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Methodology and Measurement of Adenylate Energy Charge Ratios in Environmental Samples

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Abstract

A method for measuring ATP, ADP and AMP levels in environmental samples was devised, and applied to seawater and bacterial cell extracts. This procedure is specifically designed for measuring the extremely low concentrations of total adenine nucleotides ($[A_T]$ = [ATP] + [ADP] + [AMP]) that are apt to occur in most natural ecosystems (i.e., ≤10 ng A_T ml^{-1} of sample extract). Although the current assay methodology can be used with purified firefly luciferase reagents, it has been suitably modified to accept crude luciferase preparations as well. ATP, ADP and AMP levels have been measured, and the corresponding energy charge (EC) ratios determined for seawater samples collected off the Southern California coast. The EC ratios ranged from 0.50 to 0.89, with peak values corresponding to the subsurface maxima in ATP and chlorophyll *a* concentrations, and the minimum values corresponding to the deepest water sampled (1500 m). The measurement of adenylate energy charge ratios in environmental samples can be a useful indicator of mean community metabolic activity and potential for cell growth.

Introduction

Within the past decade, adenosine triphosphate (ATP) measurements have been used extensively to estimate the total microbial biomass in marine (Holm-Hansen and Booth, 1966; Holm-Hansen, 1969; Hobbie *et al.*, 1972; Manuels and Postma, 1974; Herbland and Pages, 1975; Devol *et al.*, 1976; Hodson *et al.*, 1976; Karl *et al.*, 1976), estuarine (Christian *et al.*, 1975; Erkenbrecher and Stevenson, 1977), freshwater (Rudd and Hamilton, 1973; Holm-Hansen *et al.*, 1976; Paerl *et al.*, 1976), and terrestrial (Conklin and MacGregor, 1972; Ausmus, 1973) ecosystems. Although quantitative biomass estimates are extremely useful for many ecological studies, they do not provide for an assessment of the biochemical activities of the represented microorganisms.

The adenine-containing nucleotides (ATP, ADP and AMP) are ubiquitous in living cells, and are responsible for coupling intracellular energy-producing and energy-requiring metabolic reactions. Detailed laboratory studies concerning the regulation of enzyme function and the control of biosynthetic processes have resulted in the formulation of the adenylate energy charge (EC) concept. As defined by Atkinson and his colleagues (Atkinson and Walton, 1967; Atkinson, 1969; Chapman *et al.*, 1971), the adenylate EC is equal to one-half of the number of anhydride bound phosphate groups per adenine moiety,

$$EC = \frac{[ATP] + 1/2\ [ADP]}{[ATP]+[ADP]+[AMP]} ,$$

and is therefore a linear measure of the amount of metabolic energy momentarily stored in the adenine nucleotide pool. Although the theoretical range of EC ratios is from 0.0 (all AMP) to 1.0 (all ATP), extensive laboratory studies indicate that the EC ratio in growing cells is stabilized at a value of between 0.8 to 0.9 (Chapman *et al.*, 1971, and references contained therein). In principle, measurement of the EC parameter in environmental samples might be useful for estimating and comparing the overall energetic state of naturally occurring microbial populations. Several investigations have recently been conducted where EC ratios were determined in natural seawater (Wiebe and Bancroft, 1975; Karl, 1977) and plankton samples (Båmstedt and Skjoldal, 1976; Skjoldal and Båmstedt,

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1976). Although the absolute growth rate cannot be predicted from EC measurements alone, the rate of protein synthesis and the capacity for cell growth have both been shown to be more closely correlated to changes in the EC ratios than to changes in the absolute concentrations of intracellular ATP, ADP or AMP (Swedes *et al.*, 1975).

The methods that are most frequently employed for adenine nucleotide determinations involve enzymatic conversions of ADP and AMP to equivalent levels of ATP, followed by a quantitative analysis of the ATP via the firefly bioluminescent reaction. Although detailed procedures have already been described for adenine nucleotide determinations in plant tissue and bacterial cell extracts (Pradet, 1967; Chapman *et al.*, 1971), several modifications were necessary in order to adapt these techniques for measuring the relatively low adenine nucleotide levels ($[A_T] \leq 10$ ng ml^{-1} extract) that occur in most marine environments. In addition, the analytical techniques described in this paper are compatible with either purified or crude luciferase enzyme preparations, thereby leaving the choice of reagents up to the individual investigator.

Materials and Methods

Sample Collection and Nucleotide Extraction

Seawater samples were collected at various stations using 5 l Niskin bottles (General Oceanics, Miami, Florida, USA). Prior to sample collection, the bottles were scrubbed with 70% ethanol and rinsed thoroughly with filtered (0.45 µm) seawater. Immediately upon shipboard arrival, the water samples were prefiltered through a 183 µm Nytex mesh into acid-washed glass bottles. Various volumes of each individual water sample, ranging from 20 to 500 ml (depending upon sample depth), were filtered through microfine glass-fiber filters (Reeve Angel, 24 mm diameter, 984-H), and the particulate material retained by the filter was extracted for adenine nucleotides using the boiling Tris method previously described by Holm-Hansen (1973). All extracts were stored frozen (-20°C) prior to analysis.

Escherichia coli Experiment

Escherichia coli cultures were prepared on the day of the experiment by inoculating 50 µl of an overnight culture into 5 ml of sterile medium containing glycerol (5 g l^{-1}), peptone (3 g l^{-1}), yeast extract (3 g l^{-1}), $Na_2HPO_4 \cdot 7H_2O$ (7 g l^{-1}) and $(NH_4)_3PO_4$ (0.5 g l^{-1}). At various stages of growth, a small portion of the medium was rapidly removed from the culture tube using a sterile syringe sampler, and was immediately injected into 5 ml of boiling Tris buffer. An equivalent volume of cell-free medium (0.45 µm filtrate) was also extracted at each sampling period in order to monitor, and correct for, extracellular adenine nucleotides. Cell growth was monitored using optical density measurements (Coleman Jr. II Spectrophotometer).

Charcoal Column Experiments

A series of charcoal-celite columns were prepared as described by Hodson *et al.* (1976) using disposable glass Pasteur pipets (15 x 7 mm). Various solutions were prepared by mixing known concentrations of ATP, ADP and AMP together to produce a wide range of expected EC ratios (0.5 to 1.0). A portion of each mixture was pipetted onto the appropriate column and the nucleotides were bound, rinsed, eluted, dried and reconstituted with Tris buffer to the exact starting volume, as described by Hodson *et al.* (1976). An additional portion of each mixture served as a control sample and was assayed at the same time as the column extracts.

Enzymatic Conversions of ADP and AMP to ATP

For each sample, 200 µl of the Tris buffered extract are pipetted into a series of 4 disposable glass culture tubes (12 x 75 mm) labeled A, B, C and D. In addition to the sample extracts, a Tris buffered reagent blank, a set of 6 to 8 ATP standards ranging in concentration from 1 to 50 ng ATP ml^{-1}, and a series of solutions containing various ratios of ATP, ADP and AMP are also prepared and processed simultaneously. For ATP determinations (Tube A), 50 µl of a solution containing $MgCl_2$ (15 mM) and sodium phosphate buffer (75 mM, pH 7.4) are pipetted into each tube. For ATP plus ADP determinations (Tube B), 50 µl of a solution containing $MgCl_2$ (15 mM), sodium phosphate buffer (75 mM, pH 7.4), phosphoenolpyruvate (PEP, 0.5 mM), and pyruvate kinase (PK, 20 µg) are pipetted into each tube. For ATP plus ADP plus AMP determinations (Tube C), 50 µl of a solution containing $MgCl_2$ (15 mM), sodium phosphate buffer (75 mM, pH 7.4), PEP (0.5 mM), PK (20 µg) and adenylate kinase (AK or myokinase, MK, 25 µg) are pipetted into each tube. Tube D is included in the current assay methodology in order to determine the efficiency of the adenylate kinase reaction. As will be presented and discussed in the "Results and Discussion" section of this report, the efficiency of the adenylate kinase reaction is affected by the total adenine nucleotide concentration in the sample. To promote this reaction, 50 µl of a solution containing $MgCl_2$ (15 mM), sodium phosphate buffer (75 mM, pH 7.4), PEP (0.5 mM), PK (20 µg), AK (25 µg), and ATP (10 ng) are pipetted into each tube. All reaction tubes were incubated at 30°C for 30 min, immersed into a boiling water bath (100°C) for 2 min, and allowed to adjust to room temperature (ca. 25°C) prior to the ATP assays.

ATP Assay

Lyophilized firefly lantern extracts are obtained commercially and stored frozen and desiccated (-20°C) prior to use. When required, each vial is reconstituted with 5 ml of distilled water as described by the manufacturer. After an aging period of 2 to 3 h at 25°C, the enzyme is further diluted to 25 ml with equal volumes of $MgSO_4$ (0.04 M) and $KHAsO_4$ buffer (pH 7.4, 0.1 M). When a large number of determinations are to be conducted, the contents of several vials are pooled to yield a single luciferin-luciferase reaction mixture. For each ATP assay, 1.0 ml of the enzyme solution is pipetted into a disposable glass vial (20 x 40 mm) and the vial is inserted into the ATP photometer (Model 2000, SAI Technology Co., Sorrento Valley Blvd., San Diego, California). Two-hundred microliters of sample are withdrawn from the appropriate culture tube using the autopipet supplied by the photometer manufacturer (kinetics kit, SAI Technology Co.). The pipet is positioned onto the photometer, the endogenous background light emission recorded, and the analog recorder is started. The sample is injected into the enzyme mixture, and the peak light emission (0 to 3 sec) is displayed on the digital output. It is essential that the samples are reproducibly injected into the enzyme and that proper mixing of the reagents occurs. These prerequisites were evaluated by statistical analysis of successive injections using our injection system, and by comparing these results to the method of integrated light flux measurements.

Chemicals and Supplies

The sodium salts of ATP, ADP, AMP, GDP and GTP were all obtained from Sigma Chemical Company (St. Louis, Missouri). Stock nucleotide solutions (2 µM) were prepared in Tris buffer (pH 7.7, 0.02 M), proportioned into clean glass vials (2 ml per vial) and stored frozen (-20°C) prior to use. When required, individual vials were thawed and further diluted with Tris buffer to obtain a set of experimental standards (approximately 10^{-9} to 10^{-7} M). Phosphoenolpyruvate was purchased from Sigma Chemical Company as the trisodium salt, and stock solutions (1.5 mM) were prepared in Tris buffer, and stored frozen (-20°C) in 2 ml aliquots. Adenylate kinase (1000 to 1500 units mg^{-1} protein from rabbit muscle), pyruvate kinase (350 to 500 units mg^{-1} protein from rabbit muscle) and firefly lantern extracts (FLE-50) were all obtained from Sigma Chemical Company. All other chemicals used in this study were analytical grade reagents.

Data Reduction and Calculation of the EC Ratio

Net light emission was determined by subtracting the appropriate blank value from each of the total light emission determinations. Standard curves were prepared by plotting net peak light emission on the ordinate versus ATP concentration on the abscissa, for each of the three sets of standard data. From these curves, the ATP concentration in each of the three reaction mixtures (ATP; ATP plus ADP; ATP plus ADP plus AMP) was determined, and the amounts of ATP enzymatically produced from ADP and AMP were calculated as differences between these measured values. By correcting for the proportion of the sample actually assayed, and the volume of water originally filtered, the ATP, ADP and AMP values can be expressed on a per liter basis. The value A_T represents the total ATP equivalent (in ng l^{-1}) of all three adenine nucleotides. The adenylate EC ratio was determined using the formulation of Ball and Atkinson (1975) in order to reduce the propagation of errors.

Results and Discussion

Although a number of different instruments have been used to measure light emission from the firefly bioluminescent reaction (see Strehler, 1968), we selected a commercial ATP photometer which had the capability of measuring the peak height of the luminescent reaction. When recording peak emission data, it is imperative that the samples are reproducibly injected into the enzyme mixture and that complete mixing of the reagents occurs. Fig. 1 shows the results of a mixing experiment that was conducted in order to determine the correct reaction volumes for our assay system. In these

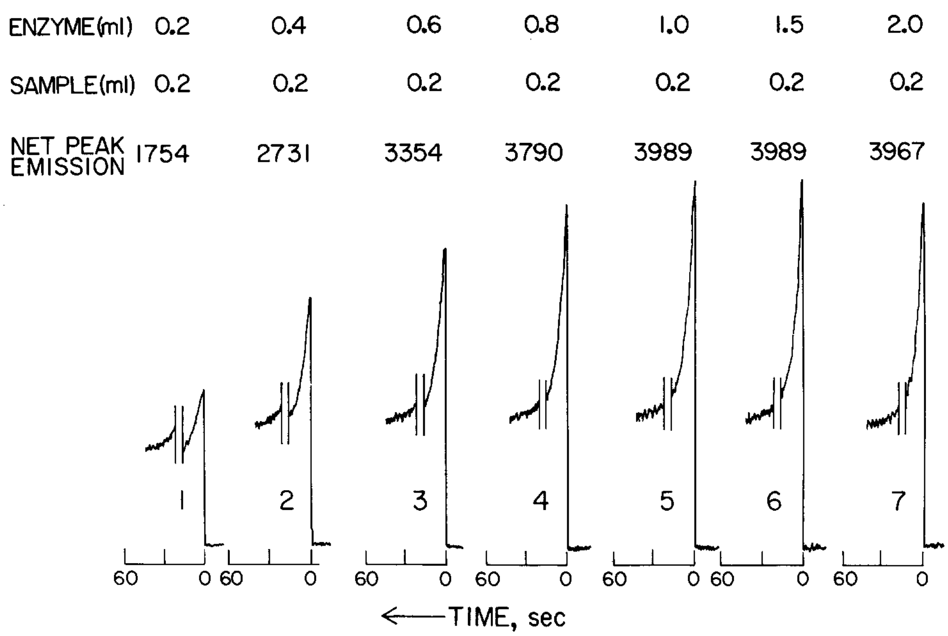

Fig. 1. Kinetics of the firefly bioluminescent reaction as a function of enzyme volume. The volume of the sample, an ATP standard solution (ATP = 5 ng ml^{-1}), was kept constant and was injected into various volumes of crude luciferase as described in "Materials and Methods"

Table 1. Comparison of peak height emission and integrated light flux measurements for quantitative ATP determinations

ATP (ng per sample)	Sample	Peak (counts x 10^{-3})	Integral (CPM x 10^{-3})
2	a	4.01	25.72
	b	4.03	25.32
	c	4.06	24.49
	d	3.94	24.99
	e	3.94	25.30
	f	3.94	25.20
	g	3.94	25.58
	h	3.90	24.80
	i	3.89	25.37
	j	3.96	25.25
		$\bar{x}$ = 3.96	$\bar{x}$ = 25.20
		s = 0.05	s = 0.36
		$s/\bar{x}$ = 1.26%	$s/\bar{x}$ = 1.43%
20	a	40.79	347.2
	b	40.54	360.4
	c	41.15	368.2
	d	41.87	364.5
	e	41.93	369.5
	f	41.06	379.2
	g	41.17	373.2
	h	42.10	379.2
	i	41.73	377.9
	j	41.47	375.6
		$\bar{x}$ = 41.39	$\bar{x}$ = 370.0
		s = 0.52	s = 10.09
		$s/\bar{x}$ = 1.26%	$s/\bar{x}$ = 2.73%

experiments, a series of vials was prepared containing variable volumes of the firefly luciferase preparation. Each vial was placed into the photometer, and the analog recorder was turned on. At time zero, 0.2 ml of an ATP standard (5 ng ATP ml^{-1}) was injected into the vial and the kinetics were recorded. Fifteen seconds after sample injection the vial was removed from the photometer, mixed gently by hand, and replaced into the photometer. The first three light emission curves (Fig. 1, 1-3) display discontinuities in the reaction decay kinetics upon manual mixing, indicating that proper initial mixing had not occurred. The remainder of the light emission curves (Fig. 1, 4-7) indicate proper mixing kinetics. For all of our adenine nucleotide analyses, 0.2 ml of the appropriate sample extract or nucleotide standard solution was injected into 1.0 ml of the luciferase preparation.

Table 1 shows a comparison of the statistical variation for two ATP standards when assayed by the peak height emission and by the more conventional integrated light-flux measurements. In both cases, the reproducibility of the peak data was equal to, or better than, the integrated determinations. In addition to the speed (approximately 30 sec/sample) of the peak assay, the ease of operation, and high level of reproducibility (± 1 to 2%), peak measurements are essential for obtaining accurate adenine nucleotide determinations (see subsequent "Results and Discussion").

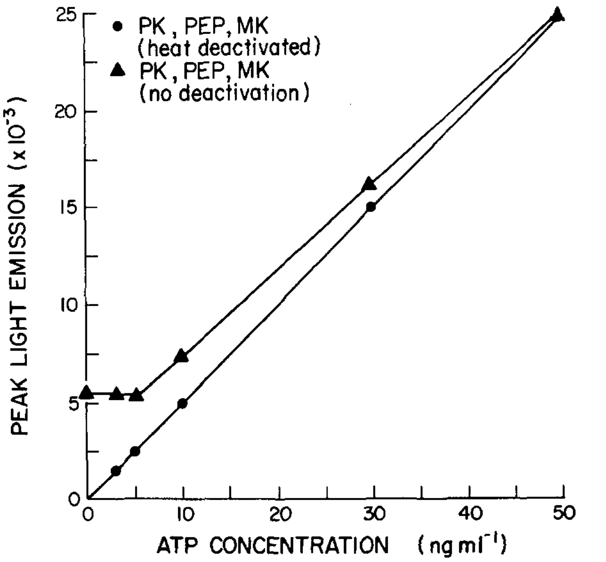

Fig. 2. Effects of heat deactivation on sensitivity and linearity of standard ATP solutions. MK: myokinase (adenylate kinase)

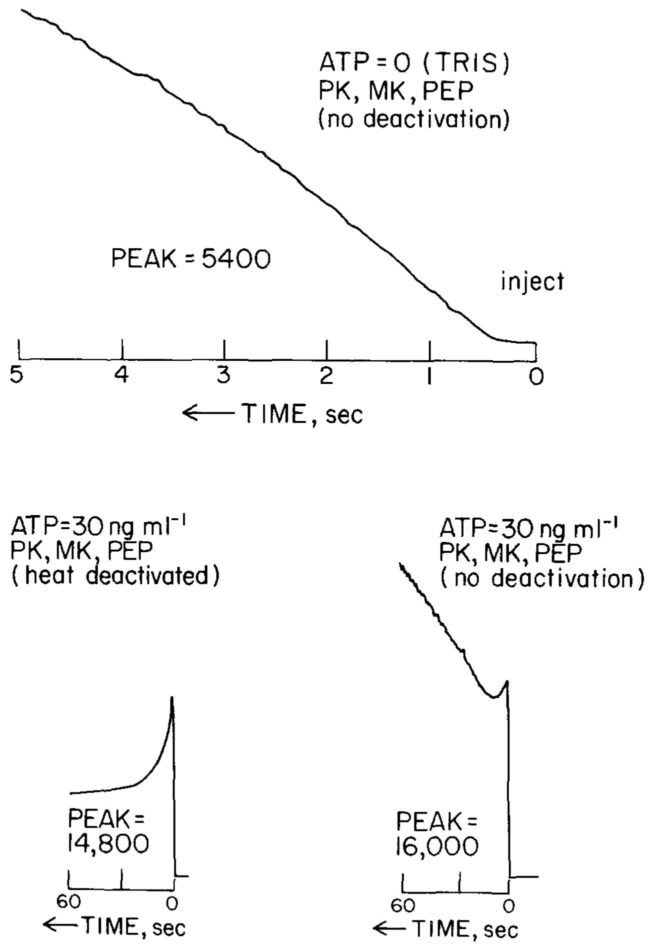

Fig. 3. Kinetics of ATP production in absence of the heat deactivation procedure, and a comparison of reaction kinetics of a standard ATP solution with and without heat deactivation. MK: myokinase (adenylate kinase)

The heat deactivation step proposed in the current assay methodology has not been utilized in previous energy charge studies; however, we found that it was essential for measuring adenine nucleotide concentrations of less than 50 ng A_T ml^{-1} of sample extract. Since most environmental extracts contain between 1 to 30 ng A_T ml^{-1}, this additional procedural step is essential. Fig. 2 presents a pair of ATP standard curves, each containing PK, AK and PEP, the only difference being that the samples represented by the lower curve were heat-deactivated (2 min, 100°C) prior to the peak emission analyses. It is evident that in the absence of heat deactivation, both the linearity of the standard curve and the lower limit of ATP detection are greatly affected. This discrepancy between the two curves is caused by the immediate production (0 to 3 sec) of ATP in the presence of PEP and pyruvate kinase, presumably from ADP contained within the crude luciferase preparations. Since pyruvate kinase is a heat-labile protein, a 2 min heating period is sufficient to denature the enzyme and produce the expected linear standard curve (Fig. 2, circles). The kinetics of ATP production resulting from the addition of pyruvate kinase and PEP are reproduced in Fig. 3. When a reagent blank (Tris buffer) containing only pyruvate kinase, adenylate kinase and PEP (but no ATP) is injected into a vial containing firefly luciferase, less than 0.5 sec elapse before light emission commences. The lower portion of Fig. 3 compares the light emission kinetics for an ATP standard solution (30 ng ATP ml^{-1}) containing pyruvate kinase, adenylate kinase and PEP, with and without a 2 min heat deactivation step. These data once again establish that ATP is produced within the sample-enzyme mixture, and that this ATP rapidly reacts (<0.5 sec) with the firefly luciferase causing an elevated peak emission. If, however, the original sample extracts contain >50 ng A_T ml^{-1}, the heat deactivation step is not necessary since the analytical interference resulting from the pyruvate kinase activity is overwhelmed by the magnitude of the ATP-dependent peak light emission. At all concentrations of ATP ≥50 ng ml^{-1}, the peak light emission was comparable for samples with or without a prior heat deactivation step.

Although the heat deactivation step is essential for low-level adenine nucleotide analyses (A_T <50 ng ml^{-1}), it introduces an additional methodological consideration to quantitative adenine nucleotide determinations. Unlike pyruvate kinase, adenylate kinase is an extremely heat- and acid-stable protein (Noda, 1973). Less than 10% of its catalytic activity is destroyed during the proposed heating period (2 min, 100°C). The selective denaturation of pyruvate kinase tends to alter the final reaction equilibrium, resulting in the backproduction of ADP from ATP in solution (AMP + ATP ⇌ 2 ADP). The equilibrium of this reaction is achieved within approximately 25 min at 25°C. In practice, a series of ATP standards are carried through all of the enzymatic reactions, and separate standard curves are then plotted. From Fig. 4 it is evident that all three of the standard curves are linear and pass through the origin, the only difference being in the values of their slopes. For data reduction, the ATP concentrations in Tubes A and B are calculated from the upper curve, and the ATP concentrations in Tubes C and D from the lower curve (see Fig. 4). If individual standard curves are not prepared, the experimental results will often indicate negative values for the concentration of AMP, as has been reported previously in the literature (Båmstedt and Skjoldal, 1976). In addition, the preparation of these standard curves enables the investigator to detect and correct for any adenine nucleotides that may be contaminating the commercial pyruvate kinase and adenylate kinase preparations, and will also correct for any contaminating activities (e.g. adenine deaminase, ATPase, etc.) that would tend to alter the final levels of ATP. For this reason, these curves should be constructed even if the heat deactivation procedure is not utilized.

Several previous investigators have been concerned with estimating the efficiency of the coupled pyruvate kinase-adenylate kinase reaction (i.e., AMP ⟶ ATP). Although many published reports indicate coupled enzymatic efficiencies of 100% (Johnson *et al.*, 1970; Weiss *et al.*, 1972; Kimmich *et al.*, 1975; Lundin and Thore, 1975), the concentrations of adenine nucleotides used in these studies were generally in the micromolar range (500 to 1000 ng A_T ml^{-1}). As mentioned previously, most environmental samples contain only 0.1 to 1% of these levels, and kinetic information concerning the coupled enzymatic reactions at these concentrations is lacking. Fig. 5 shows peak light emission data resulting from the enzymatic conversion of three concentrations of AMP in the presence of varying concentrations of exogenous ATP. It is apparent that as the total concentration of ATP in each sample increases, there is a greater efficiency of conversion of AMP to ATP by the coupled reaction (Fig. 5). In other words, by increasing the ATP concentration, the apparent K_M of adenylate kinase for AMP is lowered. These experimental results are consistent with the random bi-bi reaction mechanism for adenylate kinase recently pro-

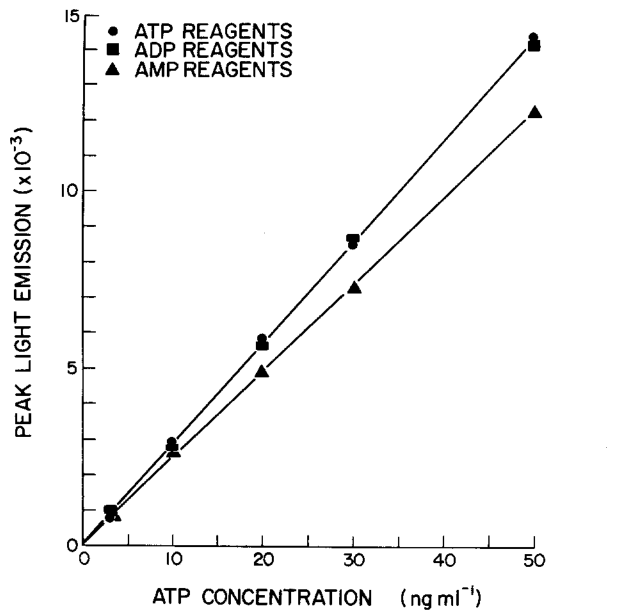

Fig. 4. Representative standard curves for adenylate energy charge determinations

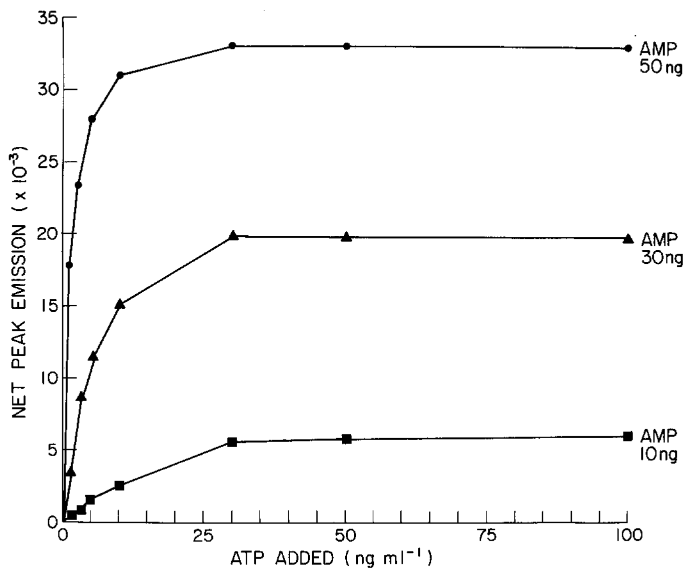

Fig. 5. ATP-dependent conversion of AMP to ATP via the coupled pyruvate kinase/adenylate kinase reactions. All samples were incubated for 30 min at 30°C and heat deactivated (100°C, 2 min) prior to the ATP assay. All ATP determinations are corrected for exogenous ATP

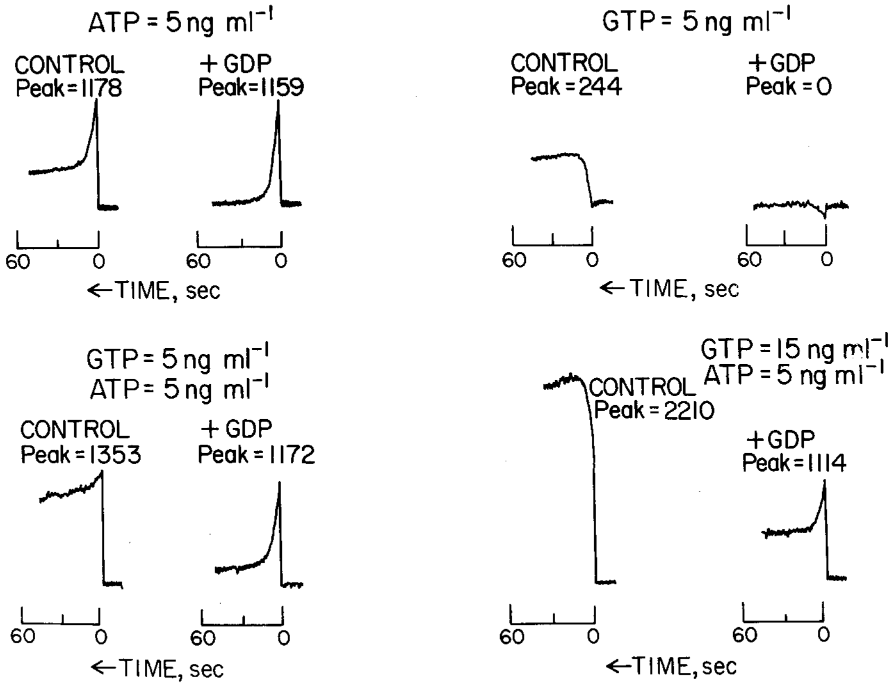

Fig. 6. Effects of addition of exogenous GDP on kinetics and reactivity of ATP, GTP and mixed nucleotide extracts

posed by Rhodes and Lowenstein (1968). The addition of 10 ng ATP to each of the D tubes (see "Materials and Methods") ensures a more efficient conversion of AMP to ATP within the reaction mixture. Although this refinement in methodology may not be necessary for all environmental energy charge determinations, especially since AMP is generally a minor portion of the total adenine nucleotide pool in growing cells (1 to 10%), it does enable one to determine a more reliable estimate of the total AMP (and A_T) concentration, and therefore a more correct EC ratio.

When utilizing enzymatic techniques to determine various compounds quantitatively, it is essential to evaluate the substrate specificity for each of the enzymes utilized, and to determine the extent of analytical interference (if any) resulting from closely related compounds. Although crystalline firefly luciferase is specific for ATP (McElroy and Green, 1956; DeLuca, 1976), a number of other ribose and deoxyribose nucleotides, especially guanosine triphosphate (GTP), will stimulate light emission in most commercial luciferase preparations (i.e., crude as well as "purified" commercial reagents; Karl, unpublished results). The pyruvate kinase reaction (ADP + PEP $\rightleftharpoons$ ATP + pyruvate) is relatively non-specific, and will catalyze the transfer of phosphate from PEP to ADP, GDP, IDP, dADP, UDP, CDP and dCDP in decreasing order of reactivity (Kayne, 1973). Adenylate kinase, on the other hand, is very specific for AMP as the phosphoryl acceptor (AMP + NTP $\rightleftharpoons$ ADP + NDP), even though the specificity for the phosphoryl donor is much more relaxed (Noda, 1973). Peak height measurements substantially reduce the analytical interference resulting from non-adenine nucleotide triphosphates (GTP, UTP, CTP and ITP), although if the concentration of GTP is significant, relative to the level of ATP (i.e., [GTP] $\geq \frac{1}{2}$ [ATP]), the final calculated adenylate EC ratio will be in error, unless the appropriate corrections are made. When GTP (or any other NTP) is injected into the crude enzyme mixture, ATP is produced from ADP in a reaction catalyzed by the enzyme nucleoside diphosphate kinase (NDPK) present within the firefly luciferase preparations (ADP + NTP $\rightleftharpoons$ ATP + NDP; K_{eq} = 1.0). A simple procedure that can be routinely followed in order to eliminate this source of interference is the addition of GDP to the crude enzyme preparation prior to the ATP determination. Fig. 6 shows the effect of GDP addition (400 ng per sample) on the kinetics and reactivity of several nucleotide preparations. Since the firefly NDPK reaction appears to be relatively non-specific, the addition of GDP will inhibit ATP production from UTP, ITP and

CTP as well as from GTP (Karl, unpublished results).

Although the assay methodology presented in this report is routinely standardized with test solutions of adenine nucleotides (see "Materials and Methods") in order to monitor the enzymatic conversion efficiencies, an experiment was conducted with the bacterium *Escherichia coli* to compare our results with those of previously published reports. Fig. 7 indicates that the EC ratio is maintained at a value between 0.8 and 0.9 throughout the exponential growth phase, results that are nearly identical to those reported by Chapman *et al.* (1971) and Swedes *et al.* (1975).

The primary analytical consideration for determining environmental EC ratios concerns the extremely low levels of adenine nucleotides that occur in most ecosystems. Hodson *et al.* (1976) described a charcoal adsorption technique as a means of concentrating ATP extracts from marine environments. We investigated the possibility that the charcoal adsorption methodology might be useful for other adenine nucleotide determinations as well. A charcoal column adsorption experiment was conducted as described in "Materials and Methods", and the results are presented in Table 2. When a solution of ATP was applied to an activated charcoal column, bound, rinsed, eluted, evaporated and reconstituted with Tris buffer as described by Hodson *et al.* (1976), both ATP and ADP were detected in the final solution (Table 2). However, in the control sample, only ATP was detected (Table 2, Sample 1). These results indicate that ATP hydrolysis had occurred at some point during the charcoal column procedure. A similar effect can be seen for Samples 3, 4, and 5 of Table 2. Moreover, the actual quantitative recovery of each of the three adenine nucleotides varied considerably. The recovery of ATP and AMP were approximately 56 and 35%, respectively; whereas the "apparent recovery" of ADP varied from 70 to 94%, depending upon the amount of ADP that was produced from the hydrolysis of ATP. These observations are consistent with the extensive experimental results of Ireland and Mills (1966), where the binding and elution properties of activated charcoal were examined using a variety of purines and purine nucleotides. Moreover, they discovered that the percent recovery varied with the amount of nucleotide applied to the column, and that the recovery was also affected by the presence of other organic substances (Ireland and Mills, 1966). Although the charcoal column procedure may be useful for certain ecological studies, it cannot be used for quantitative adenine nucleotide determinations in its present form. In order to determine the correct EC ratio for a particular sample extract, the recovery of each of the three adenine nucleotides would have to be determined independently and corrected for, and the effect of hydrolysis (as well as other chemical reactions such as deamination, etc.) would have to be carefully evaluated. The fact that ATP hydrolysis does occur during the charcoal adsorption procedure suggests that extreme caution should be used whenever this technique is applied

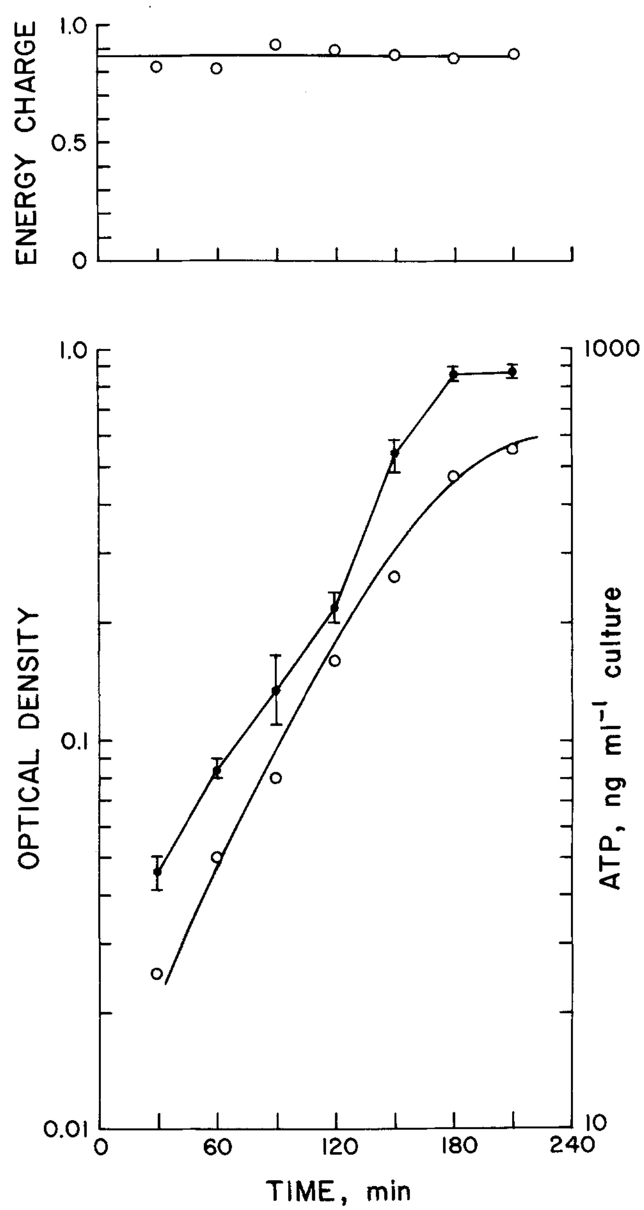

Fig. 7. *Escherichia coli.* Adenylate energy ratios during growth in batch culture. Open circles: optical density; filled circles: ATP

Table 2. Charcoal columns and adenine nucleotide determinations

Sample no.	Nucleotide content (ng ml^{-1})[a]	Adenine nucleotide determinations					
		Control			Column		
		ng ml^{-1a}		% Recovery	ng ml^{-1a}		% Recovery
1	30 ATP	ATP	29.0	97	ATP	17.1	57
		ADP	0	-	ADP	4.0	-
		AMP	0	-	AMP	0	-
			A_T = 29.0	97		A_T = 21.1	70
2	30 ADP	ATP	0	-	ATP	0	-
		ADP	30.8	103	ADP	21.1	70
		AMP	0	-	AMP	0	-
			A_T = 30.8	103		A_T = 21.1	70
3	15 ATP	ATP	14.3	95	ATP	8.1	54
	10 ADP	ADP	10.5	105	ADP	9.4	94
	5 AMP	AMP	4.7	94	AMP	2.0	40
			A_T = 29.5	98		A_T = 19.5	65
4	15 ATP	ATP	14.0	93	ATP	8.4	56
	15 AMP	ADP	0	-	ADP	1.9	-
		AMP	14.5	97	AMP	4.7	31
			A_T = 28.5	95		A_T = 15.0	50
5	15 ATP	ATP	14.1	94	ATP	8.7	58
	15 ADP	ADP	14.8	99	ADP	12.8	85
		AMP	0	-	AMP	0	-
			A_T = 28.9	96		A_T _ 21.5	72

[a]All nucleotides are expressed as ATP equivalents.

to quantitative analysis of ATP. The recommended use of ATP-γ-^{32}P in order to determine the efficiency of each column (Hodson *et al.*, 1976; Azam and Hodson, 1977) is only valid if the observed ATP hydrolysis occurs during the nucleotide adsorption step. If the hydrolysis (and release of $^{32}PO_4$) occurs during or subsequent to the elution procedure, then the $^{32}PO_4$ will be detected and radioassayed as if it were ATP-γ-^{32}P. A more reliable internal standard would seem to be the addition of non-radioactive ATP to a subsample of each extract, since the final analysis of this internal standard will be subjected to the same set of reactions and interferences as the original ATP in solution.

Perhaps the most critical methodological consideration concerns the effect of vacuum filtration on the determination of the adenylate energy charge value. It has been previously shown (Cole *et al.*, 1967) that centrifugation had a detrimental effect on the amount of ATP recovered from growing bacterial cells. An experiment was conducted at sea to determine whether or not vacuum filtration had a similar effect on natural microbial populations. The results are presented in Fig. 8. It is evident from these data that, although the total amount of ATP per sample when normalized to a per liter basis decreased as a function of the volume filtered, there was no significant change in the concentration of A_T. This indicates that the cells are rearranging their intracellular adenine nucleotides, presumably in response to metabolic stress imposed by vacuum filtration. This hypothesis is supported by the fact that the EC ratio consistently decreased as a function of the volume of water filtered (Fig. 8). The exact magnitude of this "filtration effect" is related to the density and types of organisms present within each sample, and is also related to the *in situ* EC of the water sample. Our experience has indicated that the higher the initial EC ratio, the greater the "filtration effect". It is also apparent from these data that the effect is not a simple linear function of the volume of water filtered, but that an asymptote is approached, presumably corresponding to some level of maximum metabolic stress (EC = 0.5 to 0.6, Fig. 8). A recent report by Sutcliffe *et al.* (1976) described an unusual problem with ATP determinations in coastal waters, which we now feel might be at least partially explained by the EC results presented in Fig. 8. The "filtration effect" could not be alleviated by reducing the pressure differential during vacuum filtration (our usual ΔP = 20 cm Hg), nor could the effect be lessened by convert-

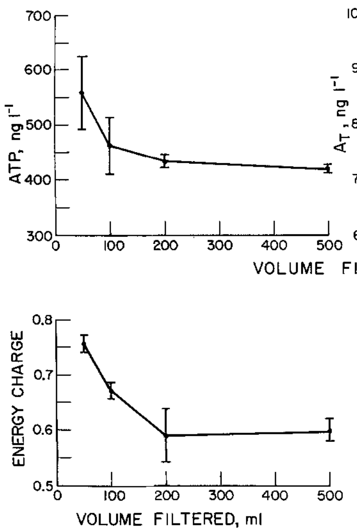

Fig. 8. Effect of vacuum filtration on ATP, A_T and energy charge ratio determinations. This water sample was obtained at 33°45.5'N; 118°47.6'W from a water depth of 9 m

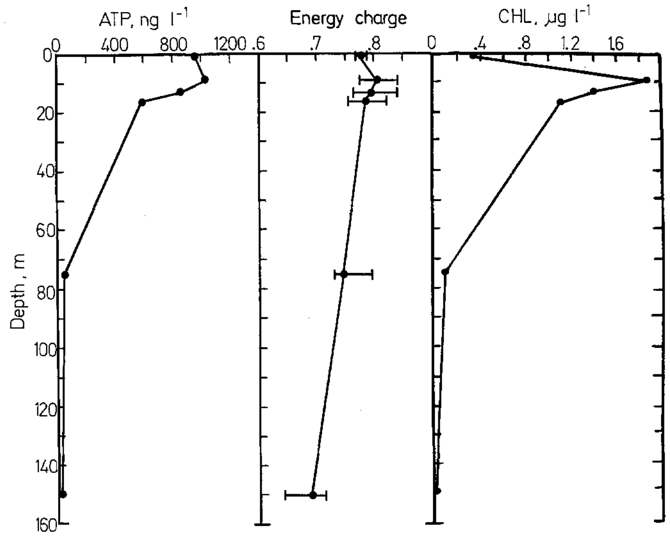

Fig. 9. ATP, energy charge ratios and chlorophyll *a* concentrations for a station located within the Southern California Bight (33°15'N; 117°41'W) approximately 5 km offshore. Total water depth was 200 m

ing from vacuum to pressure filtration. It is very difficult to define this problem quantitatively in order to apply the necessary correction factor. As a compromise, minimal sample volumes should be filtered and extracted (i.e., 25 to 100 ml for coastal oceanic waters <100 m; 250 to 1000 ml for deeper samples). If additional reaction sensitivity is required in the ATP assay procedure, D-luciferin can be added to the enzyme preparation as described by Karl and Holm-Hansen (1976).

Fig. 9 presents the results obtained from seawater samples collected within the Southern California Bight, approximately 5 km offshore. The EC ratios were calculated from minimum sample volumes and, although they might represent slight underestimates of the true *in situ* EC values, we feel they are representative of the true community levels. The EC ratios ranged from 0.69 to 0.81, with maximum ratios corresponding to the subsurface maxima in ATP and chlorophyll *a* concentrations (i.e., 10 m, Fig. 9). These data suggest that this portion of the water column has a greater community metabolic activity and greater potential for cell growth than the water located directly above or below it. Below approximately 20 m, there is a gradual decrease in the EC ratio, suggesting reduced growth potential. These data and interpretations are supported by measurements of $H^{14}CO_3$ uptake calculated from these same water samples (data not given). Since phytoplankton cells probably account for the major portion of the microbial biomass in euphotic oceanic waters, any community parameter such as the adenylate EC ratio should reflect the growth and metabolic characteristics of the most predominant organisms.

Fig. 10 presents ATP, A_T and energy charge determinations measured within water samples collected approximately 100 km off the Southern California coast to a depth of 1500 m. The concentrations and vertical distribution of ATP within the water samples are similar to the results previously presented by Holm-Hansen and Booth (1966) for a similar station located within the Southern California Bight. An interesting feature of the vertical profiles is the existence of a subsurface maximum in ATP and A_T centered at approximately 300 m. This elevated biomass is accompanied by an increase in the EC ratio to a value of 0.89, the highest ratio measured. These data suggest that certain portions of the water column may contain an elevated level of active microbial cells, relative to the water column directly above or below them. These environments may arise due to density discontinuities within the water column resulting in the accumulation of sinking organic matter as described by Karl *et al.* (1976); however, regardless of their origin, the regions of increased biomass and metabolic activity must be important as sites of nutrient regeneration required

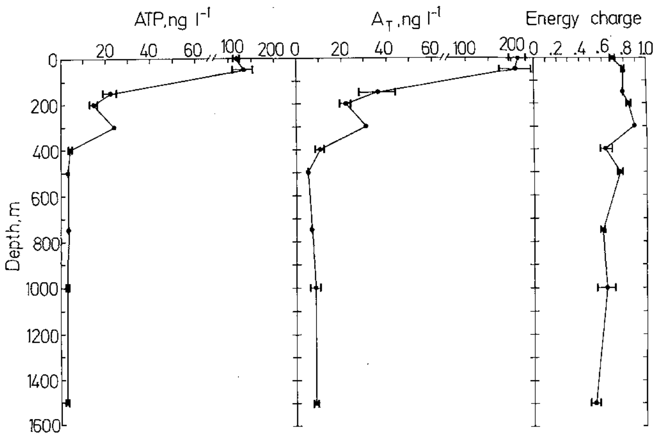

Fig. 10. ATP, A_T and energy charge determinations for a station located at 32°31.6'N; 118°07.0'W, approximately 100 km offshore. Total water depth was 1850 m

for sustained phytoplankton growth. The most significant feature of the vertical distribution of energy charge ratios is the apparent change from a relatively high ratio (EC = 0.78 to 0.89) within the surface portion of the water column (50 to 300 m), to relatively low ratios (EC = 0.55 to 0.64) within the deeper portions of the water column (>600 m). Although the absolute growth rate cannot be determined from energy charge ratios alone, these results support the notion of a general slowdown in mean community metabolic activity with increasing water depth in the ocean. The water sample collected at 1 m, however, exhibited a lower energy charge ratio (and presumably a lower growth potential) than the water sample collected from a depth of 50 m. These results are identical to the data presented in Fig. 9, and may be the result of inhibition of phytoplankton growth due to nutrient depletion or ultraviolet and photo-inhibition, or both.

The methodology presented in this report has also been successfully applied to the analysis of microbial populations within the water column of the Black Sea (Karl, unpublished results), to various coastal marine sediments, and to a variety of multicellular marine and terrestrial organisms. Although the results of these latter experiments are the subject of a separate report (Karl *et al.*, 1978), one important consideration merits attention. In order to extract ATP efficiently from microbial cells attached to solid particles, or from multicellular organisms, additional factors must be evaluated, namely the rate of cell death and the effectiveness of the various extraction techniques for preserving the intracellular adenine nucleotides at *in vivo* levels. The EC methodology described in this report offers a new approach for the critical evaluation of various extraction methodologies. Karl and Holm-Hansen (1977) reported that the EC ratio measured in beach sand varied as a function of the extraction procedure used. The cold sulfuric acid-EDTA procedure of Karl and LaRock (1975) yielded consistently higher [ATP]/[A_T] ratios and higher EC ratios (indicating a more efficient extraction procedure) than when the sand was extracted with either boiling Tris or boiling sodium bicarbonate buffers (Karl and Holm-Hansen, 1977). These results support the theoretical thermal gradient hypothesis previously proposed by Karl and LaRock (1975).

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Literature Cited

Atkinson, D.E.: Regulation of enzyme function. A. Rev. Microbiol. *23*, 47-68 (1969)

- and G.M. Walton: Adenosine triphosphate conservation in metabolic regulation. J. biol. Chem. *242*, 3239-3241 (1967)

Ausmus, B.S.: The use of the ATP assay in terrestrial decomposition studies. Bull. ecol. Res. Comm. Stockholm *17*, 223-234 (1973)

Azam, F. and R.E. Hodson: Dissolved ATP in the sea and its utilisation by marine bacteria. Nature, Lond. *267*, 696-698 (1977)

Ball, W.J. and D.E. Atkinson: Adenylate energy charge in *Saccharomyces cerevisiae* during starvation. J. Bact. *121*, 975-982 (1975)

Båmstedt, V. and H.R. Skjoldal: Studies on the deep-water pelagic community of Korsfjorden, western Norway. Adenosine phosphates and nucleic acids of *Euchaeta norvegica* (Copepoda) in relation to its life cycle. Sarsia *60*, 63-80 (1976)

Chapman, A.G., L. Fall and D.E. Atkinson: Adenylate energy charge in *Escherichia coli* during growth and starvation. J. Bact. *108*, 1072-1086 (1971)

Christian, R.R., K. Bancroft and W.J. Wiebe: Distribution of microbial adenosine triphos-

phate in salt marsh sediments at Sapelo Island, Georgia. Soil Sci. *119*, 89-97 (1975)
Cole, H.A., J.W.T. Wimpenny and D.E. Hughes: The ATP pool of *E. coli*: measurement of the pool using a modified luciferase assay. Biochim. biophys. Acta *143*, 445-453 (1967)
Conklin, A.R. and A.N. MacGregor: Soil adenosine triphosphate: extraction recovery and half-life. Bull. envir. Contam. Toxicol. *7*, 296-300 (1972)
DeLuca, M.: Firefly luciferase. *In:* Advances in enzymology, Vol. 44. pp 37-68. Ed. by A. Meister. New York, N.Y.: John Wiley & Sons 1976
Devol, A.H., T.T. Packard and O. Holm-Hansen: Respiratory electron transport activity and adenosine triphosphate in the oxygen minimum of the eastern tropical North Pacific. Deep-Sea Res. *23*, 963-973 (1976)
Erkenbrecher, C.W. and L.H. Stevenson: Factors related to the distribution of microbial biomass in salt-marsh-creeks. Mar. Biol. *40*, 121-125 (1977)
Herbland, A. et J. Pages: L'adênosine triphosphâte (ATP) dans le dome de Guinêe distribution verticale et signification êcologique. Cah. O.R.S.T.O.M., Sêr. Ocêanogr. *13*, 163-169 (1975)
Hobbie, J.E., O. Holm-Hansen, T.T. Packard, L.R. Pomeroy, R.W. Sheldon, J.P. Thomas and W.J. Wiebe: A study of the distribution and activity of microorganisms in ocean water. Limnol. Oceanogr. *17*, 544-555 (1972)
Hodson, R.E., O. Holm-Hansen and F. Azam: Improved methodology for ATP determination in marine environments. Mar. Biol. *34*, 143-149 (1976)
Holm-Hansen, O.: Determination of microbial biomass in ocean profiles. Limnol. Oceanogr. *14*, 740-747 (1969)
- Determination of total microbial biomass by measurement of adenosine triphosphate. *In:* Estuarine microbial ecology, pp 73-89. Ed. by L.H. Stevenson and R.R. Colwell. Columbia, S.C.: University of South Carolina Press 1973
- and C.R. Booth: The measurement of adenosine triphosphate in the ocean and its ecological significance. Limnol. Oceanogr. *11*, 510-519 (1966)
-, C.R. Goldman, R. Richards and P.M. Williams: Chemical and biological characteristics of a water column in Lake Tahoe. Limnol. Oceanogr. *21*, 548-562 (1976)
Ireland, D.M. and D.C.B. Mills: Detection and determination of adenosine diphosphate and related substances in plasma. Biochem. J. *99*, 283-296 (1966)
Johnson, R.A., J.G. Hardman, A.E. Broadus and E.W. Sutherland: Analysis of adenosine 3', 5'-monophosphate with luciferase luminescence. Analyt. Biochem. *35*, 91-97 (1970)
Karl, D.M.: ATP and energy charge measurements in the Black Sea. A. Mtg microbiol. Soc. Am. Abstr. *N-6*, p. 229 (1977)
-, J.A. Haugness, L. Campbell and O. Holm-Hansen: Adenine nucleotide extraction from multicellular organisms and beach sand: ATP recovery, energy charge ratios and determination of carbon/ATP ratios. J. exp. mar. Biol. Ecol. (In press). (1978)
- and O. Holm-Hansen: Effects of luciferin concentration on the quantitative assay of ATP using crude luciferase preparations. Analyt. Biochem. *75*, 100-112 (1976)
- - Adenylate energy charge measurements in natural seawater and sediment samples. *In:* ATP Methodology Seminar, Vol. II. pp 141-169. Ed. by G.A. Borun. Sorrento Valley Blvd., San Diego, Ca.: SAI Technology Co. 1977
- and P.A. LaRock: Adenosine triphosphate measurements in soil and marine sediments. J. Fish. Res. Bd Can. *32*, 599-607 (1975)
- -, J.W. Morse and W. Sturges: Adenosine triphosphate in the North Atlantic Ocean and its relationship to the oxygen minimum. Deep-Sea Res. *23*, 81-88 (1976)
Kayne, F.J.: Pyruvate kinase. *In:* The enzymes, Vol. VIII. pp 353-382. Ed. by P.D. Boyer. New York, N.Y.: Academic Press 1973
Kimmich, G.A., J. Randles and J.S. Brand: Assay of picomole amounts of ATP, ADP and AMP using the luciferase enzyme system. Analyt. Biochem. *69*, 187-206 (1975)
Lundin, A. and A. Thore: Comparison of methods for extraction of bacterial adenine nucleotides determined by firefly assay. Appl. Microbiol. *30*, 713-721 (1975)
Manuels, M.W. and H. Postma: Measurements of ATP and organic carbon in suspended matter of the Dutch Wadden Sea. Neth. J. Sea Res. *8*, 292-311 (1974)
McElroy, W.D. and A. Green: Function of adenosine triphosphate in the activation of luciferin. Archs Biochem. Biophys. *64*, 257-271 (1956)
Noda, L.: Adenylate kinase. *In:* The enzymes, Vol. VIII. pp 279-305. Ed. by P.D. Boyer. New York: Academic Press 1973
Paerl, H.W., M.M. Tilzer and C.R. Goldman: Chlorophyll *a* versus adenosine triphosphate as algal biomass indicators in lakes. J. Phycol. *12*, 242-246 (1976)
Pradet, A.: Étude des adênosine-5'-mono, di et triphosphates dans les tissus vêgêtaux I. Dosage enzymatique. Physiologie vêg. *5*, 209-221 (1967)
Rhodes, D.G. and J.M. Lowenstein: Initial velocity and equilibrium kinetics of myokinase. J. biol. Chem. *243*, 3963-3972 (1968)
Rudd, J.W.M. and R.D. Hamilton: Measurement of adenosine triphosphate (ATP) in two Precambrian shield lakes of northwest Ontario. J. Fish. Res. Bd Can. *30*, 1537-1546 (1973)
Skjoldal, H.R. and V. Båmstedt: Studies on the deep-water pelagic community of Korsfjorden, western Norway. Adenosine phosphates and nucleic acids in *Meganyctiphanes norvegica* (Euphausiacea) in relationship to the life cycle. Sarsia *61*, 1-14 (1976)
Strehler, B.L.: Bioluminescence assay: principles and practice. *In:* Methods of biochemical analysis, Vol. 16. pp 99-181. Ed. by D. Glick. New York, N.Y.: Interscience Publishers 1968

Sutcliffe, W.H., E.A. Orr and O. Holm-Hansen: Difficulties with ATP measurements in inshore waters. Limnol. Oceanogr. *21*, 145-149 (1976)

Swedes, J.S., R.J. Sedo and D.E. Atkinson: Regulation of growth and protein synthesis to the adenylate energy charge in an adenine-requiring mutant of *Escherichia coli*. J. biol. Chem. *250*, 6930-6938 (1975)

Weiss, B., R. Lehne and S. Strada: Rapid microassay of adenosine 3'-5'-monophosphate phosphodiesterase activity. Analyt. Biochem. *45*, 222-235 (1972)

Wiebe, W.J. and K. Bancroft: Use of adenylate energy charge ratio to measure growth state of natural microbial communities. Proc. natn. Acad. Sci., U.S.A. *72*, 2112-2115 (1975)

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