Biogeochemistry 2: 299–312 (1986)

Rapid microbial metabolism of non-protein amino acids in the sea

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Key words: amino acids, microbial activity, sea water, β-alanine

Abstract. The non-protein amino acids, β-alanine and γ-aminobutyric acid, frequently dominate the amino acid composition of deep-sea sediments. This accumulation is most likely due to the slower decomposition of non-protein amino acids by microorganisms or to the preferential adsorption of non-protein amino acids by clay minerals. We investigated relative rates of heterotrophic uptake of alanine, β-ala, and γ-aba in sea water to see if there were different rates of microbial assimilation and respiration between these protein and non-protein amino acids. Heterotrophic uptake was rapid for all three amino acids with turnover times of hours in productive coastal waters and days in more oligotrophic open-ocean waters. Uptake of the non-protein amino acids was significantly slower than uptake of alanine, particularly in anoxic waters. However, the difference in uptake rates is probably not great enough to cause significant preferential accumulation of non-protein amino acids.

Introduction

The amino acid composition of deep-sea marine sediments is dominated by two non-protein amino acids, β-alanine (β-ala) and γ-aminobutyric acid (γ-ala). These two amino acids can account for over half of the total amino acid pool in sediments (Degens et al. 1964; Schroeder 1975; Whelan 1977). Both β-ala and γ-aba are found as free amino acids in plants, but usally in low concentration relative to other amino acids (Meister 1965). γ-aba has been found in relatively high concentrations in the intracellular pools of some dinoflagellates (Kittredge et al. 1962) and in marine macroalgae (Scheuer 1980), but is not a constituent of plant structural proteins. Both compounds have been reported in marine particulate matter consisting mostly of partially decomposed phytoplankton and zooplankton fecal pellets (Lee and Cronin 1982). Although their concentration is generally low, both β-ala and γ-aba have been found as dissolved free amino acids in natural waters (Mopper and Lindroth 1982; Henrichs and Williams 1985). Carlucci et al. (1984) reported that β-ala may constitute up to 20% of the total dissolved free amino acids released by phytoplankton in Pacific surface waters.

β-ala and γ-aba can be formed as microbial decarboxylation products from aspartic and glutamic acids, and β-ala also results from the decomposition of

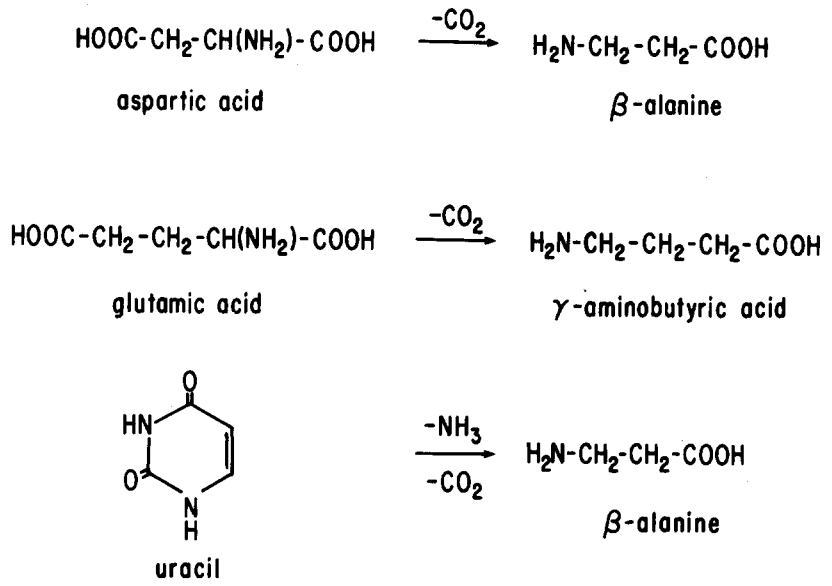

Figure 1. Structure and modes of formation of β-ala and γ-aba, two non-protein amino acids (modified from Mahler and Cordes 1971).

uracil (Figure 1). Degens et al. (1964) suggested that β-ala and γ-aba are formed in marine sediments as a result of enzymatic decarboxylation of detrital aspartic and glutamic acids. Aizenshtat et al. (1973) found relative increases of both β-ala and γ-aba with depth in a sediment core. In addition, Hare (1973) found that absolute concentrations of β-ala and γ-aba increased with depth in a sediment core from the Cariaco Trench while the protein amino acids decreased in concentration with depth. The fact that β-ala and γ-aba accumulate in sediments suggests that they may be decomposed more slowly than other amino acids. Lee and Cronin (1982) suggested that the relative concentration of β-ala in marine particulate matter might reflect the extent to which the particles have decomposed. As a comparison of relative decomposition rates of non-protein and protein amino acids, we investigated the microbial assimilation and respiration of free radiolabelled β-ala, γ-aba, and alanine at natural concentrations in brackish, coastal, and open ocean waters.

Methods

Sampling locations

Sea water was collected from several coastal and open-ocean sites for incubation experiments and for measurements of concentration. Siders Pond is a meromictic, brackish-water, coastal pond in Falmouth, Massachusetts (Caraco 1986; Caraco and Valiela 1983). Vineyard Sound (Massachusetts)

samples were collected from the Chemotaxis dock of the Woods Hole Oceanographic Institution. Samples from Vineyard Sound and Siders Pond were pumped directly into flasks by a hand-operated peristaltic pump (all plastic parts). This pumping system allows samples to be taken from low oxygen or anoxic waters without aeration. Surface samples were collected directly in the flasks. Ocean samples were taken from Nantucket Shoals (40 °10′ N, 71 °W), a warm core ring (38 °42′ N, 72 °53′ W), the Sargasso Sea 33 °57′ N, 73 °18′ W) and the Gulf Stream (33 °44′ W, 76 °08′ W) during Cruise 94 of R/V Knorr from 19 May through 4 June 1982. Water samples for shipboard incubation experiments were taken from 10 or 15 m depth using 10-or 30-liter Niskin bottles or hand collected in 2-gallon cubitainers by scuba divers, more than 200 m away from the ship.

Amino acid concentrations

Water samples were filtered through pre-ashed Whatman GF/F filters in Swinnex holders. No vacuum was used for the open-ocean samples; approximately 50 ml was filtered through each filter, including the rinses. The filter holders were connected directly to the Niskin bottles or cubitainers and the filtration occurred under the force of the hydraulic head (about 0.09 atm). The filtrate was collected directly into pre-cleaned glass vials which had been rinsed with the sample and were frozen immediately. The concentration of individual dissolved free amino acids was determined by HPLC (Mopper and Lindroth 1982; Jones et al. 1981) using a Varian 5000 pumping system, Altex Ultrasphere ODS column (25 cm, 5 μm), and Schoeffel FS 970 fluorometer (340 nm excitation, 470 nm emission). Due to high alkalinity, deep water samples from Siders Pond required an adjustment of the pH before addition of the fluorescent reagent.

Incubation experiments

Incubation experiments were performed to determine net assimilation and respiration of radiolabelled tracer. Water samples were placed in sterile, pre-cleaned 240-ml polycarbonate Erlenmeyer flasks. Each flask was rinsed (3 x) with sample prior to filling; the volume of each flask was determined after the incubation. During filling and handling, the flasks were held in an opaque cooler filled with ambient sea water to reduce light and temperature shock.

Aliquots of labeled amino acids (10 to 600 μl) were added to the full flasks, using an automatic micropipet with pre-sterilized polypropylene tips inserted below the surface of the water. The amino acids were added within 10 minutes of sample collection; final concentrations ranged from 6×10^{-5} nM for the lowest ^{3}H experiments to 30 nM for the highest ^{14}C experiments. We varied the incubation times from 10 minutes to over 30 h in time-course experiments and used 2 to 4 h incubations for end-point determinations. The flasks were incubated *in-situ* in Siders Pond and Vineyard Sound; open-ocean

samples were incubated on-deck in large-volume containers which were maintained near ambient temperature by a continuous flow of surface sea water.

Net assimilation measurements

Following the incubation, the entire contents of each flask were vacuum-filtered (Millipore HA filters, 0.13 atm). Each flask was rinsed with 20 ml filtered sea water and shaken vigorously to dislodge adherent particles; rinse water was filtered through the same filter. Radioactivity on the filters was measured using a Beckman LS 330 scintillation counter using the appropriate ^{3}H or ^{14}C quench set. Both the channel's ratio and external standard-channel ratio methods were used to estimate counting efficiency. Estimates of efficiency from the two methods were averaged for calculations. We used a toluene-based fluor (2:1 toluene: 2-methoxyethanol plus 7 g PPO/1). For the lower level ^{3}H experiments, we varied the counting time until at least 2000 (usually $>$ 10,000) counts had accumulated. For the samples with higher activity, we counted to 1% precision.

Respiration measurements

We measured the production of $[^{14}C]CO_2$ from the ^{14}C-labeled substrate after the incubation by extracting all of the dissolved inorganic carbon from solution by gas sparging (Cole and Likens 1979). After incubation, the sample was poisoned with an alkaline toxin (containing phenol, azide, and NaOH: Cole 1982) to stop respiration and prevent the escape of CO_2. The solution was then acidified to pH 1.0 in the incubation flask and extracted for 8 hours. We used CO_2-free air as the carrier gas and 2.0 ml of 1:1 2-methoxyethanol: ethanolamine as the trapping solution. The efficiency of recovery of added $[^{14}C]HCO_3$ was 90% under these conditions.

Controls

Each series of incubation experiments included several types of controls. For sterile controls, sample water was autoclaved in polycarbonate flasks prior to substrate addition. For filtered controls, sample was filtered aseptically through 0.22-um Milipore filters prior to substrate addition. For formalin-poisoned controls, formalin was added to 0.1 to 0.5% (v/v) final concentration at the time of substrate addition. Respiration blanks used alkaline toxin to arrest substrate mineralization. The toxin was added at the same time as the substrate.

Substrates

All ^{14}C and ^{3}H compounds were purchased from New England Nuclear as sterile aqueous solutions in 0.01N HCl. The ^{3}H compounds were obtained at very high specific activities (β-alanine, β-[3-^{3}H (N)] at 109 Ci/mmol; L-alanine, [3-^{3}H] at 75 Ci/mmol) while the ^{14}C-labeled compounds were at

Table 1. Concentration of dissolved free β-alanine and dissolved free alanine in coastal and open-ocean stations

	Sample depth (m)	Alanine (nM)	β-ala (nM)
Coastal:			
Siders Pond	1	1270	< 115
(Falmouth, MA)	3	660	< 60
	4	210	< 19
	6	170	< 15
	8	120	< 11
	13	120	< 11
Vineyard Sound (Falmouth, MA)	1	19	< 1
Open Ocean:			
Nantucket Shoals	10	42	1.5
Gulf Stream (28 May)	10	41	1.1
Warm Core Ring (21 May) 0700	5	52	1.6
Warm Core Ring (21 May) 1300	5	38	< 1
Warm Core Ring (3 June)	10	32	1.1
Sargasso Sea (24 May) 0900	10	2	0.4

[a]In Siders Pond, β-ala was not detectable relative to alanine. Because alanine concentrations were so high, the alanine peak obscured β-ala in the chromatograms; thus, we report only the detection limits under the conditions used for each sample.

much lower specific activities (γ-aminobutyric acid [^{14}C] at 208 mCi/mmol; β-alanine [1-^{14}C] at 50 mCi/mmol; and L-alanine [^{14}C (uniform)] at 160 mCi/mmol). The stock solutions were diluted with twice glass-distilled water (amino-acid free), sealed in pre-ashed glass ampoules and autoclaved (90 °C, 10 minutes, twice). The amino acids were all heat stable under these conditions and the purity and concentration of some of our diluted preparations were checked by high pressure liquid chromatography.

Cleaning procedures

To reduce contamination from metals or organic compounds, each flask was cleaned by a serial procedure modified from Fitzwater et al. (1982). Flasks were scrubbed, then soaked, and rinsed with deionized water followed by 2N reagent grade H_2SO_4 and a final rinse (10x) with deionized water. Flasks were then partially filled with deionized water and autoclaved (40 minutes, 20 °C) prior to use. The same procedure was used for the filtration glassware.

Table 2. Turnover time of ^{14}C-labeled β-ala and γ-aba in Siders Pond, Vineyard Sound, and Nantucket Shoals. Added concentrations of β-ala were 3.1 nM in Siders Pond and ranged from 0.1 to 0.0001 nM in Vineyard Sound. Added concentration of γ-aba was 0.01 nM in Siders Pond and ranged from 0.1 to 0.001 nM in Nantucket Shoals. Values are means for multiple (3 minimally) incubations; turnover times for formalin-poisoned controls are also shown for Siders Pond. Note that turnover time for poisoned controls was 10–100 times slower than in untreated samples.

	Depth (m)	Temp. (°C)	Oxygen (mg/l)	Turnover time (hours) β-ala	Poisoned control (β-ala)	γ-aba	Poisoned control (γ-aba)
Siders Pond	0.5	24.8	8.1	4.93	76	6.27	895
	3	19.9	7.8	6.41	118	4.04	–
	4	17.7	0.4	14.1	152	8.42	–
	5	17.0	0.2	4.03	374	–	–
	6	16.0	0.0	20.3	250	–	–
	8	16.2	0.0	18.1	432	3.40	2200
	13	9.0	0.0	40.9	–	5.54	–
Vineyard Sound	0.5	22.0	8.6	54.4	812	–	–
Nantucket Shoals	0.5	11.6	12.1	24.0	1200	12-30	1490

Results

Concentration of dissolved free β-alanine and alanine

Measured concentrations of alanine and β-ala are shown in Table 1. In Siders Pond the free amino acid concentrations were much higher (10x) than normally found in coastal or open ocean sea water but were similar to other local salt ponds where DOC concentrations can be >10 mg liter^{-1} (Lee, unpublished results). Surface alanine concentrations in Siders Pond were 10–20 times higher than open ocean samples and concentrations decreased exponentially with depth.

In Siders Pond, the concentration of β-alanine was not detectible relative to alanine because the concentration of alanine was high and the ratio of β-alanine to alanine was very low (Table 1). β-ala was easily detectible in the open-ocean samples and the ratio of β-ala/ala was higher than in the coastal or coastal pond samples. Water from the Sargasso Sea, the least productive area sampled, had the lowest concentration of DFAA and the highest β-ala/ala ratio.

Respiration of ^{14}C-labeled β-alanine

In our incubation experiments with natural sea water, the carboxyl-C of β-ala was rapidly converted to CO_2 and was not significantly incorporated into biomass (particulate matter). In general, we found that the ^{14}C incorporated into particulate matter was less than 10% of the amount converted to ^{14}C-CO_2 during 0.5 to 5-h incubations. In sterile or poisoned

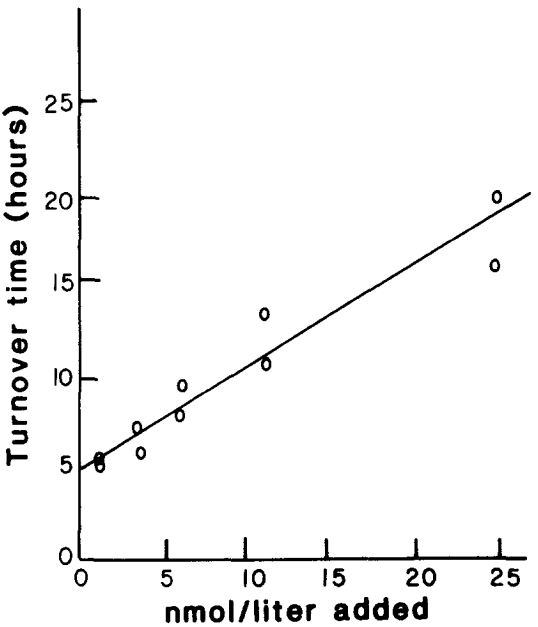

Figure 2. Decarboxylation of ^{14}C-labeled β-ala in Vineyard Sound water. Specifically labeled [1-^{14}C]-β-ala was added to Vineyard Sound water at a range of final concentrations (X-axis). After an in-situ incubation of 2.5 h, the evolved ^{14}C-CO_2 was measured. The data are plotted so that the turnover time for the carboxyl carbon is the y-intercept. Regression: $y = 0.58x + 4.71$; $r^2 = 0.94$. Temperature was 24 °C. Turnover times in the presence of formalin averaged 420 hours for the lowest addition of β-ala.

controls, β-ala was not decarboxylated significantly (Table 2).

In surface sea water, respiration of β-ala followed typical saturation kinetics that could be described by the usual Michaelis-Menten model (Figure 2). When β-ala was added in the 1 to 25 nM range (final concentration) the calculated V_{max} was about 2 nmol liter^{-1} h^{-1} and calculated turnover time was about 5 hours. These values are within the usual range reported for amino acids in moderately warm coastal waters (Keller et al. 1982). The ambient substrate level ($K_t + S_n$) calculated kinetically (8 nM) was somewhat higher than the measured level (about 1 nM) suggesting either that K_t is very large or, more likely, that the Michaelis-Menten equation is an imperfect kinetic model in this case (see Azam and Hodson 1981). In Siders Pond the turnover times for the carboxyl carbon of β-ala varied from about 5 h in warm (25 °C), oxic surface waters to 40 h in cooler (9 °C), anoxic water at 13 m depth (Table 2, Figure 3). Over the same gradient, the turnover time for alanine respiration varied from 4 to 12 h (Figure 3).

Assimilation of ^{3}H-β-alanine

In order to determine the net assimilation of β-ala at ambient substrate levels, we added very small amounts (10^{-4} nM, final concentration) of β-ala with

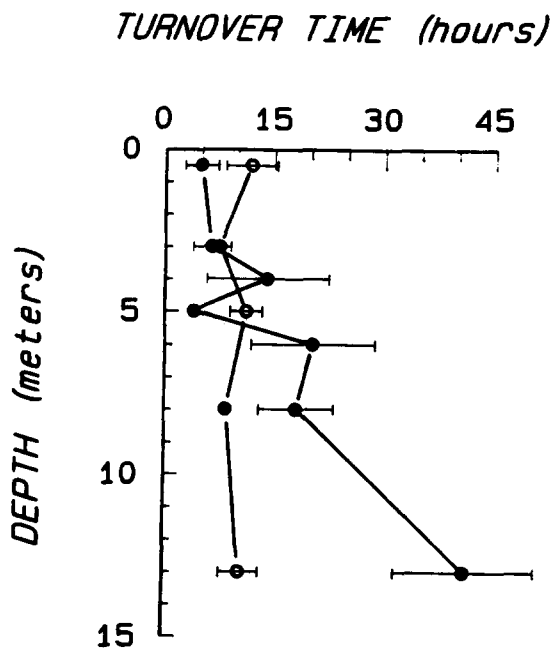

Figure 3. Turnover time for ^{14}C-labeled alanine (open circle) and β-alanine (solid circle) during August 1982 in Siders Pond, a meromictic coastal pond. The turnover times are plotted for the carboxyl carbon in both cases; mean and standard deviations of multiple (3 to 10) incubations are shown. The oxycline in Siders Pond was at 6 meters.

high specific activity (109 Ci/mmol). When β-ala was added to Gulf Stream water and incubated at ambient light and temperature, the amount of ^{3}H assimilate increased linearly over time for up to 30 h (Figure 4). Very little label was incorporated into particulate matter in our sterile or poisoned controls, again suggesting that uptake of β-ala was due to biologic processes (Figure 4).

Over a very broad range of low substrate concentrations, the net assimilation of ^{3}H was directly proportional to the amount of labeled substrate added (Figure 5). These results demonstrate that additions of β-ala in this concentration range were well below kinetic saturation, and indicate that one transport system is operating over the entire range of concentrations. Thus our estimates of turnover time are not biased. Further, the results clearly show that β-ala is removed from solution at or near natural concentrations. Thus, the concentration of dissolved free β-ala in natural waters must represent a dynamic balance between synthesis and catabolism. The turnover times calculated from these experiments ranged from 24 h (Nantucket Shoals) to 227 h (Sargasso Sea) (Figure 6a). In no case was measurable uptake observed in formalin-poisoned or sterile controls.

To compare turnover of β-ala with the turnover of a more common amino acid, we added ^{3}H-labelled alanine in the same concentration ranges under

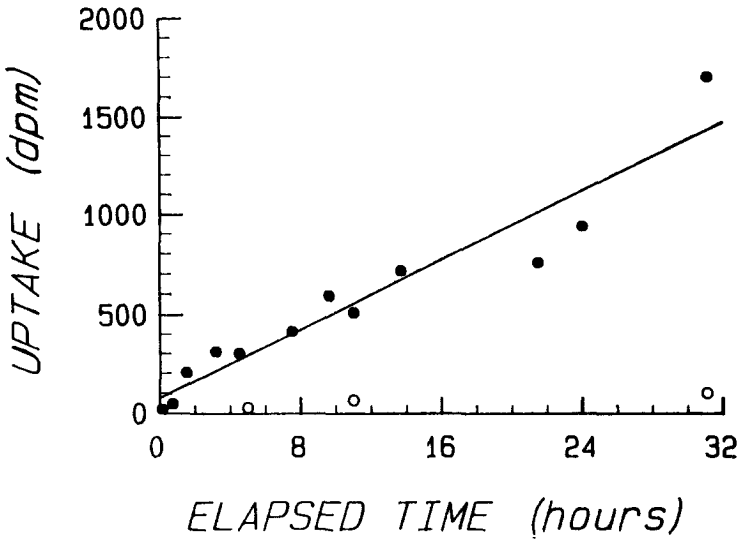

Figure 4. Time sequence of the uptake of β-[3-^{3}H (N)] ala at very high specific activity in Gulf Stream water. The final added concentration was 3.6 x 10^{-4} nM and incubations were at ambient temperature. Solid circles are samples: Regression line: $y = 43.7\ x + 75.8$; $r^2 = 0.91$. Open circles represent formalin-poisoned controls which were treated identically to samples.

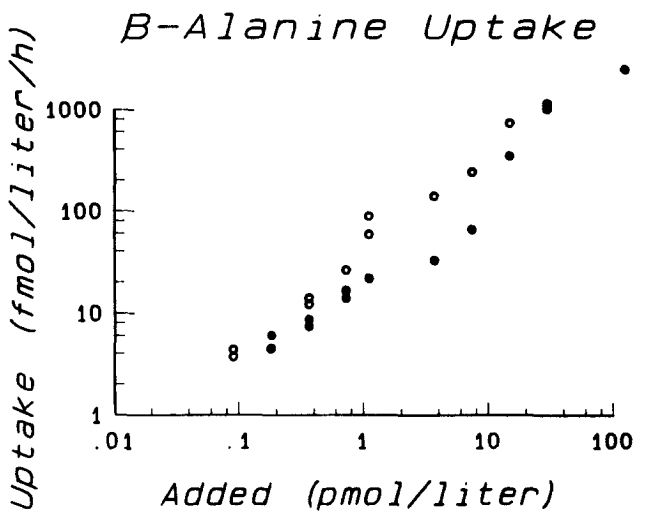

Figure 5. Concentration series experiments for the net assimilation of [3-^{3}H (N)] β-alanine at Sargasso Sea (solid circles) and Gulf Stream (open circles). β-alanine was added at a specific activity of 109 Ci/mmol and the incubations were 5 hours (Gulf Stream) and 5.28 hours (Sargasso Sea). The rate of uptake is expressed for the labelled moiety only and does not include the ambient, unlabelled pool.

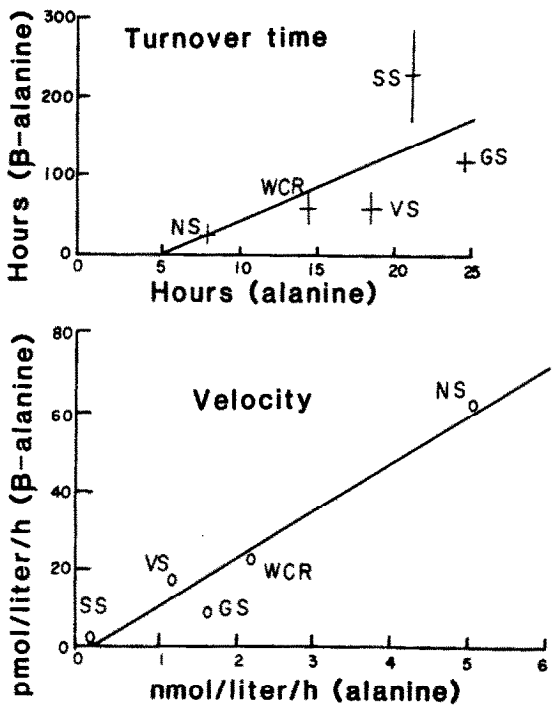

Figure 6. Covariance of metabolism of alanine and β-alanine for the ocean stations. A-turnover time is plotted for both substrates as means; error bars represent standard deviations in both directions. B-Velocity (calculated from turnover rate and measured substrate concentration) is plotted for the same data set. SS-Sargasso Sea; GS-Gulf Stream; VS-Vineyard Sound; WCR-warm core ring; NS-Nantucket Shoals. Regression line for turnover times: $y = 8.87x - 58.2$; $r = 0.69$. Regression line for velocities: $y = 12.3x$ 2.74; $r = 0.87$.

identical conditions. Alanine had turnover times that were consistently faster, by 32-to 10-fold than those for β-ala. The range of turnover times for alanine (8 to 25 h) was smaller than that for β-ala but tended to covary with it between stations (Figure 6a).

Respiration of ^{14}C-labeled γ-aminobutyric acid

We made a limited examination of net assimilation and respiration of ^{14}C-labelled γ-aba and report data from three environments, Nantucket Shoals, Vineyard Sound and Siders Pond (Table 2). In all three cases, γ-aba was respired at a rate similar to that of β-alanine; turnover times for the carboxyl carbon ranged from 4 to 30 h.

Discussion

Turnover of non-protein amino acids

Both β-alanine and γ-aminobutyric acid were assimilated and catabolized by microorganisms in surface waters under both aerobic and anaerobic conditions at natural concentrations. In every case, alanine was metabolized more rapidly than β-ala. The difference between turnover times of alanine and β-ala increased with depth in Siders Pond, and was greater at the lower productivity open-ocean sites. Nevertheless, the turnover times for β-ala (20 to 200 h) are generally comparable to those reported for other amino acids (Ferguson and Sunda 1984, Keller et al. 1982). Carlucci et al. (1984) report turnover times for ^{3}H-ala of 36 h in California coastal waters in general agreement with our results here. They found that in oligotrophic waters off southern California, the turnover time of β-ala was about 4 time slower than that of glutamic acid. These results demonstrate that dissolved free β-ala is not particularly refractory to microbial attack as has been previously suggested, but may be degraded more slowly than other amino acids.

The turnover times for alanine and β-ala covaried (Figure 6), suggesting that β-ala follows the same pattern of assimilation as other more common amino acids. Turnover times were longest at the oligotrophic, open-ocean Sargasso Sea station and shortest at the coastal, more productive Nantucket Shoals site, despite lower temperatures at Nantucket Shoals. This trend in turnover time was paralleled by lower alanine concentrations in the Sargasso Sea and higher concentrations in waters nearer the coast. The concentration of β-ala was also lower in the Sargasso Sea than in the more coastal waters, but the ratio of β-ala to alanine was higher. This high ratio could be due to the turnover time of β-ala being 11 times longer than the turnover time of alanine at the Sargasso Sea station, but only 3 times longer at Nantucket Shoals (Figure 6a). Because the oligotrophic sites had both long turnover times and low concentrations, the velocity of β-alanine net assimilation showed a strong trend with station (Fig. 6b).

Concentrations of amino acids

The concentrations of alanine that we found in the Gulf Stream and Warm Core Ring stations are 5–10 times higher than those reported for the Baltic Sea by Mopper and Lindroth (1982). On the other hand, Braven et al. (1984) measured amino acids in the English Channel on shipboard immediately upon sample collection and obtained very high values for alanine (60 to 220 nM). These differences could be due to true natural variation, or to differences in filtration or sampling methods. For example, Fuhrman and Bell (1985) found that the glass fiber filters used in our studies cause more cell leakage (due to shear stress) than do membrane filters used in some other studies. The measured concentration of DFAA can, thus, be affected by both filtration method and algal cell density. An inter-calibration experiment is

currently being conducted between several laboratories measuring free amino acids to address some of these questions.

Non-protein amino acids in deep sea sediments

If dissolved β-ala and γ-aba are readily taken up and catabolized by marine bacteria, why do marine sediments contain such large relative amounts of these amino acids? Natural concentrations of any compound result from the balance between production and consumption processes. Both β-ala and γ-aba are produced in the marine environment. γ-aba in marine sediments has two likely sources: (1) γ-aba present as the free compound in phytoplankton (Scheuer 1980; Kittredge et al. 1962) which falls to the sea floor, and (2) γ-aba from the *in-situ* microbial decarboxylation of glutamic acid (Degens et al. 1964, Meister 1965). Both γ-aba and β-ala are also found bound in the mureide complex of bacterial cell walls (Osborn 1969). Neither bound β-ala nor γ-aba commonly occur in marine plankton (Brown et al. 1972; Lee unpublished). Free β-ala has been found in some marine bacteria and benthic organisms (Awapara 1962; Henrichs 1980; Lee unpublished). β-Ala may also be formed in sediments from microbial decarboxylation of aspartic acid; but enzymatic loss of CO_2 usually occurs at the beta rather than the alpha-carboxyl group, forming alanine rather than β-ala (Meister 1965). In the marine environment, β-ala may more likely be produced from other pathways such as the degradation of the pyrimidine uracil or the polyamine spermidine (Awapara 1962; Meister 1965; Doelle 1975). Although both γ-aba and β-ala have many possible sources in marine sediments, these compounds could not accumulate or increase in concentration with depth unless they are decomposed only slowly or not at all.

There are at least two possible reasons why catabolism of non-protein amino acids might be slower than degradation of protein amino acids. One is that microbes might not be able to use non-protein amino acids as efficiently and quickly as protein amino acids. A second possibility is that some non-protein amino acids are preferentially absorbed onto clays and are no longer available or as easily available to microorganisms. Our data indicates that both β-ala and γ-aba are rapidly taken up and respired by marine bacteria in sea water. However, in our experiments, β-ala turnover times were always longer than those of alanine. Thus, if microbial uptake rates in sediments follow this same pattern, the differential rates might partially account for the accumulation of β-ala in sediments. The Siders Pond data suggested that under low oxygen or anoxic conditions, uptake of β-ala could be very much slower than uptake or alanine. Thus, under such conditions, differential rates of catabolism could account for significantly more accumulation of non-protein amino acids. However, most of the sediments containing large relative amounts of non-protein amino acids are oxic deep-sea sediments with high clay content. Coastal anoxic sediments contain smaller proportions of these amino acids (Henrichs, 1980; Henrichs et al., 1984). For these reasons, we

think it likely that another mechanism such as preferential adsorption onto clays is responsible for accumulation of non-protein amino acids in sediments.

Weliky (1983) found that in abyssal red clay sediments with high proportions of β-ala and γ-aba, the non-protein amino acids were concentrated in the smallest size fraction; she attributed this to adsorption onto clays. In laboratory experiments, clay minerals remove non-protein amino acids from solution much faster than protein amino acids are removed (Friebele et al., 1980). This increase in attraction to clays is apparently due to increasing basicity of amino acids with increasing distance between amino and carboxyl groups (gamma > beta > alpha; Sieskind and Wey, 1959). Thus, preferential accumulation of non-protein amino acids in sediments is likely due to preferential adsorption by clays and only to a lesser extent to differential rates of bacterial decomposition.

Acknowledgements

We thank L. Madin and the crew of the R/V Knorr for making space available to us on WHOI cruise 94. We thank N.F. Caraco and A.H. Puccoon of the R/V Kneither for their assistance during WHIFR cruises 103–107 on Siders Pond. J. Fuhrman kindly made useful comments on the ms. Funding was provided by a post-doctoral fellowship to J. Cole from the Woods Hole Oceanographic Institution and from The Ecosystems Center, Marine Biological Laboratory and from grants to C. Lee from the Nation Science Foundation (OCE 84–2179) and the Office of Naval Research. WHOI Contribution No. 6076.

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