Growth and Maintenance of Microbes in Natural Soil with Special Reference to Their Energetics

A dissertation submitted to the graduate school of science, University of Tokyo, in partial fulfilment of the requirements for a ph. D. degree

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Contents

General Introduction

The ultimate source of energy used by all living beings on the earth is the sun. Primary producers (mainly higher plants) convert the solar energy into the chemical energy in organic matter for their growth and reproduction. Then the consumers (animals) and decomposers (microbes) utilize the organic matter through the process of eating and being eaten. Through these food chains, the energy is gradually converted into heat and the organic materials are mineralized. The producers incorporate these mineralized materials again into the organic substances using the solar energy. This one-way flow of energy and balanced circulation of materials are the important characteristics of the ecosystems (Odum, 1971).

In terrestrial ecosystms, the detritus food chain is the main pathway of the energy and material conversion. According to Whittaker (1970), about 90% of net primary production falls to soil as litter and is decomposed. The biochemical pathway of decomposition of each organic substance has been studied by many investigators. The empirical compartment models of material conversion also have been proposed. But the soil biomass decomposing the organic matter had not been accurately determined because it was contained in heterogeneous structure of soil. In 1970's two effective methods for determination of soil biomass were developed by Jenkinson and co-workers: One is $CHCl_3$-fumigation method and the other is ATP method. Using these

methods, Jenkinson and Powlson (1976) and Jenkinson and Oades (1979) showed that the microbial biomass amounted to over 200 g dry matter m^{-2}, while the amount of consumers is only about 5 g m^{-2} (Odum, 1971). Thus the microbes in soil take the largest portion of the heterotrophs in terrestrial community which concern organic matter decomposition.

From the viewpoint of autoecology, not continuation of the whole ecosystem but survival of an individual is important to the members of ecosystem and the survival strategy of an individual characterizes the ecosystem functioning. Therefore, we cannot understand the cause of stability of ecosystem functioning unless the life of an individual is revealed. However, the dynamics and metabolic conditions of soil microbes, which would be the important features of their life, were not studied (Anderson and Domsch, 1985).

The question I intend to answer in this thesis is how the microbes are living in soil. The answer to this question would also explain what is the survival strategy of soil microbes. For this purpose, I tried to clarify the features of microbial life in soil, concentrating on acquisition and utilization of energy by soil microbes, since energy is one of the most important factors that affect the biological processes.

A limiting factor in growth of microbes is one of the important features. In Part I, changes in the limiting

factor are shown through the measurement of formation and turnover of microbial biomass in the organic matter. Plant materials of Polygonum cuspidatum are incubated in the laboratory and changes in biomass-C of soil microbes are measured. The turnover time of biomass is estimated from the amount of biomass-C and the release rate of CO_2-C.

Metabolic condition maintained by energy utilization is important. In Part II, the metabolic condition of microbes in natural soil is studied using a biochemical indicator, adenylate energy charge. First, the adenine nucleotide form and amount are determined in glucose-amended and unamended soil. Second, a chemostat system with organism feedback (Pirt and Kurowski, 1970) is applied to simulate the soil ecosystem. The metabolic condition of microbes in soil and the response of biomass to energy supply are discussed.

Microbes in natural soil obtain their energy source by degradation of soil organic matter with their extracellular enzymes. In Part III, the rate limiting of this reaction is clarified through measurement of reaction velocity in substrate- or enzyme-amended soils to evaluate the effort for energy acquisition by soil microbes. Cellulase and protease are used as the typical soil enzymes.

Lastly, synthesizing the results, I will answer the question and discuss the relationship between the survival strategy of an individual and the function of a whole ecosystem.

Part I. Growth and Turnover of Microbial Biomass in Organic Matter Decomposition Process

Introduction

In terrestrial ecosystems, the consumers graze only about 10% of dry matter production of plants and the remaining 90% falls down as litter (Whittaker, 1970). In natural soil, microbes are major component of soil biomass (Jenkinson and Powlson, 1976; Anderson and Domsch, 1978) and utilize the organic matter for their energy and nutrient source. They convert part of organic matter into new biomass and mineralize the remainder to obtain energy. Hence growth and decay of microbial biomass and its turnover are the important process of energy flow and nutrients cycles in the terrestrial communities.

However, only few authors studied the dynamics of soil microbial biomass through organic matter decomposition (Jenkinson and Rayner, 1977; Ladd et al., 1981), although decomposition rates of total organic matter have been estimated in many field studies (Jenkinson, 1965, 1971, 1977a,b; Smith and Douglas, 1971; Shields and Paul, 1973; Nyhan, 1975; Jenkinson and Ayanaba, 1977) and biochemical pathways of decomposition of each component have been clarified by many investigators. Especially the dynamics of soil microbes have not been studied in the early stages of organic matter decomposition in which large amount of organic matter is lost.

Of all the elements converted by soil microbes, carbon is the most important, because it is the major component of organic matter and is used as both nutrient and energy source.

In part I, changes in biomass-C and turnover of soil microbes in the course of organic matter decomposition are studied with special reference to those in the early days.

Materials and Methods

Organic matter

Fresh leaf, fresh stem, newly fallen dead leaf and standing dead stem of Polygonum cuspidatum were collected on Mt. Fuji (1400 m altitude) in August 1984 to study the difference of decomposition process between fresh and dead materials. The materials were air dried at room temperature and crushed with a mill (<14 mesh). The carbon and nitrogen contents were measured with a NC-Analyzer (Sumika). C/N ratios of the fresh leaf, fresh stem, dead leaf and stem were 24, 72, 48 and 110, respectively.

Soils

Soil contains organic matter processed by soil biomass for a long period. To compare the turnover time of biomass-C in organic matter decomposition with those in

soils, eleven organic soils were collected from H layer: Three were sampled from the oak forest on sand dune of Azigaura, three from a coniferous forest on scoria of Mt. Fuji and five from the campus of the University of Tokyo, in November 1984. These soils were collected from different types of soil to generalize the tendency. But soils from deep layers were not used, because it was difficult to determine the small biomass in deep soil quantitatively. The soils were wrapped with polyethylene film to avoid evaporation and stored at 4°C until experiment. Part of soils was air-dried and used for the determination of soil properties. Table 1-1 shows properties of the soils.

Incubation

Plant materials: 10 g of wet soil (even mixture of the eleven soils which were collected for comparison) was added to 500 ml of water and stirred for 10 min. After precipitation of the soil particles, 2.4 ml of the supernatant was placed in a glass test tube (105 mm length, 18 mm diameter). Then 600 mg of air dried organic matter was put into the tube. After 1 h, moistened organic matter was sticked on the wall of the tube (about 2 mm thickness, 40 mm length) by a spatula to maintain aerobic condition. The tube was loosely capped with a sheet of aluminum foil to prevent both suffocation and evaporation.

Soils: The soils were sieved (2 mm mesh) and the water content was adjusted (50 % of the water holding capacity).

Table 1-1. Properties of the organic soils.

Soil	WHC (%)	pH	C (%)	N (%)	Biomass-C (g C/kg)
1	186	5.9	16.8	1.23	1.76
2	206	5.7	12.1	0.75	1.41
3	136	6.9	11.6	0.74	1.52
4	93	4.2	7.2	0.39	0.34
5	240	6.7	15.7	1.53	2.13
6	251	4.4	23.1	1.54	2.30
7	241	4.3	21.1	1.62	1.89
8	189	4.5	18.1	1.06	1.24
9	66	4.7	7.0	0.42	1.29
10	65	4.5	9.1	0.51	1.80
11	60	4.7	5.3	0.30	1.21

Soil 1-5 from the campus of the University of Tokyo, soil 6-8 from Mt. Fuji, soil 9-11 from Azigaura.

a, water holding capacity.

50 g of the wet soil was put into a 100 ml polyethylene vial. The vial was wrapped with a thin polyethylene film.

Incubation of the plant materials and soils was done at 25°C. Moisture content was kept constant, adding water to the tubes and vials every two weeks.

Analytical method

Dry weight of organic matter was determined after drying at 80 C for a week. CO_2 evolution from the incubated organic matter and soils was determined by the alkaline absorption method (Stotzky, 1965).

Microbial biomass was determined by the ATP method (Jenkinson and Oades, 1979). This method is based on the close link between ATP content in soil and the amount of living organisms (Lee, et al., 1971). ATP in the soils and organic matter was extracted with the trichloroacetic acid-phosphate-paraquat solution and 10% trichloroacetic acid, respectively. For the assurance of the recoveries, known amount of ATP was added separately to the extractant. The extract was stored at -20°C and ATP content was determined within a week, following Karl and Holm-Hansen (1978). An Aminco Chem Glow Photometer J4-7441 was used to detect the light emitted from luciferine-luciferase of firefly (FLE-50, Sigma) with ATP. The recoveries of added ATP were 53.4-94.0% in the organic matters and 40.8-82.2% in the soils. Although the recoveriers were incomplete, the reproductivity of

recovery was good. The corrections were made using the recoveries. The biomass-C was estimated from 171*ATP (Tate and Jenkinson, 1982).

Calculation of turnover time

Calculation of turnover time followed Chapman and Gray (1986) with some modifications: The substrate-C utilization rate in a chemostat is

$$-\frac{dS}{dt} = \frac{1}{Y}\frac{dx}{dt} + ax \qquad (1)$$

where S, x, Y and a represent substrate-C (carbon and energy source), biomass-C, true growth yield and specific maintenance rate, respectively (Marr et al., 1963). Organisms use part of substrate for maintenance of cells and the remainder for synthesis of new cells under growth yield, Y. The maintenance energy is used for protein and nucleotide turnover, osmoregulation, motility, etc. (Tempest and Neijssel, 1984).

Chapman and Gray (1986) pointed out that dead cells return to the substrate pool in soil. Their carbon flow diagram in soil is shown in Figure 1-1 with slight modification, assuming steady state. Substrate-C (S) is supplied from the pool of soil organic matter by enzymatic degradation. μx, carbon in dead microbes, is added to S. μ represents the specific growth rate of biomass, which is equal to the specific death rate under the steady state condition. Biomassuses -dS/dt plus μx for their growth and

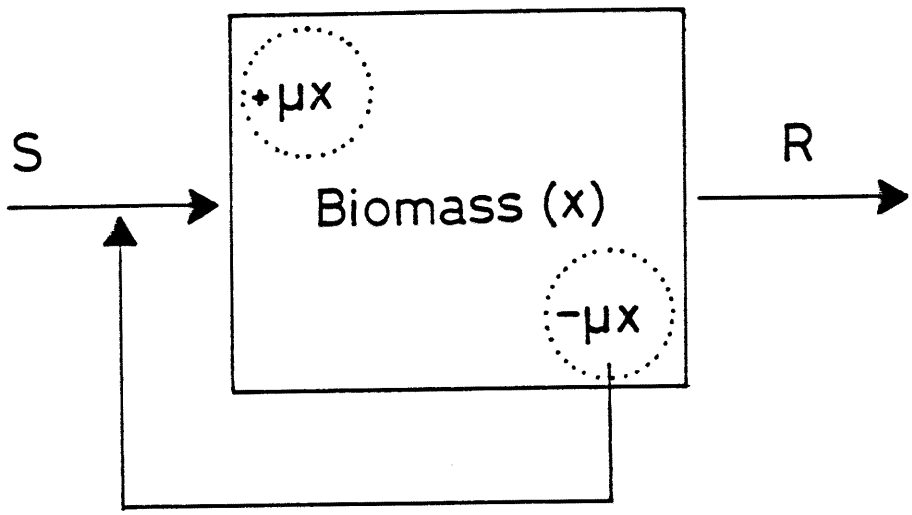

Figure 1-1. Carbon flow diagram in soil used for estimation of turnover time of microbes. S, substrate-C for soil microbes; R, lost material-C by respiration; x, microbial biomass-C; μx, synthesized biomass-C; -μx, dead biomass-C. Under steady state, S is balanced to R.

maintenance. R represents carbon lost by respiration. From equation (1),

$$-\frac{dS}{dt} + \mu x = \frac{1}{Y} \cdot \mu x + ax \qquad (2)$$

In the steady state, -dS/dt is equal to dR/dt. By substitution and rearrangement, we obtain μ:

$$\mu = \frac{Y}{Y - 1}\left(a - \frac{1}{x} \cdot \frac{dR}{dt}\right) \qquad (3)$$

μ^{-1} gives the turnover time. The value of Y used is 0.5 (Payne, 1970). 0.000 and 0.001 h^{-1} were used for a (Babiuk and Paul, 1970).

Results

By day 250, the microbes decomposed 28-39% of dry matter (Figure 1-2). The decomposition rates were relatively high in the initial few days, especially in fresh leaf and decreased slowly with time. The higher the initial C/N ratio, the larger the amounts of material remaining on day 250. The release rate of CO_2-C is closely related to the decreasing rates of the plant materials (Figure 1-3). The respiration rate was high in early days, especially in fresh leaf and fresh stem. Then the rate decreased and after 70 days no significant changes were observed in all series.

The microbial biomass-C increased and attained the maximum level on day 7 in fresh stem and on day 14 in fresh leaf (Figure 1-4). The biomass-C increase in dead leaf and stem ceased within 4 days. The amount of carbon utilized by

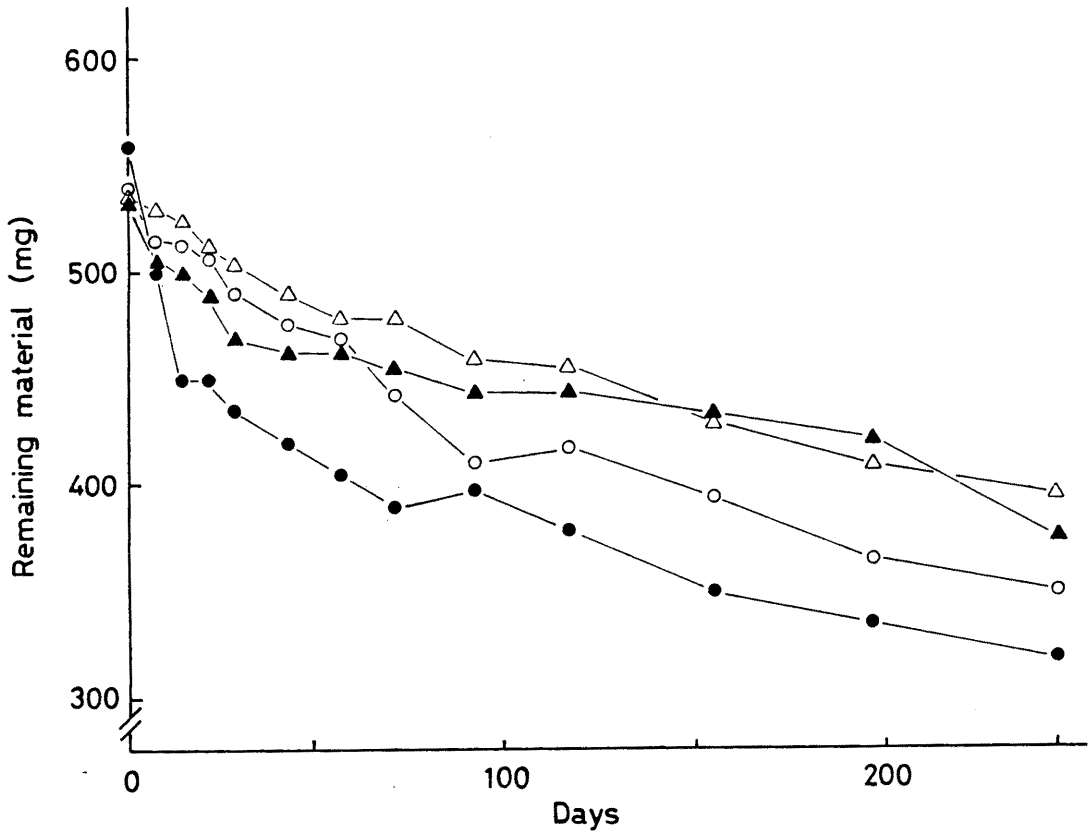

Figure 1-2. Remaining dry matter of fresh leaf (●), fresh stem (▲), dead leaf (○) and dead stem (△) in a test tube. Mean of duplicate samples. maximum range was 18 mg.

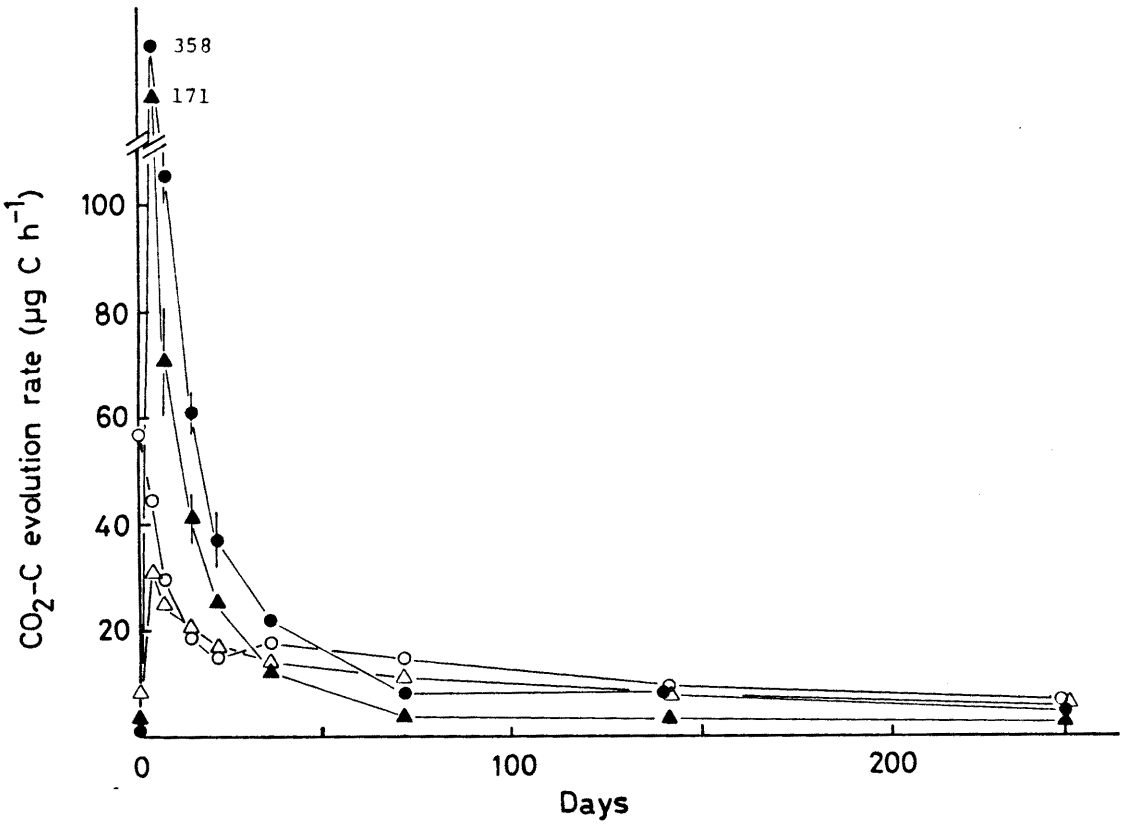

Figure 1-3. Changes in release rate of CO_2-C from a test tube during incubation of fresh leaf (●), fresh stem (▲), dead leaf (○) and dead stem (△). N = 3, mean ± SD.

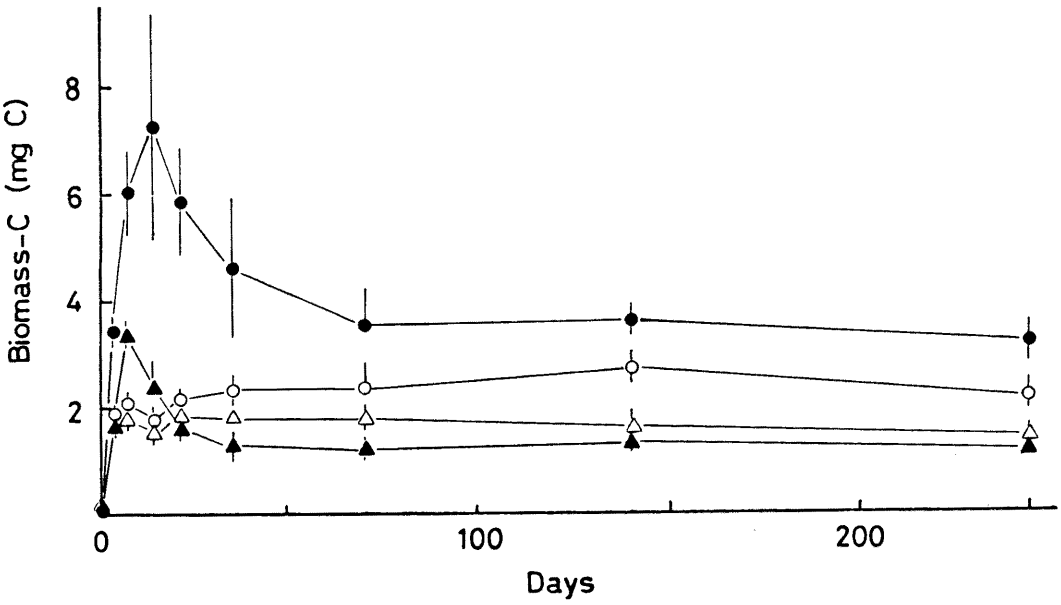

Figure 1-4. Changes in biomass-C in a test tube during incubation of fresh leaf (●), fresh stem (▲), dead leaf (○) and dead stem (△). N = 3, mean ± SD.

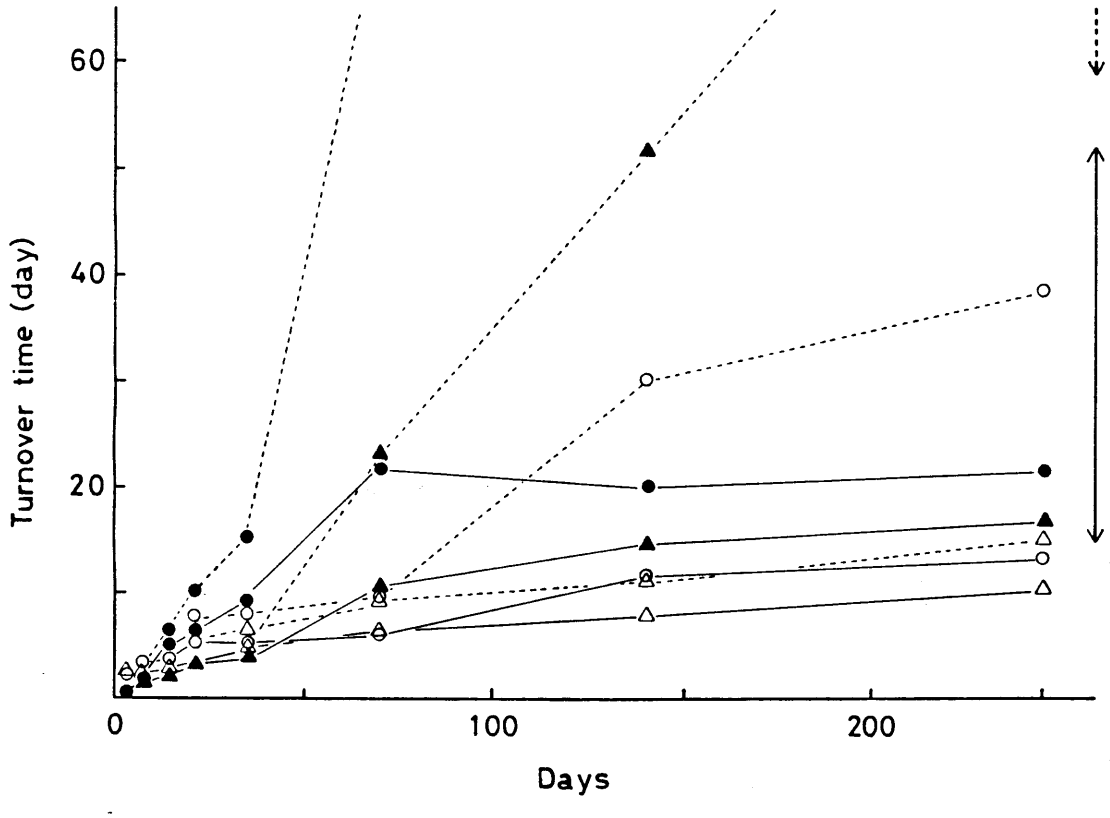

Figure 1-5. Estimated turnover time of microbial biomass in fresh leaf (●), fresh stem (▲), dead leaf (○) and dead stem (△). Solid line and dotted line represent the values when specific maintenance rate, a, is 0.000 h^{-1}, and 0.001 h^{-1}, respectively. Vertical bar in the right side of the figure represents the turnover time in natural soil. When a = 0.001 h^{-1}, turnover did not occur in some soils and fresh leaf after 70 days, because all energy was used for maintenance of cells.

the microbes can be estimated from the synthesized biomass-C plus the evolved CO_2-C. Then by the day when the increase in biomass-C ceased, 11.2%, 16.0%, 38.7% and 41.1% of the utilized carbon were incorporated into the biomass-C in fresh leaf, fresh stem, dead leaf and dead stem, respectively. Thereafter the biomass-C settled down to stable levels in fresh leaf and fresh stem and did not change in dead leaf and dead stem. These stable biomass and stable respiration rates after 70 days indicates that the organic matter available for the microbes was constantly supplied by the degradation of plant materials. In the fresh leaf and fresh stem succession of fungi was observed until day 70.

Figure 1-5 shows the changes in turnover time of the microbial biomass. Although the steady-state model may not be applied in the early days owing to the variation of the biomass-C and the respiration rate, the turnover time was estimated in all growth stages with this model. The shortest turnover time (10 h) was observed on day 4 in fresh leaf. This value was similar to generation time of many microbial species in a favorable condition (Brock, 1966). After the cessation of increase in the biomass-C, the turnover time became longer in all series with incubation. On day 250, the value was longer than 9 days when a = 0.000 h^{-1} and approached those in the natural soils (longer 15 days for the same a). This a gives the shortest time, because all energy is used for synthesis of new cells. When

$a = 0.001\ h^{-1}$, the time is longer than 17 days in the decomposition experiment on day 250 and longer than 59 days in the natural soils. The growth yield Y, as well as the maintenance energy a, affects the turnovertime estimated with this model. When Y is small, the turnover time became longer.

Discussion

Microbes in natural soil cannot grow actively due to energy limitation (Babiuk and Paul, 1970). In the plant corpses after 70 days and the all natural soils examined in the present experiment, the turnover time was much longer than that of actively growing organisms. This long turnover time suggests that available energy is too small to grow actively. Although longer than actively growing ones, the turnover time was shorter than that obtained by several other authors. Hunt (1977) found the average turnover time to be 0.5 yr in grassland. Jenkinson and Ladd (1981) found that turnover time was 1.25 yr and 2.5 yr for an Australian clay soil and Rothamsted soil, respectively. The differences may be ascribed to the following reasons: (1) The incubations of this experiment were done in the laboratory under favorable condition, while the others were done in the field where low temperture and water stress might have limited microbial growth. (2) Compared with whole soil system, the organic matter and soils studied were young and immature. (3) Differences in the values, Y and a,

affected the turnover time greatly (Chpapman and Gray, 1986). Indeed, the Ychanges widely (Linley and Newell, 1984). However, at least, it is sure that soil microbes cannot grow rapidly at the late stage of organic matter decomposition process.

In contrast, rapid growth and turnover of biomass were observed in the early stages of organic matter decomposition. Since the model can be applied to steady state condition only, the turnover time at the early days might not be correct. However the model can be applied to this stage, the turnover time is affected by Y and a. For example, when Y is 0.1, the turnover of biomass in the fresh leaf does not take place. In spite of these reservations, rapid increase in total biomass indicates the occurrenceof rapid division of cells at this stage.

These results suggest that the survival strategy of microbes changes with incubation. Rapid growth may be essential in the early stages of decomposition, while tolerance for energy deficiency is indispensable for survival in the later stages. If soil microbes must trade the ability to grow rapidly for that to tolerate energy-deficiency, the rapidly growing species will be succeeded by the tolerating species in the course of organic matter decomposition. In fact, several authors reported the microbial succession in organic matter decomposition (Hudson, 1958; Dickson and Pugh, 1974; Flankland, 1981). In the fresh leaf and fresh stem of this experiment, fungal

succession was observed at the early days. Since the rapid growth and turnover ceased within two weeks (Figure 1-4 and 1-5), it is probable that the tolerating species dominate in soil ecosystems.

Part II. Metabolic Condition of Microbes in Soil

Chapter 1

Form and Amount of Adenine Nucleotide in Soil

Introduction

The studies on energy flow have shown that microbes in natural soil cannot grow actively due to energy deficiency (Babiuk and Paul, 1970; Jenkinson and Ladd, 1981; Chapman and Gray, 1986). The results of Part I also showed that the average turnover time of microbes in soil was rather long probably due to energy deficiency except for those in the early few days of organic matter decomposition. However, this average long turnover time would not reveal the metabolic condition of each microbe tolerating energy deficiency. The following two cases may occur when the turnover time is long. First, a large portion of the microbes may be in energy-depleted condition, while a small portion grows actively. Second, almost all of the microbes are slowly growing or maintaining themselves due to limitation of energy supply.

Generally organisms convert the chemical energy in organic matter into that in high-energy phosphate, including various nucleoside phosphate and polyphosphate, before utilization for biological processes. Of all the high energy phosphates, the adenine nucleotide form and content

in organisms are useful indicators of their metabolic condition. Atkinson and Walton (1967) defined the adenylate energy charge (AEC) ratio as AEC = ([ATP]+1/2[ADP])/([ATP]+[ADP]+[AMP]). In in vitro experiments, actively growing organisms have high AEC ratios (above 0.8) while energy-depleted organisms and spores have a low AEC ratio (Chapman et al., 1971; Chapman and Atkinson, 1977; Karl, 1980). The biomass-C to ATP (C/ATP) ratio is another indicator of metabolic condition. Actively growing organisms have low C/ATP ratios (below 500), while energetically stressed ones have high ratios (Karl, 1980).

The aim of this chapter is to clarify the metabolic condition of microbes in soil through measurement of form and amount of adenine nucleotide in soil. For this purpose, the amount of adeine nucleotide and AEC ratios in glucose-amended and unamended soil were measured in the laboratory. The glucose amendment increased the biomass temporally (Paul and Johnson, 1977; Nannipieri et al., 1978). At this increasing stage, soil microbes are sufficient in energy. On the other hand, when the amended glucose was used up, the temporally increased biomass were in strict energy deficiency more than that in unamended soil, because supplied energy per unit biomass decreases as the biomass increases, assuming that degradation rate of soil organic matter is constant (see Part I). Then response of biomass and AEC ratios to energy sufficiency and strict energy deficiency is clarified by the glucose-amendment.

Materials and methods

The soil was taken on 30 September, 1983 from the humus layer of Himalayan ceder plantation in the campus of the University of Tokyo. It contained 0.96% total N and 15.7% organic C. The soil pH was 6.7. The CO_2 evolution rate was 5.64 mg C kg^{-1} dry soil h^{-1} at 25°C. The soil was sieved (2 mm mesh) and dried in air at room temperature until the water content was reduced to 88.7% (67.5% of water holding capacity). After preincubation for 7 days without evaporation at 25°C, two courses of incubation were started after 1 ml addition of one of the following solutions to each bottle containing 10 g moist soil.

1. +glucose: 150 mg glucose, 7.0 mg K_2HPO_4, 2.0 mg KH_2PO_4, 0.1 mg $MgSO_4$ and 20.0 mg $(NH_4)_2SO_4$ in 1 ml (pH 7.0).
2. -glucose: no glucose addition. Other salts were at the same level as in 1 (pH 7.1).

The solutions contained sufficient mineral N and P for microbial growth. The incubation was done under aerobic conditions at 25°C and the moisture content maintained by adding water every two or three days.

Soil extracton with TCA-phosphate-paraquat and enzymatic conversion of ADP and AMP to ATP were done by the original method of Brookes et al. (1983) with slight modification. The adenine nucleotides were extracted in triplicate and the extracts were stored at -20°C. At the

end of incubation, AEC ratios were analyzed in one batch. ADP and AMP were converted to ATP with pyruvate kinase and pyruvate kinase plus myokinase, respectively. ADP was completely converted to ATP, but the rate of AMP conversion was low (48.8%). ATP was measured by peak height determination with an Aminco Chem Glow photometer J4-7441, following Karl and Holm-Hansen (1978). Known amounts of ATP, ADP and AMP were added separately to the extractant. Their recoveries, after soil extraction, were 55.1%, 50.6% and 49.0%, respectively. Corrections were made based on the recoveries. Glucose remaining in the amended soil was determined by the glucose oxidase method with a Wako's Glucose B-test kit.

Results

Figure 2-1-a shows the changes in ATP and glucose during incubation. In the glucose amended soil, ATP level increased rapidly and attained a maximum after two days. Added glucose was consumed within two days and ATP level decreased thereafter. In contrast, ATP level remained lower but constant in the unamended soil. Since ATP is a good indicator of biomass (Oades and Jenkinson, 1979), these results indicate that biomass increased up to 2 days, and then declined in the amended soil, but was stable in the unamended soil. In the glucose-amended soil, white hypha covered the soil surface on day 3 and disappeared by the end

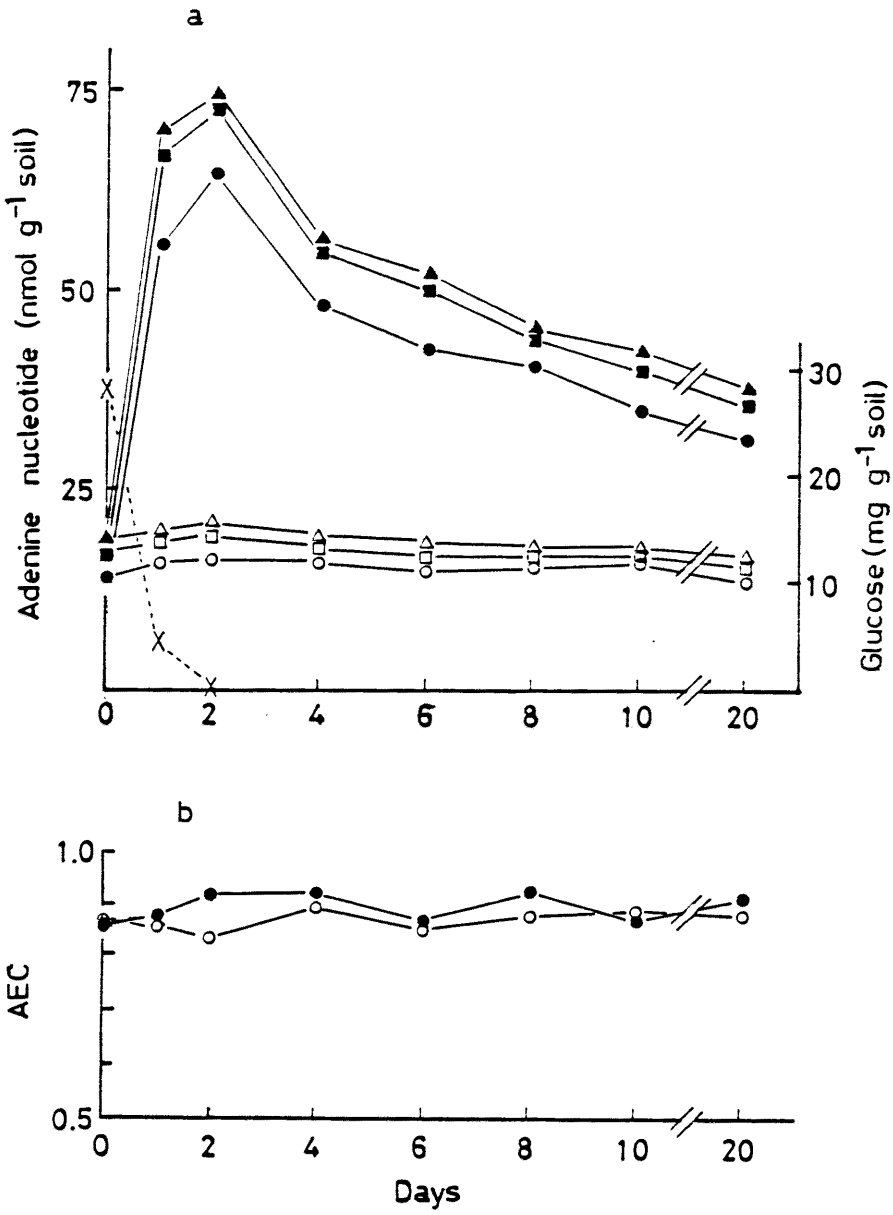

Figure 2-1. (a) Adenine nucloetides and glucose in the glucose-amended and unamended soil. Symbols: ●, ATP in glucose-amended soil; ■, ATP+ADP in glucose-amended soil; ▲, ATP+ADP+AMP in glucose-amended soil; ○, ATP in unamended soil; □, ATP+ADP in unamended soil; △, ATP+ADP+AMP in unamended soil; χ, glucose remaining in glucose-amended soil. Mean of three samples. (b) AEC ratio in the glucose-amended soil (●) and unamended soil (○). Mean of three samples.

of incubation. No significant difference in AEC ratio was found between the amended and unamended soil (Figure 2-1-b).

Discussion

In natural soil, the microbes have low C/ATP ratios (Oades and Jenkinson, 1979; Ross et al., 1980; Sparling, 1981; Tate and Jenkinson, 1982; Sparling and Eiland, 1983), and high AEC ratio (Brookes et al., 1983; Brookes et al., 1987b), although an exception was reported by Martens (1985), who found low AEC ratio and low C/ATP ratios in natural soil. However, Brookes et al. (1987a) did not appreciate the method of adenine nucleotide extraction used by Martens (1985) because of its incomplete denaturalization of ATPase. In our experiment, high AEC ratios were also observed in the unamended soil throughout the incubation period. In this respect, generally microbes seem to be actively growing in soil. However, the results of Part I indicates that microbes in natural soil cannot grow actively.

The AEC ratio in glucose amended soil take a suggestion to solve this inconsistency. In the glucose-amended soil the high AEC and low C/ATP ratios were observed at the growing stage. This is easily expected from the information in vitro. But the microbes had the high AEC and low C/ATP ratios even at the decreasing stage which succeeded the consumption of amended glucose. At this stage the microbes must be strictly energy-deficient and

cannot grow actively. Accordingly, the adenine nucleotide behavior of soil microbes is different from that observed in the laboratory and they maintain the high AEC and low C/ATP ratio in energy-deficient conditions. Probably maintainance of high AEC and low C/ATP ratio is indispensable for survival in natural soil.

Chapter 2

Adenine Nucleotide Behavior of Soil Microbes in a Chemostat with Organism Feedback

Introduction

The results of Chapter 1 suggested that the adenine nucleotide behavior of soil microbes is different from that observed in the expertiments *in vitro*. Therefore adenine nucleotide behavior measured previously in *in vitro* experiments by many authors is not sufficient to estimate the metabolic conditions in microbes in natural soil. Probably the reason is that the behavior *in vitro* has been measured only in energetically extreme states, i.e. abundant or depleted states, while in natural soil energy is not abundant but supplied continuously (see Part I). Therefore, to understand the metabolic conditions of microbes in natural soil, the behavior of soil microbes *in vitro* must be examined in a state similar to those of natural soil.

In Chapter 2, a chemostat system with a limited carbon-energy source and organisms feedback (Pirt and Kurowski, 1970) was used to simulate the soil ecosystems. In this system, organisms are always retained and energy, though small, is supplied continuously.

Materials and methods

Culture system

In the chemostat system, the fresh sterile medium was sent to the culture bottle (10 l) by a perister pump and the waste medium was drawn through the internal filter (Millipore HA 0.45 μm) to retain the organisms in the culture bottle (Figure 2-2). The culture medium (pH 7.0) contained the following inorganic salts: $Na_2HPO_4.12H_2O$ 42 mM, KH_2PO_4 22 mM, $(NH_4)_2SO_4$ 23 mM, $CaCl_2$ 10 μM, $MgSO_4.7H_2O$ 1 mM, $CuSO_4.5H_2O$ 26 μM, $FeSO_4.7H_2O$ 4 μM, $MnCl_2.4H_2O$ 4 μM, $ZnSO_4.7H_2O$ 5 μM. The flow rate of the medium was 10 ml h^{-1}. The volume of the medium in the culture bottle was 5500 ml. Glucose was used as the carbon-energy source of the organisms and the supply rate of glucose was controlled by changing the glucose concentration in the fresh medium. For stirring and maintaining the aerobic condition, air was vigorously blown into the bottle. The incubation was done at 25°C.

The mixed soil microbes were extracted from the soil of the deciduous forest in Tokyo University of Agriculture and Technology. Water, 100 ml, was added to 10 g of fresh soil, and the mixture was stirred for 10 min. The supernatant, 2 ml, of the mixture was added to the bottle on day 0 and day 30. On day 0, 5500 ml of the inorganic culture medium was prepared in the bottle. The initial supply rate of glucose-C was set at 16 mg glucose-C h^{-1}. From day 103, the supply

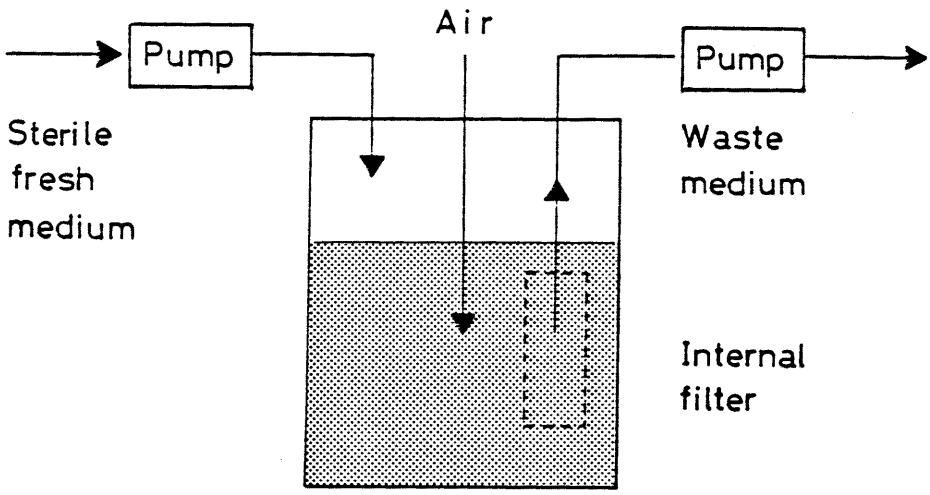

Figure 2-2. The chemostat system with organism feedback. The internal filter (Millipore HA) removes biomass before waste medium is removed from the culture bottle.

rate was reduced to 1/4 of the initial rate and, from day 110, no glucose was supplied.

Analytical method

Organic-C in the medium was measured by the sodium persulfate oxidation method (Seto and Tange, 1980). The particulate organic carbon (POC) in the culture bottle, including living biomass-C, dead biomass-C and particulate debris, was estimated from the total organic-C in the culture bottle minus the organic-C in the waste medium. The released CO_2-C was estimated from the supplied glucose-C minus the accumulated POC minus the organic-C in the waste medium.

The adenine nucleotides were extracted with trichloroacetic acid (TCA). Five milliliters of the medium was added to 5 ml of 20% TCA solution on crushed ice. After 15 min, the extract was frozen and stored at -20°C. Within 1 week, the stored samples were melted and centrifuged (10,000*g, 10 min). ADP and AMP in the supernatant were enzymatically converted to ATP with pyruvatekinase and myokinase, and the ATP was determined following Karl and Holm-Hansen (1978) with the modifications of: (1) The extract was neutralized with 1 N NaOH; (2) myokinase was dialyzed in Mg-free 0.1 N phosphate buffer (pH 7.4) for 12 h at 4°C to prevent precipitation caused by $(NH_4)_2SO_4$ in myokinase solution (Brookes et al., 1983); (3) the standard curve was calculated by the internal standard

method. ADP was completely converted to ATP, whereas only about half of AMP was converted to ATP. The adenine nucleotides of organisms were estimated as the adenine nucleotides in the culture bottle minus those in the waste medium.

Organic-C and adenine nucleotides were determined in duplicate.

Results

Figure 2-3 shows the time course of the POC in the culture bottle until day 102. Until day 30, the supply of fresh medium was not stabilized because of the trouble in the pumping system. The POC attained a stationary level in 80 days and the level did not change thereafter. At this stationary stage, the release rate of CO_2-C per POC was less than 0.0037 h^{-1}. Throughout the incubation period, almost all of the supplied carbon was utilized by the microbes and the organic-C in the waste medium was below 30 ug C ml^{-1}. At the early days white fungi dominated the incubation bottle but disappeared by day 60. Thereafter bacterial species dominated in the bottle.

Figure 2-4-a shows the changes in POC and the total adenine nucleotide of the biomass from day 88. Reducing the carbon-energy source to 1/4 caused a decrease in the POC and the total amount of adenine nucleotide. No addition

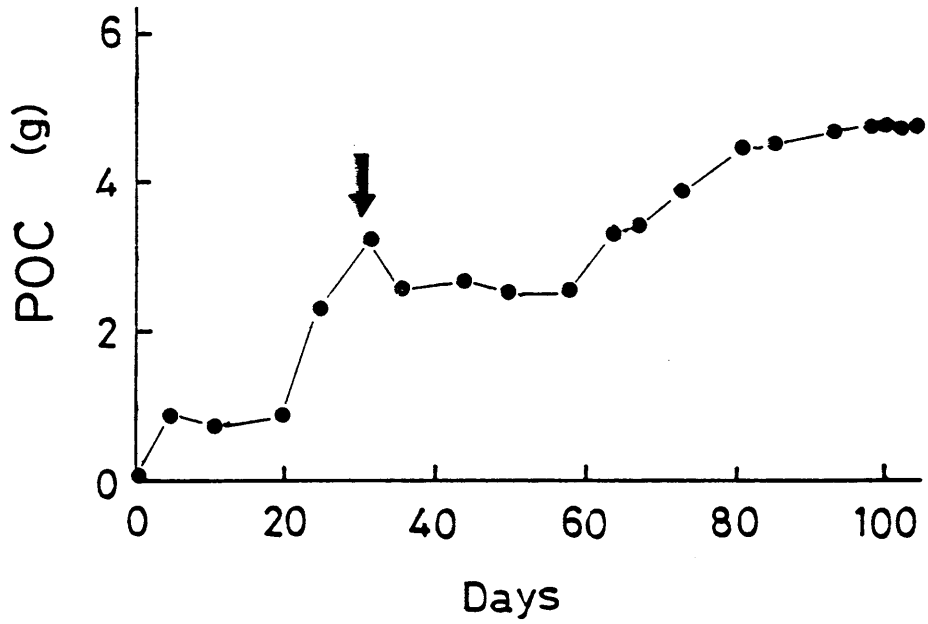

Figure 2-3. Changes in POC in the chemostat until day 102. Soil suspension was inoculated on day 0 and day 30 (⬇).

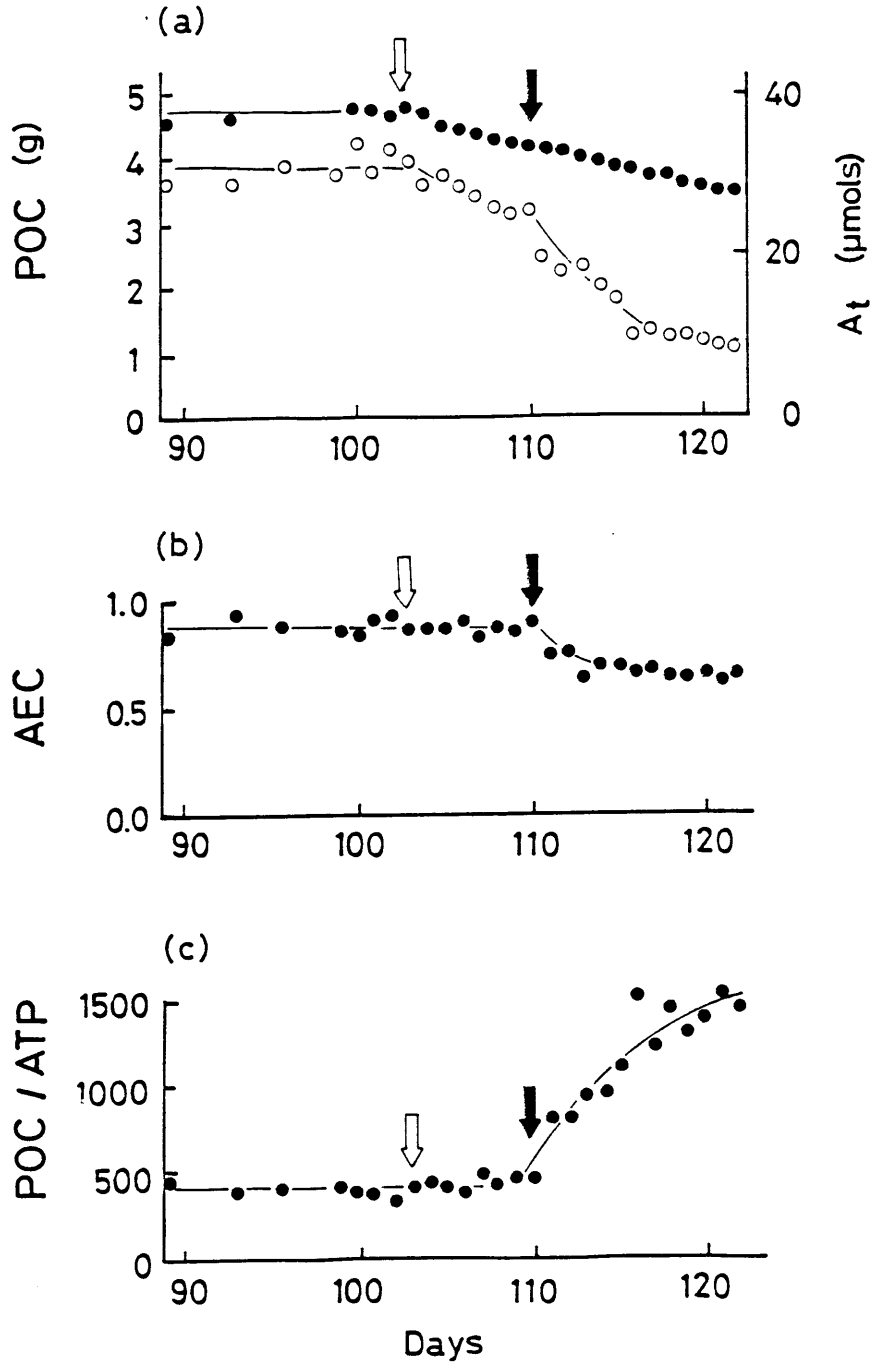

Figure 2-4. (a) Changes in POC (●) and total adenine nucleotide, A_t (○) from day 88 of incubation. (b) Changes in the AEC ratio of biomass from day 88. (c) Changes in POC/ATP ratio from day 88. From day 103 (⇩) to day 110 (⬇), the glucose supply rate decreased to 4 mg glucose-C h^{-1} (1/4 of the initial rate). From day 110 to 122, no glucose was supplied.

of glucose after day 110 caused a more rapid decrease in POC. After day 110, the total adenine nucleotides decreased more rapidly than POC. Throughout the incubation period, no adenine nucleotide was detected in the waste medium except for trace AMP.

Figure 2-4-b shows the changes in the AEC ratios of the biomass. At the stationary stage, these ratios were high (above 0.81), which is similar to the findings with growing organisms. The high AEC ratios did not change when the glucose-C supply rate was reduced to 1/4, but did decrease when the glucose supply was stopped.

Figure 2-4-c shows that the POC/ATP ratios was between 313 to 407 at the stationary stage. The living biomass-C/ATP ratios at this stage must have been much lower than these values, because the POC included dead cell and deblis. These low levels, similar to those of actively growing organisms, did not change until day 110. Thereafter, the ratio increased gradually.

Discussion

Meaning of AEC and C/ATP ratios of soil microbes

At the stationary stage in this experiment, the turnover time was estimated using equation (3) in part I. The Y value used was 0.5 (Payne, 1970). The living biomass-C was assumed to be the half of POC. When a = 0, the turnover time is 5.5 days; this is the shortest period

because all of the energy is used to synthesize new cells. When a = 0.001 h^{-1}, the turnover time is 6.5 days. Table 2-1 shows the specific maintenance rates reported by several authors. These values suggest that the actual a is larger than 0.001 h^{-1} and that the energy larger than that assumed for the calculation is used for maintenance of the cells. Therefore it is probable that the turnover time is longer than 6.5 days. These turnover times are much longer than those of actively growing organisms in the laboratory (a few hours in many species). If all of POC is the living biomass-C, the turnover time becomes longer. At this stage, the AEC ratios of the biomass were maintained at high levels (above 0.81) and the C/ATP ratios were lower than 313 to 407. These values are similar to those of actively growing ones (listed in Chapman et al., 1971; Karl, 1980). This does not suggest that a high AEC ratio and a low C/ATP ratio are the indicators for actively growing organisms.

Table 2-2 shows the relationship between metabolic conditions and adenine nucleotide behavior in vitro. A high AEC ratio and a low C/ATP ratio indicate that the organisms are not completely depleted of energy. A low AEC ratio and a high C/ATP ratio indicate that the organisms are not supplied with energy source from the environment. In other words, high AEC and low C/ATP ratio indicates that the organisms maintain their metabolic homeostasis independently of their growth rates. In contrast, low AEC and high C/ATP ratio indicate that the organisms cannot maintain the homeostasis owing to complete energy depletion.

Table 2-1. Maintenance energy of microbial species based on glucose as substrate.

Species	Maintenance energy (g glucose-C g^{-1} biomass-C h^{-1})
Arthrobacter globiformis (1)	0.008
Enterobacter cloacae (2)	0.075
Escherichia coli (3)	0.004
Penicillium chrysogemum (4)	0.018

(1) Chapman and Gray, 1981; (2) Pirt, 1965; (3) McGrew and Mallete, 1962; (4) Righelato et al., 1968.

a, assuming that 50% of the dry weight is organic carbon.

Table 2-2. Relationship between metabolic condition and adenine nucleotide behavior *in vitro*.

Metabolic condition	AEC	C/ATP
Energy-sufficient	high	low
Energy-limited (Turnover time is longer than 5.5 days.)	high[a]	low[a]
Energy-depleted	low[a]	high

a, the results of this experiment.

Rey (1956) showed that earthworm muscle, which did not grow, had high AEC ratio in resting stage, while it had low AEC ratio after electric stimulation for 20 min. Therefore the estimation of AEC and C/ATP ratio made in this experiment may be applied to estimate the metabolic condition of various organisms besides soil microbes.

Metabolic condition of microbes in natural soil

In natural soil, the microbes have low C/ATP ratios (Oades and Jenkinson, 1979; Ross et al., 1980; Sparling, 1981; Tate and Jenkinson, 1982; Sparling and Eiland, 1983), and high AEC ratios (Brookes et al., 1983; Brookes et al., 1987a, 1987b). In Chapter 1, high AEC ratios were also observed in the unamended soil throughout the incubation period. If completely energy-depleted organisms hold a large portion of biomass, it leads to low AEC and high C/ATP ratios for total biomass, which does not agree with the high AEC and low C/ATP ratios generally found in actual soils. Therefore, in this respect, the dominant organisms in soil are those which can maintain metabolic homeostasis, including actively growing and maintaining ones. On the other hand, the results of Part I do show no possibility of soil microbes growing actively from the energetical point of view except for early days of organic matter decomposition. Hence it is finally concluded that soil microbes cannot grow actively due to energy limitation but can maintain their cells using small but continuously supplied energy-source

from soil organic matter.

Response of biomass-C and AEC ratio to energy supply

In glucose-amended soil in Capter 1, a temporally increased soil biomass began to decline slowly after consumption of the amended glucose with the AEC ratio remaining at high levels, while the biomass does not change in unamended soil. In the chemostat in Chapter 2, the biomass-C with high AEC ratios decreased when the glucose supply rate was reduced to 1/4 of the initial rate. These decreases in biomass caused by strict energy deficiency suggest that the soil microbes cannot preserve a biomass that requires more energy than that supplied constantly, which leads to decomposition of the surplus biomass. Probably the survivors used the decomposed biomass as their energy source for maintaining the metabolic homeostasis. In contrast, when the biomass is relatively small and the energy is sufficient for the growth, the biomass increases to higher levels (Figure 2-3). In both cases, the biomass will attain a level just enough for maintenance.

Generally spore formation has been considered to be advantageous in stressed environment. However, since spores have low AEC ratios (Chapman et al., 1971), high AEC ratios always observed in soils suggest that microbes in natural soil did not form spores even in strictly energy-deficient condition which led to decrease in biomass. Spore formation does not always seem to give a higher chance of

survival in soil, because the spores formed in soil are generally short-lived (Petras and Casida, 1985). In natural soil, probably, maintaining vegetative cell at any cost is the best way for survival for a long period, although spores tolerate some extreme stresses: a long drought, etc..

Part III. Rate Limiting of Organic Matter Degradation in Soil

Introduction

The results of Part I and II showed that almost all of microbes in natural soil cannot grow actively due to energy deficiency and only maintain their cells with the small but continuously supplied energy-source. This supply of energy source results from degradation of soil organic matter by extracellular enzymes produced by soil microbes (Skujins, 1967). In the enzymatic process, either the amount of enzyme or its substrate limits the velocity when the other physical environmental factors -- temperature, pH, moisture content, etc. -- are normally maintained. Enzyme limitation indicates that soil microbes are energy-deficient owing to low enzyme production. In contrast, substrate limitation indicates that soil microbes obtain energy, though small, with maximum effort by producing sufficient amounts of enzymes.

The scheme for the action of an enzyme is

$$S + E \underset{k2}{\overset{k1}{\rightleftharpoons}} ES \xrightarrow{k3} P + E$$

where E is an enzyme, S is a substrate, P is a product, and ES is an enzyme-substrtate complex; k1, k2 and k3 are rate constants. The amount of total enzyme, Eo, is the sum of the free enzyme E plus the combined enzyme ES. Similarly, the total amount of substrate, So, is the sum of S and

ES. McLaren and Packer (1970) reported two extreme cases, assuming a steady state in a soluble enzyme-soluble substrate system. When the substrate is in excess, the reaction velocity, v, is described as:

$$v = k3 \frac{Eo\ So}{km + So} \quad (1)$$

This equation indicates that, if the amount of substrate is large, only the amount of enzyme limits the velocity. On the other hand, when the enzyme is in excess,

$$v = k3 \frac{So\ Eo}{km + Eo} \quad (2)$$

Equation (2) indicates that, if the amount of enzyme is large, only the amount of substrate limits the velocity. In both cases, km is given by

$$km = \frac{k2 + k3}{k1}$$

Using these equations, the rate-limiting condition in the enzymatic reaction can be determined based on the following considerations. If enzyme amendment increases the velocity and substrate amendment does not, the amount of enzyme limits the reaction and the substrate is present in excess. If the substrate amendment increases the velocity and the enzyme amendment does not, the amount of substrate limits the reaction with excess enzyme present. This analysis has not been ever done in the field of soil science.

The aim of part III was to clarify the limiting factor in degradation of soil organic matter by measuring the reaction velocity in unamended and substrate- or enzyme-

amended soils. Cellulase and protease were used as the typical soil enzymes, because cellulose-like and protein-like components are the major components of soil organic matter (Bremner, 1949; Lowe, 1978). Toluene was used to inhibit the microbial uptake of the enzyme product and the reaction velocity was estimated by measuring the accumulation rate of the product under toluene treatment. Also the velocity in sonicated soils was measured to study the effect of physical dissociation of soil organic matter on its degradation rate.

Materials and Methods

Toluene treatment of microbes

Soil microbes were extracted from soil from an oak plantation of Tokyo University of Agriculture and Technology to test the effect of toluene on microbial uptake of glucose and amino acid. Water, 100 ml, was added to the 10 g of fresh soil with stirring for 10 min. The supernatant of the soil suspension, 10 ml, was added to 1000 ml of the incubation medium containing the following inorganic compounds: 42 mM $Na_2HPO_4.12H_2O$, 22 mM KH_2PO_4, 23 mM $(NH_4)_2SO_4$, 10 μM $CaCl_2$, 1 mM $MgSO_4.7H_2O$. Glucose, 2 g, and casamino acid (Difco), 0.5 g, were added to the medium as the carbon-energy source. The pH of the medium was 7.0. After incubation for 3 days at 25°C, the medium was

centrifuged (5000*g, 10 min) and the precipitated microbes were suspended in 500 ml of the incubation medium from which glucose and amino acid had been removed. Four treasments were used:

1) Glucose without toluene, 10 ml microbe suspension with 10 ml glucose solution.
2) Glucose with toluene, 10 ml microbe suspension with 10 ml glucose solution and 2 ml toluene.
3) Amino acid without toluene, 10 ml microbe suspension with 10 ml amino acid solution.
4) Amino acid with toluene, 10 ml microbe suspension with 10 ml amino acid solution and 2 ml toluene.

The glucose solution contained 2 mg glucose ml^{-1} and the amino acid solution contained 3 mg casamino acid ml^{-1} . Four glass bottles (50 ml) were prepared for each treatment. The bottles were incubated at 25°C. On each sampling occasion, 1 ml of the solution was drawn from each bottle, and the microbes were precipitated by centrifugation (5000*g, 10 min). The glucose and amino acid contents in the supernatant were determined. Glucose was determined by the glucose oxidase method, using the Glucose B-test (Wako). Amino acid was measured by the photometric ninhydrin method (Moore and Stein, 1954). Leucine was used as the standard amino acid. The amount of amino acid was calculated on the assumption that average molecular weight was 100. Before amino acid determination, ammonium in the supernatant was removed by distillation with 1 ml of 5 N NaOH for 1 h to prevent it from interfering with the

measurement.

Soils

Ten organic soils from H layer were used for the experiments and their characteristics are given in Table 3-1. Soil 1 - 7 were sampled from kuroboku soil in the campus of the Tokyo University of Agriculture and Technology, and soil 8 was from the flood plain of Tama River. Soil 9 and 10 were sampled from brown forest soil of Mt. Ohira. To generalize the results, the soils were sampled from different soil types. The soils at field moisture content were sieved (2 mm mesh) and incubated for 2 months at 25°C before the experiments. Water contents of the soils were kept constant, adding water every week.

Measurement of reaction velocity

Table 3-2 and 3-3 show the experimental design for the measurement of reaction velocity in unamended soil and substrate- or enzyme-amended soil. Carboxymethyl cellulose (CMC) and casein were used as the substrate of cellulase and protease, respectively. These substrates react with enzymes more easily than cellulose and protein in natural soil owing to their higher solubility. Therefore the velocity in substrate-amended soils is regarded as the maximum velocity of enzymes. Cellulase (Sigma, Type 7, from

Table 3-1. Properties of the soils studied.

Soil No.	pH	C (%)	N (%)	WC (%)	"Cellulose" ($g\ kg^{-1}$ soil)	" Protein" ($g\ kg^{-1}$ soil)
1	6.7	20.8	1.33	111.2	30.0	46.4
2	6.2	18.2	1.16	105.5	25.8	58.8
3	5.3	14.1	0.87	83.1	17.7	43.8
4	4.9	13.7	0.77	90.8	20.4	31.2
5	6.2	12.1	0.85	79.4	14.1	46.8
6	6.5	11.1	0.72	81.0	17.2	26.8
7	5.8	12.4	1.01	97.2	23.9	47.8
8	7.4	4.1	0.29	33.7	6.1	12.0
9	5.7	14.8	0.78	72.5	27.0	35.6
10	5.6	17.8	9.94	97.7	37.1	49.0

a, determined by the proximate analysis method (Stevenson, 1965).

b, determined by the alkaline hydrolysis method (Bremner, 1949). A known amount of casein was added to the soils as the internal standard of protein.

c, water content.

Table 3-2. Experimental design for measuring the reaction velocity of cellulase.

Treatment	Wet soil (g)	Toluene (ml)	Enzyme (ml)	Substrate (ml)	Buffer (ml)
1	0.5	0.0	0.0	0.0	3.0
2	0.5	0.3	0.0	0.0	3.0
3	0.5	0.3	0.0	3.0	0.0
4	0.5	0.3	0.3	0.0	2.7
5	0.5	0.3	0.3	0.0	2.7
6	0.0	0.3	0.3	0.0	X
7	0.0	0.3	0.0	3.0	Y

a, 2.7 ml + water contained in 0.5 g of wet soil.

b, water contained in 0.5 g of wet soil.

c, 0.5% (W/V) enzyme in the buffer.

d, 1.5% (W/V) CMC in the buffer.

e, pH was adjusted to 6.0 by addition of saturated Na_2HPO_4 solution to 0.1 M KH_2PO_4.

f, Soil suspension, 0.4 ml, was added to 2 ml of 2% CMC solution every day. After incubation for 12 h, the glucose produced was determined. Total accumulation of glucose was estimated by totaling all determinations.

g, excluding the glucose contained in enzyme powder.

h, excluding the glucose in CMC.

Table 3-3. Experimental design for measuring the reaction velocity of protease.

Treatment	Wet Soil (g)	Toluene (ml)	Enzyme (ml)	Substrate (ml)	Buffer (ml)
1	0.5	0.0	0.0	0.0	2.0
2	0.5	0.3	0.0	0.0	2.0
3	0.5	0.3	0.0	2.0	0.0
4	0.5	0.3	0.1	0.0	1.9
5	0.5	0.3	0.1	0.0	0.9
6	0.0	0.3	0.1	0.0	X
7	0.0	0.3	0.0	2.0	Y

a, 1.9 ml + water contained in 0.5 g of wet soil.

b, water contained in 0.5 g of wet soil.

c, 0.05% (W/V) enzyme in the buffer.

d, 0.5% (W/V) casein in the buffer. Casein, 1%, in 0.05 M Na_2HPO_4, which was dissolved in a water bath, was added to the same volume of the buffer.

e, pH was adjusted to 6.8 by adding of saturated Na_2HPO_4 solution to 0.1 M KH_2PO_4.

f, 1 ml of 1% casein in 0.05 M Na_2HPO_4 was added and after incubation for 2.4 h, the amino acid produced was determined. Total accumulation of amino acid was estimated by totalling all determinations.

g, excluding the amino acid contained in enzyme powder.

h, excluding the amino acid in CMC.

Penicillium funiculosum) and protease (Sigma, proteinase K, from Tritirachium album) were used as amending enzymes. The pH of the incubation medium was adjusted to 6.0 for cellulase and 6.8 for protease. KH_2PO_4 - Na_2HPO_4 buffer was used for pH adjustment. The reaction velocity was determined as follows:

2 minus 1, reaction velocity of unamended soil

3 minus 1 minus 7, reaction velocity of substrate-amended soil

4 minus 1 minus 6, reaction velocity of enzyme-amended soil

5 minus 1 minus 6 minus 7, reaction velocity of substrate- and enzyme-amended soil (potential activity of amending enzyme)

The reaction velocity in substrate-amended soil was measured for all soils. The velocity in enzyme-amended soil was measured for soil 2 and 3.

Some of soil 2 and 3 were processed by a sonicator (Yamato, at 50 W) for 1 or 2 min on crushed ice to dissociate the physical binding of soil organic matter. After sonication, the soil was incubated immediately.

All incubations were done at 25°C. The soil suspensions were slowly shaken throughout the incubation period. For the measurement of the cellulase activity, the soil suspension was centrifuged (5000*g, 10 min) at the end of the incubation and the glucose content of the supernatant was determined. To measure the protease activity, the

reaction was stopped by adding 1 ml 20% trichloroacetic acid solution to the test tube. After soil and denatured protein had been precipitated by centrifugation (5000*g, 10 min), the amino acid level in the supernatant was determined. Ammonium in unamended and enzyme-amended soil was removed to prevent the interference in the amino acid determination.

Results

Effect of toluene treatment

Glucose and amino acid in the toluene treated medium did not change in amount until day 5, but they disappeared within 6 h from toluene-free medium (Figure 3-1-a and 3-1-b). Microbes utilized glucose and amino acid readily under natural condition and toluene completely inhibited the process as well as the synthesis of enzyme (Skujins, 1977). Thus, the velocity of glucose and amino acid accumulation under toluene treatment indicates the velocity of cellulase and protease reaction, respectively.

Reaction velocity in substrate-amended soil

The reaction velocity in CMC-amended soils, which indicates the potential activity of soil cellulase, was 5 to 30 times larger than those in unamended soils (Table 3-4).

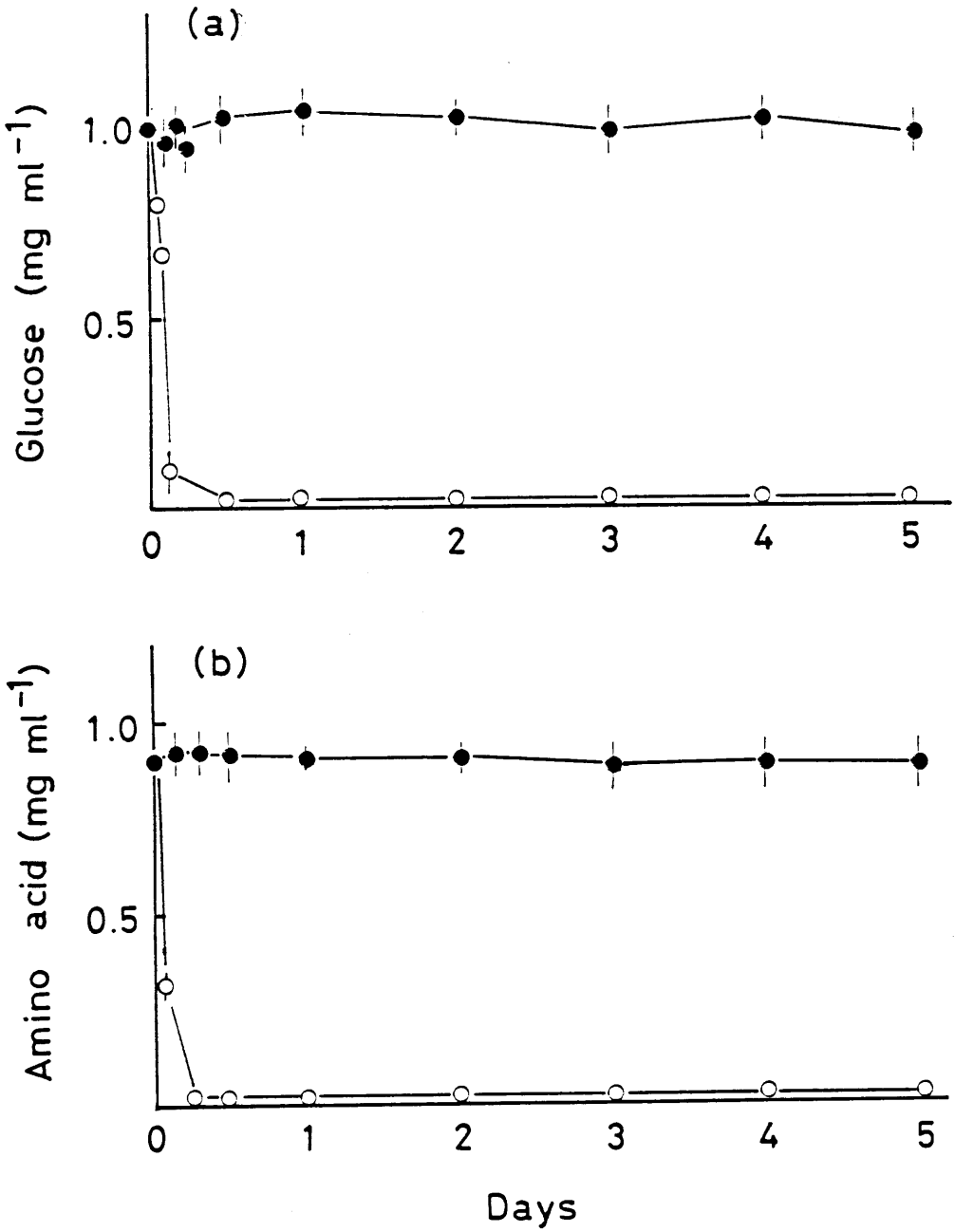

Figure 3-1. Changes in glucose (a) and amino acid (b) concentration in the toluene treated (O) and untreated (O) medium (n = 3, mean $\pm$ SD).

Table 3-4. Reaction velocity of cellulase in substrate amended and unamended soil.

Soil No.	Reaction velocity (mg glucose kg^{-1} h^{-1})		
	unamended	amended	amended/unamended
1	2.0	59.8	30
2	3.3	58.0	18
3	4.8	34.3	7
4	5.0	53.8	11
5	9.0	44.5	5
6	4.8	47.3	10
7	4.5	59.0	13
8	1.8	30.0	17
9	7.3	56.5	8
10	7.3	53.5	7

Incubation time was 24 h.

Table 3-5. Reaction velocity of protease in substrate amended and unamended soils.

Soil No.	Reaction velocity (mg amino acid kg^{-1} h^{-1})		
	unamended	amended	amended/unamended
1	30	496	17
2	26	432	17
3	14	76	5
4	10	48	5
5	24	278	12
6	16	342	21
7	8	110	14
8	10	228	23
9	22	72	3
10	14	122	9

Incubation time was 24 h.

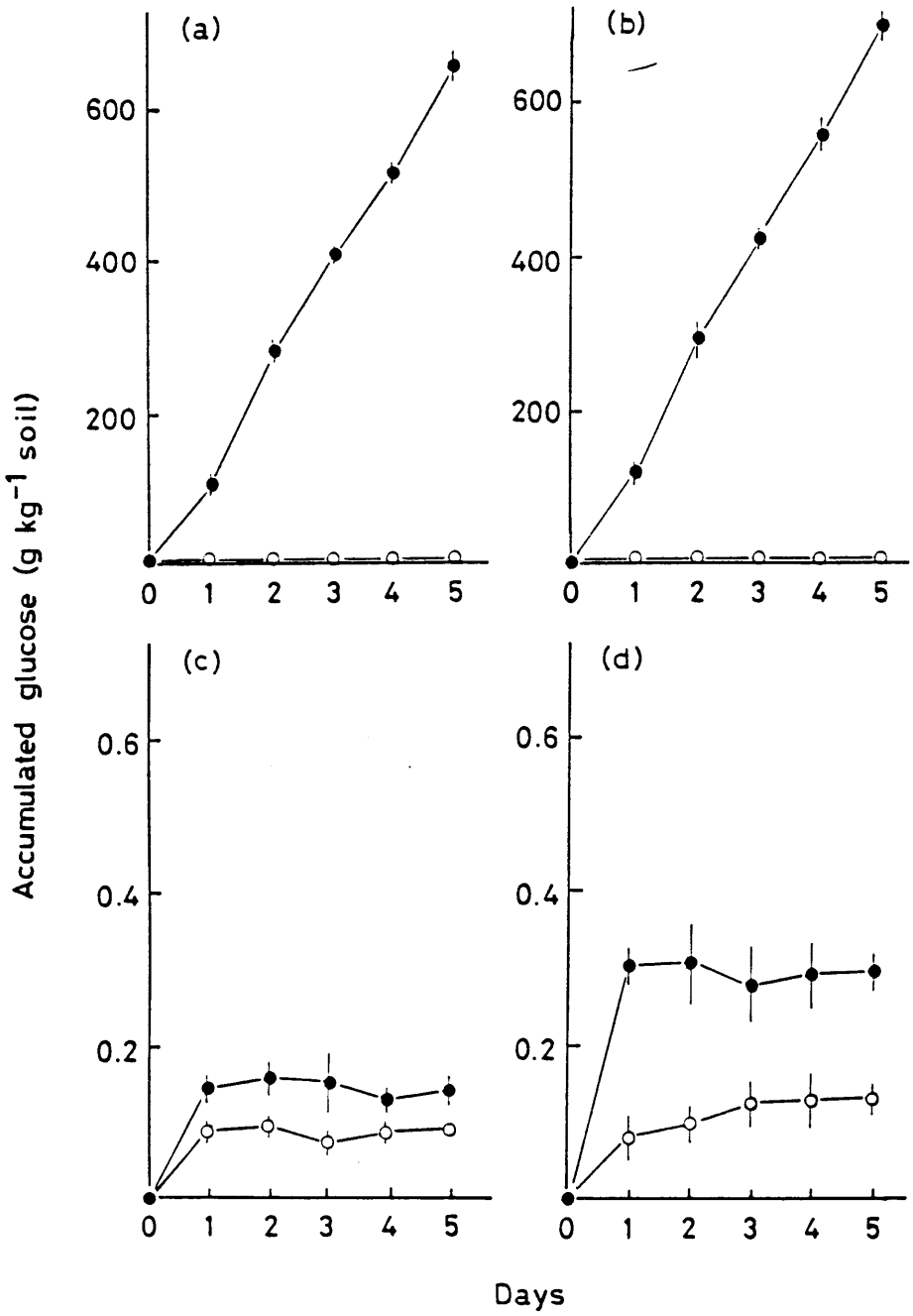

Figure 3-2. Glucose accumulation with (O) or without (O) cellulase amendment in soil 2 and 3. (a) Potential velocity in cellulase-amended and unamended soil 2 determined with adequate CMC. (b) Potential velocity in soil 3. (c) Actual velocity in cellulase-amended and unamended soil 2 determined without CMC. (d) Actual velocity in soil 3. The slope represents the reaction velocity. N = 3, mean $\pm$ SD.

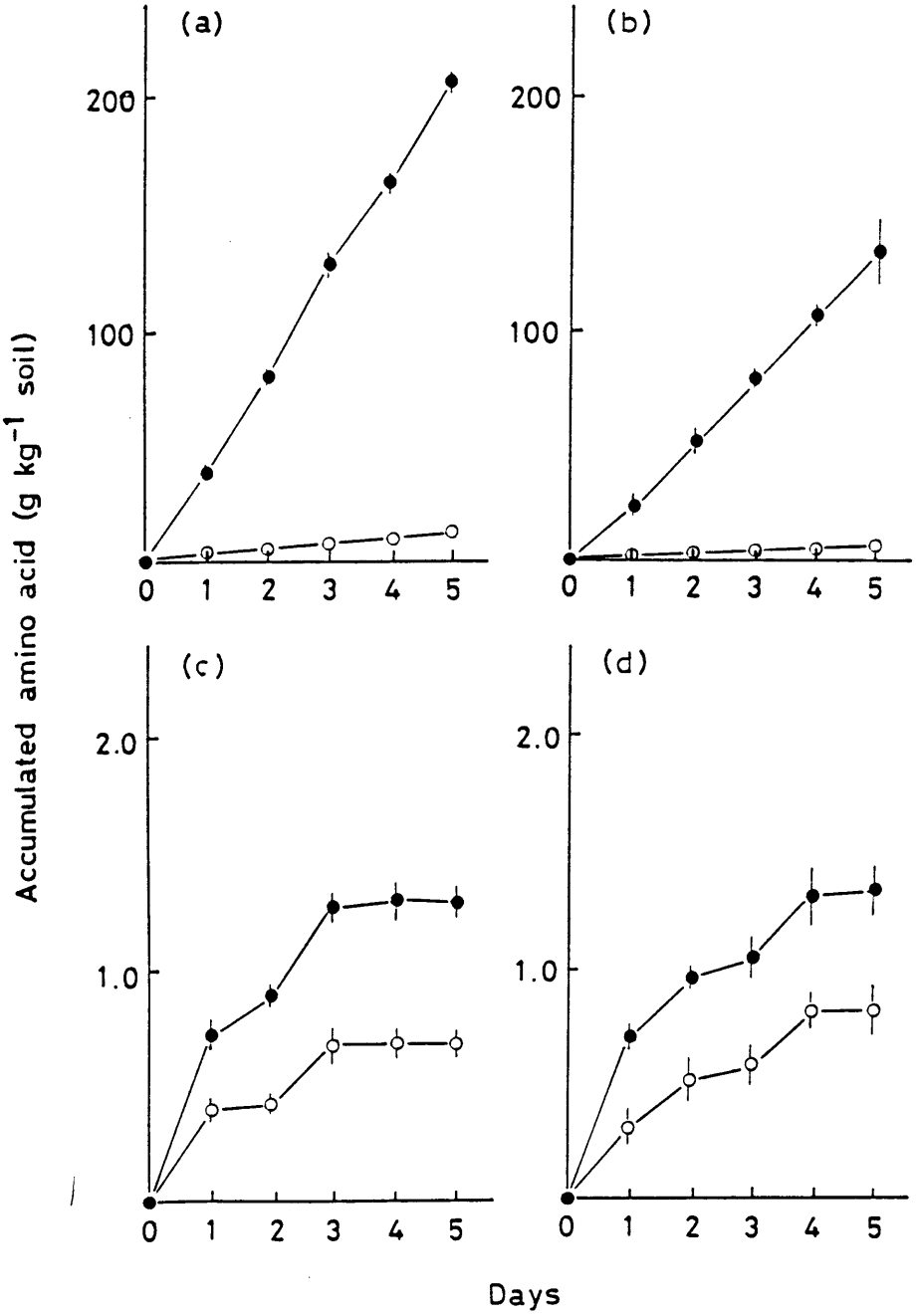

Figure 3-3. Amino acid accumulation with (O) or without (O) protease amendment in soil 2 and 3. (a) Potential velocity in protease-amended and unamended soil determined with adequate casein. (b) Potential velocity in soil 3. (c) Actual velocity in protease-amended and unamended soil 3 determined without casein. (d) Actual velocity in soil 3. The slope represents the reaction velocity. N = 3, mean $\pm$ SD.

Table 3-6. Relative reaction velocity of cellulase and protease in sonicated soils.

Soil No.	Cellulase			Protease		
	Sonication time (min)					
	0	1	2	0	1	2
2	100	212	278	100	95	107
3	100	116	119	100	126	129

Incubation time was 24 h.

Similarly, the reaction velocity in casein-amended soil was 3 to 23 times larger than that in unamended soils (Table 3-5). The higher velocities of the enzyme reaction with substrate amendment suggest that only small part of the enzyme reacts in natural soil because of the low availability of substrate.

Reaction velocity in enzyme-amended soils

Figure 3-2-a and 3-2-b show glucose accumulation in cellulase-amended and unamended soils to which adequate CMC had been added. The slope indicates the potential reaction velocity. The potential activity of amended cellulase was about 100 times larger than that in unamended soil and did not change throughout the incubation period. However, in the soil to which no substrate had been added, cellulose contained in natural soil was the only substrate, and glucose accumulation ceased on day 1 in both enzyme-amended and unamended soil (Figure 3-2-c and 3-2-d). Although the velocity in enzyme-amended soil was about 2-4 times larger than that in unamended soils on day 1, no significant increase in reaction velocity was observed in enzyme-amended soils from day 2 until day 5.

Figure 3-3-a and 3-3-b show the accumulation of amino acid in protease-amended and unamended soils to which adequate casein had been added. The potential reaction velocity in enzyme-amended soils was about 20 times larger than that in unamended soil. In the soil to which no

substrate had been added, the rate of amino acid accumulation in enzyme-amended soil was about twice that in unamended soil on day 2. From day 3 to day 5, the accumulation rates in both soils decreased and no difference in reaction velocity was observed in both treatments (Figure 3-3-c and 3-3-d).

No significant increases in the velocity with enzyme amendment in natural soil suggest that an adequate amount of enzymes is produced by soil microbes for degradation of cellulose and protein in soil.

Reaction velocity in sonicated soils

Soil sonication partially increased the reaction velocity of the cellulase and protease in natural soils except for protease in soil 2 with 1 min sonication (Table 3-6). To some extent, sonication improved the probability of substrate-enzyme contact in natural soils by dissociating the physical complex of soil organic matters.

Discussion

Since enzymatic reaction in soil takes place at the solid/liquid interfaces, the kinetics used for soluble enzymes in solution are not always applicable to heterogeneous systems like soils (Skujins, 1977). However, McLaren and Packer (1970) indicated that the equation (2)

can be applied to a soluble enzyme-insoluble substrate reaction with excess enzyme. Equation (1) can be applied to the soluble enzyme-insoluble substrate system, if the substrate is in excess (Sarda and Desnuelle, 1958). McLaren and Packer (1970) and Sarda and Desnuelle (1958) used the surface area of the substrate instead of the total substrate. These studies suggest that equation (1) and (2) can be applied to a heterogeneous system such as soil by interpreting So as the available substrate, assuming that the soluble enzyme-insoluble substrate system holds in natural soil. In natural soil, the enzymes as well as the substrates are insoluble as the case may be (Stotzky and Burns, 1982). In this case, Equation (1) and (2) can be applied by interpreting So as the available substrate and Eo as the available enzyme.

The results of the present experiments suggest that the enzymatic reaction in natural soil is limited by the substrate supply to the enzymatic reaction and not by the amount of enzyme (Figure 3-4). However, Lowe (1977) showed that cellulose is 2.8 - 26.1% of total organic matter in soil. Bremner (1949) showed that a third of the total soil nitrogen is in a protein-like component. Analysis of the soils examined in this study also showed that the total amount of cellulose and protein is the major component of soil organic matter(Table 3-1). The abundance of total cellulose and protein and the lack of available ones suggest that a large part of the soil organic substances forms a structure resisting enzymatic degradation. Fuller and Norman

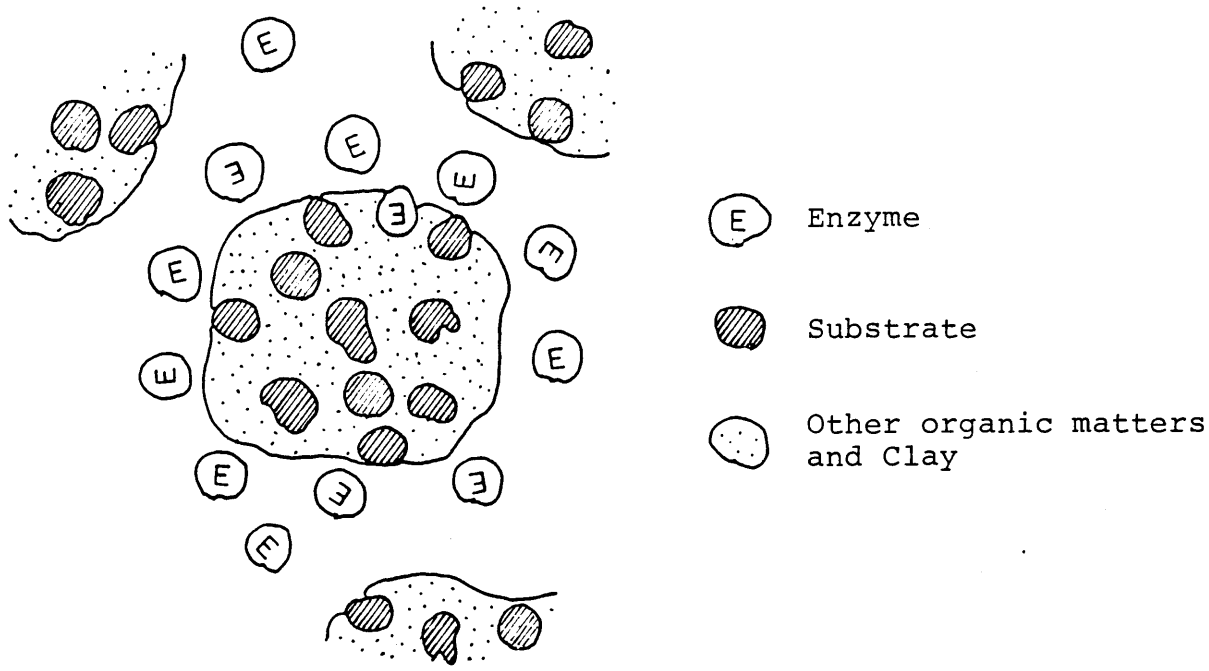

Figure 3-4. Scheme for enzyme-substrate reaction in soil. The amount of available substrate limits the reaction and the enzyme is present in excess.

(1943) showed that the decomposition of cellulose is inhibited by associated lignin. Verma et al. (1975) showed that when ^{14}C-labelled proteins, peptides and amino acid were linked to model phenolic polymers, their rates of mineralization in soil were reduced by 80 to 90%. Furthermore, protein is adsorbed and bound to clays (Harter and Stotzky, 1971). The partially increased velocities of reaction by soil sonication (Table 3-6) support the existence of a physically associated structure of substrate and other materials. In natural soils, this association of organic substances would reduce the substrate available to enzymes and reduce the energy-source supply to soil microbes.

Although not only amount of substrate for enzymes but also temperature and moisture content affect enzyme activity in the field, yet the excess production of degrading enzymes suggests that organic matter is decomposed as rapidly as possible in any physical condition.

General Discussion

Relationship between survival strategy of an individual and function of ecosystem

In the present thesis, the features of microbial life in natural soil (especially in Ao layer) were studied from the viewpoint of acquisition and utilization of energy. The results of the present study are summarized as follows.

(1) In the course of organic matter decomposition, rapid growth and turnover of biomass ceased by day 14. In natural soils and organic matter incubated for over 70 days, the turnover time was rather long. This suggests that supply of energy source limits the microbial growth except for early stage of organic matter decomposition.

(2) AEC of soil biomass was maintained at high levels both in unamended and glucose-amended soil. After the comsumption of amended glucose, the temporally increased biomass dacayed slowly. Soil microbial biomass in a chemostat also decreased slowly maintaining high AEC ratio, when glucose supply rate was reduced to 1/4. This suggests that even in energy-deficient condition in soil, maintaining vegetative cells is indispensable for survival.

(3) Soil cellulase and protease were present in excess. The degradation of soil cellulose and protein was limited by available substrate. This suggests that soil microbes

degrade soil organic matter with maximum effort by producing large amounts of soil enzymes.

These results relate to each other as follows: The long turnover time and high AEC of soil microbes suggest that they are deficient in energy but have to maintain vegetative cells in order to survive in soil ecosystems. Maintenance of vegetative cells requires energy necessarily. Therefore, soil microbes devote themselves to obtain their energy source.

From these findings, I would like to propose the survival strategy of dominant microbes in Ao layer: obtaining energy source at any cost and maintaining vegetative cell using this energy source. Since organic mattrer is decomposed mainly in Ao layer, this strategy would be the main one in the whole soil system.

How does this strategy of soil microbes concern the function of a whole ecosystem? In natural ecosystems, production is usually balanced to decomposition and continues regularly year after year. The optimal strategy of producers for survival in terrestrial ecosystems is to maximize their progenies. For this purpose, they must increase their growth rate. Hirose (1986) showed that they use nitorgen effectively to maximize the growth rate. On the other hand, the survival strategy of decomposers is to obtain their energy source for maintaining vegetative cells. For this purpose, they must degrade soil organic matter

with muximum effort. If spores, which do not require energy, dominate in natural soil, soil organic matter is not decomposed. This reduces the inorganic nutrients available to the producers and leads to decrease in the production of organic matter. If the producers do not grow effectively, the litter supply decreases. Therefore, such survival strategy of producers and decomposers enables the regualr production and decomposition in terrestrial ecosystems.

Generally an evolutionary process is considered to be driven by effects on individual fitness and not by effects on stability of ecosystem. Probably, the biological features of soil system have been shaped by this evolutionary process. However, although selected by effects on individual fitness, we must note that these features ensure the continuation of whole ecosystem.

Future research needs

One of the ploblems to be solved is the circumstantial examination of assumptions used in this study. For example, the parameters in model greately affected the turnover time (See Part I). Therefore the experimental study should be done in the future. The recovery of adenine nucleotide should be also improved for accurate measurement of biomass and AEC.

The other is the question why grazing pressure is weak in soil. The long turnover time of soil biomass indicates that nematoda and protozoa, cannot act as the grazers of

soil microbes effectively, although the turnover time of microbes in deep layers was not estimated. In contrast, as well known, the turnover of biomass in aquatic ecosystems is short, indicating the strong grazing pressure. The difference between these two systems is of interest. We need further study to clarify the cause of this difference.

Conclusion

Advances are being made in the field of soil chemistry and biochemistry. Structure of many organic substances in soil was determined and biochemical properties of soil enzymes were studied by many investigators. Isolated soil microbes were physiologically and biochemically characterized in many *in vitro* studies.

In this thesis, the growth of microbes in soil was studied with special reference to energy acquisition and utilization based on the physiological and biochemical achievements. The growth model showed that microbes in soil cannot grow actively except for the early stage of organic matter decomposition probably due to energy limitation. However, adenylate energy charge of soil biomass showed that microbes maintain vegetative cells even in the energy-deficient soil. This suggests that only the micbobes which can acquire the energy, though small, for maintenance of vegetative cell survive in natural soil. The excess production of soil cellulase and protease suggests that microbes devote themselves to obtain their energy source to survive in soil. This supports the importance of energy acquisition for soil microbes. These results suggest that energetical viewpoint is the effective approach to microbial ecology in soil. We need further study from this angle to understand the relationship between the life of individual microbe and the ecosystem functioning.

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