Bacterial 5′-Nucleotidase in Aquatic Ecosystems: A Novel Mechanism of Phosphorus Regeneration

Abstract. *Zooplankton excretion and algal alkaline phosphatase are presumed to be responsible for phosphorus recycling in aquatic ecosystems; the role of bacteria has been unclear. High levels of bacterial cell-surface 5′-nucleotidase were discovered in samples of picoplankton from California coastal waters. 5′-Nucleotidase rapidly generated orthophosphate from 5′-nucleotide added in nanomolar amounts and could supply half the orthophosphate required by plankton. Unlike alkaline phosphatase, 5′-nucleotidase was not inhibited by orthophosphate at any concentration found in aquatic environments. Initial results indicate even greater 5′-nucleotidase activity in fresh water (Lake Hodges, California) and brackish water (Baltic). Release and uptake of orthophosphate were tightly coupled.*

Global oceanic productivity depends on rapid regeneration of orthophosphate (P_i) by metabolic activities of plankton within the euphotic zone (*1*). Existing models represent excretion of P_i by zooplankton as the dominant mode of phosphorus mineralization in rich coastal waters (*1*). In phosphorus-depleted oceanic waters, phytoplankton synthesize cell-surface alkaline phosphatase, which liberates P_i from dissolved organic phosphorus in seawater (*2*). However, alkaline phosphatase is often repressed by P_i (*2, 3*) and may play only a minor role in global phosphorus cycling. Interest in the picoplankton (organisms less than 1 μm in diameter) component of marine ecosystems (*4*) has led to questions about its role in phosphorus cycling. Although the microplankton (organisms less than 100 μm in diameter and including the picoplankton) can regenerate 50 to 100 percent of the P_i required for growth of phytoplankton (*5*), specific organisms or mechanisms have not been identified. Thus, the role of bacterioplankton in P_i regeneration is unclear (*1*).

We now report that natural marine and freshwater bacterial plankton have a cell-surface 5′-nucleotidase that rapidly hydrolyzes 5′-nucleotides and regenerates P_i. This enzyme appeared to be distinct from alkaline phosphatase in substrate specificity and was not repressed by P_i. Hydrolysis of 5′-nucleotides by 5′-nucleotidase and uptake of the hydrolyzed P_i were coupled, and this coupling increased with decreasing concentrations of P_i in the environment (*6*).

Our 5′-nucleotidase assay was based on hydrolysis of ^{32}P-labeled P_i ($^{32}P_i$) from adenosine [γ-^{32}P]triphosphate ([γ-^{32}P]ATP) and measured both release of P_i and uptake of released P_i (*7*). If ATP hydrolysis was due to alkaline phosphatase, it should be competitively inhibited by other substrates of alkaline phosphatase. However, substrates such as glucose 6-phosphate, β-glycerophosphate, 3-*O*-methylfluorescein phosphate (3-OMFP), and 4-methylumbelliferyl phosphate (4-MUP) inhibited ATP hydrolysis by only 0 to 5 percent (Fig. 1) [3-OMFP and 4-MUP are commonly used to assay alkaline phosphatase in natural waters (*2, 8*)]. All 5′-nucleotides tested inhibited ATP hydrolysis and were thus active substrates. This structural specificity indicated that the enzyme was a 5′-nucleotidase; all other organic phosphates tested were inactive substrates (Fig. 1). Similar 5′-nucleotidase substrate specificity is found in marine and enteric isolates (*9*).

High 5′-nucleotidase activity occurred in both nearshore and offshore samples (Table 1), and it was measured at depths of up to 50 m in samples taken 100 km off shore [1.09 percent hydrolysis of 2 n*M* ATP per hour (*10*)]. Size-fractionation (*11*) of the plankton (Table 1) showed that most of the activity was due to bacteria (the picoplankton fraction). Little activity was free in solution (<0.22 μm). 5′-Nucleotidase must be located on the cell surface or in the periplasmic (not intracellular) space because bacteria do not take up intact ATP (*12*). This was

Table 1. Size fractionation of 5′-nucleotidase activity (*7*) and related parameters in seawater samples taken from the Scripps pier and 3 km off shore (bottom depth, ~100 m). Subsamples were filtered through 1.0-μm Nuclepore filters (by gravity alone) or through washed 0.22-μm Millipore filters (by gentle vacuum) before size fractionation studies. Pier samples were incubated indoors under 340 μE of illumination per square meter per second, and offshore samples were incubated outdoors under simulated in situ conditions (20°C and 25 percent surface light intensity). The concentration of the substrate varied (see note below). Amounts of P_i, dissolved organic phosphorus (DOP), chlorophyll a, and bacteria were determined by standard methods (*17*). Results are expressed as the mean ± standard deviation (n = 2 for P_i and DOP, n = 3 for 5′-nucleotidase, n = 2 or 3 for chlorophyll a, and n = 1 for bacteria). Numbers in parentheses indicate the percentage found in that size fraction. N.D., not determined.

Date	Size fraction	P_i (μm)	DOP (μm)	5′-Nucleotidase activity		Chlorophyll a†	Bacteria per liter ($\times 10^9$)
				Percent per hour	Nanomoles per liter per hour*		
			Pier (depth, 0 m)				
7 May 1982	Total	0.19 ± 0.00	0.27 ± 0.02	8.81 ± 0.27	0.48 (100)	2.36 ± 0.02 (100)	1.93 (100)
	<1.00 μm			7.18 ± 0.20	0.40 (81)	0.07 ± 0.02 (3)	1.89 (98)
	<0.22 μm			0.96 ± 0.06	0.05 (11)	N.D.	0 (0)
14 May 1982	Total	0.18 ± 0.00	0.25 ± 0.00	12.41 ± 0.26	0.88 (100)	4.36 ± 0.49 (100)	2.57 (100)
	<1.00 μm			8.92 ± 0.25	0.49 (72)	0.14 ± 0.04 (3)	2.59 (101)
	<0.22 μm			1.38 ± 0.25	0.08 (11)	N.D.	0 (0)
6 June 1982	Total	0.27 ± 0.02	N.D.	7.40 ± 0.34	0.81 (100)	1.65 ± 0.05 (100)	1.09 (100)
	<1.00 μm			5.41 ± 0.09	0.59 (73)	0.06 ± 0.01 (4)	1.04 (95)
			Offshore (depth, 5 m)				
28 October 1983	Total	0.20 ± 0.00	0.21 ± 0.00	3.80 ± 0.50	0.038 (100)	0.23 ± 0.01 (100)	0.79 (100)
	<1.00 μm			2.56 ± 0.06	0.026 (67)	0.01 ± 0.01 (4)	0.89 (113)
			Offshore (depth, 20 m)				
28 October 1983	Total	0.31 ± 0.00	0.22 ± 0.00	2.57 ± 0.10	0.026 (100)	0.14 ± 0.09 (100)	0.70 (100)
	<1.00 μm			1.17 ± 0.07	0.012 (46)	0.02 ± 0.01 (14)	0.70 (100)

*Calculated from the percent per hour times the concentration of added [γ-^{32}P]ATP: experiments on 7 and 14 May 1982 used 5.5 n*M* ATP; on 6 June 1982, 11 n*M* ATP; and on 28 October 1983, 1 n*M* ATP. †Addition of phaopigments increased the amount of total pigment in the <1 μm fraction to 4 to 6 percent in the pier samples and to 18 to 20 percent in the offshore samples.

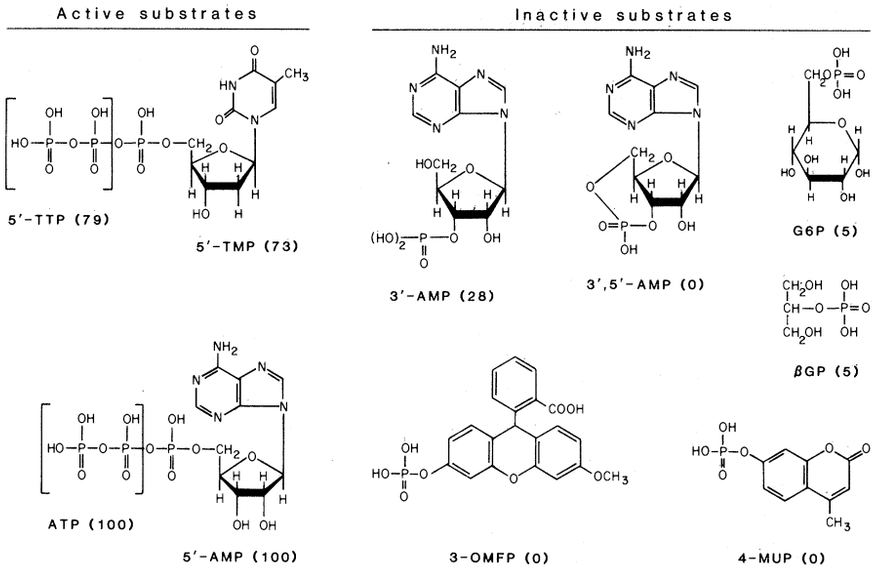

Fig. 1. 5′-Nucleotidase substrate specificity. Specificity (*7*) in samples from Scripps pier (32°53′N, 117°15′W) was determined by simultaneous incubation of [γ-^{32}P]ATP (1 n*M*) and various unlabeled competitors (10 n*M*). Samples were incubated at 18° to 20°C and 83 μE of light per square meter per second. Numbers in parentheses represent the relative ability (in percent) of each competitor to inhibit substrate hydrolysis (one to three determinations). The largest absolute standard deviation was 16 percent (n = 2) for adenosine 3′-monophosphate (3′-AMP); all others were below 10 percent. Competition results with thymidine 5′-[α-^{32}P]triphosphate (5′-TTP, three P_i's) as the substrate were nearly identical to those with [γ-^{32}P]ATP (three P_i's), although the control activity with the former was only half that with the latter. This lower activity with the α-labeled substrate and chromatography studies showing P_i as the major hydrolysis product suggest that 5′-nucleotidase hydrolyzed the P_i groups from ATP sequentially, beginning with γ-P_i. Other abbreviations: 5′-AMP, adenosine 5′-monophosphate (one P_i); 5′-TMP, thymidine 5′-monophosphate (one P_i); 3′,5′-AMP, cyclic adenosine monophosphate; G6P, glucose 6-phosphate; βGP, β-glycerophosphate; 3-OMFP, 3-*O*-methylfluorescein phosphate; and 4-MUP, 4-methylumbelliferyl phosphate.

confirmed by the extracellular release of $^{32}P_i$ by 5′-nucleotidase; uptake of this released $^{32}P_i$ was competitively inhibited by added P_i (Fig. 2). Activity in the microplankton fraction may occur in a few highly active attached bacteria (*13*) or may be due to algae or the related cyanobacteria, although this enzyme has not been reported in photoautotrophs.

An ecologically important property of the bacterial plankton 5′-nucleotidase was its insensitivity to high concentrations of P_i (Fig. 2). There was no inhibition at P_i concentrations below 100 μ*M*; therefore, this enzyme should be fully active at all P_i concentrations found in the oceans. 5′-Nucleotidase may be an important mechanism for regeneration of P_i in coastal waters, where P_i concentrations are measurable and alkaline phosphatase is probably repressed by P_i (*2, 10*). In contrast, in the euphotic zone of the open ocean both enzymes could be active. Rapid regeneration of P_i in situ is important in both open ocean and coastal waters (*1*). However, the coupling between P_i regeneration and primary production is less stringent in coastal waters.

For 5′-nucleotidase to have a role in the hydrolysis of 5′-nucleotides in seawater, it must have a high affinity for its substrates. Concentrations in seawater of the individual 5′-nucleotides adenosine monophosphate, adenosine diphosphate, and ATP approach 1 n*M* (*13, 14*). Therefore, the total concentration of all 5′-nucleotides in the euphotic zone is probably 10 to 20 nmol per liter. The results of two 5′-nucleotidase kinetics experiments showed a high affinity for

Table 2. Rate of uptake of $^{32}P_i$ from [γ-^{32}P]ATP by 5′-nucleotidase (*7*) compared to the rate of uptake of added $^{32}P_i$ in parallel samples. N.D., not determined.

Date	P_i (μ*M*)	DOP (μ*M*)	$^{32}P_i$ uptake (percent per hour)		Ratio *A*:*B*
			From released $^{32}P_i$ (*A*)*	From added $^{32}P_i$ (*B*)†	
		Fresh samples			
9 December 1981	0.14	N.D.	14.47	0.55	26
16 December 1981	0.15	0.18	9.94	0.59	17
21 January 1982	0.43	0.21	8.77	0.22	40
6 June 1982	0.27	N.D.	11.63	1.18	10
		P_i-depleted samples			
5 June 1982	0.00	N.D.	51.39	N.D.	
11 June 1982	0.00	N.D.	50.71	14.05	4

*Added [γ-^{32}P]ATP concentration ranged from 1.6 to 13 n*M*. †Added at tracer concentrations (3 to 900 p*M*).

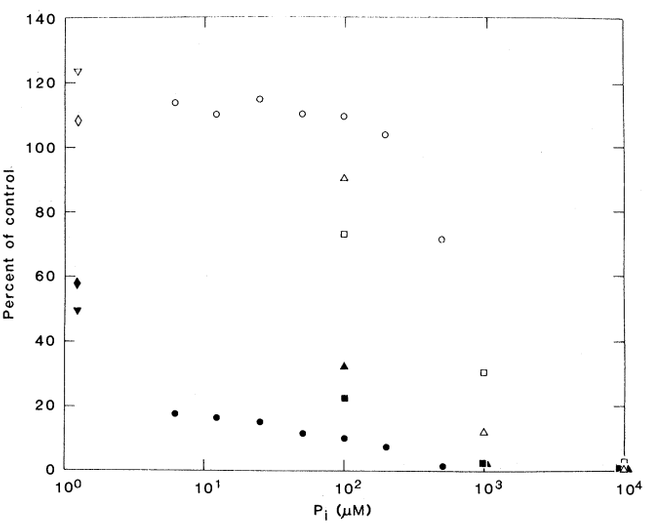

Fig. 2. Effect of P_i on both 5′-nucleotidase activity (*7*) (open symbols) and uptake of $^{32}P_i$ released by 5′-nucleotidase (closed symbols); results are from five experiments. Concentrations of [γ-^{32}P]ATP (added substrate) ranged from 1 to 11 n*M*. Results are the means of three replicate filtrations; control rates ranged from 3 to 16 percent per hour for 5′-nucleotidase activity and from 1 to 8 percent per hour for uptake. With the exception of one uptake control, all 5′-nucleotidase and uptake controls had standard deviations less than or equal to 4 percent of the mean.

ATP, with multiple or multiphasic (*15*) kinetics (Michaelis constants, 3 and 67 n*M* and maximum velocities, 0.06 and 4.07 nmol per liter per hour, respectively).

We wondered whether 5′-nucleotidase could supply a substantial fraction of the P_i required for phytoplankton growth. In California coastal waters, a typical rate of P_i consumption was 1 to 2 nmol per liter per hour (Table 2). If we assume a total concentration of 5′-nucleotides of 10 nmol per liter and a hydrolysis rate of 10 percent per hour (Table 1), then the rate of P_i release was 1 nmol per liter per hour or 50 to 100 percent of the consumption rate. Although 5′-nucleotidase activity decreased in offshore samples (Table 1), P_i consumption also decreased (*10*); thus, 5′-nucleotidase remained a substantial source of regenerated P_i for primary productivity.

From 10 to 15 percent of the P_i resulting from 5′-nucleotidase activity did not mix with bulk-phase P_i but was taken up by the bacteria that produced it. The rate of uptake of this hydrolyzed P_i was 10 to 40 times greater than that of bulk-phase P_i (Table 2). At seawater concentrations of P_i (Table 2), this coupling between hydrolysis and uptake was loose, and the remaining 85 to 90 percent of the P_i hydrolyzed by 5′-nucleotidase was released into the environment. When P_i in seawater was depleted, however, coupling increased to 50 percent, and half the hydrolyzed P_i was taken up (Table 2).

Phosphorus controls primary productivity in many fresh and brackish waters (*3*). Picoplankton samples from fresh water (Lake Hodges, California) and brackish water (Northern Baltic Sea; salinity, 4 to 5 parts per thousand) hydrolyzed added ATP faster than marine picoplankton (*16*). Rates often exceeded 100 percent per hour, and initial competition data suggest 5′-nucleotidase to be responsible. From 10 to 50 μ*M* added P_i had little effect on hydrolysis but significantly inhibited uptake. Coupling between release and uptake of P_i was tight; often 100 percent of the released P_i was taken up.

Most of the ATP hydrolysis was due to the picoplankton fraction (probably bacteria), but most of the uptake of released P_i was in the microplankton fraction (probably phytoplankton). Apparently the phytoplankton rapidly took up the P_i released by the bacteria. 5′-Nucleotidase (unlike alkaline phosphatase) was not inhibited by P_i; it is therefore likely to be more widely distributed in aquatic ecosystems than alkaline phosphatase. Our results may necessitate a revision of the current concept of P_i regeneration in aquatic ecosystems so that the role of bacterial plankton may be incorporated.

JAMES W. AMMERMAN
FAROOQ AZAM
Institute of Marine Resources, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California 92093

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6. We have assumed that the molybdenum-blue method measured P_i. Actual concentrations of P_i, particularly in fresh water, were probably lower (*3*).
7. 5′-Nucleotidase activity in different aquatic environments was assayed by adding 10 p*M* to 13 n*M* [γ-^{32}P]ATP to water samples. Samples were incubated (0.3 to 6 hours) and filtered onto 0.2-μm Nuclepore filters to measure uptake of $^{32}P_i$ released from the substrate by 5′-nucleotidase (*A*). A portion of the filtrate was assayed for ^{32}P (*B*), and the remainder was acidified with 0.06*N* H_2SO_4, mixed with about 20 mg of activated charcoal, and filtered through a 0.45-μm Millipore filter. The ^{32}P in this second filtrate was the $^{32}P_i$ released from [γ-^{32}P]ATP by 5′-nucleotidase but not taken up by organisms (*C*). 5′-Nucleotidase activity was the sum of *A* and *C* divided by the total amount of label added (*A* + *B*). Blanks were boiled or zero-time samples. All measured enzyme activities and rates of uptake were the means of three replicate filtrations; values for the replicates almost always fell within 5 percent of the mean.
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11. In samples from the Southern California Bight, bacterial plankton can be separated from phytoplankton on the basis of size; most bacteria pass 1-μm Nuclepore filters, whereas most phytoplankton (measured as chlorophyll a) are retained (Table 1).
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Heterogeneity of 5*S* RNA in Fungal Ribosomes

Abstract. Neurospora crassa *has at least seven types of* 5S *RNA genes* (α, β, γ, ϵ, δ, ζ, *and* η) *with different coding regions. A high resolution gel electrophoresis system was developed to separate minor* 5S *RNA's from the major* 5S *RNA* (α). *A study of several* Neurospora crassa *strains, four other species in the genus* Neurospora, *members of two closely related genera, and three distantly related genera demonstrated that* 5S *RNA heterogeneity is common among fungi. In addition, different* 5S *RNA's are present in* Neurospora *ribosomes. The finding that fungal ribosomes are structurally heterogeneous suggests that ribosomes may be functionally heterogeneous as well.*

Eukaryotic cytoplasmic ribosomes contain four RNA molecules. The three largest ribosomal RNA's are made by posttranscriptional processing of a single high molecular weight transcript. The smallest ribosomal RNA, 5*S* RNA, is transcribed by a different polymerase and coded separately (*1*). *Neurospora crassa* has approximately 100 5*S* RNA genes, widely dispersed over at least six of its seven chromosomes (*2, 3*). Of the 21 distinct 5*S* genes that we have characterized, 13 encode α 5*S* RNA, which constitutes about 70 to 80 percent of *Neurospora* 5*S* RNA. Four of the genes encode β 5*S* RNA, which differs in sequence from α 5*S* RNA in 15 out of 120 nucleotides. We have also identified several other presumptive 5*S* RNA genes (γ, δ, ζ, and η) which, if functional, would have the capacity to produce RNA's different from α or β by 12 to 34 nucleotides. The sequences flanking 5*S* genes are extremely heterogeneous, suggesting that the various 5*S* genes may be differentially regulated. Sequencing studies of total 5*S* RNA have shown that both α and β 5*S* RNA genes are expressed in vegetative hyphae, perithecia, and aerial conidia and that at least two other *Neurospora* 5*S* RNA's are made (*2*). We have developed a high resolution polyacrylamide gel electrophoresis system for 5*S* RNA to detect expression of variant 5*S* genes which may be present in the genome as single copies.

The 5*S* RNA from *Aspergillus nidulans* has been separated into two bands in a 15 percent polyacrylamide gel containing 4*M* urea (*4*). We have found that, under certain conditions, *Neurospora* 5*S* RNA can also be resolved into two or