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Fluorometric Determination of Adenosine Nucleotide Derivatives as Measures of the Microfouling, Detrital, and Sedimentary Microbial Biomass and Physiological Status

WILLIAM M. DAVIS AND DAVID C. WHITE*

Department of Biological Science, Florida State University, Tallahassee, Florida 32306

Adenosine, adenine, cyclic adenosine monophosphate (AMP), AMP, nicotinamide adenine dinucleotide, adenosine diphosphate, and adenosine triphosphate (ATP) were recovered quantitatively from aqueous portions of lipid extracts of microfouling, detrital, and sedimentary microbial communities. These could be detected quantitatively in the picomolar range by forming their 1-N^6-etheno derivatives and analyzing by high-pressure liquid chromatography with fluorescence detection. Lipid extraction and subsequent analysis allowed the simultaneous measurement of the microbial community structure, total microbial biomass with the quantitative recovery of the adenine-containing cellular components, which were protected from enzymatic destruction. This extraction and fluorescent derivatization method showed equivalency with the luciferin-luciferase method for bacterial ATP measurements. Quick-freezing samples in the field with dry ice-acetone preserved the ATP and energy charge (a ratio of adenosine nucleotides) for analysis at remote laboratories. The metabolic lability of ATP in estuarine detrital and microfouling communities, as well as bacterial monocultures of constant biomass, showed ATP to be a precarious measure of biomass under some conditions. Combinations of adenosine and adenine nucleotides gave better correlations with microbial biomass measured as extractable lipid phosphate in the detrital and microfouling microbial communities than did ATP alone. Stresses such as anoxia or filtration are reflected in the rapid accumulation of intracellular adenosine and the excretion of adenosine and AMP into the surrounding milieu. Increases in AMP and adenosine may prove to be more sensitive indicators of metabolic status than the energy charge.

Adenosine triphosphate (ATP) has been widely utilized as a measure of the microbial biomass. The extraction of ATP by boiling tris(hydroxymethyl)aminomethane (Tris) buffer and its subsequent assay by firefly luciferin-luciferase (17) have been very successful when applied to water column samples. When this method is applied to sediments or the microfouling community, modifications in the methodology are required (1, 2, 23, 28, 31, 40).

There are at least two problems in the recovery of adenosine nucleotides from environmental samples. The first is the quantitative extraction of the adenosine nucleotides from the organisms and the substrate. A control for recovery using added authentic ATP is necessary to correct for errors such as hydrolysis and incomplete extraction. The second problem involves the inhibition of the luciferin-luciferase assay system by reagents in the extraction or sample components, particularly metals, that contaminate the extraction of the nucleotides. To overcome the latter problem, the extracts are diluted 100-fold, and ethylenediaminetetraacetic acid (EDTA) is titrated into the extraction mixture to chelate the metals (23, 40). The addition of EDTA must be carefully controlled, as magnesium is essential for the luciferin-luciferase activity. Another solution to the extracted contaminants is to bind the nucleotides to charcoal (16) or ion-exchange columns (1, 29) to allow washing before elution. The problems of quantitative recovery from charcoal have been discussed (22). The luciferin-luciferase enzyme itself must be aged to minimize the effects of contaminating nucleotide triphosphate (41), titrated with luciferin for maximum activity (21), and purified to prevent reactivity with non-adenosine nucleotide triphosphate (19).

The need to assay the biomass and metabolic status of the microfouling community in aluminum pipes prompted the development of an alternative assay procedure. The acid extractions of the microfouling community of aluminum pipes release contaminating metals that require removal before the luciferin-luciferase assay can be used (1). To be useful in environmental samples, the alternative assay proce-

dures must be sensitive to ATP in the picomolar range. This precludes use of the ultraviolet absorbance of the purine rings for detection. The formation of a third conjugated ring by reaction with chloroacetaldehyde gives the fluorescent 1-N^6-etheno derivatives (ϵ-derivatives) which are detectable at the requisite sensitivity (30, 39). This derivatization provides additional advantages. Only purines with a primary amine in the 6-position react. Cytosine, which is present at lower concentrations in the microbes, does not form an ϵ-derivative efficiently at the conditions used for derivatization (27, 39). Guanine nucleotides are not reactive (38); therefore, the reaction is essentially specific for adenosine derivatives.

A second advantage of the fluorescent adenosine nucleotide assay is the release from the inhibition of the luciferin-luciferase assay system. This allows the use of sufficient chloroform to rapidly terminate cellular metabolism and release the adenosine nucleotide pool (10). Thus, a single extraction which quantitatively releases cellular lipids as well as the nucleotides can be used. Analysis of the extractable lipids has been shown to provide information on the biomass and community structure of the detrital and sedimentary microbiota (7, 15, 24, 37, 42–47).

The third advantage of the fluorescent ϵ-nucleotide assay lies in the easy determination of all of the adenosine-containing components in the same analysis. The determination of adenosine monophosphate (AMP) and adenosine diphosphate (ADP) by the luciferin-luciferase method requires enzymatic conversion of the nucleotides to ATP (11). These enzymatic processes are subject to the same types of inhibitions as the luciferin-luciferase assay. By directly isolating and measuring the nucleotides, the controls for luciferase inhibition are eliminated. Adenine, adenosine, and cyclic AMP would require different reactions. There are several advantages to measuring all adenosine nucleotides. ATP has extraordinary metabolic reactivity. The total ATP content of growing bacterial monocultures turns over many times per second (18, 33). The ATP content of nongrowing bacteria can change two- to fivefold very rapidly (26, 41). This lability suggests that combinations of adenosine components might be a better measure of microbial biomass than the ATP content. If the metabolic lability of ATP can be "quenched," then the determination of the three nucleotides allows calculation of the energy charge (3), which is a potentially useful measure of the metabolic states of the microflora (20, 25, 36, 48). Because this method involves actually isolating the adenosine-containing components from the cells, pulse-chase radioactive labeling experiments that yield information about the kinetics of nucleotide formation and degradation can be done.

This study describes the extraction, derivatization, separation, and assay of the ϵ-derivatives of the adenine-containing components from detrital, sedimentary, and microfouling environmental samples.

MATERIALS AND METHODS

Materials. Highest-purity commercially available solvents (nanograde or glass distilled; Burdick and Jackson, Muskegon, Mich.) were used. Firefly luciferin-luciferase, enzymes, nucleotides, ϵ-nucleotides, and biochemicals were supplied by Sigma Chemical Co., St. Louis, Mo., unless otherwise noted. High-pressure liquid chromatography (HPLC)-grade potassium phosphate and sodium acetate (Fisher Scientific Co., Pittsburgh, Pa.) and deionized, and then glass-distilled, water were used in preparing the extraction and HPLC buffers.

Extraction of nucleotides. A modified Bligh and Dyer (5) extraction procedure (46) was used to recover the lipids and the adenosine nucleotides. To prevent enzymatic degradation of the ATP for energy charge measurements in fouled pipes and in detrital or sedimentary samples, the samples were placed in contact with a one-phase solution of 10 mM potassium phosphate buffer, pH 6.1, containing 0.59 mM EDTA, methanol, and chloroform (0.8:2:1, vol/vol/vol) and mixed thoroughly. After 15 min at 25°C, equal volumes of water and chloroform were added to a final buffer-methanol-chloroform content of 0.9:1:1 (vol/vol/vol). The suspension was then mixed thoroughly and allowed to stand for 15 min. The chloroform layer was filtered through fluted Whatman 2V filter paper and analyzed for phosphate and lipid composition (44, 46). A 10-ml portion of the aqueous phase was pipetted into a 250-ml round-bottom flask and quick-frozen on the walls in a dry ice-acetone bath. The methanol was removed by lyophilization with a liquid nitrogen-cooled trap inserted between the samples and the Thermovac (Copiague, N.Y.) lyophilizer. The water was then removed with the lyophilizer, and the samples were stored at −70°C before derivatization. If enzymatic hydrolysis of ATP proved to be a problem in particular samples, the EDTA content of the initial extracting buffer could be increased 100-fold without affecting the subsequent analysis.

To extract 10-in. (25.4-cm) sections of aluminum pipes for analysis of the microfouling community, one end was plugged with a Teflon–stainless-steel expandable plug (6), and 57 ml of a mixture containing 12 ml of EDTA-phosphate buffer, 30 ml of methanol, and 15 ml of chloroform was added, the upper end was closed, and the pipe was shaken. After 15 min one end was opened, the mixture was transferred to a separatory funnel, and 15 ml of water and 15 ml of chloroform were added. A portion of the aqueous phase was removed and lyophilized as described above. The chloroform phase was used for lipid analysis as described (44, 46).

Adenine nucleotides analyzed by the luciferin-luciferase methods were extracted with rapid dilution into perchloric acid, followed by neutralization with ice-cold KOH, separation from $KClO_4$, and dilution (11)

or dilution into boiling Tris buffer containing EDTA (23). These are the most effective extraction methods when using the enzymatic assays (31).

Preparation of chloroacetaldehyde. Chloroacetaldehyde dimethyl acetal, 99% (Aldrich Chemical Co., Milwaukee, Wis.) in 100-g bottles is stable at room temperature. The free aldehyde was prepared by gently refluxing in a 1:5 (vol/vol) mixture of diacetal–5% (vol/vol) aqueous H_2SO_4 for 1 h. After cooling, the flask was transferred to an all-glass distilling apparatus, a partial vacuum was applied, and the free aldehyde was collected in a dry ice-acetone-cooled receiver. The vacuum was adjusted so that the chloroacetaldehyde distilled at 45 to 53°C. The distillate was a 1 to 1.6 N chloroacetaldehyde solution in water containing methanol at a pH greater than 4.5 (by pH paper). If the pH were lower than 4.5, it was brought to 4.5 with 1 N NaOH. With care in the distillation essentially no sulfuric acid was transferred and pH adjustment was not necessary. The chloroacetaldehyde can be stored in a glass-stoppered flask for 1 month at 4°C without loss of reactivity. This preparation is a modification of that described by Secrist et al. (39). Chloroacetaldehyde is a mutagen (32), so the distillation and subsequent derivatization procedures should be conducted in a fume hood.

Formation of the 1-N^6-ethenoadenosine derivatives. The lyophilized samples were quantitatively transferred from 250-ml round-bottom flasks to 13-mm Teflon-lined screw-capped test tubes, using three portions of water (2, 1, and 1 ml for each sample). Then 2.0 ml of the chloroacetaldehyde was added, and the tube was capped, mixed on a Vortex mixer for 30 s, and incubated in a 60°C water bath for 2 h. The derivatization was quantitative for AMP, ADP, ATP, adenosine, adenine, nicotinamide adenine dinucleotide, and cyclic AMP as reported previously (49). The kinetics of derivatization of the adenine nucleotides is illustrated in Fig. 1. The pH is a compromise between the alkaline lability of chloroacetaldehyde and the ionization of the primary amino group at the 6-position of the adenosine ring. Under these conditions, cytosine, but not guanine, has slight reactivity (27, 35, 38). The solvents were removed, and the sample was taken to dryness under reduced pressure at 35°C in a rotary evaporator (Calab model 5101, California Laboratory Equipment Co., Emeryville, Calif.). The derivatized samples were redissolved in 1 ml of water, and 0.1 ml was transferred to the high-pressure liquid chromatographic injector.

The 1-N^6-etheno nucleotide derivatives (ε-derivatives) have the ultraviolet absorbance spectra, fluorescence excitation, and emission spectra illustrated in Fig. 2 and the chromatographic mobility on Eastman cellulose sheets in isobutyric acid-ammonia-water (75:1:24, vol/vol/vol) (39) of the authentic compounds. The ε-adenosine was recovered and chromatographed with the authentic standard at R_f 0.60 on cellulose thin-layer plates (Eastern Chromatogram no. 13255), using distilled water as the solvent.

HPLC. In this study derivatives were separated by HPLC on a 4.60-mm-inside diameter stainless-steel column 25 cm long packed with Whatman Partisil 10 SAX (a microparticulate strong anion-exchange resin of 10-μm particle size) with a guard column (5 by 0.56 cm) packed with Whatman AS pellicular SAX resin. The chromatograph was operated at a pressure of 80 kg/cm² with a flow rate of 80 ml/h. It has proved prudent to change the guard column packing after 20 runs with environmental samples.

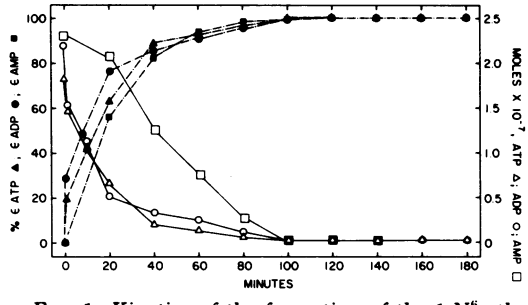

FIG. 1. *Kinetics of the formation of the 1-N^6-ethenoadenosine nucleotides (indicated as ε) from AMP, ADP, and ATP during incubation at pH 6.1 and 60°C in the presence of freshly distilled chloroacetaldehyde. The ε-nucleotides were detected by their fluorescence and the underivatized nucleotides were detected by their absorbance at 260 nm after separation by ion-exchange HPLC.*

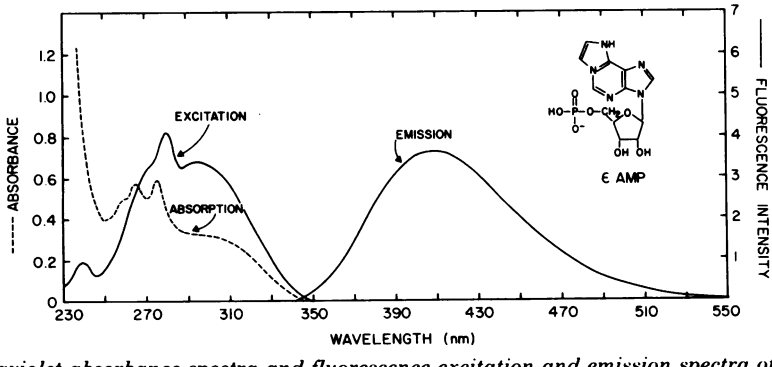

FIG. 2. *Ultraviolet absorbance spectra and fluorescence excitation and emission spectra of ε-AMP measured at pH 4.6 at an ionic strength of 0.01 M (conditions of elution). The spectra of ε-ADP and ε-ATP are essentially similar, but with the expected lower molar yields.*

The apparatus consisted of a Rheodyne model 70-10 injection valve with a 100-μl sample loop (Rheodyne Inc., Berkeley, Calif.) and the ISCO model 1440 dual-syringe pump liquid chromatograph with a Dialagrad controller for gradient elution (Instrument Specialties Co., Lincoln, Neb.). The derivatives were detected with a Fluorochrome detector, using a deuterium excitation source and quartz optics (Varian Associates, Inc., Sunnyvale, Calif.). The quartz flow cell has a 25-μl (12.5-μl fluorescing volume), 2-mm path length. A 280-nm interference filter was in the excitation beam, and filters passing 420 to 560 nm were in the emission beam. Maximum sensitivity was achieved by keeping the photomultiplier activated continuously and allowing a 1-h warm-up for the deuterium lamp. The components of the HPLC were connected with 316 stainless-steel microbore tubing, 0.007-in. (ca. 0.018-cm) inside diameter, and zero dead volume fittings (Whatman Inc., Clifton, N.J.). The nucleotide derivatives were eluted with a programmed 8-min isocratic elution with 10 mM KH_2PO_4 plus 20 mM sodium acetate (pH 4.5), followed by a 32-min linear gradient with the first solvent and 250 mM KH_2PO_4 plus 500 mM sodium acetate (pH 4.5). This is essentially a modification of the system reported by Brown (8, 14). The chromatographic separation of an environmental sample is shown in Fig. 3. The fluorometric detector is interfaced with a Hewlett-Packard 18652A analog to digital converter into a programmable 21MX data system.

Recently we have separated the bonded ϵ-adenosine nucleotides on a reversed-phase octadecylsilane column (8-mm inside diameter by 10 cm long) in the Radial Compression Module (RCM-100), using an isocratic solvent of 10% (vol/vol) acetonitrile-water containing 5 mM tetrabutylammonium phosphate counter ion, pH 7.5 (PIC reagent A, Water Associates, Milford, Mass.) at a rate of 6 ml/min in 12 min per sample. This greatly speeds up the assay and simplifies the apparatus because no gradient or high-ionic-strength elutants are required.

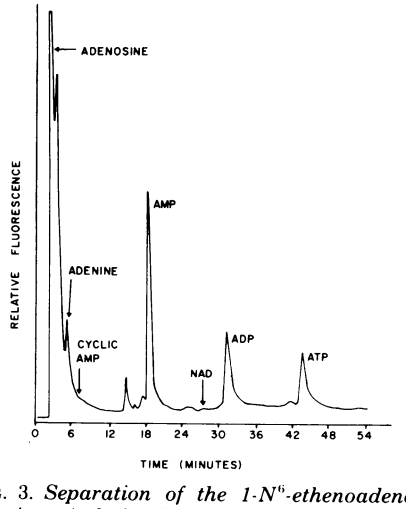

FIG. 3. *Separation of the 1-N^6-ethenoadenosine derivatives (ϵ-derivatives) from an environmental sample on a 10-cm microparticulate strong anion-exchange resin. The concentrations of nucleotides were 8.1 × 10^{-12} mol of ϵ-adenosine, 4.0 × 10^{-12} mol of ϵ-AMP, 2.5 × 10^{-12} mol of ϵ-ADP, and 3.3 × 10^{-12} mol of ϵ-ATP. The elution times for authentic ϵ-adenine, cyclic ϵ-AMP, and ϵ-nicotinamide adenine dinucleotide (ϵ-NAD) are indicated.*

Enzymatic assay of adenosine nucleotides. The rapid sampling of respiring *Staphylococcus aureus* suspensions involved use of a 50-ml syringe, the barrel of which contained an oxygen electrode and from which rapid removal of aliquots into perchloric acid could be achieved (11).

To compare the equivalency of the enzymatic and fluorometric methods of ATP analysis, portions of exponentially growing *Escherichia coli* were pipetted into boiling Tris-EDTA buffer and then analyzed as described (23). The analyses were performed by P. A. LaRock and D. A. Bare. Replicate samples of the *E. coli* culture were simultaneously extracted for analysis by the fluorometric procedure.

Bacterial monoculture. *S. aureus* S-2 was grown to exponential growth phase in nutrient broth, washed, and resuspended as described (11). *E. coli* strain B (the gift of J. H. Stuy, Florida State University, Tallahassee) was grown to stationary phase in nutrient broth, centrifuged, washed, quick-frozen, and lyophilized or harvested in exponential phase as described in Results.

Detrital samples. Strings of 100, 5-cm^2, 2.5-mil Teflon were incubated for 4 weeks in Alligator Harbor, North Florida, in the fall of 1979. The strips were transferred to the laboratory and incubated in aerated estuarine water at a salinity of 12‰, to which 0.05% (wt/vol) nutrient broth was added every other day. The strips were sampled, subjected to an argon atmosphere, and then reoxygenated to determine the effects on the energy charge. Oxygen content was measured polarographically (34).

Microfouling community. Samples of the microfouling community were analyzed in 5052 aluminum pipes of 1-in. (2.54-cm) internal diameter exposed to seawater flowing at 6 ft. (ca. 183 cm)/s in the Ocean Thermal Energy Conversion test facility at Panama City, Fla., or at the Florida State University Marine laboratory (6).

To measure the effects of anoxia and recovery from anoxia, 10-in.-long sections of the pipes were cut with a pipe cutter while the seawater was running to prevent heating stress to the fouling community. Some sections were filled with argon-purged seawater and analyzed after 10 and 45 min. Other sections were filled with argon-purged seawater, allowed to stand for 45 min, and then replaced in the stream of flowing seawater and sampled by cutting 10-in. pipe sections from the flowing stream at the designated intervals. Control samples were taken from the pipes with the water flowing. Anaerobiosis was checked by inserting a Clark oxygen electrode into the pipes. The pipe samples were stoppered, quick-frozen in a dry ice-acetone bath, and returned to the laboratory for analysis.

Determination of sedimentary extracellular adenine compounds. Several cores of marine sands from the Florida State University Marine Laboratory estuary were mixed in 1/10 volume of seawater, and 5-

g replicate samples were removed. Half of the 5-g sediment samples were mixed with 5 ml of filter-sterilized seawater with the Vortex mixer for 3 min and then centrifuged at 25,000 × *g* for 15 min at 4°C. The supernatant and suspended sediment were poured into a chloroform-methanol solution to form the one-phase chloroform-methanol-water extraction. The pellet was extracted. The adenine components were recovered from aqueous portions of the lipid extractions of the uncentrifuged sediment, the supernatant, and the pellet of the centrifuged samples and analyzed fluorometrically.

RESULTS

Sensitivity and linearity of the fluorometric assay. Concentrations of 3.8×10^{-13} mol of ϵ-adenosine, 2.7×10^{-13} mol of ϵ-AMP, 5.6×10^{-13} mol of ϵ-ADP, and 1.9×10^{-12} mol of ϵ-ATP gave peak areas of at least 100 integrator units above background, with an amplitude at least 2.5 times the noise level. The response to the ϵ-adenosine derivatives was linear with the concentration between 10^{-12} and 10^{-10} mol/12.5-μl fluorescing volume.

Chromatographic separation of the ϵ-nucleotides from an environmental sample is illustrated in Fig. 3. The efficiency of the column calculated as 5.54 (retention time/half peak width)^2 for ϵ-ATP was 7,000 theoretical plates.

Reproducibility. Replicate samples of adenosine, AMP, ADP, and ATP standards and of ATP from 10-in. aluminum pipes gave reproducibilities with standard deviations of 9 to 12% of the means. The recoveries (± standard deviation) of added ATP, ADP, and AMP were 97.5 ± 3.9, 104 ± 3.0, and 94 ± 10%, respectively (n = 6). The recovery of the added nucleotides, using the modified lipid extraction described above, was as efficient as with classical extraction methods of perchloric acid (12), cold sulfuric acid (23), chloroform and base (40).

Extraction procedure. In an extraction procedure it is necessary to protect the ATP from hydrolytic enzymes that may be present in the sediment or activated in the recovery from the microbiota. The addition of sufficient EDTA to inactivate adenosine triphosphatases and shortened exposure to the standard lipid extraction procedure used for detrital, microfouling, and sedimentary samples allowed the recovery of added adenosine nucleotides extracted in the presence of *E. coli* preparations or in the presence of resuspended *E. coli* that had been lyophilized previously (Table 1). The nucleotides in fresh *E. coli* can also be recovered quantitatively from marine sands (Table 1).

Preservation of the energy charge. With many environmental samples analysis in the field is not possible; therefore, preservation can be important. It proved possible to preserve the energy charge in microfouling samples by draining the pipe section, stoppering the ends of the tubing with rubber stoppers, and rapidly submerging the pipe section in a bath of dry ice-acetone for 3 min. Storage at −70°C after the initial treatment for up to 1 month had no effect on the recovery. Ten-inch sections of aluminum pipes through which seawater had been pumped at 2 ft. (ca. 61 cm)/s for 6 weeks were cut and extracted or frozen within 15 s after removal from the flowing water stream. The pipe samples extracted immediately without freezing contained, in moles × 10^{-11}/10-in.-long pipe sample (± standard deviation): AMP, 1.85 ± 0.18; ADP, 4.03 ± 0.22; ATP, 8.57 ± 0.62; and total adenine nucleotides, 14.4 ± 0.79. In quick-frozen samples, these values were: AMP, 1.85 ± 0.31; ADP, 4.32 ± 0.28; ATP, 8.62 ± 0.44; and total adenine nucleotide, 14.77 ± 0.83. The energy charge for control and frozen samples was 0.73 ± 0.15 and 0.73 ± 0.17. The lipid phosphate for the control

TABLE 1. *Recovery of adenosine nucleotides measured fluorometrically from E. coli preparations and E. coli plus marine sands*

Sample	Added nucleotide (mol × 10^{-9})	mol × 10^{-9} (±SD)[a]			% Recovery[c]
		Recovered nucleotide[b]	*E. coli*	Total nucleotide (standard + sample)	
Fresh *E. coli* sample	ATP (20)	19.7 (0.59)	2.36 (0.31)	22.7 (0.13)	96
Standards plus lyophilized *E. coli*	AMP (3.08)	2.88 (0.30)	6.29 (0.37)	8.76 (0.19)	94
	ADP (11.67)	10.6 (0.46)	12.0 (0.88)	23.7 (0.93)	100
	ATP (9.38)	10.2 (0.27)	5.66 (0.19)	13.3 (0.74)	86
Fresh *E. coli* plus marine sand	AMP		12.1 (1.1)	11.2 (0.091)	93
	ADP		12.0 (0.96)	11.6 (0.082)	95
	ATP		19.1 (0.14)	18.7 (0.065)	98

[a] SD, Standard deviation. n = 8.
[b] Nucleotides added to phosphate buffer plus EDTA, derivatized, and assayed fluorometrically.
[c] Actual recovery (standards added + nucleotides in sample).

was 0.96 ± 0.15 μmol per sample, and that for the frozen samples was 0.96 ± 0.17 μmol per sample.

Equivalency of fluorescent derivatization and luciferin-luciferase measurement of ATP. *E. coli* B growing in nutrient broth with a doubling time of 42 min was simultaneously pipetted into boiling Tris buffer for luciferin-luciferase assay or into phosphate-EDTA buffer for immediate chloroform-methanol extraction with subsequent derivatization and HPLC assay. The density at harvest was 0.2 ± 0.02 μmol of lipid phosphate per ml. Enzymatic assay gave 1.25 ± 0.02 × 10^{-10} mol of ATP per ml compared with 1.26 ± 0.04 × 10^{-10} mol of ATP per ml assayed fluorometrically. Each value represents six replicates. Portions of the culture were filtered through 0.45-μm membrane filters (Millipore Corp., Bedford, Mass.), and the filters were washed twice with 2 volumes of sterile nutrient broth. The vacuum was <10 mmHg (ca. <1.3 kPa). The organisms on the filters were extracted and assayed by both methods. The enzymatic assay yielded 1.06 ± 0.02 × 10^{-10} mol of ATP per ml contrasted to 1.37 ± 0.09 × 10^{-10} mol of ATP per ml measured after fluorometric derivatization.

Metabolic lability of ATP. In bacterial monocultures suspended in a syringe device that allows sampling in 0.5 s, the ATP concentration per milligram of protein can increase or decrease twofold in <1 min under conditions where the biomass remains unchanged. There is a corresponding reciprocal change in ADP (Fig. 4).

In the estuarine detrital microbiota (Teflon sheets incubated in the subtropical estuary) it is also possible to demonstrate lability of ATP. In these experiments the estuarine water in which the detritus was suspended was deoxygenated by bubbling with argon. This produced a reversible decrease in the energy charge (energy charge = ATP + ½ ADP/ATP + ADP + AMP) from 0.7 to 0.46 under conditions where the extractable lipid phosphate remained unchanged at 0.28 ± 0.15 μmol per Teflon square (Fig. 5).

The microfouling community formed in aluminum pipes in rapidly flowing seawater shows metabolic lability of ATP when the community is subjected to anoxia for 45 min (Table 2). There was a significant decrease in the energy charge, ATP, and total adenine-containing components. On being replaced in the flowing seawater stream for 10 min, the energy charge rebounded to high levels which were maintained. The microbial biomass measured as the extractable lipid phosphate was 0.068 ± 0.017 μmol/10-in. pipe section.

Adenosine nucleotides as measures of biomass. The metabolic lability of ATP suggests that combinations of adenosine nucleotides might be better indicators of biomass than ATP concentration alone. In linear regressions of adenosine nucleotides versus the lipid phosphate, the following determination coefficients (r^2) were attained: lipid phosphate versus ATP, 0.74; ATP plus ADP, 0.81; ATP plus ADP plus AMP, 0.93 (n = 10). These data were obtained from the experiment with the estuarine detritus illustrated in Fig. 5.

Comparing the lipid phosphate of *E. coli* grown in nutrient broth with the total adenosine nucleotides gave r^2 linear determination coefficients of 0.92 for ATP, 0.90 for ATP plus ADP, 0.89 for ATP plus ADP plus AMP, and 0.99 for

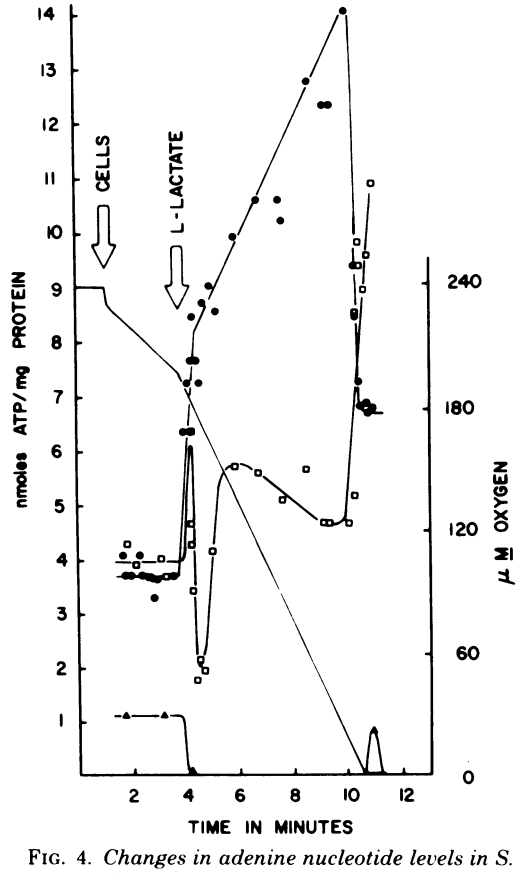

FIG. 4. *Changes in adenine nucleotide levels in S. aureus with modification in the electron transport status. S. aureus was suspended in phosphate buffer in a syringe with an oxygen electrode in the barrel. The sample contents could be rapidly dispersed into perchloric acid for analysis of the adenine nucleotides. ATP (●), ADP (□), and AMP (▲) via luciferin-luciferase in the presence of appropriate enzymes. The oxygen tracing shows the addition of the bacteria (cells), the endogenous respiratory rate, and the rate in the presence of 10 mM sodium lactate.*

adenosine plus ATP plus ADP plus AMP. For the filtered cells the linear determination coefficients, r^2, were 0.73 for ATP, 0.89 for ATP plus ADP, 0.99 for ATP plus ADP plus AMP, and 0.97 for cellular adenosine plus ATP plus ADP plus AMP ($n = 10$). In each situation combinations of the adenosine and the nucleotides gave closer correlations to the biomass measured by the lipid phosphate than did the ATP alone.

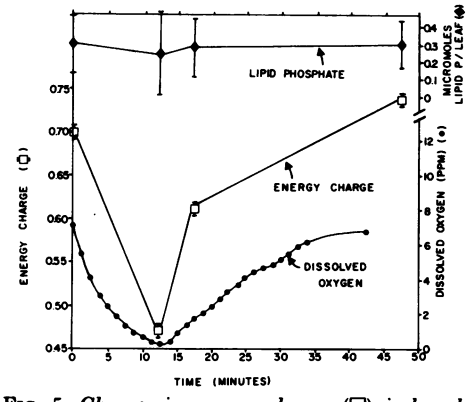

FIG. 5. *Change in energy charge (□) induced in the detrital microbiota by anoxia. Teflon squares were extracted and derivatized, and the nucleotides were analyzed fluorometrically: Energy charge = ATP + ½ADP/ATP + ADP + AMP. The oxygen utilization (●) was determined polarographically, and the lipid phosphate (◆) was determined colorimetrically from the lipid portion of the nucleotide extraction. Bars indicate standard deviations.*

Extracellular nucleotides. A possible complication of using several adenosine nucleotides to measure cellular biomass is the facility with which some microbes secrete AMP and adenosine to maintain the energy charge in times of stress (9). Ninety-five percent of the AMP and 80% of the adenosine recovered in exponentially growing *E. coli* is extracellular (Table 3). About 35% of the AMP and 46% of the adenosine can be recovered in the washings of marine sediments, which contain essentially no microorganisms as measured by the lipid phosphate (Table 4). In neither of these experiments was "extracellular" ATP or ADP detected.

The stress of filtration of exponentially growing *E. coli* is not reflected in the energy charge or the total cellular adenosine nucleotides, which remained $1.78 \pm 0.18 \times 10^{-10}$ mol/ml, but was reflected in the twofold increase in cellular adenosine (Table 3).

Relationship among energy charge, ATP, and cellular adenosine. The twofold increase in cellular adenosine with the stress of filtration of exponentially growing *E. coli* (Table 3) suggested that the ability to measure cellular aden-

TABLE 2. *Adenine nucleotides and energy charge in the microfouling community subjected to anoxia*

Treatment (duration, min)	mol × 10^{-10}/μmol of lipid phosphate[a]					Energy charge
	AMP	ADP	ATP	ATP + ADP	Total[b]	
Control[c]	6.5 (1.6)	4.9 (1.0)	14.2 (2.8)	19.0 (3.7)	59.0 (7.7)	0.65 (0.02)
Anaerobic[d] (10)	4.3 (0.6)	3.5 (1.0)	9.7 (1.6)	13.3 (2.7)	41.5 (9.8)	0.66 (0.01)
Anaerobic (45)	5.1 (0.3)	3.5 (0.6)	7.7 (0.4)	11.0 (1.0)	39.6 (8.4)	0.58 (0.01)
Recovered[e] (5)	2.4 (0.4)	3.0 (0.4)	13.6 (1.0)	16.9 (1.4)	41.1 (6.9)	0.78 (0.02)
Recovered (30)	2.5 (0.9)	3.3 (1.0)	15.9 (1.8)	19.2 (3.9)	45.5 (8.1)	0.81 (0.04)
Recovered (60)	3.1 (0.4)	2.6 (0)	13.5 (2.3)	16.0 (2.5)	55.1 (5.1)	0.77 (0.04)

[a] Number in parentheses is ± standard deviation ($n = 6$).
[b] Sum of adenosine plus the adenine nucleotides.
[c] Pipe sections removed from flowing seawater.
[d] Pipe sections removed, filled with argon-purged seawater.
[e] Pipe sections exposed to argon-purged seawater for 45 min and then returned to flowing oxygenated seawater.

TABLE 3. *Adenosine derivatives in exponentially growing E. coli measured fluorometrically*

Sample[a]	mol × 10^{-10}/ml (±standard deviation) ($n = 6$)							
	Total AMP	Cellular AMP[b]	ADP	ATP	Energy charge	Total adenosine	Cellular adenosine	Lipid phosphate
Culture	5.42 (0.26)	0.32 (0.14)	0.17 (0.03)	1.26 (0.04)	0.77 (0.01)	4.77 (0.14)	0.99 (0.06)	199 (18)
Cells		0.02 (0.02)	0.31 (0.03)	1.37 (0.09)	0.74 (0.01)		2.05 (0.25)	216 (2)
Medium	5.09 (0.06)		<0.01	<0.01		4.15 (0.10)		<1.0

[a] Exponentially growing *E. coli* were sampled directly (culture) and after filtration (cells) and filtrate (medium).
[b] Cellular AMP and adenosine − total in culture − total in medium.

TABLE 4. *Recovery of extracellular adenosine and AMP from marine sands*

Treatment[a]	mol × 10^{-11}/g		Lipid phosphate (mol × 10^{-7}/g)
	Adenosine	AMP	
Sediment	3.50 (0.26)[b]	3.48 (0.30)[b]	5.4 (1.4)[b]
Intracellular	2.11 (0.21)	2.00 (0.21)	3.5 (0.04)
Extracellular	1.64 (0.06)	1.21 (0.08)	<0.001

[a] Five-gram portions of marine sands were extracted (sediment) or suspended in 5 ml of filter-sterilized seawater for 3 min and centrifuged at 26,000 × *g* for 15 min, and the pellet (intracellular) and supernatant (extracellular) were sampled.

[b] Numbers in parentheses are ± standard deviation ($n = 3$).

osine might be useful in predicting the metabolic status of environmental samples. The cellular adenosine/ATP ratio gives a measure that is relatively independent of differences in biomass because it is a ratio. With a slight decrease in energy charge (from 0.77 to 0.74) the ratio of cellular adenosine to ATP increased from 0.79 to 1.50 when the exponentially growing *E. coli* were filtered (Table 3). In environmental samples the ratio of cellular adenosine to ATP shows correspondence to energy charge. The linear regression determination coefficient, r^2, between the energy charge and the cellular adenosine/ATP ratio for the microfouling community subjected to anoxia (Table 2) was 0.87.

DISCUSSION

Advantages of the fluorescent derivatization method of analysis. The development of an assay for adenine-containing components of environmental samples that is free of the constraints caused by the inhibition of the luciferin-luciferase enzyme system but has the required picomolar sensitivity has been achieved with the formation of the ϵ-adenosine derivatives that are readily separable by HPLC. In a single 50-min analysis, all of the major adenosine-containing components can be measured. With recent developments the HPLC separation can be achieved in 12 min in a simpler isocratic system. A single extraction that is quantitative, preserves the ATP and energy charge, and provides the lipids for complementary analysis of the community structure (7, 44, 48) both conserves samples and simplifies field collection. The method uses chloroform-methanol to lyse the cells, thus avoiding acid, heat, or base which complicate further analyses, particularly when sampling metallic surfaces. If multiple adenine-containing components are measured, the fluorescence method becomes cost effective (1, 23).

The quantitative chromatographic isolation of the adenosine-containing components by HPLC possibly provides the major advantage of this method. It will now be possible to study the rate of synthesis and catabolism of these adenine-containing components, using radioisotopes to gain insight into the regulation of metabolic activity in environmental samples.

Using an isocratic HPLC separation of the ϵ-derivatives on a reverse-phase column, the cost of the pump, column, and fluorescence detector becomes comparable to the more sensitive luminescence detectors. The chief disadvantage of this method lies in the necessity of a laboratory for the derivative formation and chromatography, although quick-freezing of samples preserves the energy charge.

Advantages of multiple nucleotide analysis. It is possible to change the ATP level in microbial communities of essentially constant biomass (Fig. 4 and 5; Table 2). Consequently, total adenine nucleotides (AMP + ADP + ATP) or total adenine components (adenosine + AMP + ADP + ATP) gave much better correlations with extractable lipid phosphate than ATP alone in the analysis of the detrital or microfouling communities. Extractable lipid phosphate has been shown to be a measure of the living cellular (membrane) biomass (46).

In sediments it is important to measure the intracellular levels of adenosine and AMP, as these nucleotides are lost from the cells and can be recovered in washes of the sediments (Table 4). If washing is not practical, then the measurement of ADP plus ATP gives a better correlation with the membrane biomass than ATP alone. With the fluorescence methods, extracellular ATP or ADP has not been detected in washes of the benthic, detrital, or microfouling communities, although 10^{-9} to 10^{-10} M extracellular ATP has been detected in seawater (4). In other studies the total adenosine nucleotides showed better correlation with bacterial dry weight than ATP alone in chemostat-grown monocultures (13).

One of the advantages of ATP and lipid phosphate as biomass measures for the microbial community is their association with viable cells. The rapid changes in the intracellular levels of the other adenosine nucleotides and adenosine (Table 2; Fig. 4 and 5) suggest that the added accuracy of using multiple adenine component combinations would not obscure their utility as measurements of viable microbes. Measurement of multiple adenosine components also yields insights into the metabolic status of the microbes.

Measurements of the metabolic status. The measurement of the adenosine nucleotides allows calculation of the energy charge, which is

known to be a sensitive reflection of the metabolic status (3, 25, 48). Indeed, the homeostatic preservation of energy charge with various stresses leads to the rapid excretion or destruction of AMP (25). The sensitivity of these shifts in metabolism of nucleotides to maintaining energy charge is demonstrated in the doubling of intracellular adenosine and the large amounts of extracellular adenosine and AMP in exponential *E. coli* subjected to filtration stress (Table 3).

The ratios of intracellular adenosine to ATP show much greater change than the energy charge when exponentially growing *E. coli* are filtered (Table 3). In the microfouling community there is a good correlation between the ratio of cellular adenosine to ATP and the energy charge.

Extracellular AMP has been detected in several growing monocultures (31). Resuspension and centrifugation of the estuarine sediments in this study show extracellular AMP and adenosine (Table 4), suggesting that excretion of adenosine and AMP help to maintain the energy charge.

Anoxia induces decreases in energy charge in both the detrital and microfouling communities (Table 2; Fig. 5) and increases in the intracellular adenosine content. The rapid increase in cellular adenosine may prove to be a very sensitive measure of metabolic stress.

That the actual adenine-containing compounds are isolated in this method makes possible studies using ^{14}C-labeled precursors to follow rates of change, which should greatly increase the knowledge of the metabolic activities of complex environmental assemblies.

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