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[8] Biomass and Adenylate Energy Charge Determination in Microbial Cell Extracts and Environmental Samples

By OSMUND HOLM-HANSEN and DAVID M. KARL

In both laboratory and field studies it is often desirable to determine the total amount of living cellular material (biomass) as well as the activity or metabolic state of the cells. Conventional methods (i.e., fresh or dry weight determinations, rate of increase of cell numbers, etc.) usually cannot be used owing to (1) lack of sensitivity in the analytical procedures, (2) the heterogeneous assemblage of cellular materials, (3) the presence of dead cells, and (4) the presence of detrital material not associated with the living cells. Estimation of cellular biomass by measurement of adenosine triphosphate (ATP) is generally not limited by any of these considerations.

The rationale for using ATP to estimate biomass is the ubiquitous distribution of ATP in all living cells, the rapid loss of ATP from dead cells, and the fairly uniform concentration of ATP in the protoplasm of all microbial cells. Data on ATP concentrations can thus be extrapolated to biomass parameters, such as cellular organic carbon or dry or fresh weight.[1-3]

In many studies it is desirable to determine the biochemical activities or metabolic states of the cells in addition to total biomass. One promising approach to the investigation of the energetic state of cells is to determine the concentrations and ratios of the adenine ribonucleotides (ATP, ADP, and AMP), which are involved in stoichiometric energy transduction between most metabolic reactions, and thus are of great significance with respect to regulation of cellular metabolism. Atkinson and his colleagues have defined adenylate energy charge (EC) as

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

whereby the EC is a measure of the metabolically available energy stored in the adenine nucleotides. Since cells in normal growth conditions have control systems that maintain the concentrations of these nucleotides within narrow range limits, marked decreases in the EC may reflect en-

[1] O. Holm-Hansen and C. R. Booth, *Limnol. Oceanogr.* **11**, 510 (1966).
[2] O. Holm-Hansen, *Plant Cell Physiol.* **11**, 689 (1970).
[3] O. Holm-Hansen, *in* "Estuarine Microbial Ecology" (L. H. Stevenson and R. R. Colwell, eds.), p. 73. Univ. of South Carolina Press, Columbia, South Carolina, 1973.

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vironmental stress or growth conditions that interfere with the normal functioning of the organism.

In this chapter we describe the methodology for quantitative determinations of ATP, ADP, and AMP in cell extracts and environmental samples, by use of the firefly luciferin–luciferase bioluminescent reaction. Difficulties and sources of error are discussed in regard to the analytical phase as well as the preliminary steps of sampling and extraction of the nucleotides from living cells.

Assay Principles

$$ATP + LH_2 + O_2 \xrightarrow{FL} AMP + PP_i + CO_2 + P + h\nu \quad (2)$$

$$ADP + PEP \xrightarrow{PK} ATP + \text{pyruvate} \quad (3)$$

$$AMP + ATP \xrightleftharpoons{AK} 2\ ADP \quad (4)$$

where FL = firefly luciferase (EC 2.7. .), PK = pyruvate kinase (EC 2.7.1.40), AK = adenylate kinase (EC 2.7.4.3), LH_2 = reduced luciferin, P = product, PP_i = inorganic pyrophosphate, PEP = phosphoenolpyruvate.

Firefly luciferase is specific for ATP,[4,5] and hence it is not necessary to separate ATP from other nucleotides in the extracts. The light-emission reaction catalyzed by luciferase [Eq. (2)] is more complicated than shown above (see this volume [1]); as there is one quantum of visible light emitted per ATP molecule hydrolyzed, quantification of the light flux enables one to calculate the ATP concentration in the extract.

To determine ADP and AMP, these nucleotides must first be converted to ATP, which is then measured as in Eq. (2). To determine ADP, subsamples of the extract are incubated with excess PEP and pyruvate kinase [see Eq. (3)]. The ATP formed in this reaction is measured as in Eq. (2) and, after subtraction of the initial concentration of ATP, is equivalent to the original concentration of ADP in the sample extract. The concentration of AMP in another subsample is likewise determined by converting the AMP to ADP via the adenylate kinase reaction [Eq. (4)], and simultaneous conversion of the ADP formed to ATP via the pyruvate kinase reaction [Eq. (3)].

The most practical and convenient way to relate the measured light flux to ATP concentrations is to use a series of internal ATP standards. A variety of light-detecting instruments can thus be used, as it is necessary

[4] A. Green and W. D. McElroy, *Biochim. Biophys. Acta* **20**, 170 (1956).
[5] M. DeLuca, *Adv. Enzymol.* **44**, 37 (1976).

only to measure relative changes in light emission rather than absolute energy fluxes.

Reagents and Equipment

Reagents. Unless otherwise indicated, catalog numbers or information refer to items sold by Sigma Chemical Company.[6]

Firefly lantern extract (FLE-50)
Sodium arsenate buffer, 100 m*M*, pH 7.4
ATP, ADP, and AMP, sodium salts, 2 μM solutions in Tris buffer, dispensed into small aliquots (3–5 ml), and stored frozen (−20°)
Magnesium sulfate solution, 40 m*M*
Tris(hydroxy)aminomethane−HCl buffer, 20 m*M*, pH 7.75
Potassium phosphate buffer, 225 m*M*, pH 7.4
Magnesium chloride solution, 45 m*M*
D-Luciferin, optional (L 9504)
Pyruvate kinase, 350–500 units per milligram of protein (P 1506)
Adenylate kinase, 1000–1500 units per milligram of protein (M 3003)
Phosphoenolpyruvate (P 7002)

Equipment

Light-detection instrument: Any one of a number of commercially available general instruments, such as fluorometers, spectrophotometers or liquid scintillation counters, are suitable for measuring light emission. However, in order to obtain efficient and reliable data, with maximum sensitivity, a specially designed ATP photometer is often required. Instruments specifically designed for ATP analyses are marketed by the American Instrument Company (Chem-Glow photometer), E. I. DuPont, Inc. (Luminescence Biometer), and SAI Technology Company (ATP Photometer). A detailed description and comparison of these three instruments can be found in this volume [43].
Strip-chart recorder with adjustable ranges from 10 mV to 10 V
Water baths: one at 30°, one at 100°
Glassware: All glassware used for nucleotide analyses should be thoroughly cleaned in order to eliminate the possibility of contamination; if possible, disposable glassware should be used: (a) Pyrex glass flasks, 50 and 250 ml, (b) vials, 18 × 40 mm (Kimble, No. 74400), and (c) glass culture tubes, 12 × 75 mm (Scientific Products, No. T-1285-3).

[6] References to Sigma Chemical Company or to other manufacturers do not represent any endorsement of these sources, but are cited only for the convenience of the reader.

Pipettes: (a) 5- and 10-ml glass or disposable plastic, (b) automatic pipette 0.1 to 1.0 ml, with disposable tips, (c) automatic pipettes of 50-μl and 200-μl volume, with disposable tips

Automatic injector: For use in conjunction with the light-detection instruments. Must be capable of rapid and reproducible injection of sample into the enzyme preparation; disposable tips are recommended.

Assay Methods

The procedures described below assume that the investigator has a nucleotide-containing extract that does not contain any inhibitors that would interfere with the reactions shown in Eqs. (2)–(4); later sections will discuss extract preparation and sources of analytical interference.

Luciferase Enzyme Preparation

Each vial of lyophilized firefly lantern extract (50 mg/vial) is reconstituted by adding 5 ml of distilled water, 10 ml of 100 m*M* sodium arsenate buffer (pH 7.4), and 10 ml of 40 m*M* $MgSO_4$. The enzyme mixture is allowed to stand for 3–4 hr at room temperature (23°–25°) in order to reduce the level of background light emission. After aging, the insoluble residue is removed by passage through a filter or by centrifugation. The clear filtrate or supernatant is transferred to a clean flask, and the enzyme preparation is now ready for use. Since considerable variation occurs between individual vials of firefly lantern extract, the contents of several vials should be pooled whenever large volumes of enzyme are required.

Standard Nucleotide Solutions

Internal standards are used to calculate absolute concentrations of ATP, ADP, and AMP in sample extracts; a fresh set of standards must be prepared for each day's work. A small tube of the frozen nucleotide is thawed and sequentially diluted with Tris buffer to give a series of solutions ranging in concentration from 200 n*M* to 1 n*M*. All thawed standards are discarded immediately after use. These precautions are necessary to minimize hydrolysis of the nucleotides during storage and also to minimize bacterial contamination of working solutions. There should be no significant loss of nucleotides in these standard solutions during an 8-hr period.

Light Measurement

There are several ways to quantitate the amount of ATP when using the luciferase bioluminescent reaction. The two methods most commonly used

are (1) to record the peak height of the light emission, which should occur within 3 sec after injection of the sample, and (2) to integrate the light flux for a set time period. The major advantages of the integrated mode are (a) increased sensitivity, (b) ease and reliability of mixing, and (c) no need to depend upon a peak-height response, which may be difficult or impossible to measure with certain instruments. Reliability of the peak-height analysis depends upon a very rapid and complete mixing of all reactants. This is best accomplished by use of an automatic pipette, which ensures consistent mixing velocities for all samples. The peak-height mode of analysis offers the advantages of speed of assay and minimum interference from other enzymes or substrates that may affect the rate of the luciferase-catalyzed reaction.[7,8] This is particularly relevant when determining AMP concentrations, as adenylate kinase [Eq. (4)] and PEP are heat stable and cause a continual synthesis of ATP when injected into the luciferase preparation. For this reason, all work described in this chapter will utilize the peak-height mode of light measurement. As our work has been done with the ATP Photometer and Automatic Pipette produced by SAI Technology Co. (San Diego, California), volumes of enzyme and sample as well as size of the reaction vials to be used refer to that instrument.

ATP Determination

A glass vial (18 × 40 mm) containing 1.0 ml of the luciferase enzyme preparation is placed in the sample holder of the ATP photometer. The automatic pipette, containing 0.2 ml of the sample to be analyzed, is placed on top of the sample holder and the background light emission of the enzyme preparation is recorded by pushing the assay switch on the photometer. After clearing the digital display, the release mechanism on the automatic pipette is pushed, causing injection of the sample into the enzyme preparation and starting the timing circuits. The maximum peak height is obtained as a digital readout on the photometer. Although it is not necessary, it is informative to connect the ATP photometer to a strip-chart recorder in order to monitor the kinetics of the light-emission reaction. This is particularly useful in recognizing possible interferences with the luciferase reaction (effects of inhibitors, other nucleotides, etc.) as well as any instrument malfunction (e.g., leaky pipette). With any one batch of samples (up to about 100), a series of standards is generally run before and after the samples. The standard ATP solutions should have the same ionic composition as the sample solutions to ensure that the reactivity of ATP is similar in all determinations. Calculations of ATP concentrations are described in a later section.

[7] H. Rasmussen and R. Nielsen, *Acta Chem. Scand.* **22**, 1745 (1968).
[8] A. Lundin and A. Thore, *Anal. Biochem.* **66**, 47 (1975).

Enzymic Conversion of ADP and AMP to ATP

Although detailed procedures have already been described for adenine nucleotide determinations in plant tissues[9] and bacterial cell extracts,[10] several modifications were necessary in order to adapt these techniques for the relatively low levels of adenine nucleotides in most environmental samples.[11]

Each batch of samples to be analyzed for ATP, ADP, and AMP must be prepared and processed together with (a) a Tris-buffered reagent blank, (b) a set of 6–8 ATP standards ranging from 1 n*M* to 200 n*M* and (c) a series of solutions containing various ratios of ATP, ADP, and AMP. For each test sample, 200 μl of the Tris-buffered extract are pipetted into each of 4 disposable glass culture tubes (12 × 75 mm) labeled A (for ATP), B (for ATP + ADP), C and D (for ATP + ADP + AMP). The following additions are then made to these tubes:

Tubes A: Add 50 μl of a solution containing $MgCl_2$ (15 m*M*) and potassium phosphate buffer (75 m*M*, pH 7.4).

Tubes B: Add 50 μl of a solution containing $MgCl_2$ and potassium phosphate buffer (as above) in addition to phosphoenolpyruvate (0.5 m*M*) and pyruvate kinase (20 μg).

Tubes C: Add 50 μl of a solution containing $MgCl_2$, potassium phosphate buffer, PEP, and PK (as above), in addition to adenylate kinase (25 μg).

Tubes D: Add 50 μl of a solution similar to that added to C tubes, except that the solution also contains 10 ng of ATP.

Blanks, ATP standards, and the standard mixtures of ATP, ADP, and AMP are all treated as tubes A–D above. The reason for having the D tubes is that the efficiency of the adenylate kinase reaction is affected by the total adenine nucleotide concentration in the sample,[11] and inclusion of a series of D tubes (with increased concentrations of ATP) will indicate the efficiency of the adenylate kinase reaction in the sample extracts.

All the tubes are incubated for 30 min at 30°, placed into a boiling water bath (100°) for 2 min, and allowed to adjust to room temperature (~30 min). The samples are now ready for ATP analyses according to the methods described above.

Data Reduction and Calculation of the EC Ratio

Net light emission is determined by subtracting the appropriate blank value from each of the total light-emission determinations. Standard curves

[9] A. Pradet, *Physiol. Veg.* **5**, 209 (1967).
[10] A. G. Chapman, L. Fall, and D. E. Atkinson, *J. Bacteriol.* **108**, 1072 (1971).
[11] D. M. Karl and O. Holm-Hansen, *Mar. Biol.*, in press (1978).

are 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 + ADP, ATP + ADP + AMP) is determined, and the amounts of ATP enzymically produced from ADP and AMP are calculated as differences between these measured values. The adenylate EC ratio is determined using the formulation of Ball and Atkinson[12] to reduce the propagation of errors.

If the integral mode of light detection is used, calculation of ATP concentrations becomes more difficult for the following reasons: (1) The addition of phosphoenolpyruvate to crude luciferase preparations results in a significant level of light emission, which increases with time after injection, owing to the presence of pyruvate kinase and ADP in the enzyme preparations. (2) Since adenylate kinase is a heat-stable protein, it remains active following the heat deactivation period and competes with luciferase for the ATP in solution. (3) The plot of ATP versus integrated light flux does not result in a linear plot as expected, especially at ATP concentrations greater than or equal to 20 n*M*. Although graphical analyses of integrated light values have been used to calculate ATP concentrations, greater precision is achieved through the use of a computer program, such as the one described by Booth.[13]

As discussed previously, peak height analyses may be conducted without any of the above considerations, and therefore are strongly recommended for adenine nucleotide determinations.

Discussion

Sampling of Cells and Extraction of Nucleotides

As ATP is rapidly turned over in growing cells,[14] and rapid and dramatic variations result from changes in environmental conditions,[15] it is essential that the cells be extracted as quickly as possible after sample collection. The most direct method of sampling bacterial or algal cultures is through direct injection of the sample into the extraction medium. If this procedure is utilized, it is imperative to measure the levels of soluble (cell-free) nucleotides in the culture medium as well, and to correct the original adenine nucleotide determinations for these dissolved concentrations. The most convenient method for determining cell-free adenine nucleotides is to

[12] W. J. Ball and D. E. Atkinson, *J. Bacteriol.* **121**, 975 (1975).
[13] C. R. Booth, *in* "Proceedings of ATP Methodology Seminar" (G. A. Borun, ed.), p. 104. SAI Technology Company, San Diego, California, 1975.
[14] D. E. F. Harrison and P. K. Maitra, *Biochem. J.* **112**, 647 (1969).
[15] J. Coombs, P. J. Halicki, O. Holm-Hansen, and B. E. Volcani, *Exp. Cell Res.* **47**, 315 (1967).

filter a portion of the culture medium through a membrane filter (pore dimensions dependent upon mean cell diameter) and to extract and assay an equivalent volume of the filtrate.

With most environmental samples, however, a preliminary concentration of the cellular material is required before the extraction procedure. The most practical and effective method for cell concentration is through vacuum filtration. The choice of filter is dictated by the size spectrum of cells. For marine bacterial cells, microfine glass fiber filters (Reeve Angel, 984H), or membrane filters such as Millipore (GS) or Nucleopore (0.2 μm), are most commonly used. For phytoplankton and larger unicells, Whatman (GF/C) glass fiber filters are effective. Immediately after the final amount of liquid has passed through the filter, the vacuum should be released and the filter should be quickly immersed into the extraction medium. It should be mentioned that vacuum filtration has been shown to have a detrimental effect on the concentration of ATP, and on the relative concentrations of ATP, ADP, and AMP recovered from naturally occurring microbial populations[11]; therefore, caution should be taken whenever preliminary concentration of the samples is required. The effect of vacuum filtration can be demonstrated by filtering increasing volumes of a particular water sample and then normalizing the results to a particular volume (i.e., normalize all samples to nanogram of ATP liter^{-1}). As the sample volume is increased, the concentration of ATP liter^{-1} and the value of the adenylate energy charge both decrease. As a compromise, minimal sample volumes should be extracted in order to reduce the effect of vacuum filtration.[11]

Azam and Hodson[16] have described an additional method for ATP determinations from dilute suspensions of microorganisms. This procedure involves the addition of H_2SO_4 directly to the water sample, followed by adsorption of the nucleotides onto activated charcoal and subsequent elution with ammoniacal ethanol. The samples are then evaporated to dryness and reconstituted in Tris buffer (pH 7.7, 20 m*M*). It is necessary to correct the measured ATP concentrations for the concentration of dissolved ATP in each sample. An equal volume of cell-free (0.2 μm filtrate) liquid must also be extracted as above. Since the efficiency of ATP binding to and elution from activated charcoal is highly variable, it is necessary to correct all the determinations using an ATP internal standard. Furthermore, since the binding and elution characteristics of the various adenine nucleotides are not identical,[11,17] and also owing to the possibility of charcoal-catalyzed ATP hydrolysis, this procedure is not recommended for energy charge determinations.

[16] F. Azam and R. E. Hodson, *in* "Proceedings of the Second Biannual ATP Methodology Symposium" (G. A. Borun, ed.), p. 109. SAI Technology Company, San Diego, California, 1977.

[17] D. M. Ireland and D. C. B. Mills, *Biochem. J.* **99**, 283 (1966).

A number of different extraction media and methodologies are currently in use for adenine nucleotide analyses. These include: boiling buffers,[1,18-20] sulfuric acid,[21] perchloric acid,[22] nitric acid,[23] trichloroacetic acid,[24] *n*-butanol,[25] chloroform,[26] and *n*-bromosuccinimide.[27] It has been our experience that excellent results are obtained with boiling Tris buffer (pH 7.7, 20 m*M*) for aqueous suspensions of microbial cells, and with cold sulfuric acid for multicellular organisms and soils or sediments. When using boiling Tris buffer, it is essential that the temperature be maintained at 100° to deactivate the ATPases. Moreover, when samples are directly injected into the boiling Tris, it is necessary to keep the injection volume low relative to the extractant volume (i.e., $\leq 2\%$) to minimize temperature drops. After continued heating for 4–5 min, the samples are removed from the water bath, restored to the original volume with distilled water, and stored frozen (−20°) until time of analysis.

When extracting multicellular organisms or soil or sediment samples, boiling buffers generally result in low ATP recoveries. This is due in part to the effects of thermal gradients associated with fluid–solid mixtures[28] and in part to loss of ATP through adsorption at neutral or alkaline pH. These difficulties can be averted through the use of cold sulfuric acid as the extraction medium.[21,28] After an extraction period of 15–20 min at 4°, the nucleotides are separated from the solid phase by centrifugation or vacuum filtration, and the pH of the acid extracts is adjusted to 7.7 with sodium hydroxide. The neutralized extracts are stored frozen (−20°) until time of analysis. Since both Na^+ and SO_4^{2-} ions inhibit the luciferase reaction, the ATP standards must be prepared in the same extraction and neutralization fluids.

Purity of Luciferase Preparation

Considerable variation exists among laboratories concerning the purity, preparation, handling, and use of the firefly luciferin–luciferase prepa-

[18] E. Beutler and M. C. Baluda, *Blood* **23**, 688 (1964).
[19] S. Lin and H. P. Cohen, *Anal. Biochem.* **24**, 531 (1968).
[20] K. Bancroft, E. A. Paul, and W. J. Wiebe, *Limnol. Oceanogr.* **21**, 473 (1976).
[21] C. C. Lee, R. F. Harris, J. D. Williams, D. E. Armstrong, and J. K. Syers, *Soil Sci. Soc. Am., Proc.* **35**, 82 (1971).
[22] R. E. Strange, H. E. Wade, and F. A. Dark, *Nature (London)* **199**, 55 (1963).
[23] J. W. Deming, M. W. McGarry, E. W. Chappelle, and G. L. Picciolo, *in* "Proceedings of the Second Biannual ATP Methodology Symposium" (G. A. Borun, ed.), p. 465. SAI Technology Company, San Diego, California, 1977.
[24] E. Beutler and K. Mathai, *Blood* **30**, 311 (1967).
[25] E. W. Chappelle and G. V. Levin, *Biochem. Med.* **2**, 41 (1968).
[26] A. M. Dhople and J. H. Hanks, *Appl. Microbiol.* **26**, 399 (1973).
[27] N. H. MacLeod, E. W. Chappelle, and A. M. Crawford, *Nature (London)* **223**, 267 (1969).
[28] D. M. Karl and P. A. LaRock, *J. Fish. Res. Board Can.* **32**, 599 (1975).

rations. In general, the specific application of the methods will dictate the choice of the reagents. Crude reagents are less sensitive and less specific than purified preparations, but they are also considerably less expensive. Both crude and partially purified enzyme preparations are available commercially; a number of laboratory techniques are also available for purification of the firefly reagents. These include: $(NH_4)_2SO_4$ precipitation followed by adsorption onto calcium phosphate gels,[29] Sephadex G-100 chromatography,[7] DEAE-cellulose chromatography,[30] and isoelectric focusing.[31] The techniques described in this chapter can be used with either crude or partially purified reagents.

Reaction Specificities

Firefly Luciferase. Although crystalline firefly luciferase is specific for ATP,[4,5] a number of ribose and deoxyribose nucleotides, especially guanosine triphosphate (GTP), will stimulate light emission in most commercial luciferase preparations (crude, as well as "purified"). The degree of analytical interference will depend upon the particular enzyme preparation used (see previous section), the method used to detect light emission (peak vs integral), and the [ATP]/[NTP] ratio of the cell extract. Since the time course of light emission resulting from non-adenine nucleotide triphosphates is slower than the ATP-dependent light-emission kinetics (see this volume [9]), peak height measurements will substantially reduce this source of interference.

Pyruvate Kinase. The pyruvate kinase reaction [Eq. (3)] is relatively nonspecific and will catalyze the transfer of ~P from PEP to GDP, IDP, UDP, and CDP, as well as ADP.[32] The corresponding nucleotide triphosphates that are formed can then serve as substrates for the nucleoside diphosphate kinase reaction, resulting in light emission in impure luciferase preparations.

Adenylate Kinase. The reaction given in Eq. (4) is very specific for AMP as the phosphoryl acceptor, but relatively nonspecific for ATP as the phosphoryl donor[33] (i.e., AMP + NTP $\rightleftharpoons$ NDP + ADP).

[29] W. D. McElroy, this series, Vol. 2, p. 854, 1955.

[30] I. Bekhor, J. K. Pollard, and W. Drell, *in* "Proceedings of the Second Biannual ATP Methodology Symposium" (G. A. Borun, ed.), p. 27. SAI Technology Company, San Diego, California, 1977.

[31] A. Lundin, A. Rickardsson, and A. Thore, *in* "Proceedings of the Second Biannual ATP Methodology Symposium" (G. A. Borun, ed.), p. 205. SAI Technology Company, San Diego, California, 1977.

[32] F. J. Kayne, *in* "The Enzymes" (P. D. Boyer, ed.), 3rd ed., Vol. 8, p. 353. Academic Press, New York, 1973.

[33] L. Noda, *in* "The Enzymes" (P. D. Boyer, ed.), 3rd ed., Vol. 8, p. 279. Academic Press, New York, 1973.

Analytical Interferences

In addition to the problems discussed above in the section entitled Sampling of Cells and Extraction of Nucleotides, other sources of analytical interference may be encountered during the enzymic determination of ATP. These include: (1) the presence of inorganic and organic ions in the sample extracts, resulting in loss of ATP in solution (i.e., through chelation) or in decreased luciferase activity, (2) the presence of humic acid-like substances in the sample extracts that impart a yellow color to the solution, thereby resulting in attenuation of the emitted light, (3) turbidity of the final extracts resulting in light scattering and absorption, (4) the presence of a high concentration of inorganic particulate material in the final extracts resulting in loss of ATP through adsorption, and (5) the presence of contaminating enzymes, in either the sample extracts or the luciferase preparation, that compete with luciferase for the ATP in solution (e.g., ATPase or adenylate kinase) or that result in the production of ATP through transphosphorylase reactions (e.g., nucleoside diphosphate kinase or pyruvate kinase).

Most of the above sources of error can be detected, and corrected for, through the use of an ATP internal standard, as discussed by Strehler.[34] The internal standard may be added in the form of an ATP salt solution, as live or lyophilized bacterial cells, or as radiolabeled ATP. To minimize the effects of ionic interference, it is imperative that the standard ATP solutions be prepared in an ionic medium identical to that of the samples. Peak-height measurements significantly decrease the analytical interference due to the presence of non-adenine nucleotide triphosphates and therefore, are strongly recommended. If available, a strip-chart recorder or oscilloscope should be interfaced with the photometer in order to detect deviations from the standard ATP-dependent light-emission reaction kinetics.

Sensitivity and Reproducibility

The sensitivity of this assay procedure is governed to a large extent by the instrumentation used to detect light emission, and also by the purity of the luciferase preparation. Using crude commercially available luciferase preparations available from Sigma Chemical Company (FLE-50) prepared as described in this chapter, and a commercial ATP photometer (SAI Technology Co.), the lower limit of ATP detection (i.e., >200 counts above background light emission) is about 1 n*M* ATP. If additional sensitivity is required, exogenous luciferin may be added to the enzyme prepa-

[34] B. L. Strehler, *Methods Biochem. Anal.* **16**, 99 (1968).

rations, thereby enabling the detection of 1 p*M*.[35] When extremely sensitive determinations of ATP, ADP, and AMP are to be conducted, it may be necessary to reduce the level of adenine nucleotides contained within the pyruvate kinase and adenylate kinase enzyme preparations. This is most easily achieved via equilibrium dialysis or gel filtration techniques.

The lower limit of ADP detection is governed by the rate of the pyruvate kinase reaction. Using the enzyme and substrate concentrations described in this report, the conversion of ADP to ATP is complete at least down to a concentration of 1 n*M* ADP. At ADP concentrations of < 1 n*M*, the amount of pyruvate kinase used per sample should be increased.

The rate-limiting step for the conversion of AMP to ATP via the coupled adenylate kinase/pyruvate kinase reaction is the initial conversion of AMP to ADP (i.e., AMP + ATP $\rightleftharpoons$ 2 ADP). The apparent K_m of adenylate kinase for AMP is a function of the concentration of ATP in solution; therefore, the overall reaction rate is dependent upon the concentration of ATP in the reaction mixture.[11] If a sufficient concentration of ATP is added to the extract, 1 n*M* AMP may easily be detected. At lower concentrations, the light emission resulting from the conversion of AMP to ATP becomes a small portion of the total light emission, owing to the requirement for exogenous ATP.

The reproducibility of the peak assay procedure as routinely performed in our laboratories is ± 1–2% of the mean ($n = 8$) throughout the entire range of ATP standards.

Relationship of ATP Concentrations to Biomass

In many ecological studies it is often convenient to express ATP levels in terms of cellular organic carbon, which in turn can be extrapolated to wet-weight or dry-weight determinations, approximate caloric content, etc. There is a considerable body of data in the literature indicating that the ATP concentration in unicellular algae and bacteria is a good measure of the total cellular organic carbon.[2,36] For most microbial cells, the ratio of organic carbon to ATP averages about 250 (i.e., ATP = 0.4% of the cellular organic carbon). In contrast, multicellular organisms, such as crustaceans, bivalves, and annelids, appear to have more ATP per unit carbon, thereby resulting in carbon to ATP ratios of less than 100 (i.e., ATP $\geq$1% of the cellular organic carbon).[37–41] Therefore, in order to obtain the most reliable estimates of biomass in environmental samples, it is essential to remove the

[35] D. M. Karl and O. Holm-Hansen, *Anal. Biochem.* **75**, 100 (1976).
[36] R. D. Hamilton and O. Holm-Hansen, *Limnol. Oceanogr.* **12**, 19 (1967).
[37] N. Balch, *Limnol. Oceanogr.* **17**, 906 (1972).
[38] H. Goerke and W. Ernst, *in* "Proceedings of 9th European Marine Biology Symposium" (H. Barnes, ed.), p. 683. Univ. Press, Aberdeen, Scotland, 1975.

larger organisms (i.e., prefiltration for water, sieving for sediments) prior to the extraction procedure, or to determine their biomass independently and correct the total biomass calculation for these measured values. For pure cultures of bacteria and algae, ATP determinations can be used as a rapid, sensitive, and accurate method of monitoring changes in growth and cell viability (see this volume [7]).

Acknowledgments

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[39] V. Bämstedt and H. R. Skjoldal, *Sarsia* **60**, 63 (1976).
[40] A. D. Ansell, *J. Exp. Mar. Biol. Ecol.* **28**, 269 (1977).
[41] D. M. Karl, J. A. Haugness, L. Campbell, and O. Holm-Hansen, *J. Exp. Mar. Biol. Ecol.*, in press.