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Kinetic Analysis of Protein Degradation by a Freshwater Wetland Sediment Community

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The mineralization of proteins by the sediment microflora of a freshwater wetland conformed to a kinetic model developed for polymer degradation. The maximum velocity of protein mineralization ranged from 2,078 to 147 nmol of protein cm^{-3} h^{-1} from May to October. The turnover time of protein was 13 to 69 h. A statistical comparison of the kinetic parameters by the standard error of the estimate demonstrated that protein degradation exhibited significant random fluctuations throughout the growing season. These fluctuations were related to changes in the concentration of readily degradable protein as estimated by K_t. Utilization of amino acids was 9 to 57 times greater than the utilization of protein, and the turnover time for amino acids was 1.6 to 3.7 h.

A significant development in microbial ecology was the application of radioactive tracers for the measurement of the utilization of organic compounds by the heterotrophic community (13). This method provided a means to investigate the heterotrophic community at near in vivo conditions. Further refinements in the methodology by Wright and colleagues (18–20) permitted various pelagic community processes to be compared and the populations to be described in terms of the kinetic activities of their enzymatic systems. The method was further adapted to measure the mineralization processes by sediment communities (10).

In the application of kinetic analyses to various heterotrophic communities, usually only simple organic compounds have been used. These organic compounds represent only a small percentage of the total dissolved organic matter pool available for heterotrophy. Most of the dissolved organic matter is present as polymers of amino acids, carbohydrates, and phenolic compounds (13, 14). In the sediment community, a large portion of the organic matter available for microbial utilization occurs in a polymer form. Neglect of polymer degradation by investigators often originates from the lack of suitably labeled organic substrates. The purpose of this study was to investigate the mineralization of polymer protein by a wetland sediment community.

MATERIALS AND METHODS

Substrate preparation. The protein used for the experiments was extracted from an early-stationary-phase *Escherichia coli* B grown in 8 liters of medium at 25°C. The medium contained (in millimoles $liter^{-1}$) glucose (5.5), $(NH_4)_2SO_4$ (6.05), KH_2PO_4 (49.9), K_2HPO_4 (48.8), $CaCl_2$ (0.09), and $FeSO_4$ (0.033) as well as 2.96 kBq (80 μCi) of [U-^{14}C]glucose (Amersham-Searle). The *E. coli* were harvested by continuous centrifugation (12,100 relative centrifugal force, 120 ml min^{-1}; Sorvall SS-3). Pellets were washed with 90% acetone, dried under an air stream, and then desiccated overnight. Lipids were removed by a 16-h Soxhlet extraction with a chloroform-methanol (1:1, vol/vol) mixture. Polysaccharides were removed from the defatted residue by refluxing in 15 ml of 10% NaCl (wt/vol) in 20-ml sealed ampoules at 107 ± 2°C for 24 h. The resulting residue and the liquid were dialyzed against five changes of distilled water. The residue and any solubilized protein that was recovered by precipitation with 50% trichloroacetic acid and centrifugation were treated with 2 mg of phosphodiesterase (Sigma Chemical Co.) in 0.1 M Tris-acetate buffer (pH 8.7) containing 0.1 M $MgCl_2$ for 12 h at 25°C to solubilize the nucleic acids. The resulting nucleotides were removed by dialysis against four changes of distilled water, and the protein in the liquid phase and residue was solubilized in 0.3 M NaOH at 2°C for 48 h. Undissolved solids were removed by centrifugation (12,100 rcf, 15 min), and the protein in the supernatant was recovered by precipitation with 50% trichloroacetic acid, centrifugation, and two washings with 100% ethanol to remove the excess trichloroacetic acid.

The specific activity of the resulting 230-mg protein fraction was 19,000 dpm mg^{-1} or 64 dpm nmol of amino-N^{-1}. Molecular weight determination by Amicon ultrafiltration revealed that 59% of the protein was larger than 30,000 daltons, 36% was between 10,000 and 30,000 daltons, and 5% was between 1,000 and 10,000 daltons.

The protein was solubilized in 0.1 M Tris-acetate buffer (pH 7.5) at a concentration of 1 mg ml^{-1} (295 μmol of amino-N ml^{-1}) and frozen in ampoules at −20°C until use. The four concentrations used for the kinetic analyses were 74, 148, 221, and 295 μmol of amino-N $liter^{-1}$.

An amino acid mixture with a specific activity of 39,900 dpm μmol of amino $acid^{-1}$ was obtained from Amersham-Searle and was diluted to 18.5 kBq ml^{-1} (0.5 μCi ml^{-1}) with glass-distilled water. The amino acids were frozen in ampoules at −20°C and thawed immediately before use. The four concentrations used for the kinetic analyses were 6.75, 13.5, 20.25, and 27 μmol of amino-N $liter^{-1}$.

Sampling sites. Sediment samples were collected biweekly from May to October in a *Typha latifolia* wetland associated with Lawrence Lake in southwest Michigan (4, 8). Sixteen 20-cm^2 cores were taken in a 0.5-m^2 area of organic sediment occurring among *Typha* tussocks. The sediment temperature often varied from 9°C in the early morning to 27°C by 1500 h.

Mineralization experiments. Mineralization experiments were performed by a modification of the method of Harrison et al. (10). Samples were returned within 30 min to the laboratory, where the overlying water was removed by aspiration. The top 2-cm section of each sediment core was

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removed with an interval core slicer (6). The core slices were pooled in a large beaker, gently mixed, and adjusted to a 90% water content by weight. Preliminary experiments determined this condition to be the natural saturation for these sediments.

Protein degradation was determined with 20 cm^3 of sediment slurry placed in 125-ml Erlenmeyer flasks, and amino acid degradation was determined with an 8-cm^3 sediment slurry in 25-ml Kontes reaction flasks (K-882360) with side arms. Degradation analyses were performed in triplicate at four concentrations of protein or of amino acids with one killed blank (1 ml of 1 N H_2SO_4 2 cm^3 of $sediment^{-1}$) for each concentration. The 125-ml reaction flasks were stoppered after isotope addition with a neoprene bung that was modified with an injection port and a suspended plastic cup (Konte) assembly containing an accordion-folded filter paper (3 by 2 cm) and 0.5 ml of monoethanolamine (Fisher Scientific Co.) to absorb respired CO_2. The smaller reaction flasks (25 ml) were stoppered after isotope addition with a serum stopper and a similar plastic cup assembly. The samples were shaken at 120 rpm on an oscillatory shaker in the dark at 23°C in an environmental chamber. The amino acid incubations were terminated after 15 min with 1.0 N H_2SO_4 (1 ml 2 cm^3 of $sediment^{-1}$). The protein incubation was terminated in a similar manner after 2 h. After the addition of the acid, the flasks were shaken for an hour before the plastic cups were removed to assure absorption of all respired CO_2. The cup assembly was added to 10 ml of scintillation cocktail (Complete Counting Cocktail 3a20; Research Products International Corp.) and 5 ml of methanol. Samples were radioassayed with a Beckman 133 or 8000 liquid scintillation spectrometer.

Calculations. The counting efficiency was determined by the addition of 0.925 kBq (0.025 μCi) of standardized [U-^{14}C]glucose to a separate set of cup assemblies, and the resulting quench curve was used to correct counts per minute to disintegrations per minute. Disintegrations per minute were further corrected for the trapping efficiency of the monoethanolamine. Trapping efficiency was determined on triplicate flasks treated the same as in the mineralization experiment except that 3.7 kBq (0.1 μCi) of $NaH^{14}CO_3$ was added instead of the protein or amino acids. The corrected number of disintegrations per minute of the sample was converted to its molar value from the specific activity of the substrate. All values were standardized to nanomoles of substrate respired centimeters of $sediment^{-3}$ $hour^{-1}$ by appropriate conversion factors.

The data were analyzed by a Lineweaver-Burk transformation with unweighted velocity measurements, a Lineweaver-Burk transformation with v^4 weighting after the method of Wilkinson (15), a Haldane transformation (20) without v^2 weighting, and the Eisenthal and Cornish-Bowden (5) nonparametric method.

The ability of each method to predict an accurate estimate of maximum velocity of protein mineralization (V_m) and natural concentration of protein at one-half V_m in sediments (K_t) was tested by using a theoretical data set for V_m = 1,600 nmol cm^{-3} h^{-1} and K_t = 20 μmol cm^{-3}. The data subset where S is equal to values between $K_t/5$ and $K_t/2$ for the rate-limiting enzymes was used to test conditions that approximated first-order reaction kinetics. Mixed-order reaction kinetics were approximated by a $K_t/2$-to-2K_t and K_t-to-3K_t data subsets. A range of values from 2K_t to 5K_t tested conditions that approximated zero-order reaction kinetics. The precision of each estimate of V_m and K_t was determined by the sum of square of error (SSE), where:

$$SSE = \sum_{i=1}^{n}\sum_{j=1}^{n}\left\{v(i,j) - [V_m \times s(j)]/[K_t + s(j)]\right\}^2$$

with $v(i,j)$ being the observed velocities for substrate (j) (3). When the simulated results were compared in detail, all latter three models gave basically the same results. The Eisenthal and Cornish-Bowden (5) transformation was selected for the present analyses because the data do not need to be homogeneously distributed and because it is less sensitive to random error than the other methods.

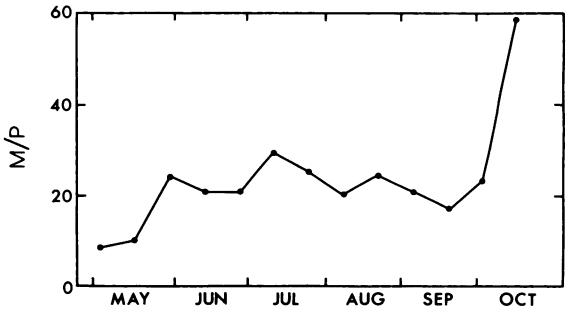

FIG. 1. M/P ratio of amino-N and protein-N degradation by a wetland sediment microflora during the active growing season of the wetland. The M/P ratio was determined from the rates of degradation of 3.5 μmol of amino acids cm^{-3} and of 3.5 μmol of protein cm^{-3}. See text for further details.

RESULTS AND DISCUSSION

Kinetic model of protein degradation. Before protein mineralization can be analyzed by coupled enzyme kinetics, the enzymes responsible for protein degradation must be shown to be effectively saturated so that only a small portion of the added protein is converted to the final product. The percentage of the added protein mineralized ranged from 6.5% h^{-1} during the period of maximum proteolytic activity to 0.6% h^{-1} during the period of minimum proteolytic activity. Approximately a 1% decrease was observed between the percentages respired at the lowest and highest concentrations of added protein at both low and high proteolytic activities. These results suggest that the reaction was not limited by substrate availability and that all enzymatic sites were effectively saturated by the concentrations of the added protein, indicative of zero-order kinetics.

These estimates of protein degradation rates require that the rate of final product formation be dependent solely on the rate of protein degradation and that it be independent of intermediate amino acid degradation. Sole dependency of the rate of final product formation on the rate of protein degradation was estimated by a monomer-to-polymer (M/P) ratio, that is, the comparison of the relative rate of monomer utilization to the relative rate of polymer utilization. The M/P ratio was determined by comparing the mineralization of amino acids to mineralization of an equivalent amount of protein (Fig. 1). Similar values were obtained from the comparison of the respective turnover times. Changes in the M/P ratio were caused mainly by the variation of the amino acid degradation rates; the protein degradation rate remained fairly constant through the growing season. Except for the first date on which the highest rate of protein mineralization was observed, the mineralization of amino acids exceeded protein mineralization by over an order of

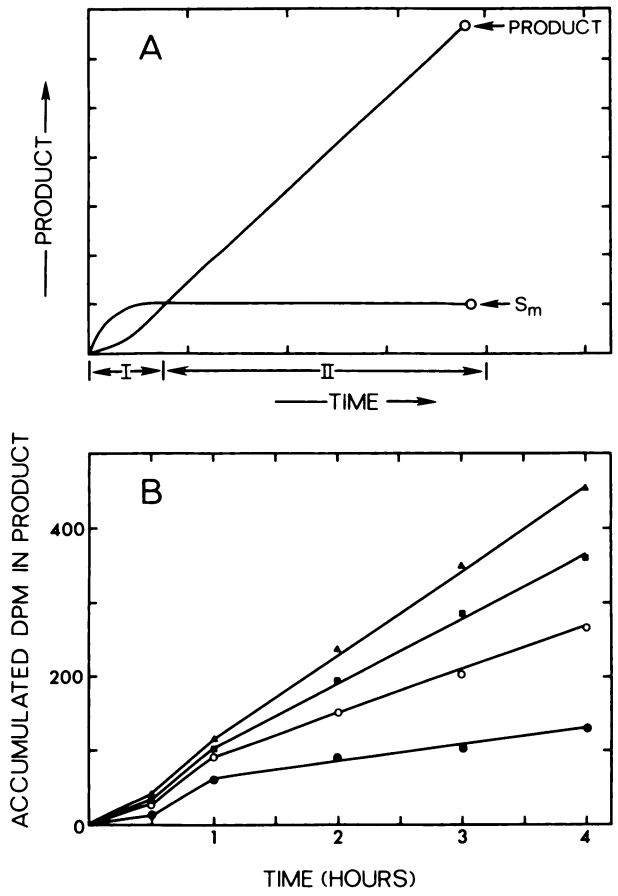

FIG. 2. A comparison of the mineralization of protein with time to the theoretical model of coupled enzyme reactions. (A) Theoretical model of coupled enzymatic reactions adapted from the work of McClure (12). S_m, Concentration of the monomer; product, formation of the product from the polymer. (B) Accumulation of $^{14}CO_2$ from ^{14}C-labeled protein versus time. Symbols (micromoles of protein-N centimeter^{-3}): ●, 3.7; ○, 7.4; ■, 11.1; ▲, 14.8.

magnitude (Fig. 1). This pattern indicated that the sediment community had the capacity to remove amino acids at a rate 10 to 30 times higher than its rate of protein degradation. Therefore, the rate of $^{14}CO_2$ formation from the added protein would be dependent on the formation of amino acid monomers rather than on the rate of amino acid degradation, and the rate of $^{14}CO_2$ evolution can be used to indicate the probable kinetics of protein mineralization by the sediment microflora.

Further evidence that the coupled enzyme kinetic model reflects polymer protein degradation is given in Fig. 2. The accumulation of $^{14}CO_2$ versus time for four concentrations of protein (Fig. 2B) is in accord with the theoretical model of coupled enzymatic reactions (12) as summarized in Fig. 2A. In the model, a time lag observed before the linear formation of product (Fig. 2A, section I) results from delays in arrival by the monomer (S_m) at a steady-state concentration by action of the enzymes responsible for the production of S_m and the utilization of S_m. The theoretical time lag can be calculated from the time required to reach a fractional steady state (12). The fractional steady state is calculated for the utilization rate of S_m and the rate of formation of S_m. After this time lag, the linear formation of the final product (Fig. 2A, section II) is dependent only on the rate of S_m formation

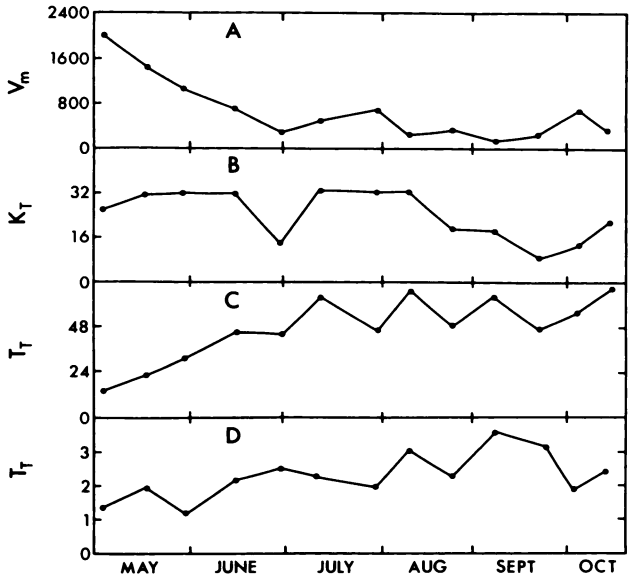

FIG. 3. Kinetic parameters of protein and amino acid degradation by the sediment microflora from May through October. (A) V_m respired of (nanomoles of protein respired centimeter^{-3} hour^{-1}); Composite of the actual uptake constant (K = 1/2 V_m) and K_t (micromoles centimeter^{-3}); (C) T_t (hour^{-1}) of protein by the sediment microflora calculated from the K_t and V_m of protein utilization; (D) T_t of amino acids calculated from the inverse of the slope of the initial velocity. See text for details.

and is independent of S_m utilization. A time lag of less than 30 min was required before a linear production of $^{14}CO_2$ was observed from the added ^{14}C-labeled protein (Fig. 2B). The time lag corresponded to a theoretical lag time of 0.2 h calculated from the relative rate constants for amino acid and protein mineralization. Incubation times of greater than 30 min would therefore be required to measure the rate of protein degradation by kinetic analysis.

The time lag likely did not arise from microbial enzyme induction, since the percentage of the added protein respired was not significantly different for all concentrations. Similarly, the percentage respired remained constant throughout the duration of the experiment. If induction had occurred, the percentage respired would exhibit a concentration dependency and would increase with time.

Protein and amino acid degradation by the microflora. Kinetic parameters of protein mineralization by the sediment microflora populations during the active growing season of a typical *Typha* wetland indicate that the largest maximum velocity of uptake (V_m) and the lowest turnover time (T_t) were observed during the early growing season before canopy closure (Fig. 3). During this period, available light to the sediment was the greatest and an obvious filamentous green algal mat covered much of the sediment surface. The epipelic algal mat was not apparent after the 29 May measurement, and the V_m of the population decreased with an increase in the T_t of protein. From June to the end of the study in October, the V_m demonstrated significant ($P < 0.01$) random fluctuations around values about 1/2 to 1/10 the magnitude of the values observed in the earlier growing season. The lowest values of V_m occurred during August and September and corresponded to a large and significant decrease in the estimate of K_t. Before the reduction of the value in August, the K_t remained at a constant value around 30 μmol cm^{-3}, with the exception of one measurement in

late June. The low estimate in June occurred after a major rainstorm that transported a large amount of particulate sediment from the system and may have removed a portion of the proteolytic microbial population or altered the supply and composition of protein. The T_t increased in a linear fashion during the early growing season to a plateau of about 53 h at the beginning of July and then exhibited small fluctuations around this value. The T_t exhibited an inverse relationship to the V_m of the system.

Amino acid degradation by the sediment community exhibited first-order reaction kinetics throughout the study. The rate of mineralization was linearly related to the added substrate concentration ($r^2 = 0.96$ or greater). As much as 20% of the added substrate was respired during the 15-min incubations. However, an estimate of amino acid turnover can be obtained from first-order kinetics, since the velocity is equal to ks where $k = V_m/K_t$. The values of T_t were calculated as the inverse of k at V_m and were statistically similar to the estimate of the turnover time calculated by the method of Christian and Wiebe (2).

The turnover time of amino acids by the sediment microflora ranged from 1.2 to 3.6 h (Fig. 3D). These values suggest that significant accumulation of amino acids in the wetland sediment is not likely. The T_t of amino acids did not follow precisely the pattern observed for protein, but the lowest T_t corresponded to the period of highest proteolytic activity, and the greatest T_t occurred during the period of lowest proteolytic activity. The relationship observed between the amino acid and protein degradation rates (Fig. 1) suggests that the proteolytic activity was necessary to support the amino acid-degrading population not directly involved in the proteolytic process.

The values obtained for amino acid T_t were 1/5 to 1/10 the values obtained for the turnover of alanine by an anaerobic salt marsh sediment, and the velocity of utilization was about 100 times greater than the maximum velocity observed by Hanson and Gardner (9). The differences may be caused by a different incubation temperature (not given), anaerobic conditions, or the method of calculation. Hanson and Gardner (9) determined the T_t for the velocity calculated from a single substrate concentration. The percentage of the added alanine respired in their analyses was similar to the percentage respired in this study (20 to 50%), but the percent respiration of aspartic acid was much lower (5 to 20%). The T_t values of amino acids in this study were quite similar to those of glucose by lake sediments (10), anaerobic salt marsh sediments (2), and in other analogous environments but more rapid than in planktonic situations (for example, see reference 7).

Sediments are structured with numerous microzones that contain distinct but metabolically interacting microbial consortia. Disruption of this structure by the slurry methods likely altered rate processes of the microbes as well as bacterivorous protozoa, although the latter are minimized by the reducing conditions. However, the methods permit only an estimation of the potential mineralization of protein by the wetland sediment microbial community. The methods were consistent and should permit effective seasonal comparisons of rates.

The rates of mineralization represent only a portion of the total utilization of compounds by the microbial community. The relationship between the amount of the substrate respired and the total uptake, assimilation, and respiration is ambiguous. The respiration of amino acids ranged from 8 to 60% of the total uptake for the planktonic community (11, 16, 17), and values of 13 to 48% were observed for alanine and aspartic acid in salt marsh sediments (9). In the sediment environment, a spatial difference observed between the percent respiration and total uptake in vertical sediment profiles (2, 9) suggests that this relationship is some combined function of the metabolism and composition of the microbial populations. The functional difference between populations may be related to the availability of carbon substrates, the growth phase of the population, and ambient redox conditions. Considering the relationship between respiration and total uptake, the rates of protein and amino acid utilization of this study (Fig. 3) are probably underestimates of the true rates. The clear relationship between protein concentration added and respired $^{14}CO_2$ (Fig. 2) indicates a relatively constant percent respiration.

Regardless of true uptake rates, the rates of mineralization of protein indicate that these compounds are important carbon sources to the microflora of this freshwater wetland. Expanding the observed rates to an areal measurement, the uppermost centimeter of sediment had the potential to convert 35 to 500 mmol of protein m^{-2} day^{-1} to CO_2 during the growing season. The origins of protein in this system are unknown but probably include massive macrophytic production (4), the microfloral community through lysis and extracellular enzymatic activity, proteinaceous releases by macrophytic rooting tissue, and epipelic algal populations. The contribution of each protein source would vary seasonally.

In addition to providing an important carbon source, protein can also represent a significant source of nitrogen for the microflora. Wetland ecosystems are often nitrogen limited (1). The utilization of protein by the microflora represents a recycling of nitrogen by exploitation of nitrogen sources not directly available to the plant community.

The utilization of protein conforms to the kinetic model for polymer degradation and permits a kinetic analysis of polymer utilization by a microbial population. Studies of polymer degradation focus on a predominant type of organic compound in the aquatic environment and allow examination of processes that regulate the degradation of organic matter.

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