EFFICIENT UTILIZATION OF DISSOLVED FREE AMINO ACIDS BY SUSPENDED MARINE BACTERIA

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Abstract:

Incorporation of a mixture of ^{14}C-labeled amino acids by bacteria averaged 79% of assimilation (incorporation plus respiration) but varied with time and location in the salinity gradient of the Newport River estuary, North Carolina, over a 9-month period. The incorporation ranged from an average of 86% at a high (34.2‰) to 74% at a low (4.1‰) average salinity location. Incorporation at the high salinity location generally was greater than values previously reported in the literature because we accounted for amino acids lost from the particulate fraction as a result of the acidification required to liberate $^{14}CO_2$ from the water. Loss of label due to acidification for estuarine water and for neritic water collected from Florida to Massachusetts was up to 79% of the incorporated amino acids and varied inversely with the amount of incorporated label. The data affirm that suspended marine bacteria efficiently utilize dissolved free amino acids and that acidification should not be used to stop the incubation of samples to be filtered for measurement of substrate incorporation.

Article:

INTRODUCTION

Dissolved free amino acids (DFAA) are an important component of marine food chains. DFAA range from ≃20 nmo1·1^{-1} in oceanic to ≥100 nmo1·1^{-1} in estuarine water (Bada & Lee, 1977; Jørgensen, 1982; Mopper & Lindroth, 1982). Although free amino acids make up only 0.2% of the total dissolved organic carbon (DOC) pools in estuaries (Crawford et al., 1974) and 0.3% of that in neritic or oceanic water, they have a rapid turnover and high nutritional value compared to the total DOC pool (Bada & Lee, 1977). The major pathway for the input of DFAA into the marine planktonic food chain is through bacteria (Hoppe, 1976; Hobbie & Rublee, 1977).

The availability to higher trophic levels of amino acids assimilated by bacteria depends on many factors, including the incorporation efficiency of amino acids by bacteria. For mixtures of radioactively labeled amino acids, average incorporation to assimilation (respiration plus incorporation) ratios range from 50% (Herbland, 1978) to 78% (Williams, 1970). The incorporation efficiency of individual amino acids varies from 50-87% but does not appear to change with varying incubation times, temperatures, or substrate concentrations (Crawford et al., 1974), while variations in percent respiration of individual amino acids and mixed amino acids may occur in different biotypes (Gocke, 1976).

Estimates of incorporation or incorporation efficiency can be affected by the method chosen to terminate the incubation. The methods generally used are to add chemical fixatives, such as inorganic acid, mercuric chloride, or formaldehyde to the sample before filtration, to flood the sample with excess substrate (Jordan et al., 1978) or to filter at the end of the incubation period (Carney & Colwell, 1976). Acids are used to reduce pH sufficiently to allow collection and measurement of respired $^{14}CO_2$ (Hobbie & Crawford, 1969), but their use limits observed substrate incorporation to that incorporated into macromolecules (Griffiths et al., 1974a,b; Baross et al., 1975; Ramsay, 1976, Hobbie & Rublee, 1977). Labeled substrate within the cells can occur both as macro-molecules and as metabolic intermediates (Britten & McClure, 1962). Loss of substrate incorporated

into metabolic intermediates due to acidification has been avoided by some researchers (e.g. Williams, 1970; Gocke, 1976; Herbland, 1978). However, it is a potential problem when using the method of Hobbie & Crawford (1969) without modification. Thus loss of substrate due to acidification may have affected other studies (e.g. Crawford et al., 1974; Hansen & Snyder, 1979). Even if acid is not used in samples that are used to measure incorporation, other methods, such as formaldehyde fixation, may have the same effect (Griffiths et al., 1974b).

Our study was initiated to determine the incorporation efficiency, of DFAA within the salinity gradient of the Newport River estuary, North Carolina. Later experiments and articles published subsequent to initiation of this work demonstrated that our initial estimates of incorporation were underestimates due to loss of labeled amino acids from the cells due to acidification of the sample prior to filtration. We examine alternative methods to terminate the incubation and provide improved estimates of the growth yield of marine bacteria utilizing amino acids in estuarine and neritic waters.

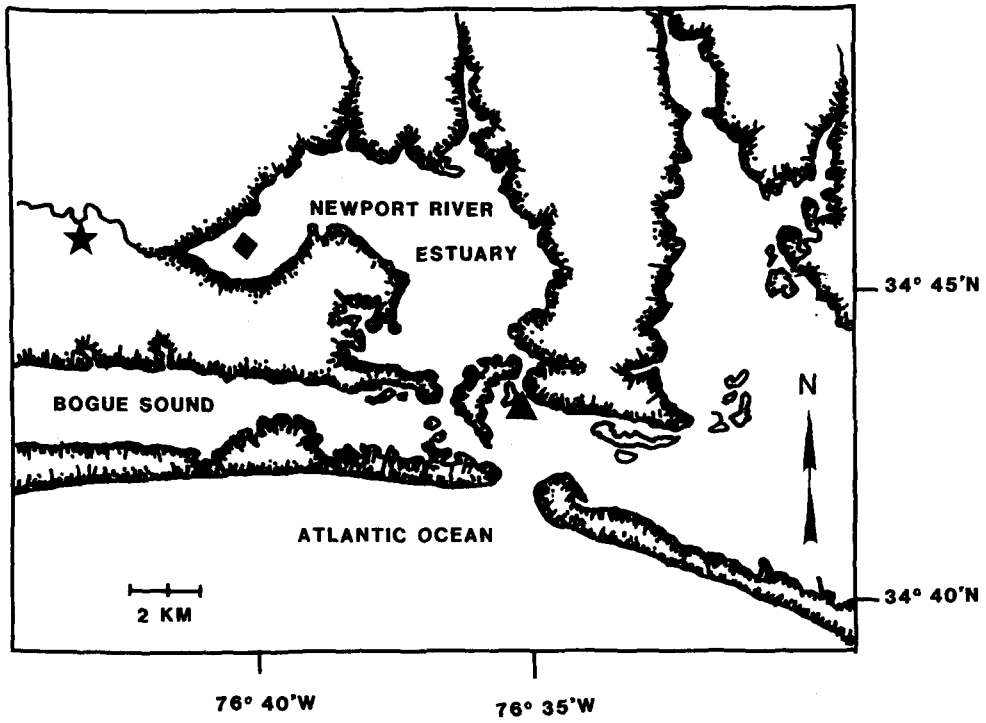

Fig. 1. Map of the Newport River estuary showing the high (▲), mid (◆), and low salinity (★) sampling locations for the 24-h respiration studies.

METHODS

We ran two series of experiments. The first series measured incorporation and respiration of a ^{14}C-labeled mixture of amino acids (New England Nuclear NEC-250) in water samples from three locations along the salinity gradient of the Newport River estuary (Fig. 1). The sampling sites were: a dock at the National Marine Fisheries Beaufort Laboratory near the mouth of the estuary (high salinity); a shallow mixing basin near the head of the estuary (sampled from an anchored boat, intermediate salinity); arid an abandoned boat ramp on the Newport River upstream of the broadened portion of the estuary (fresh or low salinity water). We attempted to minimize influence of short term changes in bacterial parameters associated with tidal and diurnal effects (Erkenbrecher & Stevenson, 1975) with our sampling design. Measurements were taken from four surface water samples collected at two high and two low tides during a 24-h period on three or four sampling dates for each station from December 1977, through September 1978, for a total of 44 samples. Incorporation and respiration of amino acids by bacteria was determined using the method of Hobbie & Crawford (1969). We added 0.025 mCi, final concentration by adding $\simeq$10 to 50 nmo1·1^{-1} of ^{14}C-labeled amino acids to each of four replicate subsamples of water (three live and one acid-killed control). Samples were incubated in a closed system for 1 h and incubation was terminated by addition of H_3PO_4, final concentration 0.4% v/v. $^{14}CO_2$ released by acidification was absorbed over an additional 1-h period by phenethylamine on chromatographic paper suspended in a sealed flask, which was gently swirled several times to facilitate CO_2 recovery. The

chromatography paper then was placed in 10 ml of a toluene-based scintillation fluid (toluene + Fisher, Omnifluor) and the radioactivity counted in a Beckman LS-200 liquid scintillation counter. The external standard method was used to correct for quench. Incorporated label was determined from the same set of flasks, and incorporation and respiration (corrected for efficiency of CO_2 recovery) for acidified samples were calculated.

On each sampling day we measured CO_2 recovery efficiency on one low and one high tide sample. Measurements were made in sets of three replicate subsamples of the water used for the measurement of respiration. Labeled (^{14}C) HCO_3^- was added to the flasks just prior to the addition of the acid, thus there was no time for photosynthesis to remove any of the label. Efficiency of CO_2 recovery under our experimental conditions in the field was variable over the course of the study, but it was very reproducible on a given water sample. The standard deviation of the CO_2 recovery efficiency on a water sample averaged 3.3% of the added label. The average recovery over the study was 38.7%. The age of the phenethylamine and differences in the pH buffering capacity and inorganic carbon content of the sample water may have contributed to the variability in CO_2 recovery (generally between 25 and 75%) over the course of the study. Other methods (e.g. Williams et al., 1976), as well as allowing a longer time for CO_2 recovery and putting the flasks on a rotary shaker, will give better CO_2 recovery.

The second series of experiments measured the effects of acidification on observed incorporation of a tritium-labeled mixture of amino acids (New. England Nuclear NET-250) and was used to obtain a posteriori corrections for loss of label due to acidification in the first series of experiments. In these experiments we compared the incorporation of ^3H-labeled amino acids in replicate subsamples after addition of 0.2 ml of a 50% v/v solution of H_3PO_4, or excess substrate, 0.2 ml of a 3 w/v solution of hydrolyzed protein (Difco, Bacto, Neopeptone). Use of ^3H-labeled substrates with a higher specific activity than available for ^{14}C-labeled substrates is necessary for tracer level additions to low productivity water.

For one estuarine water sample, we also tested termination by filtration or by use of the following reagents (and final concentrations): formaldehyde (0.6% w /v) Lugol's acetic acid (0.7% v/v) $HgCl_2$ (0.01% w; v) and H_2SO_4 (0.1% w/v). A range of ≈0.25 to 0.5 μCi in 50 μl of a freshly diluted stock solution was added to each flask in the different experiments, amounting to a final concentration increase of ≃1.5 to 3 $nmol \cdot 1^{-1}$. Incubations were terminated after 30 to 60 min. We used two, three, or four replicates and one acid or neopeptone terminated control, with 25-30 ml subsamples of water in 50-ml flasks, for each treatment. Controls were treated similarly, except that the incorporation of label was terminated at the time of addition of label. Incubations were conducted at in situ temperatures, 10-30 °C in the estuarine samples, 15-25 °C in the Georgia—Florida neritic water samples, and 4-6 °C for the Georges Bank sample.

Water from both series of experiments was filtered through 0.2-μm pore size Nuclepore filters. Filters with incorporated label were placed in scintillation cocktail (New England Nuclear, Aquasol), counted for radioactivity, and corrected for quench and background.

RESULTS AND DISCUSSION

Acidification resulted in a reduction of label incorporation relative to excess substrate addition over a wide range of temperatures (4-30 °C), salinities (0-35‰), sampling locations, and uptake rates (Fig. 2). Seventeen of the 19 pairs had greater incorporation in the neopeptone addition. Using the sign test the probability of having 17 of the 19 pairs with the same sign is <0.001 if the two treatments are equal (Connover, 1971). The reduction was up to 79% of that observed with neopeptone addition and was greatest at low levels of incorporation. There was a linear relation between the amount of label incorporated in acidified samples and the label incorporated in unacidified samples. Using these data, we computed a correction factor (CF) for the percent respiration: CF = DPM incorporated/(868.7 + 1.304 . DPM incorporated). This adjustment, i.e. dividing incorporation in acidified samples by CF, was made in the analysis of percent respiration and growth yield discussed below. The ratio of incorporated label in acidified to unacidified treatments changes with amount of incorporated label. For example, by calculation the ratio is 46% at 10^3 DPM and 77% at 10^6 DPM. Thus, error due to acidification is

particularly large in lower activity samples.

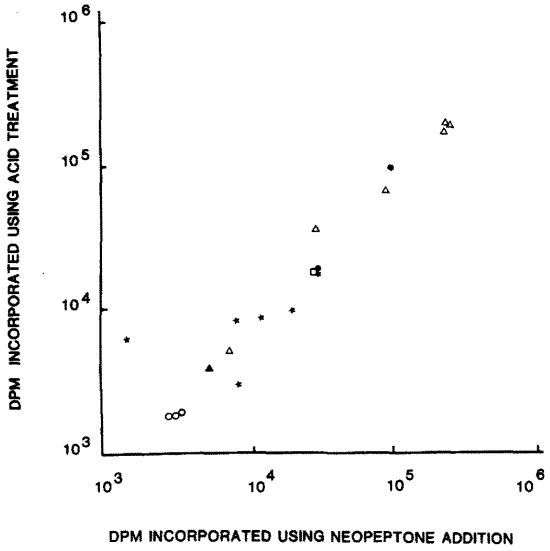

Fig. 2. Paired comparison of ^{3}H-labeled amino acid incorporation in estuarine and coastal marine waters following acidification or addition of Bacto-Neopeptone solution: data are plotted on a log–log scale only because the data cannot be resolved on a linear plot; linear regression analysis of untransformed data ($Y = 0.786 \cdot X - 674$, $r^2 = 0.9899$) indicates incorporation measured in acidified samples ranged from 45 to 79% of that in unacidified samples; ●, Newport River, 13 September 1977; △, Newport River, 27 March 1977; ○, Newport River, 15 December 1977; ★, Georgia Florida Continental Shelf Waters 5–9 October 1977; ▲, Georges Bank, 6 April 1978; □, Newport River, 23 March 1978.

Loss of substrate due to acidification is best understood in terms of substrate pools and is described by Hobbie & Rublee (1977). Britten & McClure (1962) proposed a general model of amino acid utilization derived from experimental work with Escherichia colt, which defined a loosely bound pool of metabolic intermediates as that pool released by brief exposure to 5% TCA (trichloracetic acid) at room temperature. Biochemically, the tightly-bound substrate pool is defined as the material that is combined into macro-molecules and remains as a precipitate after treatment with cold 10% TCA (Kennell, 1967), Incorporated amino acids occur in the loosely bound pool prior to polymerization into macromolecules. The loosely-bound pool may cause errors in assessing incorporation because its maintenance is dependent upon the structural integrity of the cellular membrane. Baross et al. (1975) demonstrated that measured incorporation of glutamic acid, alanine, glucose, and uracil at room temperature, using the H_2SO_4 technique (Hobbie & Crawford, 1969), was equivalent to that with cold 10% TCA treatment and, therefore, the internal pool of labeled substrate and other labeled low molecular weight metabolites were excluded. Thus, the artifact due to acidification also occurs with a carbohydrate and a pyrimidine and will affect respiration to assimilation ratios of radioactively labeled substrates in general. Our results are consistent with the substrate pool theory because, at the lower incorporation levels, the largest fraction of incorporated label was lost by acidification.

TABLE I

Effect of method of termination on incorporation of tritiated amino acids: water collected 23 March, 1978 near the mouth of the Newport River estuary, North Carolina; data expressed as mean ($\bar{X}$) and standard error (SE) of three replicates; data were analyzed using analysis of variance comparing all treatments to the control, termination by filtration.

	Final concentration	Incorporation (DPM)	
Termination method	%	$\bar{X}$	SE
Filtration		27,141	1594
Excess substrate[a]	0.02 w/v	29,121	3265
Formaldehyde	0.6 w/v	30,336	1335
H_3PO_4	0.4 v/v	18,614[b]	1924
Lugol's acetic acid	0.7 v/v	18,100[b]	363
$HgCl_2$	0.015 w/v	19,514[b]	4201
H_2SO_4	0.1 w/v	18,529[b]	1010

[a] Bacto-Neopeptone
[b] Statistically different than the control at the 95% level.

Additions of other chemical fixatives, such as Lugol's acetic acid and mercuric chloride, also result in substantial loss of incorporated label (Table I). Thus, Crawford's (1967) observation that there was no

difference between treatment with acid and Lugol's acetic acid probably resulted from loss of incorporated label due to both treatments rather than no effect of acidification. In fact, an iodine solution (Lugol's without the acetic acid) and formalin also cause ^{14}C losses from phytoplankton (Silver & Davoll, 1978). In bacteria, at final concentrations ≥1% formaldehyde, loss of label can occur (Griffiths et al., 1974b). Also, treatment with another acid, H_3PO_4 also caused significant loss of label. In our experiments, however, 0.6% formaldehyde terminated uptake without detectable release of incorporated label (Table I). Thus, lower concentrations of formaldehyde could be used in procedures where higher levels have been used in the past (e.g. Dietz & Albright, 1978).

The mean value 81% (range 59 to 99%) that we observed for incorporation efficiency (100%—% respired) in the estuary, after correction for loss of incorporation due to acidification, was close to values reported by a number of other investigators who avoided acidification of samples for incorporation. For example, Williams & Yentsch (1976) reported an average of 79% incorporation efficiency for an amino acid mixture and Dawson & Gocke (1978) reported an average of 75% incorporation efficiency. Williams et al. (1976) reported 60-90% for individual amino acids. In other studies where acidification may have affected the results, the incorporation efficiencies are somewhat lower (e.g. Crawford et al., 1974; Hanson & Snyder, 1979).

There was significant variability (analysis of variance, F tests 99% level) in the percent respiration of the mixture of ^{14}C-labeled amino acids by bacteria that could be associated with location in the estuary, sampling period, and interaction of location and sampling period. The seasonal pattern at the low salinity and mid-salinity sites is similar (Fig. 3), however, and significance of the interaction term results from the difference between these two sites and the high salinity site.

One clue to the dynamics of amino acid respiration in the estuary may be the relationship between salinity and percent respiration. A regression was computed and it had a correlation coefficient of — 0.68 significant at the 95% level, but this does not imply a cause-and-effect relationship because other factors, as discussed below, covary with salinity. Salinity at the low and mid-salinity stations varies seasonally to a greater degree than that at the high salinity site. The change in salinity either may directly affect the utilization of amino acids for growth or energy, or it may indicate changes in other factors including substrate levels or bacterial communities.

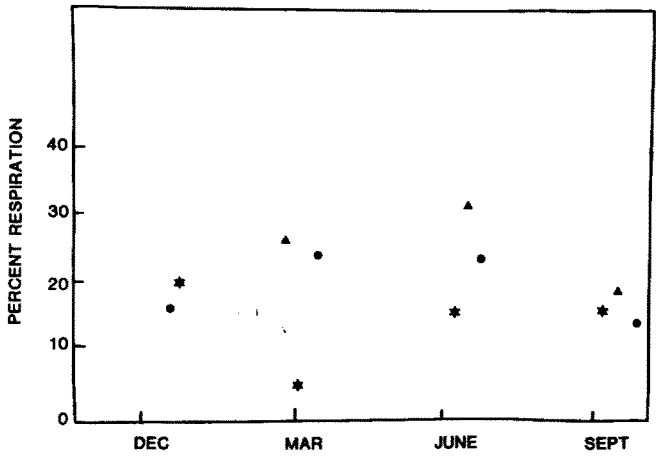

Fig. 3. Percent respiration at the high (*), mid (●), and low salinity (▲) sampling locations for the 24-h studies: uptake and respiration at the low salinity site in December were not detectable.

Utilization of DFAA may be related to the availability of inorganic and organic nitrogen to the bacteria. The Newport River is a major source of inorganic nitrogen to the estuary (Thayer, 1971), and thus inorganic nitrogen is negatively correlated with salinity. DOC also is negatively correlated with salinity in the Newport River estuary (Palumbo & Ferguson, 1978); and the distribution of dissolved organic nitrogen and of dissolved amino acids (Gardner & Stephens, 1978) follows the same pattern as DOC in coastal waters. Sediments and phytoplankton are major sources of DFAA to the water (Jørgensen, 1982) and both of these sources would lead to higher amino acid concentrations in the shallow, high productivity, low salinity region of the estuary. Thus, bacterial populations in the nitrogen-rich (low salinity) region of the estuary may be using a larger portion of the

amino acids as energy sources than populations in less nitrogen-rich (high salinity) regions of the estuary.

Changes through time in percent respiration and growth yield at the high salinity (range 31.2‰ to 35.7‰ site (Fig. 3) may reflect changes in coastal bacterioplankton populations. Gocke (1976) found geographical variations in percent respiration of nine individual amino acids and an amino acid mixture. His results from the winter of 1974 parallel ours. He found the percent respiration was lower in sea water than in fresh water, but he observed a minimum in the percent of amino acids respired in brackish water, while we always saw a minimum in sea water (Fig. 3). Griffiths et al. (1978) reported that in water of the Beaufort Sea, the percentage respiration of glutamic acid was greater in winter, 85%, than in summer, 50 to 64%.

Differences in relative flux of individual amino acids for the labeled amino acid mixture to bacteria in fresh water and sea water could also result in differences in the estimates of percent respiration we computed using radiotracer techniques. Individual amino acids are respired to different extents by bacterioplankton. Crawford et al. (1974) found that the percent respiration varied from 13% for leucine to 50% for glutamic acid and aspartic acid. Gocke (1976) also found percent respiration of individual amino acids in different biotypes varied from 1 to 15% for leucine and from 28% to 68% for aspartic acid.

The major role of bacteria in both high- and low-salinity water appears to be incorporation rather than remineralization of amino acids. The high percentage of incorporation to assimilation (86%) in samples from near the mouth of the Newport River estuary indicates that, at least in terms of amino acid dynamics, suspended bacteria in coastal waters are a source of particulate organic nitrogen. Even in low- and intermediate-salinity more nitrogen-rich waters, substantial portions (74 and 80%) of assimilated amino acids are converted into biomass. Suspended bacteria appear to be more important, therefore, as secondary producers of particulate organic nitrogen than as decomposers of DFAA in sea water. Terminating incubation with inorganic acids, mercuric chloride or with formaldehyde (≥ 1% final concentration) underestimates incorporation of amino acids relative to that observed with formaldehyde (0.6% final concentration), excess unlabeled substrate, or filtration. The relative error increases as incorporation decreases and is most severe in lower productivity sea water.

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