19: ATP, ADP, and AMP determinations in water samples and algal cultures

DAVID M. KARL AND
OSMUND HOLM-HANSEN

Institute of Marine Resources, A-018,
Scripps Institute of Oceanography, University of California, San Diego,
La Jolla, California 92093

CONTENTS

I	**Introduction**	*page* **198**
II	**Equipment and reagents**	**199**
	A. Equipment	199
	B. Reagents	199
III	**Methods**	**200**
	A. Sample collection	200
	B. Extraction	200
	C. Enzyme preparation	201
	D. Standard ATP solutions	201
	E. ATP determinations	201
	F. ATP, ADP, and AMP determinations	202
IV	**Calculations**	**203**
	A. ATP data	203
	B. ATP, ADP, and AMP data, and calculation of adenylate energy charge	203
V	**Capabilities**	**203**
	A. Sensitivity	203
	B. Accuracy and reproducibility	204
VI	**Limitations and troubleshooting**	**204**
	A. Internal standards	204
	B. Enzyme specificity	204
VII	**Acknowledgments**	**205**
VIII	**References**	**205**

I. Introduction

The ubiquitous nature of adenine nucleotides (ATP, ADP, and AMP) in living cells, and their fundamental importance as intermediates linking energy-yielding and energy-requiring functions of the ce' have provided an impetus for the development of analytical methods for their quantitative determination (Holm-Hansen 1970; Holm-Hansen and Paerl 1972). Among the various techniques for separating and measuring ATP are (1) precipitation of insoluble salts and assay by acid hydrolysis of the isolated material (LePage 1945); (2) chromatographic separations, including paper (Cohn and Carter 1950), thin-layer ion-exchange (Randerath 1966), and high-pressure liquid chromatography (Brown 1970); (3) radioisotopic assay procedures involving enzymatic transfer of the ^{32}P-labeled terminal phosphate (Schneider 1969); (4) hexokinase enzyme coupling assay (Strehler and Totter 1954); (5) adenylic deaminase enzyme coupling assay (Kalckar 1947); (6) firefly luciferase enzyme coupling assay (Strehler and Totter 1952).

Each method has its advantages and disadvantages, but the firefly bioluminescent reaction is by far the most specific, sensitive, and reproducible assay procedure known. This method makes use of the fact that firefly luciferin and luciferase react with ATP in the presence of Mg^{2+} and oxygen to yield one photon of light for every ATP molecule hydrolyzed (McElroy et al. 1969). This procedure for the quantitative determination of ATP has had wide application in phycological research, including the study of energy-transfer and energy-coupling mechanisms (both oxidative phosphorylation and photosynthesis), the regulation of enzyme function and biosynthesis, cell viability studies, phosphorus metabolism, and various ecological studies. This chapter describes the complete experimental procedures and equipment necessary for the measurement of ATP, ADP, and AMP, an discusses briefly some of the analytical principles and limitations of these techniques.

II. Equipment and reagents

A. Equipment

1. Filtration. (a) A side-armed filter flask (1- to 4-l capacity), provided with a Millipore or Gelman filter base and funnel for 25-mm diam. filters. (b) An adjustable vacuum source with a vacuum gauge. (c) Microfine glass fiber (Reeve-Angel, 984-H) or membrane (Millipore 0.45 μm pore size, HA) filters, 25 mm diam.

2. Boiling-water bath. To contain a test tube rack.

3. Special glassware. All glassware used for adenine nucleotide analyses should be cleaned in a hot chromic acid bath (1 h, 80 C) and rinsed thoroughly (10–20 times) with distilled water. Alternatively, glassware rinsed in clean distilled water can be baked in a muffle furnace (500 C, 2–3 h) to eliminate contamination. (a) Pyrex glass test tubes 5 × 100 mm) calibrated at 5-ml intervals. (b) Pyrex glass Erlenmeyer flasks, 50 and 250 ml. (c) Glass scintillation vials or round-bottom glass vials (18 × 45 mm). (4) Disposable glass culture tubes (12 × 75 mm) for ADP and AMP analyses.

4. Stainless-steel forceps

5. Pipettes. (a) Glass or disposable plastic pipettes, 5 and 10-ml. (b) Automatic pipettes of 50- and 200-μl volumes, with disposable tips. (c) Automatic pipette to deliver 0.1–1.0 ml, with disposable tips.

6. Centrifugation. (a) A microcentrifuge is required to concentrate enzyme suspensions. It should be capable of handling volumes between 0.1 and 0.5 ml. (b) A clinical centrifuge with swinging rotor to accommodate 6–8 of the 15 × 100-mm test tubes.

7. Incubation. A water bath is required for ADP and AMP analyses to maintain a uniform temperature (30 C) during the enzyme reaction period.

8. Instrumentation for quantitative light detection. Amino Chem-Glow Photometer (American Instrument), Du Pont 760 Luminescence Biometer (Du Pont), or ATP Photometer, Model 2000 (SAI Technology).

9. Strip chart recorder or oscilloscope (optional)

10. Automatic pipette for peak height analysis

Reagents

(1) Tris(hydroxymethyl)aminomethane buffer, 0.02 M, pH 7.75. (2) K_2HAsO_4 buffer, 0.1 M, pH 7.4. (3) $MgSO_4$ solution, 0.04 M. (4)

Adenosine-5′-triphosphate (ATP), disodium salt from equine muscle (Sigma). (5) Firefly lantern extract, FLE-50 (Sigma). (6) Pyruvate kinase suspension, rabbit muscle (Sigma). (7) Myokinase suspension, rabbit muscle (Sigma). (8) Potassium phosphate buffer, 75 mM, pH 7.3, containing 15 mM $MgCl_2$. (9) Potassium phosphate buffer, 75 mM, pH 7.3, containing 15 mM $MgCl_2$ and 0.5 mM phosphoenolpyruvate (trisodium salt). (10) Synthetic D-luciferin, crystalline (Sigma) (*optional*).

III. Methods

A. Sample collection

1. Subsamples from algal cultures should be gently pipetted into a clean Erlenmeyer flask and extracted immediately.
2. Environmental samples may be collected using any clean, nontoxic water sampler. Caution should be taken to reduce light and temperature shock to naturally occurring phytoplankton populations.

B. Extraction

Whenever possible, between 50 and 200 μl of the algal culture should be pipetted directly into 5 ml of boiling TRIS buffer. When direct pipetting methods are employed, it is essential also to monitor the ATP, ADP, and AMP that may be dissolved in the growth medium. Filter a portion of the culture and then directly extract between 50 and 200 μl of the filtrate. This must be done for each sampling period. If the cell density is too low, samples may be concentrated by vacuum filtration (do not exceed 25 cm Hg). Centrifugation is not recommended. Membrane filters are satisfactory (Millipore, HA), although microfine glass fiber filters (Reeve Angel, 984-H) have the advantage of a much faster flow rate. When approximately 1–2 ml of liquid remains in the filter funnel, the vacuum pump should be turned off, and the residual vacuum in the filter flask will complete the filtration operation. *Caution:* Do not suck the filter dry. Immediately after the last portion of liquid is filtered, quickly transfer the filter into a test tube containing 5 ml of boiling TRIS buffer using stainless-steel forceps. *Caution:* It is essential that the TRIS buffer is at a temperature of 100 C. Extract the filters for 3–5 min, remove the test tubes from the boiling-water bath, and allow to cool. After the extraction has been completed, restore the volume to 5 ml with distilled water and store the extracts frozen (−20 C) for subsequent analysis. Frozen extracts are stable for up to 6 months.

C. Enzyme preparation

Each vial of lyophilized firefly lantern extract (50 mg/vial) is reconstituted by adding 5 ml of distilled water, 10 ml of 0.1 M K_3AsO_4 buffer (pH 7.4), and 10 ml of 0.04 M $MgSO_4$. The enzyme mixture is then allowed to stand for 6–8 h at 23–25 C to reduce the level of endogenous ATP. After aging, the insoluble residue is removed by vacuum filtration or centrifugation. The clear filtrate or supernatant is transferred to a clean flask, and the enzyme is ready for use.

D. Standard ATP solutions

For each enzyme preparation (1 or more vials), a standard curve must be prepared. Stock ATP solutions containing 1 μg ATP ml^{-1} (ca. 2 μM) are prepared in TRIS buffer and stored frozen (−20 C) in 5-ml aliquots. Working solutions are prepared on the day of each assay by diluting the stock solution with TRIS buffer, providing 6–7 standards ranging from 0.5 ng ATP ml^{-1} (ca. 1 nM) to 50 ng ATP ml^{-1} (ca. 100 nM). If a large number of samples is analyzed in a single day, a standard curve should be analyzed at the beginning of the assay procedure and again every 35–40 samples. If fewer than 40 samples are analyzed, a single standard curve (measured halfway through the experiment) is sufficient.

E. ATP determinations

ATP can be assayed either by measuring the peak intensity of light emission or by integrating a portion of the subsequent light decay curve. All frozen samples should be thawed in a water bath (25 C) and centrifuged for 5 min at 1800 × *g* prior to analysis. *Caution:* All ATP assays should be conducted in reduced illumination, and fluorescent lighting should be avoided.

1. Peak height measurement. Pipette 1 ml of the enzyme preparation into a round-bottom glass vial. Place the vial into the photometer and record the background light emission (0–6 sec). Position the vial in the photometer. Using the automatic pipette, withdraw 0.2 ml of the sample extract or ATP standard solution and inject it into the enzyme mixture. Record the peak height digital display. Blanks are determined by injecting 0.2 ml of TRIS buffer into the enzyme mixture as described above.

2. Integrated measurement. Pipette 0.5 ml of the enzyme preparation into a clean glass scintillation vial and record the background light emission. Remove the vial from the photometer and inject 0.5 ml of sample or ATP standard solution. Mix the vial thoroughly and place into the photometer. The light flux is then integrated (either electron-

ically or manually by plotting the kinetics with a strip chart recorder and measuring the area under the curve with the use of a planimeter) over a predetermined period of the subsequent decay curve (i.e., 15–75 sec after injection of the sample). Record digital readout or calculated area. Blanks are determined by injecting 0.5 ml of TRIS buffer into the enzyme preparation as described above. *Note:* Although commercial ATP photometers (see II.A.8) are superior in terms of convenience and ease of operation, the light emitted from the firefly bioluminescent reaction can be measured using any one of a number of light-detecting instruments (Strehler 1968).

F. ATP, ADP, and AMP determinations

Each sample extract is first assayed for ATP, then after the appropriate enzymatic conversions of ADP and AMP to equivalent concentrations of ATP, the samples are reassayed. For ATP determinations, 0.2 ml of each sample extract is pipetted into a culture tube (12 × 7 mm) containing 50 μl of a solution of 75 mM potassium phosphate buffer (pH 7.3) and 15 mM $MgCl_2$. For ATP plus ADP determinations, 0.2 ml of each extract is pipetted into a culture tube containing 50 μl of a solution of 75 mM potassium phosphate buffer (pH 7.3), 15 mM $MgCl_2$, 0.5 mM phosphoenolpyruvate, and 20 μg pyruvate kinase. For ATP plus ADP plus AMP determinations, 0.2 ml of each sample is pipetted into a culture tube containing 50 μl of a solution of 75 mM potassium phosphate buffer (pH 7.3), 15 mM $MgCl_2$, 0.5 mM phosphoenolpyruvate, 20 μg pyruvate kinase, and 25 μg myokinase (adenylate kinase). Standard ATP solutions should be prepared at the same time as the samples in a manner identical to each of the three procedures described above. *Note:* Prior to use, the pyruvate kinase and myokinase enzyme suspensions should be centrifuged (2000 × *g*, 3–5 min) and the pellet resuspended in an equivalent volume of 75 mM potassium phosphate buffer (pH 7.3).

All samples are then incubated at 30 C for 30 min and then placed in a boiling-water bath for 2 min to deactivate the pyruvate kinase enzyme. This procedure prevents the rapid production of ATP from ADP present in the crude enzyme preparations. When the samples cool to room temperature, they are ready for analysis by the peak height method described previously. Enzyme blanks (TRIS buffer plus enzyme solutions) should also be determined, as certain enzyme preparations contain contaminating adenine nucleotides. *Note:* The peak emission mode is essential when measuring ADP and AMP. Reliable peak height measurements require specially designed equipment for rapid and reproducible injection velocities. To our knowledge only three instruments are commercially available for such ATP determinations (see II.A.8).

IV. Calculations

A. ATP data

Determine the net emission by subtracting the enzyme blank value from each of the sample or standard measurements. A standard curve is then prepared by plotting net peak intensities or integrated areas on the ordinate and ATP concentrations on the abscissa. From this curve, the ATP concentration in sample extracts can be calculated, and by correcting for the proportion of the sample actually assayed and the volume of medium originally extracted, the ATP ml^{-1} (or ATP $cell^{-1}$, ATP chl a^{-1}, etc.) of the original culture or natural water sample can be determined.

B. ATP, ADP, and AMP data, and calculation of adenylate energy charge

termine the net light emission by subtracting the appropriate blank values from each of the sample determinations. Standard curves are prepared by plotting net peak intensities on the ordinate and ATP concentrations on the abscissa for each of the three sets of standard data. From these curves, the ATP concentration in each of the three different reaction mixtures (ATP, ATP plus ADP, and ATP plus ADP plus AMP) can be determined. ADP and AMP concentrations are calculated by the difference between these measured values. The adenylate energy charge, as defined by Atkinson and Walton (1967), is determined from the measured adenylate concentrations.

$$\text{energy charge} = \frac{[\text{ATP}] + \frac{1}{2}[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]} \qquad (1)$$

Note: In addition to the data-reduction methods described above, at least two computer-assisted techniques for processing ATP sample data have recently been published (Booth 1975; Erkenbrecher et al. 1976). Both these methods, however, require integrated light flux measurements and are, therefore, not capable of calculating ADP and AMP concentrations or adenylate energy charge ratios in their present form.

V. Capabilities

A. Sensitivity

The lower limit of ATP detectability is influenced by the amount of .ogenous ATP in the enzyme preparation (enzyme blank) and by the sensitivity and thermionic emission (tube noise) of the photomultiplier tube. With a commercial ATP photometer (SAI Technology),

the firefly bioluminescent reaction procedure will detect ATP concentrations as low as 0.1 ng ml^{-1} (ca. 0.2 nM) using crude enzyme preparations (FLE-50). If synthetic D-luciferin is added to saturate the enzyme system, the sensitivity can be extended nearly 1000-fold (Karl and Holm-Hansen 1976). When low concentrations of adenine nucleotides (<1 ng ml^{-1}) are measured, extreme caution should be taken to ensure that all glassware has been properly cleaned (see II.A.3).

B. Accuracy and reproducibility

The maximum statistical variation for the extraction and assay of ATP from environmental samples and algal cell suspensions is ±10% at the 95% confidence interval for samples containing 12 ng ATP ml^{-1} (ca. 24 nM). For a given operator, variation in standard sample analyses is generally ±5%.

VI. Limitations and troubleshooting

A. Internal standards

The emitted light in the firefly bioluminescent reaction is influenced by the presence of interfering cations and anions, turbidity, and the color of the final cell extracts. The magnitude of this combined interference can be determined, and corrected for, through the use of an ATP internal standard. The chemical ATP standard (in TRIS buffer) can be added at any point in the extraction procedure. The concentration of the added standard should be approximately equal to the measured concentration in the extract. To determine the percent recovery of the internal standard, calculate the ATP concentration in both extracts (i.e., sample and sample plus internal standard) and use the following relationship:

$$\% \text{ recovery} = \frac{[\text{sample} + \text{internal standard}] - [\text{sample}]}{[\text{internal standard}]} \times 100 \quad (2)$$

To correct for all sources of interference, divide the final measured ATP concentration by the decimal equivalent of the percent recovery.

B. Enzyme specificity

Highly purified firefly luciferase responds only to ATP (McElroy and Green 1956), but most commercial reagents contain contaminating enzymes that will produce ATP from nucleotide triphosphate precursors in the sample extracts. The contribution of light emission from these additional reactions can be reduced by measuring the peak

height of light emission rather than using the integral mode of light detection.

VII. Acknowledgments

We thank Drs. A. F. Carlucci and R. W. Eppley for their helpful comments during the preparation of this manuscript. Much of the methodology described in this chapter was developed under ERDA Contract No. EY-76-C-03-0010, PA 20. We thank Ms. Janice Stearns for typing the manuscript.

VIII. References

Atkinson, D. E., and Walton, G. M. 1967. Adenosine triphosphate conservation in metabolic regulation: Rat liver citrate cleavage enzyme. *J. Biol. Chem.* 242, 3239–41.

ɔth, C. R. 1975. Instrument development and data processing for the ATP photometer. In Borun, G. A. (ed.). *ATP Methodology Seminar,* pp. 104–30. SAI Technology, San Diego, California.

Brown, P. R. 1970. The rapid separation of nucleotides in cell extracts using high-pressure liquid chromatography. *J. Chromatogr.* 52, 257–72.

Cohn, W. E., and Carter, C. E. 1950. The separation of adenosine polyphosphates by ion exchange and paper chromatography. *J. Am. Chem. Soc.* 72, 4273–5.

Erkenbrecher, C. W., Crabtree, S. J., and Stevenson, L. H. 1976. Computer-assisted analysis of adenosine triphosphate data. *Appl. Environ. Microbiol.* 32, 451–4.

Holm-Hansen, O. 1970. ATP levels in algal cells as influenced by environmental conditions. *Plant Cell Physiol.* 11, 689–700.

Holm-Hansen, O., and Paerl, H. W. 1972. Applicability of ATP determination for estimation of microbial biomass and metabolic activity. *Mem. Ist. Ital. Idrobiol. Dott. Marco de Marchi Pallanza Italy* 29 (Suppl.), 149–68.

Kalckar, H. M. 1947. Differential spectrophotometry of purine compounds by means of specific enzymes. *J. Biol. Chem.* 167, 445–59.

Karl, D. M., and Holm-Hansen, O. 1976. Effects of luciferin concentration on the quantitative assay of ATP using crude luciferase preparations. *Anal. Biochem.* 75, 100–12.

LePage, G. A. 1945. Methods for the analysis of phosphorylated intermediates. In Umbreit, W. W., Burris, R. H., and Stauffer, J. F. (eds.). *Manometric Techniques,* 1st ed., pp. 268–81. Burgess, Minneapolis.

McElroy, W. D., and Green, A. 1956. Function of adenosine triphosphate in the activation of luciferin. *Arch. Biochem. Biophys.* 64, 257–71.

McElroy, W. D., Seliger, H. H., and White, E. H. 1969. Mechanism of bioluɔinescence, chemiluminescence and enzyme function in the oxidation of ɾefly luciferin. *Photochem. Photobiol.* 10, 153–70.

Randerath, K. 1966. *Thin-Layer Chromatography.* Academic Press, New York. 285 pp.

Schneider, P. B. 1969. An enzymatic assay for adenosine-5′-triphosphate (ATP) and other nucleotide triphosphates and determination of the specific radioactivity of the terminal P. *Anal. Biochem.* 28, 76–84.

Strehler, B. L. 1968. Bioluminescence assay: Principles and practice. *Methods Biochem. Anal.* 16, 99–181.

Strehler, B. L., and Totter, J. R. 1952. Firefly luminescence in the study of energy transfer mechanisms. I. Substrate and enzyme determination. *Arch. Biochem. Biophys.* 40, 28–41.

Strehler, B. L., and Totter, J. R. 1954. Determination of ATP and related compounds. *Methods Biochem. Anal.* 1, 341–56.