. Estimates of N in the bacterial biomass ranged from 6·7 to 11·6 μg N/g dry weight of soil. Measurements of mycelial length of fungi indicated a decline from the commencement of incubation and the fungal biomass N was calculated to range from only 1·1 to 2·7 μg N/g dry weight of soil. The relative amounts of bacterial and fungal biomass differed from those usually recorded for grassland soils (Clark and Paul, 1970).

Nitrate-N, including both that added and that initially present in the soil, was immobilized during glucose metabolism. The nitrogen remained immobilized during the more active phase of oxidation of microbial metabolites. However, after about 15 days' incubation, net mineralization of nitrogen was noted. ^{15}N-labelled nitrate comprised an increasing proportion of the total nitrate formed until 63·5 days' incubation when it accounted for 32·5 per cent of the total nitrate present and 14·6 per cent of the nitrate initially immobilized (Fig. 3). Nitrate-N derived from unlabelled soil sources accumulated at a rate similar to that obtained when soil was moistened with distilled water only. In soil without added substrate, nitrate-N increased steadily from an initial level of 50 μg/g to 77 μg/g after 49·5 days' incubation.

Ammonium-N content of soils, incubated with or without added glucose, remained relatively constant at about 7 μg/g except for a transient increase to 16 μg/g after 2·5 days in the soil incubated with glucose. The proportion of total ammonium-N accounted for as ammonium-^{15}N was maximal (16·7–25·3 per cent) between 3·5 and 9·5 days' incubation, i.e. during the more active phase of decomposition of microorganisms and their metabolites.

Distribution of ^{15}N-labelled, acid-soluble, amino acid-N

^{15}N-labelled, acid-soluble amino acid-N probably was derived from newly-synthesized peptides and proteins. The concentrations of ^{15}N-labelled amino acid-N of several soil fractions, obtained by extraction and differential centrifugation, changed markedly during the incubation period, although the total amino acid-^{15}N remained at a relatively constant level for about 50 days (Fig. 4).

(a) *Fraction* 1. (Bicarbonate-soluble compounds of high molecular weight.) An initial increase then decline in amino acid-^{15}N coincided with changes in viable bacterial numbers. However, between 3·5 and 5·5 days' incubation, a secondary increase in labelled amino acid-^{15}N occurred, after which the level fell (half life, 13 days) to approx 30 per cent of the maximal concentration upon entering a more stable phase after about 30 days' incubation.

(b) *Fraction* 2. (Bicarbonate-soluble compounds of low molecular weight.) ^{15}N-Labelled amino acids were formed most readily even after 0·5 days' incubation when 30 per cent of the added ^{15}N-nitrate had been immobilized (Fig. 3). The concentration of ^{15}N-amino acids in fraction 2 remained near maximum between 1·5–7·5 days' incubation, then decreased rapidly (half-life 4·9 days) to a more stable level after about 20 days.

(c) *Fraction* 3. (Insoluble compounds associated with particles, $>2{\cdot}0$ μm.) After a lag of 0·5 days, the concentration of ^{15}N-labelled amino acid-N increased rapidly. In contrast to that obtained with the other fractions, amino acid-^{15}N of fraction 3 continued to increase between 1·5 and 35·5 days' incubation, but thereafter declined.

(d) *Fractions* 4 *and* 5. (Insoluble compounds associated with particles, 0·2–2·0 μm and 0·04–0·2 μm, respectively.) Like fraction 3, the amino acid-N of fractions 4 and 5 became rapidly labelled after a lag of 0·5 days. However, unlike fraction 3, the level of labelled amino acid-^{15}N decreased after 1·5 days; in the case of fraction 4, this decrease occurred

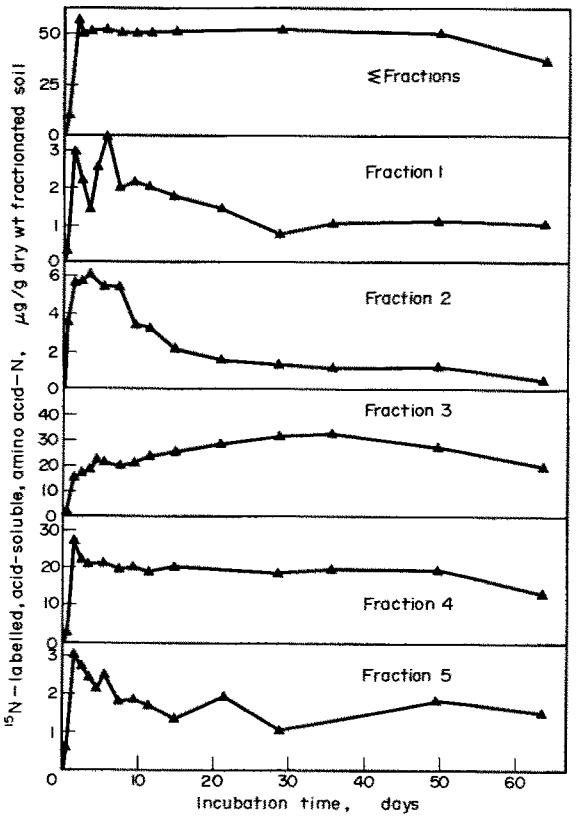

FIG. 4. Distribution of ^{15}N-labelled, acid-soluble, amino acid-N during nitrate-^{15}N immobilization.

within 4 days' incubation and the relatively stable concentration attained was about 70 per cent of the maximal for at least a further 45 days. The labelled amino acid-N of fraction 5 decreased more slowly but to a greater extent than that of fraction 4.

The content of acid-soluble amino acid-^{15}N of fractions 6 and 7 are not shown in Fig. 4; the values obtained, especially, in the early period of incubation, were highly erratic reflecting mainly large variations in values for atom percentage enrichments. However, the amounts of amino acid-^{15}N of these two fractions represented only small percentages of that of the total of all fractions.

Table 2 shows the ^{15}N atom percentage enrichment of nitrate-^{15}N and of the acid-soluble amino acid-^{15}N (fractions 1–5) throughout the incubation period. In general, changes in the atom percentage enrichments of the amino acid-^{15}N have the greatest effects on the concentration of labelled amino acid-N in each fraction.

Enzyme activities

Enzyme activities also varied with changes in incubation time after glucose and nitrogen amendment. Fig. 5 shows the net enzyme activities obtained in five of the assays. The values graphed represent differences between activities of corresponding soil samples moistened with glucose–nitrate solution and with distilled water; they thus represent the net changes in enzyme activities resulting directly or indirectly from the metabolism of the added C–N source sources. Values for the net hydrolyses of low molecular weight substrates

TABLE 2. ATOM PER CENT ENRICHMENT OF NITRATE-^{15}N AND ACID-SOLUBLE AMINO ACID-^{15}N DURING INCUBATION

Incubation period (days)	Atom per cent enrichment					
	Amino acid-^{15}N of fraction					
	1	2	3	4	5	Nitrate-^{15}N
0·5	0·189	1·941	0·039	0·241	0·364	6·747
1·5	1·360	2·435	0·243	1·533	1·407	0·455
2·5	0·809	2·551	0·314	1·478	1·318	0·082
3·5	0·751	2·371	0·359	1·411	1·228	N.D.
4·5	0·783	N.D.	0·402	N.D.	1·157	0·500
5·5	1·257	2·574	0·368	1·165	0·975	0·660
7·5	0·899	2·413	0·391	1·102	1·000	0·661
9·5	0·715	2·045	0·440	1·105	1·183	0·703
11·5	0·844	2·152	0·427	1·123	0·882	0·769
14·5	0·563	1·705	0·444	1·144	0·664	1·241
21·5	0·569	1·673	0·521	N.D.	0·781	N.D.
28·5	0·493	1·401	0·474	1·122	0·683	N.D.
35·5	0·518	1·227	0·467	1·076	N.D.	2·130
49·5	0·702	1·439	0·448	0·956	0·662	2·335
63·5	0·704	1·661	0·428	0·683	0·700	2·495

N.D. Not determined.

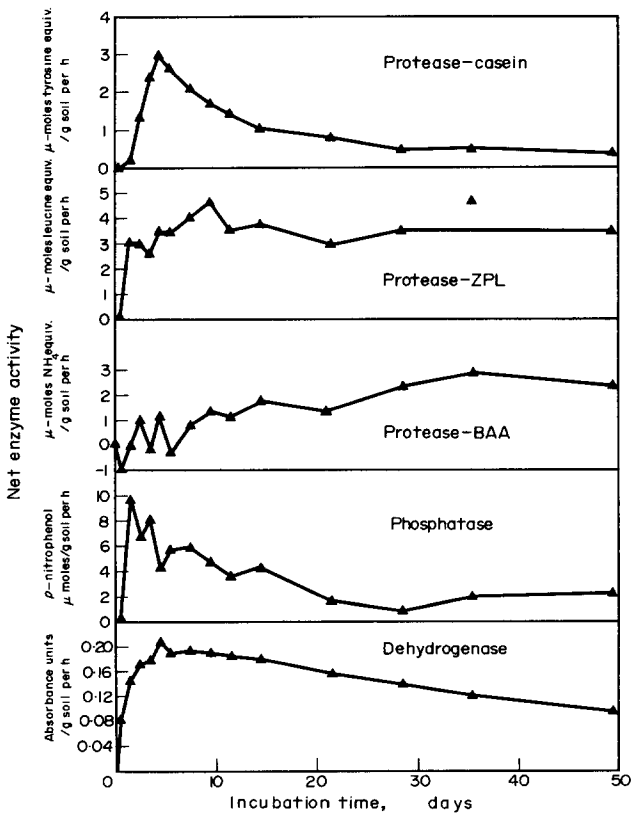

FIG. 5. Changes in net enzymic activities of unfractionated Bradwell soil during nitrate-^{15}N immobilization. Initial activities before correction were: protease (casein), 0·26 μmoles/g soil per h; protease (ZPL), 4·0 μmoles/g soil per h; protease (BAA), 10·3 μmoles/g soil per h; phosphatase, 5·6 μmoles/g soil per h; dehydrogenase, 0·13 absorbance units/g soil per h.

(ZPL, BAA, PNPP) are subject to greatest error since: (a) initial activities, even of the air-dried soil, were relatively high, necessitating assays with low amounts (0·3 g) of soil and short reaction times (up to 20 min); and (b) the percentage changes in activities were relatively low (even after glucose-nitrate addition) when contrasted with those obtained for casein hydrolysis.

(a) *Casein hydrolysis.* Net activity remained low for 1·5 days, after which a rapid increase occurred for a period of 2–3 days, coinciding with the phase of rapid decline in viable bacterial numbers. After 4·5 days' incubation, protease activity decreased rapidly (half-life, 7 days), until after 28·5 days, the net gain in activity was only 18 per cent of that of the maximal.

(b) *ZPL hydrolysis.* Net increase in activity coincided mainly with the logarithmic increase in viable bacterial numbers (0·5–1·5 days). Thereafter the gain in activity increased slightly to remain relatively constant for the latter part of the incubation period.

(c) *BAA hydrolysis.* Despite relatively large fluctuation in the early days of incubation, net activity towards BAA increased slowly and appeared to be independent of the large changes in the viable bacterial populations.

(d) *PNPP hydrolysis.* As obtained with ZPL, net gain in activity towards PNPP coincided with bacterial growth. However, the newly synthesized phosphatases rapidly lost activity on further incubation (half-life, 7·4 days), until, after 21·5 days, the net activity gain was only 18 per cent of that of the maximal.

(e) *Dehydrogenase activity.* Net activity increased rapidly from commencement of incubation, reaching a maximum after approx 5–7 days. The decline in activity was relatively slow thereafter (half-life, 45 days).

Overall, the effects of incubating glucose–nitrate with the Bradwell soil were to increase the rates of hydrolysis of casein, ZPL and BAA and the phosphatase and dehydrogenase activities to levels, which at their maximum, were 11·9, 2·4, 1·5, 3·2 and 3·2 times respectively those of the soil at the initiation of the experiment. The respective maximal levels attained by soil moistened with distilled water only were 1·7, 1·5, 1·2, 1·7 and 1·8 times those obtained initially.

TABLE 3. OXIDATION OF ^{14}C-LABELLED SUBSTRATES

Substrate	Initial activity (μg CO_2–C/g soil/h)	Maximal activity (μg CO_2–C/g soil/h)	Period for maximal activity (days)	Activity after 17·5 days' incubation (μg CO_2–C/g soil per h)
Glucose-^{14}C(U)	6·2	20·0	2·5–4·5	18·3
Succinate-^{14}C(1: 4)	5·3	29·3	2·5–4·5	24·3
Acetate-^{14}C(U)	0·7	9·3	2·5	6·1
Alanine-^{14}C(U)	1·2	11·3	2·5	4·9

Rates of oxidation of ^{14}C-labelled substrates were determined only for soil receiving glucose-NO_3 initially, so that net activities are not available. Maximal activities were attained within 2·5 days' incubation. Alanine oxidation rates declined rapidly thereafter, acetate oxidation rates were more stable, whilst glucose and succinate oxidation rates showed but little decline when sampled over a period of 17·5 days (Table 3).

Distribution of enzyme activities

Table 4 lists the recoveries and percentage distributions of enzyme activities after fractionating soil, previously incubated for 5 and 50 days following addition of glucose–nitrate.

TABLE 4. DISTRIBUTION OF ENZYME ACTIVITY

	Casein hydrolysis		ZPL Hydrolysis		BAA Hydrolysis		Phosphatase*	Dehydrogenase	
	5 days	50 days	5 days	50 days	5 days	50 days	5 days	5 days	50 days
Activity of unfractionated soil†	2·76	0·58	8·8	8·6	11·6	14·8	10·1	0·375	0·271
Sum of activities of soil fractions†	1·37	0·53	9·0	9·4	10·7	15·7	4·3	0·198	0·248
$\frac{\Sigma \text{ Activity of fractions}}{\text{Activity of unfractionated soil}}$ (%)	49·7	91·2	102·2	108·7	90·8	106·0	43·1	52·9	93·0
$\frac{\text{Activity of fraction}}{\Sigma \text{ Activity of fractions}}$ (%)									
Fraction 1 ($NaHCO_3$ sol. high M.W.)	28·8	23·9	7·0	5·8	4·4	4·7	6·0	0·8	1·9
Fraction 3 (Insol. >2·0 μm)	32·9		56·0	63·0	74·0	68·8	67·6	77·2	81·1
Fraction 4 (Insol. 0·2–2·0 μm)	22·9	50·6	26·1	22·9	15·3	15·7	16·7	20·2	13·5
Fraction 5 (Insol. 0·04–0·2 μm)	8·2		6·0	2·8	3·6	6·4	7·2	1·5	1·6
Fraction 6 (<0·04 μm. high M.W.)	7·2	25·5	4·9	5·5	2·6	4·5	2·3	0·4	3·8

* Phosphatase was not determined at 50 days.

† Activities expressed as μmoles tyrosine equiv/g soil per h (casein hydrolysis); μmoles leucine/g soil per h (ZPL hydrolysis); μmoles NH_4^+/g soil per h (BAA hydrolysis); μmoles *p*-nitrophenol/g soil per h (phosphatase); absorbance units (485 nm)/g soil per h (dehydrogenase).

Soil could be fractionated with essentially no loss in activity towards ZPL or BAA. Protease (casein substrate) and dehydrogenase activities of soils incubated for 50 days were also almost quantitatively recovered after fractionation. However, in the earlier incubation period, when increased activities of protease, dehydrogenase and of phosphatase were close to maximal or still substantial, fractionation of the soil caused large (>50 per cent) losses of activity. Thus, overall, losses of activity were least if fractionation was carried out at a stage in the incubation when rates of change of enzyme activity were low. Replacement of the extractant, 0·1 M $NaHCO_3$, with 0·1 M Tris buffer, pH 8·1 increased the total recovery of enzymic activity but the efficiency of extraction (of protease) was low.

Table 4 shows that dehydrogenase activity was almost completely retained by soil components of particle size, >0·2 μm (fractions 3 and 4). By contrast, casein hydrolysing activity was more evenly distributed, a significant proportion of the activity being located in the bicarbonate extracts (fraction 1) and in the supernatants after centrifugation at 12,000 ***g*** (fraction 6). Activities of enzymes hydrolysing the low molecular weight substrates were mainly associated with fractions of particle size >0·2 μm, but small levels of activity were present in the extracts and in particles, 0·04–0·2 μm.

The proportion of activity in the respective fractions did not change appreciably when compared at 5 and 50 days incubation. An obvious exception was the increased proportion of casein hydrolysing activity in fraction 6 after the longer incubation time.

DISCUSSION

Sodium bicarbonate solutions were used to extract amino acids, peptides and proteins, either free or loosely adsorbed by ionic bonds to soil surfaces. The extractant is regarded as being sufficiently mild not to remove large amounts of these compounds from intact cells although inevitably some extracted material would be derived from these sources. Of the extracted material, fraction 1 is anticipated to contain proteins, including enzymes produced extracellularly or released as lytic products during the period of incubation. Amino acids and peptides are confined to a separate fraction (fraction 2). Subsequent dispersion of the extracted soil was carried out at 10°C in a water-jacketed Waring blender rather than by employing an ultrasonic probe since Babiuk and Paul (1970) obtained maximal viable microbial numbers by the former procedure. Fractions 3 and 4 are thought to contain intact microorganisms, particulate cell debris and microbial metabolites adsorbed to larger soil particles. Fractions 5 and 6 (both <0·2 μm) are believed to contain no intact microbial cells but nitrogenous compounds mainly associated with soil colloids (McGill, 1971; Anderson, 1972).

Although the fractions were not completely discrete, the newly-synthesized, acid-soluble amino acids contained therein differed appreciably in their relative concentrations during the incubation period. Extractable, low-molecular weight material was formed in the early phase of nitrate immobilization. During rapid microbial growth, the contents of acid-soluble, amino acid-N of all fractions rose markedly. However, upon entering the phase of decline in viable bacterial numbers, during which microbial metabolites were the main energy sources, there was initially a rapid decline in the amounts of extractable protein and a less rapid but significant decrease in nitrogenous components associated with the fine soil colloids. Extractable amino acids and peptides remained in relatively high concentration for a slightly longer period, but this N-pool also decreased rapidly as the rate of oxidation of microbial metabolites decreased.

During the prolonged incubation period, 2·5–35·5 days, the value of the sum of the labelled amino acid-^{15}N in all fractions remained relatively constant. Increased net amounts

of labelled, acid-soluble, amino acid-N in fraction 3 balanced the decline in their concentration in all other fractions. A greater part of the decrease in acid-soluble, amino acid-^{15}N of fractions 1, 2 and 5 occurred during the active phase of oxidation of microbial metabolites (Fig. 2) when little net mineralization of labelled compounds occurred (Fig. 3) and when nearly all available ^{15}N-nitrogen was used for the synthesis of secondary microbial populations and their products (fraction 3).

^{15}N-Labelled nitrate did not form appreciably until after 35·5 days (Fig. 3) and possibly was derived to a large extent from the acid-soluble, amino acid-^{15}N of the particulate microbial cell material in fractions 3 and 4 (Fig. 4). However, the decreased content of labelled amino acid-N of fraction 3 during the latter phase of the incubation reflected an overall decline of the total amino acid-N concentration, rather than a decrease in the proportion of labelled amino acid-N present (Table 2). Hence there is no clear indication that the amino acid-^{15}N of fraction 3 constituted the source of the active organic-N pool from which nitrate-^{15}N was in part derived.

Another feature of the distribution of the acid-soluble amino acid-^{15}N was the secondary transient increase in concentration of extractable protein-^{15}N (fraction 1) after 4–5 days' incubation. This occurred after the initial flush of microbial growth but coincided approximately with the rise and fall of net proteolytic activity towards casein substrate. Since appreciable amounts of casein-hydrolysing activity are extractable (Table 4), it seems possible that extracellular proteolytic enzymes may account for much of the extractable protein, newly synthesized or released during this secondary phase. If so, the results support the suggestion of Sorensen (1969, 1972) that extracellular enzyme-N may contribute measurably to the total-N immobilized in the acid-soluble, amino acid fraction.

Casein-hydrolysing activity was short-lived. Ladd and Butler (1973) have argued that extracellular enzymes, when present in a soil microenvironment which allows their action against substrates of high molecular weight, are in a state in which they themselves are vulnerable to attack by other proteolytic enzymes. Alternatively, the enzyme proteins themselves may become protected by their association with organic or inorganic soil colloids, in which case (a) restrictions on substrate range may be imposed such that high molecular weight compounds are no longer attacked and (b) the enzymes may no longer be extractable with sodium bicarbonate.

Dehydrogenases and enzymes involved in the chain of conversion of carbon sources to CO_2, are regarded as being associated in the soil with intact cells (Skujins, 1967; Ross, 1970). The distribution data of Table 4 are compatible with this assumption. However, the relative activities of the different enzymes varied throughout the incubation period. None of these enzymes exhibited activities which were representative of the changes in total, acid-soluble, amino acid-^{15}N concentrations of fractions 3 and 4.

The states in which hydrolases of the low molecular weight substrates occur in soil are uncertain. McLaren and colleagues (Ramirez-Martinez and McLaren, 1966; McLaren and Skujins, 1971) have suggested that some of the phosphatases of soil are stabilized as extracellular enzymes since variations in phosphatase activity with the season were much less than the changes in microbial populations. Experiments with fumigated and irradiated soils show that after inoculation, enzymes hydrolysing ZPL and especially BAA, behaved similarly in this respect (Ladd, Brisbane and Butler, unpublished data). The evidence for extracellular action of these enzymes is of course, most indirect and rests on the assumptions that the plating procedures adopted were sufficiently both specific and representative to allow measurements of changes in those microbial populations which may contain the enzymes in question.

The present investigation shows that only a small proportion of the total activity towards ZPL, BAA or PNPP was associated with the soil colloids of fraction 5 (Table 4). Further, the increased activity of phosphatase and of the ZPL-hydrolysing enzyme occurring within 1·5 days' incubation was undoubtedly connected with the initial microbial proliferation. The results suggest that the newly-synthesized hydrolases may be acting intracellularly. Most of their activities were associated with fractions 3 and 4 and, like those of the oxidative enzymes, were unrepresentative of changes in the net concentration of acid-soluble, amino acid-^{15}N of these fractions.

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