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Bacterial 5′-nucleotidase activity in estuarine and coastal marine waters: Characterization of enzyme activity

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Abstract

We measured 5′-nucleotidase by following the hydrolysis of ATP and measured alkaline phosphatase by conventional fluorometric techniques. 5′-Nucleotidase (5PN) was found in all estuarine and coastal marine waters where measurements were made, regardless of the degree of eutrophication or phosphate concentrations. Its activity was usually concentrated in the bacterial size fraction. 5PN was competitively inhibited only by 5′-nucleotides and was distinguishable from alkaline phosphatase by both this substrate specificity and the lack of inhibition by low concentrations of orthophosphate. The percent of uptake of orthophosphate regenerated by 5PN was inversely related to the ambient phosphate concentration.

Over 99% of the total nitrogen (N) and phosphate (P) consumed each year by oceanic primary producers is supplied by regeneration (Harrison 1980), and 80% is provided by in situ regeneration in the upper 200 m. External sources such as riverflow and rainfall supply <1%. P metabolism is particularly dependent on rapid regeneration of orthophosphate (P_i), because there is no biological P input process analogous to N fixation (Froelich 1984). Finally, since nucleotides and most other phosphorylated compounds do not cross cytoplasmic membranes (Lugtenberg 1987), P regeneration is largely an extracellular process, which is often catalyzed by cell-surface enzymes.

In this paper, the term soluble reactive phosphate (SRP) will be used for inorganic P concentrations measured in natural waters, while P_i will be used for P additions and other situations where the chemical species is clearly orthophosphate. P regeneration is defined as hydrolysis of P_i from organic or other complex P compounds, soluble or particulate, in which the hydrolyzed P_i is released to the outside of the cell surface. This P_i could be immediately taken up by the same cell or mix with the dissolved P_i pool. This definition is comparable to that of Harrison (1983) but differs from that of Andersen et al. (1986), which includes release of dissolved organic P (DOP) as P regeneration. P regeneration will be written as P_i regeneration throughout the rest of this paper to make this distinction clear.

The role of the picoplankton (organisms <2.0 μm in size, dominated by bacteria) is particularly obscure in relation to P_i regeneration. Recent studies, however, have demonstrated the significant activity of bacterial cell-surface phosphatases in the field. These studies have suggested that bacterial alkaline phosphatase activity (AP; Chróst 1988; Chróst and Overbeck 1987), and bacterial 5′-nucleotidase activity (5PN; Ammerman 1991; Ammerman and Azam 1985; Tamminen 1989) may be significant sources of regenerated P_i. Other studies, however, suggest that bacteria sequester P, new or regenerated, and thus compete with algae (Vadstein et al. 1988). They contend that this sequestered P is made available to phy-

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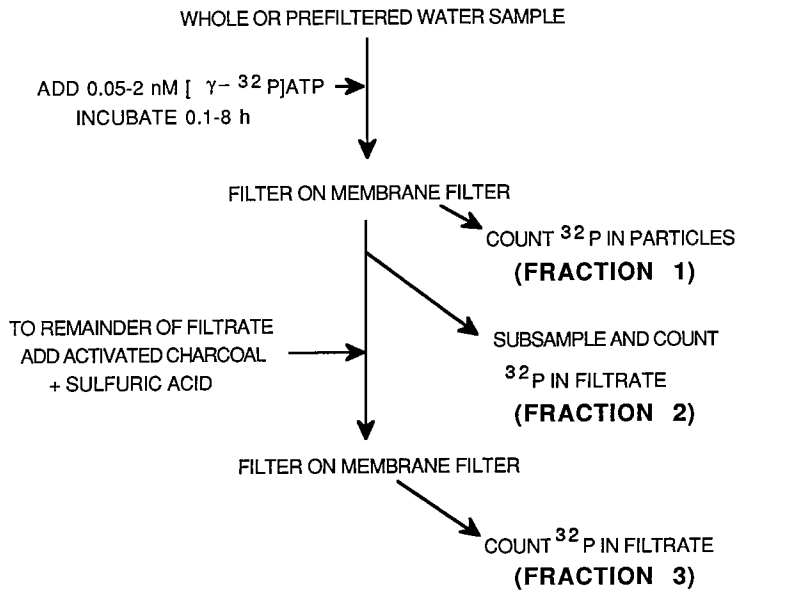

A. 5PN ACTIVITY (%/h) = (FRACTIONS 1+3/FRACTIONS 1+2) x 1/h

B. UPTAKE OF P_i RELEASED BY 5PN (%/h) = (FRACTION 1/FRACTIONS 1 + 2) x 1/h

C. % UPTAKE OF P_i RELEASED BY 5PN = B/A x 100

Fig. 1. Flow chart of 5PN method.

toplankton only by grazing on bacteria by phagotrophic protozoans (Andersen et al. 1986; Gude 1985).

Two goals of this study were to characterize bacterial 5PN activity in aquatic ecosystems and distinguish it from AP activity. Another goal was to determine the relationship between coupled uptake of P_i regenerated by 5PN and the ambient SRP concentration. We measured 5PN by following the hydrolysis of [γ-^{32}P]ATP (Ammerman and Azam 1985) and AP by conventional fluorometric techniques. Our results show that 5PN was found in all estuarine and coastal marine waters where measurements were made.

Methods

Sampling—We collected surface water samples from the pier at Scripps Institution of Oceanography (32°53′N, 117°15′W) and on a cruise in the Southern California Bight (SCB). This cruise (RV *E. B. Scripps,* 29–31 August 1984) collected vertical profiles of water samples down to 200 m along the line of stations numbered 101 to 106 in figure 1.4 of Eppley (1986).

We also collected surface samples from the lower Hudson River near the New York–New Jersey border (Nyack and Piermont) and from the western end of Long Island Sound (Mamaroneck Harbor). (*See* figure 1 of Ducklow and Kirchman 1983.) Vertical profiles were sampled in the central part of Long Island Sound (41°01′N, 73°03′W) from the RV *Privateer* by S. G. Horrigan on 4 September and 7 November 1986.

Measurements of 5PN—The enzyme was assayed by the method outlined by Ammerman and Azam (1985) and diagramed in Fig. 1. The substrate used for this assay, [γ-^{32}P]ATP, came from the following companies at different times (Amersham, ICN Radiochemicals, or NEN Research Products). During the early part of this study we used only substrate that had a specific activity of <50 Ci mmol^{-1}, because of concern about chemical breakdown. The final concentration of added [γ-^{32}P]ATP was therefore usually 1 or 2 nM, which is in the range

of natural 5′-nucleotide concentrations (Ammerman and Azam 1991). Even at this relatively low specific activity, however, a fresh batch of substrate still had a $^{32}P_i$ background of ~5%, as measured by the radioactivity not bound by activated charcoal.

More recently we have routinely used substrate with a specific activity of ~700 Ci mmol^{-1} or higher without additional background problems. These high-specific-activity substrates have the advantage that they can be added at true tracer levels (50 pM final concn). We used this tracer approach, as opposed to a saturation assay, for both 5PN and AP assays (*see below*) so that true in situ turnover rates and P_i fluxes could be determined. The turnover rates determined by this approach reflect both the enzyme activity and the ambient substrate concentration. The $^{32}P_i$ background in [γ-^{32}P]ATP stock increased with time unless it was stored at or below −20°C.

Samples for measurements of 5PN activity were incubated near in situ temperatures in a covered water bath. The incubation time ranged from 30 min in eutrophic, nearshore New York waters to 8 h in offshore southern California waters. After incubation and filtration (Fig. 1), excess [γ-^{32}P]ATP was bound by adding ~20 mg of activated charcoal and 10% vol/vol 0.03 N H_2SO_4—half the concentration of acid that we used previously (Ammerman and Azam 1985). Here we used two different types of blanks, zero-time and samples that were boiled in a water bath for 10–15 min and cooled before assay. The latter are essential where activity is high and zero-time blanks would show significant activity. 5PN activity and coupled uptake of P_i released by 5PN were calculated after subtracting blanks. Most values shown were means of two replicates.

Frequent experiments were performed to determine kinetics, substrate specificity, or the effects of P_i. All such inhibitors or other unlabeled compounds were added at the concentration listed in the particular experiment and simultaneously with the addition of labeled substrate, unless stated otherwise. In this paper, inhibition refers to a rapid decrease in enzyme activity due to the addition of end products, unlabeled substrates, or similar molecules. Repression refers to a long-term decrease in enzyme synthesis, and induction is a long-term increase in synthesis. In some cases, both inhibition and repression occur simultaneously. For size-fractionation experiments, all fractions were prefiltered through Nuclepore filters; postfiltration cannot be used to measure enzyme activity. The <1.0- and <0.6-μm fractions were filtered by gravity alone, and the <0.4- and <0.2-μm (cell-free) fractions were filtered by gentle vacuum (<25 cm of Hg).

Other methods—Temperature was measured by laboratory thermometer, reversing thermometer, or expendable bathythermograph. SRP (determined by absorbance of the phosphomolybdate complex) and Chl *a* and pheopigments (determined by fluorescence) were measured according to Strickland and Parsons (1972). Bacterial numbers were determined by epifluorescence microscopy of samples stained with acridine orange according to Hobbie et al. (1977) and cyanobacteria by epifluorescence microscopy of orange-autofluorescing (phycoerythrin-containing) cells (Waterbury et al. 1979).

AP was initially measured by the method of Perry (1972) and later by that of Hoppe (1983). The major difference between these two fluorescence methods is the substrate: 3-*o*-methyfluorescein phosphate in the first technique and 4-methylumbelliferyl phosphate in the second. The same subsaturating concentration of substrate, 100 nM, was used in both. It was the lowest concentration that could reliably detect activity and approximated a tracer assay as used for 5PN. Incubations were performed in phosphate-free containers.

For the Hoppe (1983) method, we added 1 ml of 50 mM borate buffer (pH 10.8) to 3 ml of sample before measurement, giving a final pH >10. The fluorescent breakdown product of the AP reaction, 4-methylumbelliferone, is most fluorescent at pH values of 10 or higher (Chróst and Krambeck 1986). The two different AP methods have similar sensitivities; as little as 0.5 nmol liter^{-1} h^{-1} (0.5% h^{-1} with 100 nM substrate) of AP activity can be measured, but the Hoppe method is more reproducible in our hands. This apparent difference may be due to dif-

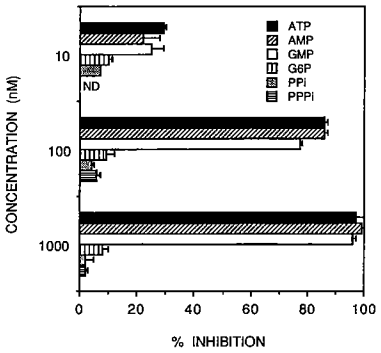

Fig. 2. Effect of six different inhibitors on 5PN activity in a sample from western Long Island Sound. Each inhibitor (except PPP_i) was tested at three different concentrations. Substrate was 75 pM [γ-^{32}P]ATP. Each value shown represents the mean ± 1 SD ($n = 2$). Abbreviations: ATP, 5′-adenosine triphosphate; AMP, 5′-adenosine monophosphate; GMP, 5′-guanosine monophosphate; G6P, glucose-6-phosphate; PP_i, pyrophosphate; PPP_i, tripolyphosphate. Control 5PN activity was 44% h^{-1}.

ferences in the substrates or the fluorometers or to the generally higher activity in New York waters.

All variables measured on the cruises were tested with the Kolmogorov-Smirnov one-sample test to see if they fit a normal distribution, and any variables that did not ($P < 0.05$) were transformed by the equation $x' = \log(1 + x)$ and retested. Variables that were normally distributed or could be successfully transformed were compared by correlation. A conservative significance level of $P < 0.001$ was used for these comparisons, most of which are presented in greater detail elsewhere (Ammerman and Azam 1991). Paired-sample t-tests were used to analyze the size-fractionation results.

Results

The apparent substrate specificity and therefore the identity of bacterial 5PN was established by the inhibition experiments of Ammerman and Azam (1985). Those experiments showed that only 5′-nucleotides were inhibitors of enzyme activity and therefore possible 5PN substrates. Those experiments, however, did not show that the 5′-nucleotides were competitive inhibitors—a necessary prerequisite for substrates (Segel 1976). Samples from the western end of Long Island Sound were planned to answer this question (Figs. 2 and 3). As previously found (Ammerman and Azam 1985), only 5′-nucleotides significantly inhibited hydrolysis of [γ-^{32}P]ATP by 5PN. Furthermore, inhibition by 5′-nucleotides increased as their concentrations increased.

We took one of the effective inhibitors, 5′-GMP, and added it at three different concentrations (plus a control with no additions) to a range of substrate ([γ-^{32}P]ATP) concentrations. GMP was chosen because it had a different purine base and a different number of phosphates than the substrate, ATP. The results have been plotted as a modified Hanes-Woolf linear transformation of the Michaelis-Menten equation, sometimes called a Wright-Hobbie plot (Wright and Burnison 1979), where the natural substrate concentration is unknown. From this plot we can calculate V_{max} (the reciprocal of the slope) and ($K_t + S_n$) (half-saturation constant plus the unknown natural substrate concentration, equal to the absolute value of the x-intercept, cf. Fig. 3). Note (Fig. 3) that V_{max} remains nearly constant while ($K_t + S_n$) increases. These results are characteristic of competitive inhibition (Segel 1976), showing that 5′-GMP is probably a substrate of 5PN.

In a previous study (Ammerman and Azam 1985), we showed that 5PN activity was generally uninhibited by $[P_i] < 100$ μM. In those experiments P_i was added simultaneously with the substrate, [γ-^{32}P]ATP. The question remained, however, whether smaller concentrations of P_i added before assay would repress enzyme synthesis. To answer this question, we added 20 μM P_i to water samples at 0, 1, 3, and 5 h in advance of the start of the 5PN assay. In two separate experiments there was virtually no inhibition of 5PN activity in any incubation period tested, though the coupled uptake of P_i released by 5PN was 86–89% inhibited due to isotope dilution. These experiments were done on samples collected from Scripps pier during the large red tide bloom described below.

Both 5PN and AP can hydrolyze [γ-^{32}P]ATP—the substrate we used for the 5PN assay. In contrast to 5PN, however, AP is inhibited by low P_i. Therefore, further experiments were done in New York waters

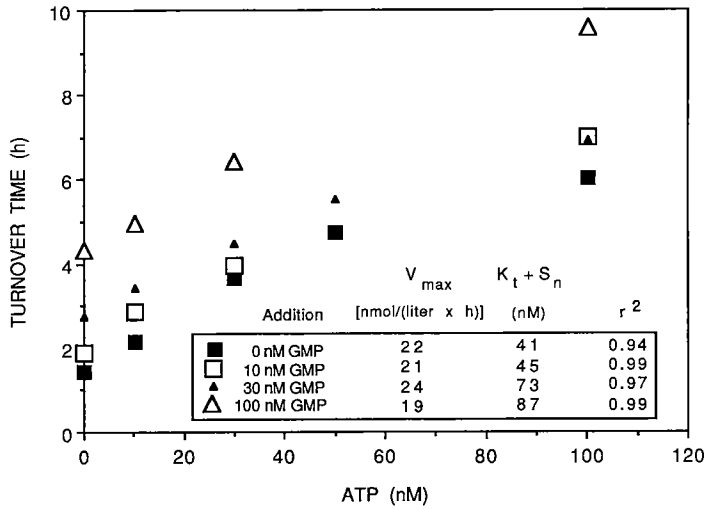

Fig. 3. Kinetics curves (Hanes-Woolf or Wright-Hobbie transformation, *see text*) of 5PN activity from a sample in western Long Island Sound. ATP was the substrate, and various concentrations of GMP were used as inhibitors. Kinetic parameters V_{max}, $(K_t + S_n)$, and r^2 are shown in the box. Values shown are means (n = 2). Abbreviations as in Fig. 2.

to distinguish 5PN from AP on the basis of P_i inhibition of enzyme activity. Though SRP concentrations ranged from 1.9 to 2.4 μM, low levels of AP activity (turnover rate $<5\%$ h^{-1}) were measurable (Fig. 4) in the lower Hudson River and western Long Island Sound during early fall 1987 (we had not previously made AP measurements in this area). In each experiment the activities of one or both enzymes were measured in the presence and absence of the indicated P_i concentrations. Incubation times were similar for both enzymes, generally 60–90 min, so that length of exposure to P_i was not a variable. AP activity was 75% inhibited by 0.1 mM P_i (perhaps a combination of inhibition and repression), whereas 5PN activity was only slightly affected at this concentration and inhibited by >50% only at 10 mM P_i.

We previously used size-fractionation experiments (gravity filtrates from 1.0-μm Nuclepore filters) to show that heterotrophic bacteria are responsible for most of the measurable 5PN activity in seawater (Ammerman and Azam 1985). There have been few reports of 5PN activity in eucaryotic algae (Flynn et al. 1986), but cyanobacteria could be an important source. To test this possibility we did detailed size-fractionation experiments with samples from Scripps pier using five different size fractions, including the total. These experiments fortuitously coincided with a large bloom of the red tide dinoflagellate, *Prorocentrum micans,* during which Chl *a* concentrations and bacterial numbers reached >10 times normal levels (46 μg Chl *a* $liter^{-1}$ and 12 × 10^9 bacteria $liter^{-1}$). This bloom

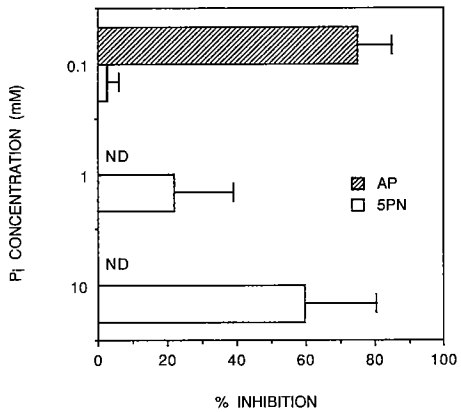

Fig. 4. Effect of various concentrations of added P_i on AP and 5PN activities in western Long Island Sound and the lower Hudson River. Values shown represent means ±1 SD of two–four combined experiments (n = 6–8). (ND—Not determined.)

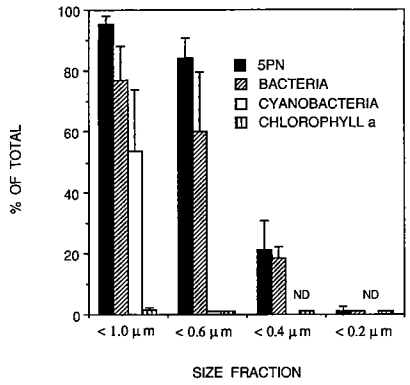

Fig. 5. Size fractionation of 5PN activity and biomass parameters in water samples from Scripps pier. Values shown represent means ±1 SD of two combined experiments (n = 1–4). (ND—Not determined.)

provided two advantages for detailed size-fractionation studies: 5PN activity was very high and therefore easy to measure with high precision, and most of the Chl *a* was in a large size fraction—the *Prorocentrum* cells—and consequently easy to separate from bacteria.

Greater than 90% of the 5PN activity was in the <1.0-µm fraction and >80% in the <0.6-µm fraction (Fig. 5). 5PN activity declined rapidly in smaller fractions; only 1% was in the dissolved fraction (<0.2 µm). Less than 2% of the Chl *a* was in the <1.0-µm fraction, and only 1% of the cyanobacteria was in the <0.6-µm fraction. In contrast, >75% of the heterotrophic bacteria was in the <1.0-µm fraction and 60% in the <0.6-µm fraction, though under normal conditions virtually all would pass through a 1.0-µm filter (Ammerman and Azam 1985). These results clearly show that bacteria (or bacteria-sized organisms), especially the cells between 0.4 and 0.6 µm in diameter, accounted for the 5PN activity. Furthermore, there was no evidence for cell-free or "dissolved" 5PN activity or 5PN activity in organisms containing Chl *a*. Cyanobacteria could account for a maximum of ~15% of the 5PN activity because nearly all cyanobacteria disappeared between the total and the <0.6-µm size fractions, but only 15% of the 5PN activity was lost between the same size fractions (Fig. 5).

In offshore samples taken at two stations (103 and 105) in the Southern California Bight, 116±18% (mean ±1 SD, n = 6) of the bacteria was in the <1.0-µm fraction, but only 55±17% (n = 6) of the 5PN activity was in the same fraction. The bacterial numbers were >100% because bacteria in <1.0-µm filtrates are often easier to count than in unfiltered samples. In the more eutrophic waters around New York, the bacteria were generally larger and all did not pass through 1.0-µm filters. In nine samples from these areas, however, the percentages (mean ±1 SD) of bacteria (75±24%) and 5PN activity (62±28%) that passed through 1.0-µm filters were comparable. Because all variables were normally distributed, we compared these two data pairs with paired-sample t-tests. For the data from California waters, we can reject the null hypothesis of no difference between the two population means ($P < 0.01$). This result suggests that a significant portion of the 5PN activity was in the >1.0-µm fraction. For the New York data, however, the null hypothesis was accepted; bacteria and 5PN activity both passed through the 1.0-µm filter in similar amounts.

We also wanted to determine the relationship between coupled uptake of P_i regenerated by 5PN and ambient SRP concentration. Of all the pairwise correlations from the SCB cruise, excluding those involving depth or temperature, only two were significant at $P < 0.001$. One was the inverse correlation between coupled uptake of P_i regenerated by 5PN and log(1 + SRP concn), the latter transformed to be normally distributed. This correlation (Fig. 6) resembles an isotope dilution curve and suggests that coupled uptake was strongly influenced by SRP concentration.

Discussion

The results of this study clearly establish bacterial 5PN as an enzyme that is widespread in estuarine and coastal marine environments and can hydrolyze exogenous 5′-nucleotides at high rates. 5PN appears distinct from AP in both substrate specificity and P_i inhibition and repression. The enzyme 5PN (also called uridine diphosphate sugar hydrolase, EC 3.1.3.5) also has been purified from *Escherichia coli* and oth-

er enteric bacteria (Neu 1968). In many strains the enzyme is released by osmotic shock, showing that it is periplasmic or on the cell surface. All the bacterial 5PN enzymes studied hydrolyze all 5′-ribo- and deoxyribonucleotides with an unsubstituted 3′-OH group (Neu 1968). No pyrophosphates are formed by the hydrolysis of nucleoside di- and triphosphates, and P_i does not inhibit enzyme activity. The enzyme also hydrolyzes uridine diphosphoglucose (UDP-glucose) to P_i, glucose-1-phosphate, and uridine (Neu 1968). The gene for *E. coli* 5PN, ushA, is a part of the pho regulon in *E. coli,* which is turned on during periods of P deficiency (Lugtenberg 1987). The gene for AP is also part of this regulon.

Marine bacteria have also been shown to have cell-surface 5PN activity (Bengis-Garber 1985). Six marine *Vibrio* or *Photobacterium* strains have 5PN specificities for 5′-nucleotides similar to the *E. coli* enzyme (UDP-glucose has not been tested). In contrast with *E. coli,* however, the 5PN enzymes in these strains appear to be bound to the cell membrane rather than free in the periplasm (Bengis-Garber 1985). Studies with *Vibrio harveyi* showed that by removing P_i from nucleotides, 5PN activity facilitates uptake of both P_i and nucleosides (or nucleoside fragments; Bengis-Garber 1985).

In this study we have shown that GMP is a competitive inhibitor of [γ-^{32}P]ATP hydrolysis (Fig. 3) and that other nucleotides tested less extensively, AMP and ATP, act similarly (Fig. 2). Therefore, all these nucleotides are probably enzyme substrates. All of the inhibitors used in Fig. 2 are *E. coli* AP substrates (Reid and Wilson 1971). Three of them, the nucleotides AMP, ATP, and GMP, are also 5PN substrates in enteric and several strains of marine bacteria (Bengis-Garber 1985; Neu 1968). Furthermore, our experiments clearly demonstrated that pyrophosphate and tripolyphosphate, which are good AP substrates (Reid and Wilson 1971), are not 5PN substrates (Fig. 2). Because the substrate for this assay, [γ-^{32}P]ATP, has three P_i groups and therefore could be hydrolyzed by an enzyme that recognized pyro- or tripolyphosphate, this finding shows the importance of the carbon moiety in substrate recognition by the enzyme. The results of this study and that of Ammerman and Azam (1985) show that only 5′-nucleotides are substrates for this enzyme, and so far every 5′-nucleotide tested has been hydrolyzed. Studies in Chesapeake Bay showed that AP hydrolyzed all P-esters and nucleotides, but 5PN hydrolyzed only nucleotides (Ammerman unpubl.).

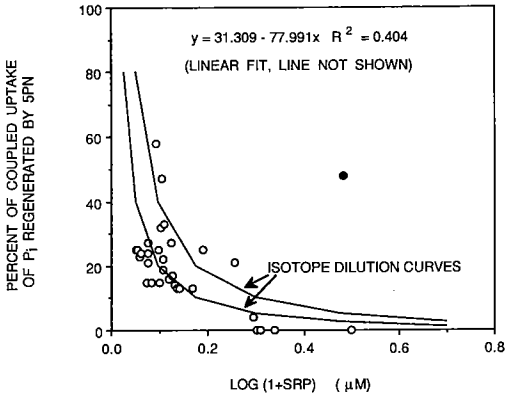

Fig. 6. Coupled uptake of P_i regenerated by 5PN vs. log(1 + SRP). The SRP data had to be log-transformed to fit a normal distribution (*see text*). The filled circle was omitted from the calculation of the correlation coefficient because the sample had very little 5PN activity and was probably unreliable. Isotope dilution curves were generated by assuming plausible initial values for percent coupled uptake at a given SRP concentration and geometrically extrapolating other values.

We found a (K_t + S_n) for 5PN of 41 nM for a natural sample from Long Island Sound with [γ-^{32}P]ATP as the substrate (Fig. 3). This (K_t + S_n) value for 5PN activity is similar to previous results (Ammerman and Azam 1985) and much lower than the 9.1 μM found in *V. harveyi* cultures by Bengis-Garber (1985) with [α-^{32}P]ATP as the substrate. Both indicate a high affinity for substrate, however, and the disparity is not surprising considering the differences in the sources of the enzyme.

This study also showed that 5PN activity was unaffected by up to 5 h of preincubation with 20 μM P_i, suggesting that there was neither inhibition of 5PN activity nor repression of 5PN synthesis by added P_i. This latter interpretation assumes that there was significant turnover of 5PN molecules in 5 h. These experiments were done during a

red tide (*see above*) when SRP depletion could have induced AP activity. (SRP and AP were not measured, however.) The fact that 20 μM P_i had no effect on ATP breakdown suggests that this breakdown was not due to AP, however, which is normally inhibited in the short term and repressed in the long term by such P_i levels (Cembella et al. 1984). In culture studies with another red tide dinoflagellate, *Ptychodiscus brevis,* Vargo and Shanley (1985) found that adding 20 μM P_i caused a 52% inhibition of AP activity within 30 s; after 30 min of incubation with 20 μM P_i, AP had dropped to 28% of its initial value. Therefore, our measured 5PN activity seems distinct from AP in its response to added P_i.

Direct comparison of the effect of added P_i and AP and 5PN activities in parallel or similar samples from New York waters (Fig. 4) extended the above results. Although AP activity was low, it was measurable and inhibited at much lower added [P_i] than 5PN. These experiments clearly separate these two enzyme activities on the basis of their P_i sensitivity and are consistent with the literature for both enzymes (*see references above*) and our own previous study of 5PN (Ammerman and Azam 1985). Similar results were found in Chesapeake Bay, where both enzymes had higher control activities than in New York waters (Ammerman unpubl.).

The size-fractionation experiments from Scripps pier (Fig. 5) extended previous results (Ammerman and Azam 1985). They show that bacteria accounted for most of the 5PN activity and rule out Chl *a*-containing organisms, including cyanobacteria, as significant sources of 5PN. Other size-fractionation results from offshore waters of the Southern California Bight suggest, however, that the bacterial size fraction accounted for only half of the 5PN activity. This result could be due to rare but highly active, attached bacteria or to inhibition of activity by the 1.0-μm prefiltration.

Highly active, attached bacteria can probably be ruled out, at least in the SCB, because 90% of the glucose assimilation in these waters is due to organisms that pass through a 1.0-μm filter in postfiltration (Azam and Hodson 1977). The most likely explanation is inhibition of 5PN activity by the 1.0-μm prefiltration, even though it was done by gravity alone. Numerous studies have shown that prefiltration can cause errors in planktonic rate measurements, the ^{14}C productivity measurements of Furnas (1987) being a recent example. Though productivity and heterotrophic uptake samples can be postfiltered (Azam and Hodson 1977), there is no current alternative to prefiltration for size fractionation of enzyme activities (Furnas 1987).

In the SCB, the coupled uptake of P_i regenerated by 5PN was strongly influenced by competition with ambient SRP (Fig. 6). It is impossible, however, to rule out a depth effect on coupled P_i uptake, because depth and SRP concentration were closely correlated. A similar correlation of coupled P_i uptake with ambient SRP was found in the *surface waters* of Chesapeake Bay (Ammerman 1991). This example eliminates the depth effect and strengthens the case for control of coupled P_i uptake by the ambient SRP concentration.

The SCB and waters around New York City are generally high in P, so AP activity was usually low and sometimes unmeasurable. 5PN activity, in contrast, was always readily measurable in these environments. There were also clear differences in the properties of these two enzymes; 5PN was competitively inhibited only by 5′-nucleotides and required much higher P_i concentrations for inhibition than AP. These two enzymes were assayed by two very different methods, however, so direct comparison of their activities is difficult. 5PN activity was assayed with the labeled form of a natural substrate, [γ-^{32}P]ATP, at tracer concentrations. AP, in contrast, was assayed by the traditional fluorometric field assay that uses a substrate analog, is less sensitive than the 5PN assay, and does not permit the fate of the hydrolyzed P_i to be followed. As a result, AP activities were probably underestimated.

5PN substrates (5′-nucleotides) are a subset of AP substrates (e.g. P-esters and nucleotides), so there is always the possibility that some of the "5PN activity" may be due to AP. This problem may not be important in this study, but would be a concern in

environments low in P and high in AP activity. There is need to develop a true tracer AP assay that would address the abovementioned problems with the current AP assay. A tracer AP assay would also permit a clearer comparison of the properties of AP with other enzymes like 5PN. The advantages of tracer methods such as the 5PN assay are obvious from these studies. The ability to follow the path of the released P_i is particularly important in determining the role of these enzymes in P_i regeneration. In the future, however, new methods in addition to enzyme assays will also be required to unravel the role of 5PN, AP, and other enzymes in P cycling and microbial metabolism.

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