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THE UTILIZATION OF DISSOLVED FREE AMINO ACIDS BY ESTUARINE MICROORGANISMS[1]

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Abstract. The importance of bacteria in the cycling of carbon in the Pamlico River estuary was studied by measuring the rates of uptake of organic compounds. Our methods allowed analysis with the Michaelis-Menten kinetics equations, and both the rates of uptake of dissolved free amino acids (DFAA) and glucose as well as the percentage of carbon subsequently respired as CO_2 were determined. In addition, the concentrations of the amino acids in the water were determined using ion exchange chromatography. Other tests included measurements of primary productivity and of the effects of the other amino acids in the water upon the uptake of one amino acid.

There was considerable variation in the heterotrophic activity over time and distance probably caused by patchiness in distribution of plankton and dissolved compounds in the water. Although there is some competition between amino acids being taken up, the effect upon kinetics measurements is probably negligible. Tests made every 3 hr showed a coefficient of variability (CV) of the measured maximum velocity of uptake (V_{max}) of aspartic acid to be only 26%, and a similar CV was found for daily samples. In several instances the uptake of one amino acid was found to be competitively inhibited by the presence of another amino acid, but the concentrations necessary to inhibit were far above natural concentrations and such effects are probably unimportant in nature. Mutual inhibition was found between the similar amino acid pairs glutamic acid and aspartic acid, threonine and serine, glycine and alanine, and leucine and alanine.

Highest V_{max} values were found during the summer months and early fall and ranged from a high of 69.42 μg C/l·hr for alanine in August to less than 0.20 μg C/l·hr for most of the substrates tested in the colder months. The V_{max} values for glucose uptake (0.06 to 9.64 μg C/l·hr) indicate that this estuarine system is one of the most microbially-active environments tested.

The DFAA were present in the water at concentrations of from 10 to 30 μg C/l; over half of this was ornithine, glycine, and serine. The DFAA were only about 0.2% of the total dissolved organic carbon in the water. Further, seasonal variations of DFAA concentrations, generally paralleling those of primary productivity, suggested that the amino acids originated from algal excretion and the decay of algal cells. The orders of abundance and concentrations of individual amino acids were similar to those reported for other bodies of water.

When the natural concentration of a substrate is known the actual velocity of uptake (v_n) or flux for that substrate may be found. Flux rates were only 1%–10% of the V_{max} values in the coldest months; the highest values were found in the warmest months.

At each experimental concentration of amino acid, a certain amount was taken up, and a percentage of this amount was oxidized to carbon dioxide. This percentage was constant for a particular amino acid in spite of varying experimental times, substrate concentrations, and temperatures. Leucine had the lowest percent respired (13%) while aspartic and glutamic acids had the highest (50%). Failure to correct uptake data for this respiratory loss introduces significant underestimation. The production of particulate material was calculated by correcting total uptake figures for each amino acid by its characteristic respiration percentage. Over 60% of the particulate production from amino acids was by uptake of alanine, leucine, valine, serine, glycine, aspartic acid, and glutamic acid. Such particulate production averaged 0.79 μg C/l·hr for the year and ranged from 0.06 to 2.37 μg C/l·hr; this is about 10% of the rate of production by algae during the summer months. This amount of particulate organic material is a significant contribution to this estuarine food chain.

Key words: Alanine; amino acids; bacteria; estuarine chemistry; glutamic acid; leucine; production, estuarine; serine; threonine.

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INTRODUCTION

Bacteria were first observed in sea water during the 17th century by Antony Van Leeuwenhoek (1676). Two centuries later the principles of pure culture and colony counts were developed and applied to the examination of aquatic microbes (Stanier et al. 1963). These same methods are still being used to enumerate bacteria and characterize the physiological potentials of the microbial populations of natural water systems. As aquatic biology developed investigators began to compare the bacterial populations of different lakes, of different parts of the same lake, and the variations caused by seasonal changes (Fred et al. 1924, Bere 1933). Such older plate-count methods are still useful for revealing the presence and numbers of bacteria and suggesting their roles and importance in cycling nutrients, but can give no data on the rates of their activities. More recently, rates of activities of the bacteria have been studied using measurements of oxygen consumption and the uptake or respiration of radioactively labeled substrates.

We have attempted in this study to quantify more precisely the importance of the aquatic bacteria in carbon cycling by measuring the actual rates of uptake of organic compounds. Both the rates of uptake of dissolved free amino acids (DFAA) and glucose, as well as the percentage of the carbon subsequently respired as CO_2 were determined. Monthly measurements were compared with primary productivity data in order to estimate the importance of the bacteria as producers of particulate organic material.

Methods of enumeration of aquatic bacteria have improved in recent years but still have large sources of error. For example, early workers employed plate counts (Fred et al. 1924) having the disadvantage of selecting for those bacteria that grow well on the media being used; this can cause an underestimation of up to four orders of magnitude (Jannasch and Jones 1959). Although there is still great underestimation, recent techniques of counting microcolonies grown upon membrane filters with less selective media have increased the percentage of bacteria counted. Plate-count techniques are also valuable in testing for the presence of bacteria having certain physiological potentials. For example, by properly formulating the selective media one may isolate and count the bacteria involved in the process being examined (Jannasch 1969). More recently, increasingly accurate counts have been made by direct microscopic observation where the bacteria are concentrated, usually on a membrane filter, and stained to aid in visualization. In addition, special microscopic techniques including fluorescent stains and ultraviolet and epi-illumination may be used (Francisco et al., *in press*, Rodina 1967). More bacteria are counted using these methods than in plate counting; however there are still serious difficulties (Jannasch and Jones 1959). One is that the bacteria exist in the water in a number of forms such as clumps, pairs, filaments—and are also associated with detritus particles (Rodina 1967). Often it is difficult to distinguish between bacteria and particles of detritus (Jannasch 1969). Such limitations seriously affect the use of such methods in determining absolute numbers of bacteria.

In addition to gaining an idea of the numbers of bacteria in aquatic systems we must find out the progressive rates of metabolic processes and the factors that control them before their ecology can be understood. Recently, various measures of the rates of physiological processes have begun to enlarge our knowledge of aquatic bacteria. For example, measures of oxygen consumption by the microplankton have given estimates of metabolic activity (Pomeroy and Johannes 1968).

Proving sensitive, radioactively labeled substrates were used widely in the study of the metabolic activities of microbes. Parsons and Strickland (1962) used ^{14}C-labeled glucose and acetate to measure the activity of marine bacteria and interpreted their data as representing the uptake of the substrate by bacterial transport systems. However, at their added substrate concentrations (0.25 mg C/l) the substrates may have been taken up by the algae as well as by the bacteria through active transport systems. To avoid this algal interference, Hobbie and Wright (1965, Wright and Hobbie 1965, 1966) developed modifications of this method using lower substrate concentrations. By applying Michaelis-Menten enzyme-kinetics equations to these transport data one can calculate the maximum velocity of uptake (V_{max}), turnover time (T_t)—the time required for the population to remove all of the substrate from the water, and a value ($K_t + S_n$)—the sum of a constant representing the affinity of the microbial population for the substrate (K_t) plus the natural substrate concentration (S_n).

It was thought that ^{14}C lost through respiration of CO_2 would be negligible (Wright and Hobbie 1966); later work, however (Hobbie et al. 1968), suggested that this was not so. Indeed, bacteria in the aquatic ecosystems tested (Hobbie and Crawford 1969*a*, *b*) respired up to 60% of the total carbon uptake from certain substrates. As a result of these findings, techniques were developed whereby the respired CO_2 could be recovered. The techniques of Hobbie and Crawford (1969*a*, *b*) have been used in this study because in combination with analyses for substrate concentration they enable one to find the V_{max}, T_t, the actual flux of a substrate through the bacteria (v_n), and the amount of respiration of a given compound.

Recently, sophisticated means have allowed quite accurate measurement of individual amino acids in

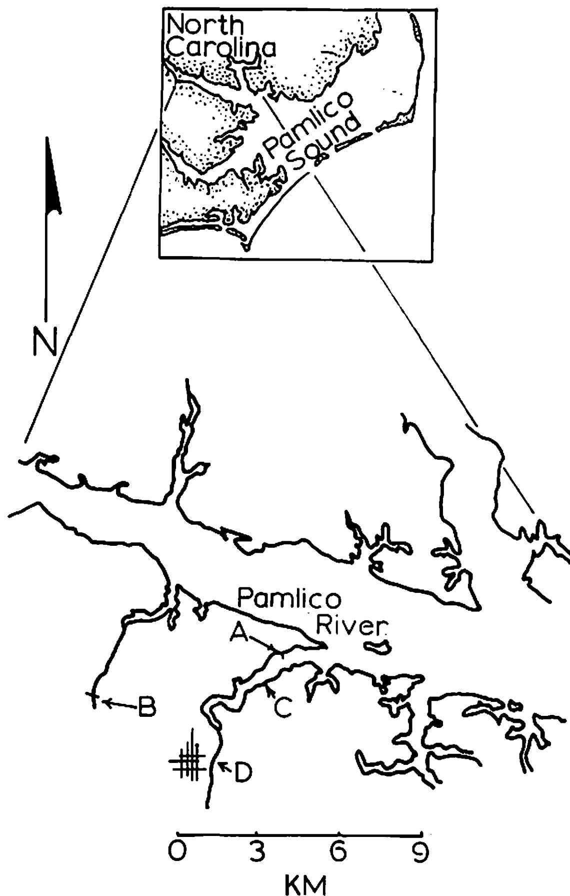

FIG. 1. The location of the sampling station in South Creek, a branch of the Pamlico River-Pamlico Sound estuary ecosystem. A—Pamlico Marine Laboratory pier; B—current meter on Durham Creek near Edward, N. C.; C—South Creek; D—Aurora, N. C.

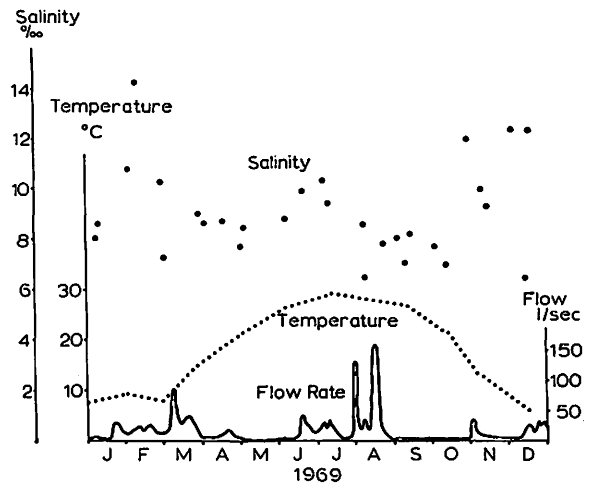

FIG. 2. Salinity and temperature at the sampling station and stream flow at nearby Durham Creek 1969.

natural waters. In the sea the amino acids have been measured by Siegel and Degens using ion-exchange column chromatography (1966), by Riley and Segar using thin layer chromatography (1970), by Litchfield and Prescott (1970) using dansyl-amino acids and thin-layer chromatography, and by Pocklington (1972) using gas chromatography. The amino acids in lake water and sediments in Germany have been reported in detail by Brehm (1967) and those in estuaries by Webb and Wood (1967) and Hobbie et al. (1968), all using ion-exchange chromatography. In most cases average values for total amino acid concentration were 5–15 μg C/l, and the orders of abundance were similar, glycine and serine generally in highest concentration.

Dissolved amino acids may enter a body of water in any of several ways including excretion by and decomposition of the phyto- and zooplankton. Various organisms living in the water have been observed to release dissolved metabolites. For example, Stewart (1963) found that two blue-green algae liberated dissolved amino acids. Up to 12.5% of the total carbon assimilated in pure cultures of Chlorophyceae appeared in soluble form in the external medium, and *Anabaena cylindricum* liberated up to 50% of the total carbon fixed as soluble metabolites, mostly polypeptides (Fogg 1953). Hellebust (1965) reported that in 22 species of unicellular marine algae, representing all groups, 3% to 6% of their assimilated carbon was released. A maximum of 38% was released by diatoms at the end of a bloom, a considerable portion of this carbon being in amino acids and peptides. Algal cells also contained pools of free amino acids that were released into the water upon the death of the cells (Fowden 1962). Planktonic crustaceans and other marine and fresh water invertebrates take up dissolved amino acids and polypeptides but some have a net loss of these compounds to the water (Johannes and Webb 1970).

In this study we have examined the rates of production of particulate organic material both by bacteria acting upon the dissolved amino acids and by photosynthetic algae. Jørgensen (1966) thought that the dissolved organic compounds played no significant part in the nutrition of higher organisms, but that the particulate organic material formed by the heterotrophic activity of bacteria may be important.

The area studied was South Creek near its junction with the Pamlico River near Aurora, North Carolina (Fig. 1). This estuary (Copeland and Horton 1970, Tenore 1972) is an oligohaline system in which the salinity ranges from 0–10 ppt and the temperatures from 2°–32° C (Fig. 2). The water is shallow, averaging 1.5 m with deep areas to 5 m, turbid (10–30 ppm of suspended material), and a moderately brown. Flow through the system is quite slow with

TABLE 1. Concentrations of added amino acids and glucose used in this study, computed for 0.01 μci/5-ml sample—for 0.02, 0.03, 0.04 μci/5-ml sample multiply by 2, 3, 4, respectively

Amino acid	Concentrations (μg C/l)
Glu	15.99
Asp	15.78
Lys	23.66
Met	3.39
Ser	9.84
Phe	31.11
Ala	3.56
Leu	21.67
Ile	16.56
Pro	14.67
Val	17.39
Tyr	39.28
Thr	17.89
Arg	33.66
Gly	0.70
Glucose	2.60

an exchange ratio of 0.073 in the river near the sampling station and a tide averaging less than 20 cm. There is considerable water movement within the estuary that tends to hold particulate material in suspension. Most of the organic fraction of this particulate material is probably produced from *Ruppia maritima* and *Potamogeton* sp. (rooted aquatic plants) found in dense stands in the shallow areas of South Creek. Some pollution enters the creek in the form of effluent from a sewage lagoon serving Aurora, North Carolina (population approximately 1,000), and some waste products from a nearby phosphate mining operation. The euphotic zone is generally limited to the upper 2 m or less of the water column, and primary productivity averages between 40 and 80 g C/m^2·year. There are two main periods of high production, one around February caused by a bloom of *Peridinium triquetrum*, and another in the autumn from a bloom of other dinoflagellates. In this estuary nitrogen is the limiting factor for phytoplankton production as phosphate and other nutrients are abundant (Harrison, *in press*).

MATERIAL AND METHODS

Monthly surface samples were collected in South Creek from the Pamlico Marine Laboratory pier 65 m from shore where water depth is about 1 m. Tests were run as soon after collection as possible; no sample was held more than 30 min before testing. All samples were collected in a clean plastic bucket rinsed with a strong HCl solution.

To measure bacterial heterotrophic activity (Hobbie and Crawford 1969*a*, *b*) different amounts of a ^{14}C-labeled substrate are added to each of a series of subsamples (Table 1). The various concentrations are determined by the specific activities of the labeled amino acids (bought in 1967, Nuclear Chicago Corp., now Amersham-Searle). The substrates were diluted so that at least 0.01 μci could be added in order to obtain sufficient uptake for counting. After incubation (with shaking, in the dark, at the in situ temperature of the sample) the plankton and respired CO_2 were collected and their ^{14}C counted by liquid scintillation. That this uptake is probably by the bacteria rather than the other members of the planktonic community was established by Hobbie and Wright (1965). Usually only 5% or less of the added substrate is taken up.

The procedure (Smith 1967) uses sealed vessels for the recovery of the respired CO_2. A 5-ml sample of estuary water was placed in a 25-ml erlenmeyer flask, and the substrate added with a micropipette. The flask was immediately sealed with a rubber serum stopper that has a plastic cup suspended from it (Kontes Co., Vineland, N. J., No. K 882320) containing a 25-mm × 51-mm piece of accordion-folded chromatography paper (Whatman No. 1). Although uptake was found to be quite linear over time (Hobbie and Crawford 1969*a*), incubation periods (2–6 hr depending upon temperature) were kept to the minimum necessary to give adequate uptake of ^{14}C to count efficiently. After incubation, 0.2 ml of a 2N solution of H_2SO_4 was injected into the water through the serum stopper. This both stopped biological activity and lowered the pH sufficiently to drive off the dissolved CO_2. Next, still working through the serum stopper, we added 0.2 ml of phenethylamine slowly to the folded paper and returned the flask to the shaker for about 50 min at room temperature. The paper was then immediately placed in 15 ml of a toluene-based scintillation counting mixture. The plankton was filtered onto Millipore filters (0.45 μ), rinsed with 10 ml of distilled water, air dried, and counted (a minimum of 1,600 counts) in the same type of counting mixture with a liquid scintillation counter. Wolfe and Shelske (1967) found that dried Millipore filters became clear in a toluene-based mixture and could be efficiently counted. For each test of each amino acid four substrate concentrations in duplicate were used as well as one control flask. The control consisted of natural water in which the plankton had been killed by the addition of 0.2 ml of 2N H_2SO_4 before addition of the labeled substrate at the highest concentration. In all, nine flasks were used for each test.

Calculations of uptake kinetics were similar to those described by Wright and Hobbie (1966) with a slight alteration to correct for respiration. The plankton counts and respiration counts for each sample flask were corrected for counting efficiency, background, and control and added together; this sum was then c in the expression $C_{\mu}t/c$ where C is the counts from one μci of ^{14}C in the counting equipment used, μ is the fraction of a μci used, and t is the incubation period in hours. The control counts, when

significant, were first corrected for background and then divided into fourths. The counts from the sample incubated at the lowest added substrate level were reduced by one-fourth of the control, the counts of the second-lowest by two-fourths, the third by three-fourths, and the fourth or highest by four-fourths. These control counts, or the uptake by acid-killed samples, were always quite low and showed little physical uptake by particulate material in the sample and little retention of the DFAA by the membrane filter. Extensive tests have shown that this single control method gives as good results as having four controls, one for each level of added substrate. The value $C_{\mu}t/c$ is graphed on the ordinate against A, the added substrate, on the abscissa and the resulting straight line computed by a regression analysis. In those instances where the relationship between substrate added and amount taken up could not be established at the 95% confidence level the data were discarded. There were 19 such failures with glycine accounting for 10. There appeared to be no relationship between the failures and other parameters of the system. The inverse of the slope of the line is V_{max}, the intercept on the abscissa is $-(K_t + S_n)$, while the ordinate intercept is T_t.

The analysis of amino acids was performed using techniques outlined by Webb and Wood (1967) consisting of desalting, lyophilizing, and analyzing on a Technicon Autoanalyzer. Samples were collected in a clean bucket, filtered through washed glassfiber filters and washed Millipore filters (0.45 μ), preserved with chloroform, and frozen in glass bottles. Bucket, bottles, and filtering glassware were all washed thoroughly in strong HCl solution and great care was taken to prevent contamination of these samples (even the amino acids in a finger print can cause error). The samples were shipped in dry ice and stored frozen until the actual analysis.

Primary production was measured in situ with the ^{14}C method. Water was put into two 125-ml glass stoppered pyrex bottles. One ml of a 2-μci/ml solution of bicarbonate-^{14}C solution (pH 9) was added, and after wrapping one bottle in aluminum foil the bottles were suspended at a depth of about 10 cm in the water. Incubation was for 6 hr (0900–1500), then the samples were formalin-killed, and a small subsample was filtered onto a Millipore filter and counted by liquid scintillation. Productivity was calculated as outlined by Strickland and Parsons (1968).

The inhibition or competition effects of other amino acids upon the uptake of one amino acid were also tested. One experiment screened many amino acids for inhibitory effects upon many others. Each labeled amino acid tested was added to twenty 5-ml samples at the highest concentration used in the kinetic experiments (Table 1). Of the samples 19 contained 100 μg amino acid/l. The 20th sample was the control, having no added unlabeled amino acid. The same experimental conditions and counting procedure were used as previously outlined. The uptake of the labeled amino acid in the presence of another unlabeled amino acid was expressed as a percentage of the uptake of the control. In addition, a more sensitive and more elaborate experiment involved testing heterotrophic uptake kinetics, as previously described, both with and without an added unlabeled amino acid. This allowed more precise appraisal of inhibition of uptake by noting the resulting changes in kinetic parameters.

Results and Discussion

The uptake of 15 amino acids was measured monthly from January through December 1969; however only 12 of these will be considered in detail. Methionine was found in measurable quantities only four times, and thus we have only a very sketchy picture of its seasonal appearance. Glycine and serine tests resulted in poor correlation with such frequency that they too were dropped for lack of data. Of the other substrates tested, all had occasional unacceptable correlation when the plotted line for $C_{\mu}t/c$ against A resulted in a negative slope or the computed correlation coefficient was far below that necessary to be significant at $P = 0.05$ (Steel and Torrie 1960).

While the uptake of 15 amino acids and glucose was measured each month along with concentrations of dissolved free amino acids, small groups of amino acids were tested at shorter periods for variability. In one diurnal study, on November 8, 1969, measurements were made every 3 hr for 24 hr. The V_{max} for aspartic acid varied from 0.32 to 0.66 μg C/l·hr (average = 0.50; coefficient of variability [CV] = 26%). There was no pattern of variation which could be correlated with the daily cycle of light and temperature (2.5°–5.0° C). Although the variations in uptake showed no direct correlation with the changes in salinity (10–13 ppt), these changes in salinity show the variability of the estuary caused by the varying inputs of both fresh and sea water with their differing microbial populations, as well as by the circulation, the influence of contact with the bottom, and the diluting effects of rainwater. The flow through the estuary is quite low (Copeland and Horton 1970) and there is a great deal of mixing caused by tidal fluctuation, wind action, and river and creek currents. The mixing results in many localized water cells having different temperatures, salinities, nutrient concentrations, and microplankton populations. In this estuary the phytoplankton biomass measurements have a CV of 25% for replicated samplings from one bucketful of water and also for samples taken every 2 hr for 24 hr (R. J. Miller, *pers. comm.*). In the same study, however, Miller found that for 3 of

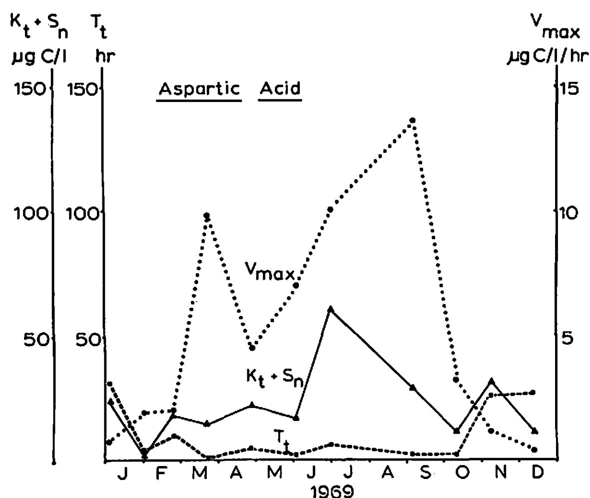

FIG. 3. The kinetic parameters of the uptake of aspartic acid; August data missing because of unacceptable correlation.

10 randomly selected groups of daily samples the CV was 50%, 103%, and 85%. In our study, tests were run at approximately the same hour each day for 7 days. Although for 6 of the 7 days levels of activity were very closely grouped (CV = 14%), on the 5th day activity dropped sharply to only 10%–30% of the previous levels (CV for all 7 days = 45%). This drop occurred at the same time as the low point in salinity for the week and is probably associated with changing microbial populations in the shifting water mass. This variability within the estuary is also illustrated by a series of V_{max} measures of glucose uptake at seven stations over a 27-km stretch (Hobbie and Crawford 1969*b*). During a July run, the values for glucose varied from 1.2 to 4 µg/l·hr whereas the November values ranged from 0.5 to 1.0.

The highest levels of activity occurred in the warm summer months. A representative annual cycle is that for aspartic acid (Fig. 3). Here the turnover time (T_t) was longer during the midwinter months than the rest of the year. In addition V_{max} was low during these winter months and increased during warmer weather. Peaks of activity, indicated by a larger increase in V_{max}, coincided with the peaks of production in the early spring (March) and later summer (August and September). The uptake of the other amino acids followed similar cycles (Table 2).

The amino acids could be divided into two quite distinct groups according to their high or low V_{max} values. The group with higher rates of activity included arginine, aspartic acid, tyrosine, alanine, and glutamic acid. When the uptake of serine and glycine was measured they were also found to be in this group of high activity. In the low group were proline, valine, lysine, threonine, methionine, leucine, and isoleucine. Phenylalanine was in the lower group in all cases except in August when it had a very large peak of 17.69 µg C/l·hr. This compares to its June value of 0.70 µg C/l·hr which was about average throughout the year. These groupings show no patterns related to structure, since both the high and low groups have all types of amino acids represented (aliphatic, acidic, basic, etc.) and all molecular sizes.

Based on the uptake of glucose the Pamlico River estuary was found to be one of the most microbially active bodies of water ever measured, as the V_{max} for glucose was 600–96,000 × 10^{-4} µg C/l·hr. Hobbie and Wright (1968) measured V_{max} values in Sweden of 3.2 × 10^{-4} µg C/l·hr in an oligotrophic lake in Lappland, and 24 to 500 × 10^{-4} µg C/l·hr in Lake Erken at different seasons. Also in Sweden, Allen (1969) found a V_{max} of 60,000 × 10^{-4} µg C/l·hr in the highly polluted Lötsjön. In addition, the oceans are areas of very low microbial activity. For example the western north Atlantic had V_{max} values of 2.0–12.0 × 10^{-4} µg C/l·hr (J. E. Hobbie, *unpubl. data*), the tropical Pacific 5.6–540 × 10^{-4} µg C/l·hr (Hamilton and Preslan 1970). The Antarctic Ocean, the western Mediterranean, and the eastern Atlantic had no detectable uptake when tested with the methods used in this study (C. C. Crawford, *unpubl. data*).

The uptake of amino acids was competitively inhibited by the presence of another amino acid of similar structure or of one entering the microbial cells via the same transport system, but this phenomenon probably has no importance in nature. The uptake of aspartic acid was inhibited somewhat by the similar amino acids glutamic acid and asparagine. Other pairs of mutually inhibiting amino acids are threonine and serine, glycine and alanine, lysine and arginine, and leucine and alanine. This pattern of inhibition is similar to that found by Hellebust (1970) in the diatom *Melosira nummuloides*, where there was one transport system for acidic, one for basic, and several for the neutral amino acids. In studies of mammalian cells Berlin (1970) found transport systems, each capable of carrying molecules of a group of sometimes unrelated amino acids into the cell, and two amino acids carried by the same system would compete for the carrier sites. He specifically excluded close comparison with bacterial systems since data are so sparse in the area. One remarkable finding of our inhibition studies was instances of increased or enhanced uptake of an amino acid upon the addition of another. In one case we found the uptake of aspartic acid was increased by alanine (246%), and the uptake of serine was increased by tyrosine (135%). This reaction has also been observed by experimenters with mammalian cells (Reiser and Christiansen 1969), and they offer no explanation.

TABLE 2. Concentration, kinetics, and respiration data for all amino acids and glucose tested at 1-mo intervals; V_{max}, μg C/l·hr; T_t, hr; $K_t + S_n$, μg C/l; S_n, μg C/l; v_n, μg C/l·hr—A = data missing due to unacceptable correlation of results, B = no substrate detectable, C = uptake not tested

		Jan. 5	Feb. 1	Feb. 27	Mar. 25	Apr. 29	June 3	July 7	Aug. 8	Sept. 9	Oct. 11	Nov. 8	Dec. 13
Ala	V_{max}	0.25	0.61	0.44	5.46	1.50	2.58	4.04	69.42	3.56	0.93	A	0.15
	T_t	71.25	5.30	17.22	2.25	6.78	2.91	3.41	3.84	1.36	2.29		43.42
	$K_t + S_n$	17.61	3.21	7.51	12.28	10.19	7.51	13.76	266.56	4.84	2.14		6.27
	S_n	1.26	1.44	0.82	1.01	1.13	0.61	0.54	0.67	1.85	0.67	2.21	0.63
	v_n	0.02	0.27	0.05	0.45	0.17	0.21	0.16	0.17	1.36	0.29		0.01
	% Resp.	47	42	36	43	40	41	35	34	37	40	35	38
Thr	V_{max}	0.20	0.32	0.46	1.43	0.68	A	A	2.55	2.16	0.69	A	0.09
	T_t	168.82	4.00	47.60	4.38	19.42			2.13	5.90	34.35		149.40
	$K_t + S_n$	33.88	1.29	21.87	6.28	13.15			5.42	12.74	23.67		13.94
	S_n	0.83	0.83	0.78	1.09	0.82	0.86	0.58	0.76	2.00	1.16	1.71	0.52
	v_n	0.0048	0.21	0.02	0.25	0.04			0.35	0.34	0.03		0.003
	% Resp.	25	28	21	34	30	35	22	34	33	34	21	20
Gly	V_{max}	A	A	A	A	A	A	1.51	3.40	A	A	A	A
	T_t							3.67	2.57				
	$K_t + S_n$							5.53	8.74				
	S_n	2.83	2.95	3.24	1.87	2.78	1.30	1.92	1.54	3.05	0.33	12.96	3.12
	v_n							0.52	0.60				
	% Resp.	43	35	36	50	40	34	27	36	42	42	34	36
Lys	V_{max}	0.21	0.39	0.23	1.22	0.55	A	1.95	A	2.00	A	0.16	0.09
	T_t	148.70	50.10	34.75	10.34	12.70		23.70		6.51		112.80	30.02
	$K_t + S_n$	31.65	19.76	8.05	12.59	7.01		46.39		13.18		18.02	2.63
	S_n	0.86	0.98	0.57	0.31	1.02	0.35	0.46	0.52	1.07	0.45	2.68	0.55
	v_n	0.01	0.02	0.01	0.03	0.08		0.02		0.16		0.02	0.02
	% Resp.	17	23	16	23	18	17	17	16	18	24	11	11
Met	V_{max}	0.17	0.26	0.06	0.33	0.20	0.22	0.56	4.07	0.60	0.15	0.04	0.18
	T_t	57.90	44.30	24.67	2.70	5.58	0.12	7.42	13.93	5.26	4.75	30.80	154.92
	$K_t + S_n$	25.87	11.61	1.49	0.90	1.10	0.03	4.13	56.74	3.14	0.71	1.19	28.25
	S_n	0.70	B	0.10	B	B	B	B	B	0.15	B	0.11	B
	v_n	0.0048		0.004						0.03		0.004	
	% Resp.	60	45	22	34	27	16	13	17	18	21	15	48
Ile	V_{max}	0.23	0.36	0.12	0.81	0.42	A	1.18	1.88	1.20	0.54	0.14	0.10
	T_t	206.65	95.00	82.51	4.01	6.87		9.34	5.25	14.02	51.81	123.80	215.40
	$K_t + S_n$	47.96	34.49	9.98	3.26	2.91		11.04	9.85	16.89	27.92	17.63	21.58
	S_n	0.41	0.54	0.44	0.40	0.56	0.35	0.30	0.23	0.84	0.25	0.79	0.26
	v_n	0.0022	0.0055	0.0055	0.10	0.08		0.03	0.04	0.06	0.0055	0.01	0.001
	% Resp.	25	14	15	21	18	10	13	12	21	15	12	21
Pro	V_{max}	0.09	0.36	A	1.41	0.52	1.00	1.12	2.18	1.31	0.44	A	0.07
	T_t	55.35	47.30		6.63	22.11	10.86	8.11	4.76	14.43	24.82		49.87
	$K_t + S_n$	5.08	17.04		9.38	11.43	10.83	9.06	10.39	18.91	10.88		3.53
	S_n	0.64	0.84	0.43	0.41	0.62	0.45	0.28	0.28	0.93	0.49	0.92	0.41
	v_n	0.01	0.02		0.06	0.03	0.04	0.04	0.06	0.06	0.02		0.01
	% Resp.	50	33	23	45	41	35	33	22	35	33	24	29
Val	V_{max}	0.21	0.30	A	1.21	0.72	0.85	1.54	A	1.87	0.55	0.14	0.09
	T_t	126.03	48.5		4.61	19.14	5.95	3.90		7.15	38.80	81.80	162.22
	$K_t + S_n$	26.18	14.68		5.58	13.85	5.08	6.02		13.35	21.49	11.29	16.04
	S_n	0.61	0.72	0.68	0.67	0.70	0.60	0.42	0.30	1.09	0.35	1.73	0.41
	v_n	0.0046	0.01		0.14	0.04	0.10	0.11		0.15	0.01	0.02	0.003
	% Resp.	23	23	10	29	24	22	18	15	27	26	10	25
Tyr	V_{max}	0.63	1.83	0.79	4.54	2.39	4.30	8.31	4.43	20.94	3.73	1.46	A
	T_t	86.60	69.00	81.94	26.28	32.19	18.62	18.61	8.29	19.14	28.69	120.80	
	$K_t + S_n$	54.96	126.46	65.06	119.39	76.87	80.16	154.65	36.72	400.84	106.88	175.89	
	S_n	0.71	0.83	0.70	0.55	1.16	0.60	0.45	0.47	1.39	0.53	1.34	0.48
	v_n	0.01	0.01	0.01	0.02	0.04	0.03	0.02	0.05	0.07	0.02	0.01	
	% Resp.	20	33	28	33	36	34	21	41	35	31	24	13

TABLE 2. (Continued)

		Jan. 5	Feb. 1	Feb. 27	Mar. 25	Apr. 29	June 3	July 7	Aug. 8	Sept. 9	Oct. 11	Nov. 8	Dec. 13
Ser	V_{max}	A	A	0.59	A	1.34	18.92	2.09	6.05	A	1.22	0.27	0.23
	T_t			20.16		8.96	2.57	0.73	2.75		5.38	36.00	58.43
	$K_t + S_n$			11.82		11.98	4.56	1.53	16.63		6.59	9.88	13.45
	S_n	2.85	2.66	2.16	1.61	2.36	1.84	1.41	1.55	6.08	2.06	6.49	1.39
	v_n			0.11		0.26	0.72	1.93	0.56		0.38	0.18	0.02
	% Resp.	37	44	36	46	42	40	29	36	40	44	35	35
Phe	V_{max}	0.14	0.27	1.12	0.96	A	0.70	1.68	17.69	1.59	0.97	A	A
	T_t	181.95	74.00	131.36	19.75		12.48	21.93	6.22	18.02	56.65		
	$K_t + S_n$	25.51	19.87	22.58	19.01		8.34	36.77	110.00	28.68	54.92		
	S_n	0.60	0.77	4.13	0.35	0.62	0.45	0.31	0.47	0.91	0.48	1.10	0.52
	v_n	0.0033	0.01	0.20	0.02		0.03	0.01	0.07	0.05	0.0066		
	% Resp.	25	18	12	25	30	21	20	16	34	26	21	4
Glu	V_{max}	0.42	1.64	1.04	7.75	2.55	3.11	4.07	A	A	1.65	0.70	0.25
	T_t	30.70	8.70	5.42	1.70	6.85	2.02	0.64			0.94	30.80	43.40
	$K_t + S_n$	13.00	14.27	5.64	13.18	17.44	6.28	2.60			1.55	21.66	10.80
	S_n	1.08	1.47	1.45	0.32	0.74	0.30	0.29	0.56	0.91	0.32	0.89	0.71
	v_n	0.04	0.17	0.26	0.19	0.36	0.15	0.45			0.34	0.03	0.02
	% Resp.	50	49	52	51	57	54	43	42	42	50	49	55
Asp	V_{max}	0.74	1.88	2.05	10.07	4.49	6.89	10.30	A	13.66	3.25	1.13	0.42
	T_t	31.25	1.70	9.25	1.49	4.76	2.58	5.81		1.92	3.61	26.00	27.05
	$K_t + S_n$	23.13	3.20	20.44	15.00	21.36	17.76	59.84		26.23	11.71	29.33	11.35
	S_n	1.01	1.00	0.49	0.56	0.93	1.09	0.45	1.20	2.51	0.93	1.96	0.62
	v_n	0.03	0.59	0.05	0.38	0.20	0.42	0.08		1.31	0.26	0.08	0.02
	% Resp.	52	55	52	53	54	45	49	46	50	53	48	53
Arg	V_{max}	C	C	0.61	3.19	0.97	2.12	A	A	5.81	1.78	A	0.24
	T_t			43.27	4.66	18.55	13.29			7.31	29.95		137.70
	$K_t + S_n$			26.51	147.36	18.05	28.11			42.46	53.44		33.53
	S_n	0.73	0.84	0.43	B	1.00	0.34	0.27	0.30	0.49	0.26	0.48	0.63
	v_n			0.01		0.05	0.02			0.07	0.01		0.005
	% Resp.			42	47	30	47	18	22	33	31	26	31
Leu	V_{max}	A	0.40	A	1.11	0.50	1.33	1.51	2.44	1.85	A	0.12	0.08
	T_t		58.50		3.49	2.00	17.81	6.87	3.80	9.56		79.00	80.05
	$K_t + S_n$		23.17		3.86	1.00	23.58	10.39	10.36	17.67		8.99	6.20
	S_n	0.55	0.65	0.69	0.51	0.72	0.45	0.36	0.24	1.04	0.37	1.00	0.37
	v_n		0.01		0.15	0.36	0.03	0.06	0.06	0.11		0.01	0.005
	% Resp.	11	37	9	21	17	24	10	7	11	15	3	7
Glucose	V_{max}	0.15	1.34	0.89	5.61	A	0.88	2.18	9.64	1.36	1.02	0.30	0.06
	T_t	5.67	2.10	5.93	2.30		1.21	1.23	2.07	0.24	5.98	8.00	22.38
	$K_t + S_n$	0.85	2.82	5.26	12.91		1.06	2.69	19.96	0.33	6.08	2.35	1.35
	% Resp.	9	11	12	12	17	14	8	15	14	14	13	12
Orn	S_n	2.40	2.20	1.50	2.32	2.42	9.33	1.01	1.05	4.30	1.59	5.63	1.03
His	S_n	0.52	0.56	0.28	0.65	0.46	0.15	0.46	0.29	0.77	0.29	1.04	0.45

Because of the high concentration of a competing amino acid necessary either to inhibit or enhance uptake of an amino acid under study, it is unlikely that this inhibition or enhancement affects uptake at natural substrate concentrations.

In another study, added amino acids caused changes in uptake kinetics that were typical of competitive inhibition. Three uptake experiments using labeled aspartic acid were run on estuary water in 1967 with either unlabeled glutamic acid, lysine, or aspartic acid added at a concentration of 1 mg amino acid/l. A fourth experiment, the control, had no competing amino acid added. Addition of lysine had virtually no effect upon the uptake of the labeled aspartic acid, thus indicating no competition for uptake sites (Fig. 4). As would be expected, addition of unlabeled aspartic acid caused strong competitive inhibition of the uptake of the labeled aspartic acid. Here, T_t and $K_t + S_n$ were each increased by a factor of approximately 5, but the maximum velocity was unchanged.

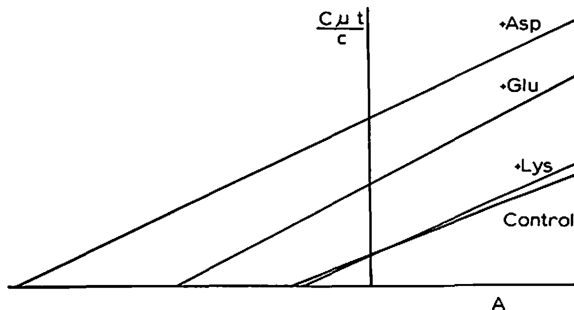

FIG. 4. Uptake of labeled aspartic acid alone and in the presence of unlabeled amino acids added at a concentration of 1 mg/l.

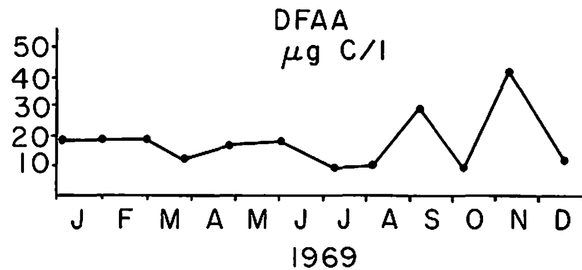

FIG. 5. Total concentration of 12 DFAA for 1969.

Addition of unlabeled glutamic acid also showed competitive inhibition with the T_t and $K_t + S_n$ values both increasing by about 3 times. Again maximum velocity did not increase. The added aspartic acid could be thought of as a "perfect" inhibitor since it was exactly the same as the labeled aspartic acid being tested. Glutamic acid, however, was a different substrate, and the uptake systems of the microorganisms were competitively inhibited by it with 1 mg/l of glutamic acid having the same effect as 0.1 mg/l of aspartic acid. These levels of inhibitor necessary to interfere with uptake measurements are so far above naturally occurring concentrations that no such competitive inhibition is thought to occur in kinetic measurements under natural conditions.

The DFAA pool was dominated by ornithine, serine, and glycine, with aspartic acid, lysine, alanine, valine, tyrosine, and threonine at intermediate levels, and the remainder at low levels (Table 2). Ornithine varied from 9.30 µg C/l, an extremely high value, to a low of 1.01 µg C/l. Midrange concentrations of individual DFAA were about 0.50 µg C/l or lower, the lowest values are represented by methionine which was frequently undetectable or present only as a trace. The lower limit of detectability for all amino acids was about 0.05 µg C/l. The very high concentrations of DFAA in November (Fig. 5) are not correlated with peaks of productivity or any other parameters. This sample may have been contaminated in some way. Lower S_n values for this month would have resulted in lower v_n and flux figures, but no attempt was made here to correct the S_n values since there are no previous data upon which to base such speculation. These concentrations and orders of abundance are generally in agreement with those found in the York River estuary (Webb and Wood 1967, Hobbie et al. 1968), Buzzard's Bay (Siegel and Degens 1966), the Irish Sea (Riley and Segar 1970), and certain lakes of Germany (Brehm 1967).

The orders of abundance of the DFAA in the water are similar to those of the amino acids in various algae and their excretion products and suggest that the DFAA in the water may largely originate from the phytoplankton. For example, Brehm (1967) analyzed various possible amino acid sources in lakes and found that dissolved organic matter excreted by algae in culture had an amino acid composition most like that of lake and estuarine water. The orders of abundance are also similar to those found in the amino acid composition of some marine diatoms (Cheucas and Riley 1969). In a study of another estuarine system, Hall et al. (1970) found that the amino acid composition of the suspended solids resembled somewhat the composition of the dissolved material found in the Pamlico River estuary, being dominated by glycine, aspartic acid, and leucine. They also found that dried *Spartina alterniflora* contained mostly lysine, leucine, and phenylalanine, completely different from the amino acid distribution in the water. Further, Webb and Johannes (1967) and Johannes and Webb (1970) found that various marine and fresh water invertebrates excreted DFAA into the water, their orders of abundance similar to those found in estuarine water. These similarities in amino acid composition suggest that the DFAA found in the estuary may originate largely from excretion and decomposition products of the phyto- and zooplankton. Degens (1970) believes that the high levels of ornithine found in seawater may be the result of the action of arginase, an enzyme found in some phytoplankton that cleaves arginine into urea and ornithine. On the other hand, ornithine, a metabolic intermediate not commonly found in protein structure, could accumulate because the bacteria fail to remove it from the water in significant quantities rather than because it enters the water in any large quantities. The incidental release of only small quantities of ornithine then would be sufficient to maintain a high concentration in a situation where it is not being removed at significant rates.

Actual v_n and K_t may be obtained from the parameters of uptake kinetics if one has the actual concentration of S_n (Fig. 6). Since the S_n values are such a small fraction of the $K_t + S_n$ obtained from the original kinetic graphs, a graphic representation of K_t alone is virtually identical to the graph for $K_t + S_n$.

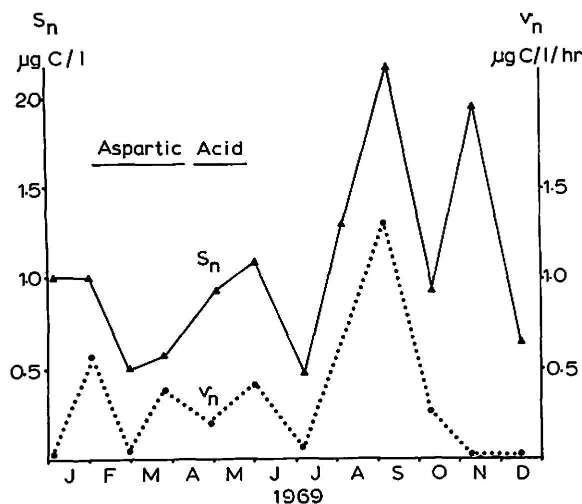

FIG. 6. S_n and v_n of aspartic acid for 1969. August v_n data missing because of unacceptable correlation.

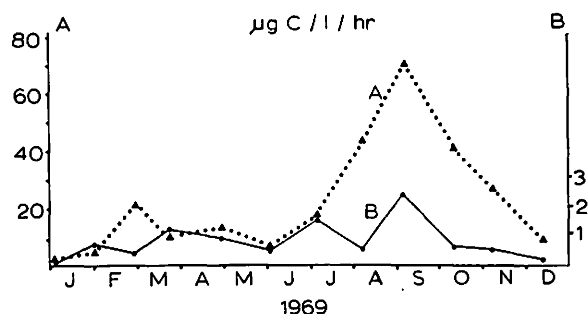

FIG. 7. Primary production (A) and bacterial production (B) for 1969. μg C/l·hr.

Those amino acids having the highest V_{max} (tyrosine, arginine) have among the lowest actual velocities of uptake and usually show a relatively small S_n and a large K_t. Four amino acids, aspartic acid, glutamic acid, alanine, and threonine, generally showed the highest actual velocities of uptake and were usually responsible for over half of the total uptake. The actual v_n represented small fractions of the maximum velocities, 1%–15% usually, and varied widely both from substrate to substrate and from month to month for the same substrate. For this reason V_{max} should not be used as an estimate of actual velocities of uptake by bacteria.

The net uptake, or the rate of production of particulate organic material attributable to the action of the microbes upon a given substrate, depends not only on velocity of uptake but also on the rate of loss of carbon through respiration; this may be as high as 50% for some substrates. Previous workers (Wright and Hobbie 1966, Vaccaro and Jannasch 1967, and others) failed to measure this loss of $^{14}CO_2$ due to respiration and therefore their estimates of total uptake in both fresh and seawater are conservative.

When the amino acid respired was expressed as a percentage of that taken up, the percentage was found to be relatively constant for a given substrate regardless of temperature, incubation period, or substrate concentration. For example, for aspartic acid tested on 29 April 1969 the respiration, measured in duplicate at four concentrations, ranged from 52% to 55% with a mean of 53.75% (SD = 1.16). Percentages respired of the total carbon taken up ranged from 13% (leucine) to 50% (glutamic acid and aspartic acid); the majority lay in the range from 20% to 50% (Table 2). These respiration rates are in general agreement with the 22% averge reported by Williams (1970) for oceanic bacteria acting upon an amino acid mixture. Probably the position of a given amino acid in relation to the energy pathways in the metabolic cycle determines how much of the carbon will be respired. Aspartic acid and glutamic acid readily enter the citric acid cycle via rather direct transformations into oxaloacetate and α-ketoglutarate, respectively. These short catabolic paths cause them to be readily respired as evidenced by the 50%-respiration values. Other amino acids show lower rates of respiration as the number of steps necessary for them to enter the citric acid cycle increases. Low values found for leucine (13%), lysine (18%), isoleucine (18%), and valine (14%) reflect their more remote relationship to the citric acid cycle (Mahler and Cordes 1966). Although glutamic acid and aspartic acid are both taken up very rapidly, their rates of respiration cause a large loss of carbon and as a result their rates of production of particulate organic material are not as high as others. Leucine and valine are in the low ranges of rate of uptake, but because of their low respiratory loss they have among the highest rates of incorporation into organic matter. About 60%–70% of the particulate organic material produced from dissolved amino acids could be attributed to alanine, leucine, valine, serine, glycine, aspartic acid, and glutamic acid. Particulate production averaged 0.79 μg C/l·hr and ranged from 0.06 μg C/l·hr (December) to 2.37 μg C/l·hr (September) (Fig. 7).

The primary productivity measured in 1969 followed a cycle (Fig. 7) similar to that reported for 1966–67 (Peters 1968), 1967 (J. E. Hobbie, *pers. comm.*), and 1970 (H. L. Davis, *pers. comm.*) with a peak of 20.3 μg C/l·hr at the end of February and another higher one of 70.6 μg C/l·hr in September. The low value was on January 5 (1.1 μg C/l·hr) and the average for the 12 monthly samples was 21.5 μg C/l·hr. For the 5 mo July–November the average was 39.3 μg C/l·hr. This annual cycle of primary productivity is matched by the cycle of abundance of particulate carbon reported by Peters (1968) who

found peaks in early spring and autumn of 100–1,100 μg C/l and low points in midwinter and summer of about 600 μg C/l. There appears to be a positive relationship between primary productivity (Fig. 7) and total amino acid concentration (Fig. 5). Increases in dissolved amino acids found during the dinoflagellate blooms could be due, at least in part, to excretion by the algae. Although the annual changes in dissolved organic carbon are not known in detail, the range is from 6–12 mg C/l. Thus the DFAA carbon is only about 0.2% of the total dissolved organic carbon.

By comparing the rates of algal and bacterial production one can easily see that the bacteria are an important source of particulate organic material (Fig. 7). It is remarkable that the production of particulate organic material by the bacteria is equal to about 10% of the production by algae during the summer months, especially since this bacterial activity was measured for only 12 amino acids.

Possibly two of the amino acids not included in the total production, glycine and serine, also have very high rates of removal. Because they also were very abundant, their uptake would greatly increase the total production of particulate materials. The reasoning is that our analysis rejected all experiments that did not show typical bacterial uptake—that is, uptake by a population of microorganisms with a low K_t. Though some algae possess transport systems for various organic compounds (e.g., Hellebust 1970), almost all of these systems have a relatively high K_t. Bennett and Hobbie (1972) pointed out that uptake by a high K_t system could not be analyzed if a concentration range similar to that given in Table 1 were used. If algae were also taking up significant quantities of the labeled substrate, their uptake would obscure that of the bacteria. Yet, this algal uptake may be an artifact of the high concentration of label used in the experiments (A), and the uptake at the natural substrate level (S_n) may be insignificant. As noted earlier, the experimental concentrations we used were dictated by the specific activity available from the manufacturer, and so with many of the amino acids we could not have the concentration of A close to that of S_n. The end result is that the bacterial uptake actually present is masked by algal uptake (or other high K_t systems) that may or may not be important at S_n. Thus, the failure of our analyses for bacterial uptake does not mean that glycine and serine are not being used at a high rate by bacteria, algae, or both.

Conclusions

The bacteria in the Pamlico River estuary were found to take up DFAA in much the same way that they have been found to take up glucose, acetate, and other organic compounds studied in this estuary and elsewhere. By this use of DFAA the bacteria contribute to the production of particulate organic material in the estuary. In addition a significant portion of the carbon taken up in the form of amino acids is subsequently released as CO_2; failure to measure this respiratory loss will result in underestimation of total uptake by the bacteria.

Over half of the DFAA, present in the water at concentrations of 10–30 μg C/l, was ornithine, glycine, and serine. Seasonal variations of DFAA concentration, generally following those of primary production, suggested that the amino acids originated from algal excretion products and the decay of algal cells.

In the eutrophic system studied the bacterial population was active in the uptake of DFAA at all times of the year, whereas this is not so in more oligotrophic systems such as the sea (Vaccaro 1969). In the sea the bacteria seem to exist in an inactive state so that they are not immediately able to take up dissolved organic compounds in measurable quantities. Vaccaro found that after exposure to a given substrate for a time, bacteria developed the ability to use that substrate. Possibly the bacteria are active at all times in the rich environment of the estuary because of a continuously high rate of supply of organic substrates.

The actual velocity of uptake (v_n) of a given amino acid was found to be a small fraction of the calculated maximum velocity (V_{max}). For this reason, it was obvious that V_{max} cannot be used as an estimate of the v_n. However, V_{max} is still useful as a comparative parameter. By comparing the V_{max} for glucose uptake in the Pamlico River estuary with the V_{max} values for a number of other aquatic environments we found that this ecosystem ranks high in the levels of activity, its values comparable to those found in quite polluted lakes. Since measurements of primary productivity alone indicate a rather low level of planktonic activity in this estuary, possibly measurements of heterotrophic activity give a more accurate picture of the overall trophic activity than measurements of primary productivity.

Methods are needed for measuring bacterial activity in situations of very low-nutrient concentration such as the sea or very oligotrophic lakes. First steps in this direction have included concentration of the plankton (Pomeroy and Johannes 1968) and induction of activity in dormant microbial populations by exposure to high concentrations of substrate prior to testing (Vaccaro 1969), but it is difficult to interpret the result of such tests.

Actual velocities of the uptake of organic substrates can be determined, when the concentration of the substrate in the natural waters is known. For amino acids such concentration can be accurately determined, but most other simple organic com-

pounds cannot be specifically measured. Further development of analytical methods to measure the organic substrate in natural water systems is needed to broaden measurements of flux in these systems.

This study was carried out in a rich estuarine ecosystem where the primary source of particulate material is probably the shallow water and surrounding land; it would be interesting to study the importance of the bacteria as a source of particulate organic material in a closed, plankton-based ecosystem such as a relatively unpolluted lake.

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