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Effects of Alkaline Phosphatase Activity on Nucleotide Measurements in Aquatic Microbial Communities†

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Alkaline phosphatase (APase) activity was detected in aquatic microbial assemblages from the subtropics to Antarctica. The occurrence of APase in environmental nucleotide extracts was shown to significantly affect the measured concentrations of cellular nucleotides (adenosine triphosphate, adenosine diphosphate, adenosine monophosphate, guanosine triphosphate, uridine triphosphate, and cytidine triphosphate), adenylate energy charge, and guanosine triphosphate/adenosine triphosphate ratios, when conventional methods of nucleotide extraction were employed. Under the reaction conditions specified in this report, the initial rate of hydrolysis of adenosine triphosphate was directly proportional to the activity of APase in the sample extracts and consequently can be used as a sensitive measure of APase activity. A method was devised for obtaining reliable nucleotide measurements in naturally occurring microbial populations containing elevated levels of APase activity. The metabolic significance of APase activity in microbial cells is discussed, and it is concluded that the occurrence and regulation of APase in nature is dependent upon microscale inorganic phosphate limitation of the autochthonous microbial communities.

Phosphorus is an essential nutrient requirement for all forms of life and comprises between 1 and 4% of the total dry weight of most microbial cells (16, 43, 58). When cultured in P-enriched media, however, many species of microorganisms exhibit luxury uptake and have the ability to condense polyphosphates, thereby disproportionately increasing their total cellular P concentration to values ≥10% of the total dry weight. The distribution of organic phosphorus under balanced growth conditions is approximately 65% as nucleic acids, 15% as phospholipids and 20% as acid-soluble fractions (58). More comprehensive separations have also been published (52). The acid-soluble fraction consists primarily of phosphate esters, and derivatives thereof, including the abundant nucleoside phosphates (e.g., adenosine mono-, di-, and triphosphate [AMP, ADP, and ATP, respectively]) and sugar phosphates (e.g., glycerol-phosphate, glucose 6-phosphate).

In nonpolluted freshwater habitats, inorganic phosphate (P_i) (as HPO_4^{2-}) availability is often regarded as the nutritional factor which limits or controls the rate of primary production. By comparison, the marine environment is generally considered to be fixed N limited, although P_i may limit marine phytoplankton growth under certain environmental conditions (44, 51).

When most microbial cells (e.g., bacteria, yeasts, algae, protozoa) are cultured in P_i-deficient medium the synthesis of a relatively nonspecific orthophosphoric monoester hydrolase (i.e., alkaline phosphatase [APase]) is derepressed (21, 22, 25, 28, 68). Under conditions of maximum synthesis, APase may represent up to 6% of the total protein synthesized by the cell (21). This cell-surface-associated enzymic activity enables the P_i-deficient cells to specifically utilize certain components of the soluble organic phosphorus pool. In most species examined, readdition of P_i to the growth medium caused a loss of APase activity and a repression of additional synthesis. By comparison, acid phosphatase activity is present at constant levels under all conditions of growth and is unaffected by internal cellular P_i concentrations (1, 41, 68).

APase activity has been detected in many freshwater (4, 5, 27, 53, 60, 61) and marine habitats (1, 38, 40, 50, 51, 57, 63, 65). Several reports have attempted to correlate the occurrence of APase activity in selected aquatic environments with conditions of P_i limitation. This practice should be approached with caution for several reasons. First, the ability to synthesize APase (even during extreme P_i deficiency) is not a universal characteristic of all microorganisms (40, 54, 65). Second, APase may also be present as a constitutive enzyme in many microbial taxa (12, 19, 41) and is therefore not obligately coupled to P_i deficiency. In many species there is a low but "normal" level of APase present in the

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cell under all growth conditions. However, during periods of P_i deficiency the activity levels may increase 5- to 120-fold (7, 18). Consequently, a more appropriate assessment of P_i deficiency might be a measure of the APase activity per unit of biomass since this might help resolve the extreme differences which have been observed between repressed (normal) and derepressed (elevated) levels of intracellular APase activity. Furthermore, since the existence of APase in microbial cells increases the spectrum of phosphorus compounds available for transport and assimilation one should be careful not to confuse orthophosphate limitation with total phosphorus limitation. This is especially important in certain aquatic ecosystems in which the total soluble organic phosphorus pool may equal or exceed the pool of P_i (56, 57, 62, 64). Comparative studies of the growth of phytoplankton cells supplemented with organic versus inorganic P have indicated comparable growth rates (39, 40, 48, 49). Therefore, microbial assemblages in nature may be P_i deficient and may contain high levels of APase activity per unit biomass but may not be P limited in terms of growth. Finally, Wilkins (71) has demonstrated that APase activity can be induced in excess P_i media by starvation for either pyrimidine or guanine. Apparently the regulation of phosphatase by P_i is indirect and the actual mechanism involves one or more cellular nucleotide species (71). This may help to explain a few of the inconsistencies in the extensive literature on the conditions of occurrence of APase in microbial cells.

During an investigation of the quantitative distribution of ATP, total adenylates (A_T = [ATP] + [ADP] + [AMP]), adenylate energy charge (EC_A), and guanosine triphosphate (GTP)/ATP ratios (i.e., "nucleotide fingerprinting") in marine and freshwater ecosystems, we encountered a significant but extremely variable analytical interference as a result of the presence of APase in certain environmental samples. In this report we describe a sensitive method for measuring APase activity in environmental samples, present data on APase activities from selected aquatic ecosystems, summarize the quantitative characteristics of APase interference on nucleotide fingerprints in nature, describe a new extraction procedure to circumvent this source of analytical interference, and discuss the possible significance of APase activity in selected habitats.

MATERIALS AND METHODS

Description of sampling sites and methods of sample collection. Water and sediment samples were collected from diverse habitats by using a variety of methods. Seawater samples from McMurdo Sound, Antarctica, the southern California Bight, and the Central North Pacific gyre were collected in ethanol-rinsed (70%, vol/vol) Niskin bottles (General Oceanics). Surface water samples from freshwater prawn (*Macrobrachium rosenbergii*) aquaculture production ponds, Oahu, Hawaii, were collected by hand in clean polyethylene plastic bottles. Surface sediment samples (0 to 5 cm) from the Oahu aquaculture ponds, from the intertidal portion of the beach at Scripps Institution of Oceanography, and from two subtidal Antarctic stations located beneath the seasonal Ross Sea ice cover (stations New Harbor and Cape Armitage) were collected by hand, the latter by using SCUBA diving techniques. Deep-sea sediment samples from stations located in the Central North Pacific gyre (~5,500 m) were obtained using a 0.25-m^2 spade-box corer. The precise geographic locations of all stations are given in the respective tables and figure captions. All samples were processed as soon as possible after collection, but at the most, after a delay of ~2 h.

Nucleotide extraction and measurement. Several different extraction procedures were evaluated in this study: the cold H_2SO_4-ethylenediaminetetraacetic acid (EDTA) method of Karl and LaRock (36), the boiling phosphate buffer-method of Bulleid (9), and a new H_3PO_4-EDTA method which was devised over the course of these studies. The last method is identical to that previously described by Karl and LaRock (36), except for the use of cold 1 M H_3PO_4 in place of 0.6 N H_2SO_4.

AMP, ADP, and ATP were measured, and EC_A was calculated as described by Karl and Holm-Hansen (35). GTP, cytidine triphosphate (CTP), and uridine triphosphate (UTP) were measured by using crude luciferase preparations (FLE-50) after addition of ADP to the reaction mixture (29). For selected samples, both the peak height of ATP-dependent light emission (0 to 3 s) and the integrated light flux (15 to 75 s) were recorded as relative measures of GTP plus UTP [i.e., ΔATP = (integrated ATP − peak ATP)α(GTP + UTP)] (30). When two or more extraction procedures are compared, it is imperative that each set of samples be measured relative to a standard curve prepared in an ionic medium identical to that of the individual sample extracts. All measurements were made using a commercial ATP photometer (SAI Technology, Sorrento Valley Blvd., San Diego, Calif.).

Firefly luciferase (FLE-50), pyruvate kinase (P-1506), myokinase (M-3003), APase (P-4377), adenosine triphosphatase (ATPase) (A-7510), and the sodium salts of ATP, ADP, AMP, CTP, UTP, and GTP were purchased from Sigma Chemical Co. (St. Louis, Mo.). All additional chemicals used in this study were analytical grade reagents.

APase assay. The presence and relative activities of APase were monitored by the rate of hydrolysis of ATP with time in selected extracts. The reaction mixture consisted of 2 ml of sample, $MgCl_2$ (~4.5 mM, final concentration) and between 100 and 500 ng of ATP. The reaction was initiated by the addition of ATP, and the mixture was incubated at room temperature (25 ± 1.5°C). Subsamples (0.2 ml) were periodically withdrawn and assayed for ATP. For selected experiments, ATP was replaced by GTP, UTP or

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CTP. For time course effects on EC_A, subsamples of the reaction mixtures were removed and immediately injected into equal volumes of phosphate buffer (pH 7.4, 200 mM) to repress further APase activity. At the end of the time course, all samples were assayed for ATP, ADP, and AMP as described above. Control samples consisted of ATP in tris(hydroxy methyi)aminomethane buffer containing $MgCl_2$. For selected experiments, the chemical composition of the reaction mixture or temperature of incubation was varied as indicated in the respective tables and figure captions. The sensitivity of this APase assay procedure was ~1 to 3 ng of ATP hydrolyzed ml^{-1} h^{-1}, but could be increased ~100-fold through the use of purified luciferase preparations.

RESULTS

Occurrence of APase in environmental samples. Figure 1 presents the partial results of a general screening of environmental samples for the occurrence of APase activity (plotted as rate of ATP hydrolysis). For each habitat examined, there was a variation in the response of samples collected from slightly different localities. At McMurdo Sound, Antarctica (Fig. 1A), APase activity was detected in sediment samples from two stations at New Harbor but was not present in either water samples collected 1 m beneath the ice at New Harbor or sediment samples collected at Cape Armitage on the western edge of McMurdo Sound. In the North Pacific Ocean, APase activity was detected in all samples collected from abyssal red clays but was notably absent from intertidal beach sand habitats (Fig. 1B; 32). The Hawaiian prawn aquaculture ponds varied substantially, in both space and time, with regard to their APase activities. On a given

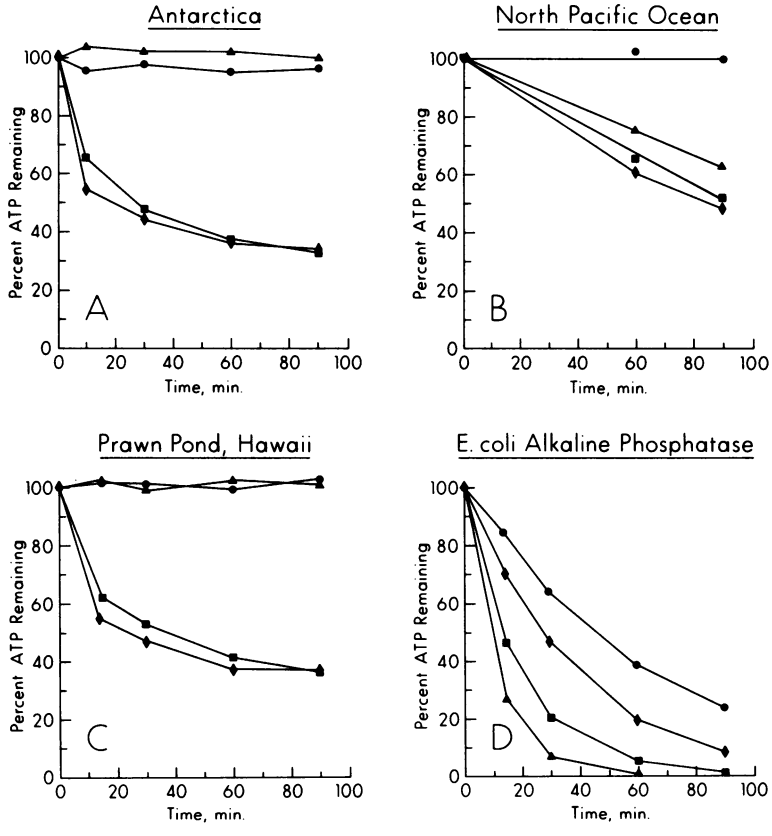


FIG. 1. *Temporal stability of exogenous ATP in a variety of H_2SO_4-EDTA-extracted environmental samples. Symbols: (A) ▲, Cape Armitage surface sediment (77° 51.7'S, 166° 40.9'E); ●, New Harbor water sample (77° 41.6'S, 163° 48'E); ■, New Harbor sediment (0 to 1 cm); ◆, New Harbor sediment (9 to 10 cm). (B) ●, SIO beach intertidal sediment (32° 50'N, 117° 17'W); ▲, Central Pacific gyre (28° 35.4'N, 155° 30.4'W) sediment (0 to 1 cm); ■, Central Pacific gyre (6 to 7 cm); ◆, Central Pacific gyre (1 to 2 cm). (C) ●, Kahuku pond no. 6, 25 October 1979 (21° 40.9'N, 157° 56.8'W); ▲, Kahuku pond no. 9, 25 October 1979; ■, Kahuku pond no. 1, 25 October 1979; ◆, Kahuku pond no. 1, 30 August 1979. (D) ●, 7 mU ml^{-1}; ◆, 11.7 mU ml^{-1}; ■, 23.5 mU ml^{-1}; ▲, 35 mU ml^{-1}.*

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sampling date the sediments of certain ponds exhibited high levels of APase activity while other ponds in the same aquaculture complex yielded no APase activity (Fig. 1C). In general, the activity was high in the spring and summer and low in late fall and winter, although numerous exceptions were evident. A plot of the reactivity and kinetics of commercially available *E. coli* APase is also included for comparison (Fig. 1D). The initial rate of ATP hydrolysis (0 to 15 min) was found to be significantly correlated ($r = -0.996$) with the concentration of APase enzyme in the reaction mixture (Fig. 2). Therefore, the initial rates of ATP hydrolysis observed in Fig. 1A to C can be used to compare the relative activities of APase in individual environmental sample extracts. To relate these measured rates to the in situ APase activities in nature (i.e., milliunits of APase per gram of sediment, or per liter of water), knowledge of the stability of APase during the extraction procedure and an estimation of the natural substrate concentrations are also required.

The nonlinear kinetics observed in this study are presumably the result of an accumulation of PO_4 in the samples with time. This apparent in vitro inhibition of APase by PO_4 is well documented in the scientific literature. It should be emphasized that the observed rates of ATP hydrolysis presented for the environmental samples in Fig. 1A to C can be assumed to be roughly equivalent to the temporal stability of ATP in these environmental extracts (i.e., after acid extraction and neutralization). Clearly, quantitative ATP measurements are not possible under these specified conditions.

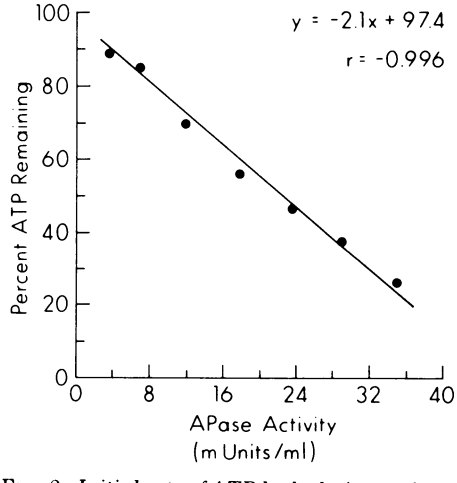


FIG. 2. *Initial rate of ATP hydrolysis as a function of APase activity. The data points represent the percentage of ATP remaining after the first 15 min of incubation. The ATP concentration at t_0 was 100 ng ml^{-1}.*

In addition to the samples presented in Fig. 1, APase activity was detected in samples collected from hypersaline lakes in the Dry Valley region of Antarctica, coral reef sediments collected on the leeward shore of Oahu, Hawaii, and in the water column of selected prawn aquaculture ponds. By comparison, no APase activity was detected in seawater samples collected from various depths in the southern California Bight (0 to 1,500 m), the Columbian Basin of the Caribbean Sea, the Black Sea, and the Galápagos Rift (37), or in seawaters off Ke-ahole Point, Hawaii.

To confirm that the observed hydrolysis of ATP was in fact enzymatic and catalyzed by APase (i.e., an APase-type enzyme), the kinetics of hydrolysis of ATP were examined at two different temperatures with and without exogenous PO_4 added (Fig. 3). In all samples tested, the rate of ATP hydrolysis was greater at 30°C than at 4°C and was repressed at both temperatures by the addition of 200 mM PO_4 (final concentration). The former result is expected of enzyme reactions at temperatures below the denaturation temperature, and the latter (i.e., PO_4 repression of ATP hydrolysis) is indicative of APase activity. Neither acid phosphatase nor ATPase activities, by comparison, are repressed by the addition of exogenous PO_4.

Effects of APase activity on ATP, ADP, and AMP determinations. The occurrence of APase in diverse environmental samples and the previously discussed interference on quantitative ATP determinations (Fig. 1) raised questions concerning the possible effect of this enzyme activity on ADP and AMP levels and on the determination of environmental EC_A ratios. An experiment was conducted to monitor APase-catalyzed temporal changes in ATP, ADP, AMP, A_T, and EC_A (Fig. 4). These data indicate that: (i) ADP and AMP are also hydrolyzed in addition to ATP, as evidenced by the simultaneous decrease in A_T (Fig. 4); (ii) the relative rates of adenine nucleotide hydrolysis are not equivalent, but rather AMP appears to be the preferred substrate for the APase reaction; and (iii) the resultant EC_A is highly dependent upon the duration of the incubation period, with an immediate decrease due to the hydrolysis of ATP to ADP but followed by a much more gradual decrease as a result of an equilibrium between the ATP → ADP and ADP → AMP hydrolysis reactions, and a constant removal of AMP at a rate that exceeds the ATP → ADP reaction. These reactions were not carried to completion (i.e, $A_T = 0$), but in theory the EC_A must proceed through another period of rapid decrease from a value of ~0.5 to 0.0 as

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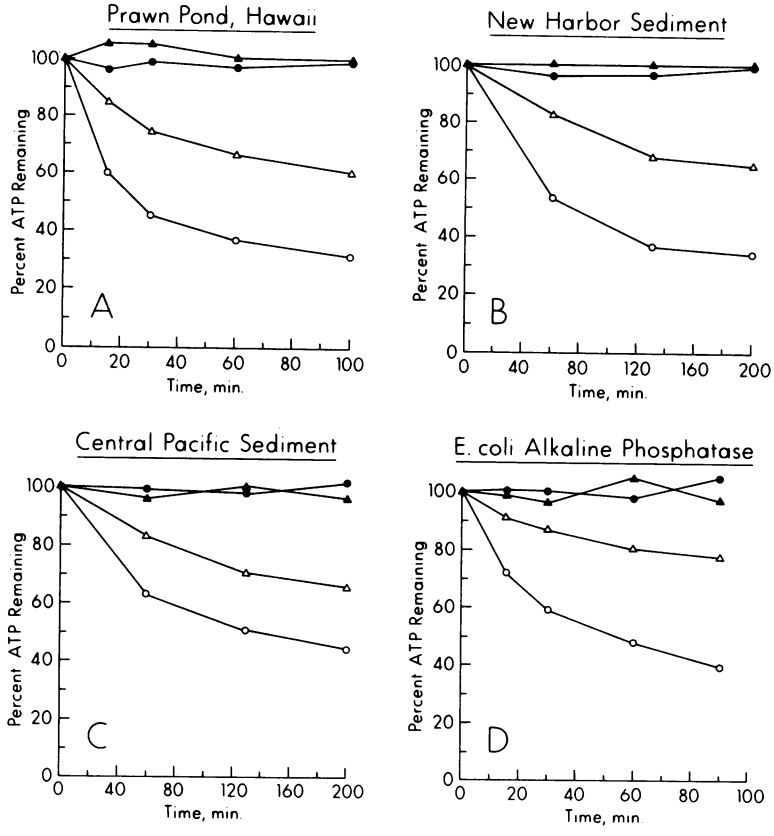


FIG. 3. *Effects of temperature and phosphate addition on ATP in representative extracts. Symbols: ▲, 4°C incubation, 200 mM PO_4 (final concentration) added; ●, 30°C incubation, 200 mM PO_4 (final concentration) added; △, 4°C incubation, no PO_4 added; ○, 30°C incubation, no PO_4 added.*

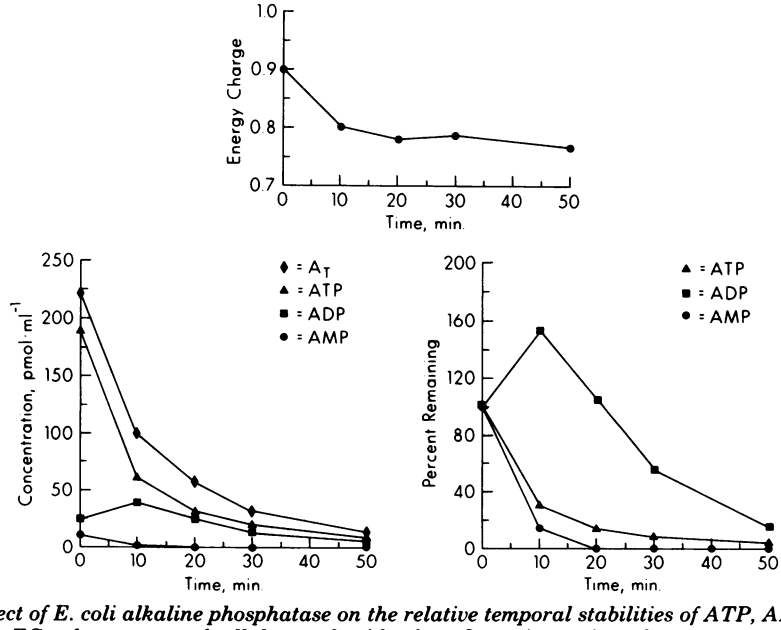


FIG. 4. *Effect of E. coli alkaline phosphatase on the relative temporal stabilities of ATP, ADP, AMP, and A_T and on the EC_A of an extract of cellular nucleotides from Serratia marinorubra.*

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ATP → 0 and ADP remains as the only nucleotide in solution. The extent to which this reaction proceeds toward completion depends upon the APase activity and the incubation period (Fig. 1D). If the sample material to be analyzed is enriched in AMP (i.e., actual $EC_A = 0.5$ to 0.6 or lower), then the initial effect of APase activity may actually be to increase the EC_A (rapid removal of AMP) followed by the subsequent decrease in EC_A depicted in Fig. 4. Consequently, depending upon the nature of the sample, APase activity may either increase or decrease EC_A ratios.

Effects of APase activity on GTP, UTP, and CTP. Figure 5 presents the results of an experiment conducted to determine the relative rates of hydrolysis of the four most ubiquitous intracellular nucleotide triphosphates. These data indicate that all four nucleotide triphosphates are hydrolyzed, with relative rates of ATP > GTP > CTP > UTP when assayed in pure solutions. Subsequent experiments conducted using mixtures of the various nucleotide triphosphates indicated that their relative reaction rates were similar to those indicated by the pure solutions. Obviously, any attempt to calculate ratios of GTP/ATP or (GTP plus UTP)/ATP in environmental samples would be frustrated by the presence of APase activity in the final sample extracts. The data presented in Fig. 5 represent the reaction specificity of APase enzyme activity from a Hawaiian prawn aquaculture sediment sample. It should be mentioned that commercially available *E. coli* APase hydrolyzed the nucleotide triphosphates in the order CTP > ATP > UTP > GTP when assayed under identical reaction conditions. This indicates that isozymes from various microbial sources might be expected to have variable reaction specificities and reactivities.

Comparison of nucleotide extraction procedures. Two currently existing techniques used for extracting ATP from sediments (e.g., H_2SO_4-EDTA and boiling PO_4 buffer) were evaluated and compared with the efficiency of extraction using an H_3PO_4-EDTA method. The criteria used for this comparison included: (i) total ATP concentration, (ii) EC_A ratios, (iii) ΔATP as a measure of the (GTP plus UTP)/ATP ratios normalized to peak ATP values [ΔATP/(peak [ATP])], (iv) an evaluation of the recovery of internal ATP standards, and (v) residual APase activity in the final sample extracts.

The first set of experiments was conducted to evaluate the efficacy of the proposed H_3PO_4-EDTA extraction method for use with sediments known to contain substantial levels of APase activity. The data presented in Fig. 6 indicate

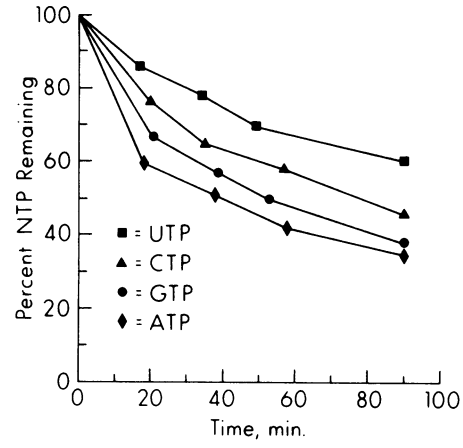


FIG. 5. *Relative temporal stabilities of a variety of nucleotide triphosphates when added to an environmental nucleotide extract exhibiting APase activity. The concentration of each nucleotide triphosphate was 200 pmol ml^{-1}.*

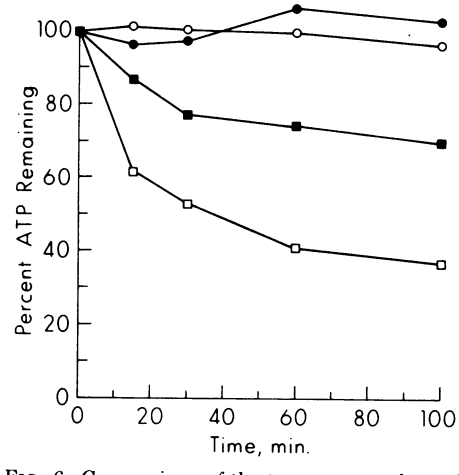


FIG. 6. *Comparison of the temperature-dependent temporal stability of ATP in H_3PO_4- and H_2SO_4-extracted Hawaiian prawn pond sediment samples. Symbols: ○, 30°C incubation, H_3PO_4 extract; ●, 4°C incubation, H_3PO_4 extract; □, 30°C incubation, H_2SO_4 extract; ■, 4°C incubation, H_2SO_4 extract.*

that the cellular ATP extracts resulting from the proposed H_3PO_4 extraction method are stable at both 4 and 30°C, whereas ATP contained within the H_2SO_4 extracts is rapidly hydrolyzed with time (Fig. 6). This indicates that the concentration of PO_4 in the final cell extracts (~150 to 200 mM) is sufficient to repress the activity of the APase enzyme present in solution. However, the concentration of PO_4 required to totally repress APase activity is dependent to a certain extent on the amount of enzyme activity present. In most of our investigations 100 mM PO_4 was sufficient to repress the in vitro activity of APase

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and to create an environment in which the NTP concentrations were stable for periods of hours to days. Long-term storage (i.e., weeks to months) of neutralized acid-extracted samples even at −20°C is not recommended since significant decreases in total adenine nucleotides and variations in EC_A ratios have been observed even in H_3PO_4-EDTA extracted samples (data not shown).

A second set of comparative experiments was conducted with prawn aquaculture sediment samples by H_2SO_4, H_3PO_4, and boiling PO_4 buffer extraction procedures (Table 1). In all cases, the ATP yield in the final extracts was greater with the H_3PO_4-EDTA procedure than with H_2SO_4-EDTA or boiling PO_4 buffer procedures. Moreover, the recoveries of the ATP internal standards were significantly lower in the H_2SO_4-extracted samples. This low recovery was due in part to adsorptive losses of ATP (as determined by [2,8-^{3}H]ATP tracer experiments) and in part due to the activity of APase in the H_2SO_4 extracts. In theory, the use of ATP internal standards should correct for adsorptive losses as well as for ATP hydrolysis by residual APase activity; however, the nonlinear kinetics of APase activity and the critical time dependence of the ATP assays resulted in inconsistent and variable results when internal standard corrections were applied. Boiling PO_4 buffer yielded a high recovery of internal ATP standards, as reported previously (9), but yielded an inefficient extraction of the microbial community as indicated by the reduced ATP yields and extremely low EC_A ratios. No APase activity was detected in either H_3PO_4 or boiling PO_4 buffer extracts. The values for ΔATP/(peak [ATP]) ratios were consistently highest in the H_2SO_4 extracts, again indicative of residual APase activity (i.e., the rate of ATP hydrolysis was greater than that of GTP and UTP hydrolysis). The ΔATP/(peak [ATP]) ratios for the H_3PO_4 and boiling PO_4 buffer procedures were substantially lower and generally comparable to each other. The data compiled in Table 1 indicate significant differences in the nucleotide fingerprint of identical

TABLE 1. *Comparison of three extraction procedures for the determination of nucleotide fingerprints in Hawaiian prawn aquaculture ponds containing APase activity*

Date	Pond	Method of extraction	ATP yield (nmol/g [dry wt] of sediment)	ΔATP/(peak [ATP]) (%)	EC_A	Residual APase activity[a]
30 August 1979	Kahuku (no. 1)	H_2SO_4	0.03 (± 0.002)[b]	155	—[c]	++
		H_3PO_4	2.52 (± 0.40)	13	—	−
30 August 1979	Kahuku (no. 3)	H_2SO_4	0.06 (± 0.004)	91	—	++
		H_3PO_4	0.57 (± 0.11)	44	—	−
13 September 1979	Kahuku (no. 1)	H_2SO_4	0.17 (± 0.016)	55	0.74	+
		H_3PO_4	1.87 (± 0.39)	7	0.56	−
13 September 1979	Kahuku (no. 11)	H_2SO_4	0.34 (± 0.072)	41	0.66	+
		H_3PO_4	2.38 (± 0.38)	6	0.60	−
25 October 1979	Kahuku (no. 3)	H_2SO_4	0.02 (± 0)	60	—	+
		H_3PO_4	0.85 (± 0.05)	25	—	−
		Boiling PO_4	0.24 (± 0.05)	30	—	−
25 October 1979	Kahuku (no. 6)	H_2SO_4	0.13 (± 0.003)	82	0.79	−
		H_3PO_4	0.88 (± 0.08)	38	0.78	−
		Boiling PO_4	0.56 (± 0.23)	28	0.45	−
25 October 1979	Kahuku (no. 9)	H_2SO_4	0.39 (± 0.06)	56	—	−
		H_3PO_4	4.12 (± 0.58)	22	0.76	−
		Boiling PO_4	3.94 (± 2.72)	10	0.43	−
26 January 1980	Pearl City	H_2SO_4	0.59 (± 0.15)	—	0.53	+
		H_3PO_4	3.67 (± 0.83)	—	0.75	−
10 April 1980	Pearl City	H_2SO_4	1.09 (± 0.12)	25	0.73	+
		H_3PO_4	11.51 (± 1.9)	6	0.81	−

[a] ++, Strong activity; +, activity; −, no activity.
[b] ±1 standard deviation.
[c] —, Not determined.

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sample materials extracted by different techniques. It is expected that the measured parameters (especially APase activity) undoubtedly vary substantially among various sedimentary habitats, and to avoid analytical interferences resulting from APase activity, samples should routinely be extracted with H_3PO_4 (or boiling PO_4 buffer for aqueous samples) to repress APase activity.

DISCUSSION

The measurement of ATP has frequently been used to detect the presence of and to estimate the biomass of viable microorganisms in diverse habitats. In recent years the ecological applications of nucleotide determinations have been extended to include measurements of EC_A ratios (31, 35, 70), GTP/ATP ratios (30, 32, 37) and rates of nucleotide metabolism and nucleic acid biosynthesis (8, 33, 66). This expanded approach, referred to as nucleotide fingerprinting (Karl, Microbiol. Rev., in press), yields corroborative data concerning in situ biomass, physiological state, and growth rates of naturally occurring microbial populations. In reviewing the literature on environmental nucleotide applications one is impressed by the overwhelming and fully warranted emphasis on comparative methodology. The extreme chemical, physical, and microbiological variability between individual habitats, especially among sedimentary ecosystems, requires that a detailed and careful comparison of methods be conducted before the selection of the most appropriate extraction procedure. No currently existing method for extracting cellular nucleotides from environmental samples has been found to be superior under all environmental conditions. Most field studies conducted to date have addressed site-specific extraction problems relating to ionic interference of the luciferase reaction (14, 36), adsorptive losses of ATP (9, 67), and incomplete extraction of cellular nucleotides resulting from the intimate association of microorganisms with nonliving organic and inorganic materials (34, 36). Although numerous investigators have reported APase activity in environmental samples from which they have attempted to extract ATP as well (6, 10, 13, 14, 44, 46, 47, 51, 60, 69), this is the first mention and presumed knowledge of the analytical interference of APase on the ATP assay procedure.

The most frequently used methods for the extraction of cellular nucleotides from environmental samples include boiling buffers, organic solvents, and mineral acids. In 1974 Davison and Fynn (15) reported the occurrence of an active APase enzyme activity in neutralized perchloric acid extracts of the bacterium *Bacillus brevis*. Lundin and Thore (42) and D. M. Karl and K. H. Nealson (36a) have also reported heat-stable (100°C) APase activities in various bacterial cell extracts. The data presented in this report demonstrate the general occurrence of a similar heat- and acid-stable enzyme activity in many diverse environmental samples. Clearly, quantitative nucleotide fingerprinting of these APase-rich microbial assemblages cannot be achieved by existing experimental procedures (Fig. 1, 3, 4, and 5; Table 1).

More recently, several investigators have suggested the use of PO_4-containing menstrua as a means for extracting ATP from microbial assemblages in nature (9, 12a, 26, 67). The increased ATP yields were unanimously interpreted to be the result of a reduction in the adsorptive loss of ATP in the presence of high concentrations of exogenous PO_4 (range of initial PO_4 concentrations from 10 to 250 mM). In light of the data presented in this report, in vitro repression of APase activity by exogenous PO_4 may have also contributed to the observed increased yields of ATP. It should be emphasized, however, that our present study indicated that a final extract concentration of ~100 mM PO_4 was generally required for the repression of APase from most environmental samples. By comparison, the final concentrations of PO_4 in the sample extracts of previous studies varied from 1 mM (67) to 32 to 52 mM (9), levels insufficient for the in vitro repression of typical residual environmental APase enzyme concentrations. Additional methods exist for the repression and/or inactivation of APase activity including arsenate, fluoride, tartrate, urea, cadmium, EDTA, and autoclaving; however, a detailed evaluation of these various options was not undertaken during the course of this study. Caution should be taken in the addition of chemicals intended to repress APase activity to ensure that these reagents do not adversely affect firefly luciferase activity or any of the enzymes required for nucleotide fingerprinting (e.g., pyruvate kinase, myokinase, UDP-glucose pyrophosphorylase, hexokinase, glucose 6-phosphate dehydrogenase, nucleoside diphosphokinase, etc.).

The currently proposed method of nucleotide extraction using 1 M H_3PO_4 as the principal reagent (final extract concentration of PO_4, >150 mM) combines the unique advantages previously discussed for cold acid extraction procedures (34, 36) with the requirement for a relatively high PO_4 concentration necessary to eliminate adsorptive ATP losses and to repress residual APase activity. Furthermore, the sparing solubility of $Ca(H_2PO_4)_2$ reduces the cation interference on the luciferase reaction in neutralized H_3PO_4 extracts of $CaCO_3$-rich sedimentary

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materials. Little or no co-precipitation of ATP with $Ca(H_2PO_4)_2$ was observed in controlled laboratory experiments (data not shown). However, internal standards can and should be used to monitor this possibility. The residual PO_4 concentration does not affect the activities of the enzymes required for nucleotide fingerprinting. Light emission from the firefly bioluminescence reaction is reduced relative to tris(hydroxymethyl)aminomethane-buffered extracts, an effect resulting from a general ionic strength inhibition of luciferase. Provided that standards are prepared in ionic solutions identical to those of the sample extracts, this effect is of no consequence to the precision, accuracy, or reproducibility of the assay procedure. One final advantage of the H_3PO_4-EDTA nucleotide extraction methods over the more commonly used H_2SO_4-EDTA procedure is the elimination of SO_4^{2-} ion interference of the uridine diphosphate-glucose pyrophosphorylase reaction of the GTP assay procedure (29, 32). This mitigates the necessity of an ~5-fold dilution of the sample extract, thereby significantly increasing the lower limit of sensitivity for the GTP assay procedure.

The use of internal standards is strongly recommended to correct for losses (or "apparent" losses) of cellular nucleotides arising from chemical hydrolysis, adsorption, co-precipitation, organic and inorganic ionic interference, turbidity, color quenching, pH spectral shifts, and other factors. Recently, it has been suggested that ATP radiolabeled with ^{14}C, ^{3}H, or ^{32}P be used to monitor and correct for the cumulative ATP losses and analytical interferences of the ATP assay. In theory, this may be a valid and seemingly accurate method of assessment, but in practice there are several substantial areas for concern. First, the liquid scintillation counting of radiolabeled ATP will only allow a quantitative assessment to be made of "real" ATP losses (i.e., adsorptive or co-precipitation losses) since the apparent losses of ATP resulting from inhibition of the firefly luciferase enzyme will not be accounted for. Furthermore, if the label is contained within the purine ring (i.e., [2-^{3}H]-, [2,8-^{3}H]-, or [U-^{14}C]ATP) or the ribose moiety, hydrolysis of ATP will not be detected. Forsberg and Lam (20) have reported a serious discrepancy between the recovery of [U-^{14}C]ATP and nonradioactive ATP standards from samples of strained rumen fluid (23 to 64% recovery from non radioactive ATP versus 91 to 93% recovery from [U-^{14}C]ATP). This result might be expected if the sample materials contained APase activity similar to that described in this report.

The proposed application of the ATP-dependent firefly luciferin-luciferase bioluminescence assay to detect and quantify APase provides an analytical system with a much-increased sensitivity when compared with previously described methods. The 3-O-methylfluorescein phosphate fluorometric assay devised by Hill et al. (23) and modified by Perry (50) for seawater analyses can effectively detect $\sim 1 \times 10^{-11}$ mol of PO_4^{3-} liberated per liter of seawater per min; however, beyond 120 min nonenzymatic hydrolysis of the 3-O-methylfluorescein phosphate was detected. By comparison, and under optimal reaction conditions, the ATP assay should be capable of detecting $\sim 2 \times 10^{-13}$ mol of ATP hydrolyzed $liter^{-1}$ min^{-1} with no restrictions on the length of the incubation period. An additional advantage of the proposed ATP method for measuring APase activity is the elimination of background or blank determinations. With certain sediments we observed highly colored extracts (presumably fulvic or humic acids) which would undoubtedly interfere with both spectrophotometric and fluorometric analyses. The increased sensitivity for the detection and measurement of phosphomonoester hydrolysis will enable experiments to be conducted at substrate levels comparable to those found in nature (≤ 0.01 μM), thus yielding more ecologically relevant data concerning APase-dependent P_i fluxes in nature.

It should be emphasized that we have no absolute proof that the enzyme whose activity we monitored in this study was actually APase. This is also true of all environmental studies published to date. We do, however, have several indirect lines of evidence, including: (i) a similarity in the kinetics, reactivity, and substrate specificity of our environmental extracts to those of commercially available *E. coli* alkaline phosphatase (Fig. 1 and 5), (ii) comparative data on the remarkable heat and acid stability of the enzyme activity from environmental extracts and commercial sources, and (iii) in vitro repression of the residual enzyme activity by exogenous orthophosphate (Fig. 3). The combined stability, specificity, and reactivity characteristics of this residual enzymic activity strongly indicate APase activity and eliminate the possibility of acid phosphatase, ATPase, and nucleotidase enzyme activities.

In light of the frequent but unpredictable spatial and temporal occurrence of APase activity in aquatic ecosystems and the observed effects on the measured nucleotide fingerprints of natural microbial assemblages, it is difficult if not impossible to determine the magnitude of this analytical interference on previously published environmental nucleotide data. However, the quantitative results of numerous ecological studies are especially questionable due to the fact that ATP values were reported from sample

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materials known to contain substantial APase activity (6, 10, 13, 14, 46, 47, 51, 60, 69). Furthermore, it has been reported that P_i limitation in microorganisms results in significant decreases incellular ATP pool levels to values as low as ~10% of the nutrient-saturated control steady-state concentrations (10, 14, 24, 51, 59). Cavari (10) reported that these low intracellular ATP concentrations did not affect the division rate of the microorganisms, and even with a C/ATP ratio of 1,700, 21% of the phytoplankton population in Lake Kinneret were growing at a rate of one division per day. Although it is well established that depletion of P_i normally induces a cessation of energy metabolism, it is difficult to reconcile ATP pool decreases of 90% in growing cells, especially considering the role of ATP in the regulation of cellular metabolism. Karl (in press) has speculated that many of these data may be in error due to the occurrence of APase activity in the P_i-deficient cultures. Cavari (10) has indicated that measurements of in situ C/ATP ratios may be used as quantitative indicators of P deficiency in natural ecosystems. However, the reported occurrence of significant APase activities in Lake Kinneret (4) coincident with Cavari's high C/ATP ratios and P limitation may simply be the result of ATP hydrolysis in the sample extracts by residual APase enzyme activity. A recent investigation of cellular ATP levels in P_i-limited phytoplankton which was conducted in full awareness of this potential source of analytical interference indicated that the P_i-limited ATP cell quotas (i.e., ATP per cell) were equal to or greater than the cell quotas under NO_3^-, NH_4^+, or light limitation (B. L. Hunter and E. A. Laws, submitted for publication).

Total environmental APase activity is undoubtedly comprised of enzymes from bacteria, algae, fungi and yeasts, protozoans, and metazoans. The data reported in Fig. 5 and Table 1 may be expected to vary depending upon the predominant source(s) of the APase activity. Berman (3) found seasonal variations in the pH optimum, substrate specificity, and K_m for total APase activity in Lake Kinneret, a result which he interpreted as representing a variable contribution of APase isozymes from various microbial taxa. Diurnal changes in the pattern of APase activity have also been reported from natural aquatic environments (3, 51).

In regard to the ecological significance of APase activity, it is useful to examine the geographical, spatial, and temporal variations in the occurrence of environmental APase. In the present study, APase was detected in the water column and sediments of certain habitats (e.g., Hawaiian prawn aquaculture ponds) at certain times of the year and only in the sediments of additional ecosystems (e.g., New Harbor, Antarctica) and was notably absent from selected environmental samples (e.g., waters and coastal sediments of the southern California Bight, the Black Sea water column and abyssal sediments, the Columbian Basin of the Caribbean Sea, etc.). Previous investigators have attempted to correlate APase activity with P_i limitation of the autochthonous microbial assemblages (4, 50, 51), or with high microbial biomass and eutrophication (27, 38, 47, 53, 65). As discussed earlier, APase activity may be constitutive in some microorganisms or totally absent from others and, consequently, need not be positively correlated with P_i limitation. Furthermore, in the present study there was no obvious correlation between total microbial biomass and APase activity. Nevertheless, we do endorse the interpretation that the appearance of APase activity (especially when expressed as specific APase activity, activity per unit of cell biomass) does indeed reflect environmental conditions of PO_4 deficiency. Since the majority of the data in the literature indicate that APase is produced in copious amounts when P_i becomes limiting in culture media, and since its synthesis is generally repressed by the readdition of P_i, the activity of this enzyme appears to be related to the cells' ability to utilize soluble organic phosphorus as a source of cellular P. This interpretation is further supported by the intracellular localization of APase activity. In bacteria, the enzyme is located in the periplasmic space, and in yeast and algae it is generally cell surface associated. This cellular localization is consistent with the presumed ecological function of the nonspecific hydrolysis of soluble organic phosphorus under conditions of P_i deficiency. Kuenzler and Perras (40) have demonstrated that the APases of many species of phytoplankton hydrolyze organic P compounds at the outer cell membrane, transport the P and leave the remaining organic carbon moiety outside the cell.

Additional studies have indicated that APase activity is only detected in waters where the N/P ratio was $\geq$30 (57), a value similar to the point of transition between N limitation and P limitation (55). Moreover, Møller et al. (45) have demonstrated that the production of APase in marine diatoms was clearly dependent upon the N/P ratio regardless of the initial PO_4 concentration. However, it should be emphasized that it is the intracellular PO_4 concentration and not that of the external environment which regulates enzyme activities. Availability of exogenous PO_4 is not always related to its concentration, espe-

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cially in sediments where sorption reactions, specifically the well-documented role of Fe, may represent a significant removal mechanism. Fitzgerald (17) reported that $Fe(OH)_3$ strongly adsorbed P_i, and less than 1% of this adsorbed P_i was available for biological uptake. Williams et al. (72) indicate that certain lake sediments may adsorb up to 5 mg of P_i g^{-1} sediment and may therefore be important in regulating total productivity of the ecosystem. The preferred chemical determination for P_i (i.e., molybdenum blue assay) requires acidification of the sample, which favors desorption and consequently results in an overestimation of the true P_i levels (11). The observation that clay sediments contained more APase activity than sand (2, this study) may be related to this sediment-specific sorption potential. This is especially true for the Antarctic samples. The sediments at New Harbor were a mixture of fine-grain glacial flour and streamborne sediment from the adjacent Taylor Valley and contained significant APase activity despite the extremely high concentrations of P_i in the overlying waters (~3 to 4 μmol liter^{-1}). By comparison, Cape Armitage sediments were comprised of large-grained volcanic rubble and ash derived from Ross Island. Although we have not measured P_i sorption of these respective sediments, it is suggested that the sorption of New Harbor sediments is much greater than that of sediments from Cape Armitage. The same scenario can be offered to explain the presence of APase activity in the abyssal red clays of the North Pacific Ocean and the complete absence of APase activity in intertidal beaches of southern California and coarse subtidal coastal sediments, even though the total microbial biomass in the latter sediments may be higher by a factor of 1,000 or more.

Microorganisms live in microenvironments which are generally defined on scales of micrometers or millimeters. The chemical composition and nutritional status of these microenvironments may be totally distinct from those of the ambient macroenvironments. The occurrence and regulation of environmental APase activity should be useful as a sensitive bioassay or metabolic indicator of microscale P_i limitation of microbial assemblages in nature.

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