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DETERMINATION OF ADENOSINE TRIPHOSPHATE ON MARINE PARTICULATES: SYNTHESIS OF METHODS FOR USE ON OTEC SAMPLES.

ABSTRACT

Adenosine triphosphate (ATP) is an indicator of living biomass in marine particulates. This report details the method used by Lawrence Berkeley Laboratory to analyze particulate ATP in samples taken from oligotrophic, tropical ocean waters. It represents a synthesis of previously published methods.

INTRODUCTION

Adenosine triphosphate (ATP) is an indicator of living biomass in marine particulates (Levin et al., 1964) and a method for ATP measurement was first described by Holm-Hansen and Booth (1966). The method is a luminescent assay using firefly luciferase enzyme to determine the concentration of ATP. This biochemical test provides a rapid means of detecting living material. The results are less restricted than other conventional biomass methods; i.e., culturing, plating, or direct counting.

Although the measurement of ATP does not differentiate between classes or types of organisms, the results can indicate total living carbon. The cellular concentration of ATP varies from 0.1 to 0.7 percent of the total cellular organic carbon content and under extreme physiological conditions can reach as low as 0.03 percent (Holm-Hansen 1973). A convenient conversion of ATP to grams of carbon (250 x ATP) has been determined (Holm-Hansen and Booth 1966, Hamilton and Holm-Hansen 1967, Holm-Hansen 1970; Eppley et al., 1971), but this universal conversion factor has been disputed by a number of investigations (Bancroft et al., 1976, Berland et al., 1972, Jassby 1975, Lee et al., 1971). A review of nucleotide measurements and application has recently been completed by Karl (1980).

OUTLINE OF METHOD

ATP supplies the energy for the light emitting reaction between luciferin (the substrate) and luciferase (the enzyme) which are contained in firefly lantern extract. In the presence of magnesium and oxygen, the reaction yields one photon of light for each ATP molecule hydrolyzed (McElroy et al., 1969). The amount of light produced is directly proportional to the amount of ATP present. From Seliger et al. (1961) the following two step reaction is suggested:

$$(1) \quad LH_2 + ATP + E \underset{\rightleftharpoons}{\overset{Mg^{++}}{\rightleftharpoons}} E\text{-}LH_2\text{-}AMP + PP$$

$$(2) \quad E\text{-}LH_2\text{-}AMP + O_2 \underset{\rightleftharpoons}{\overset{Mg^{++}}{\rightleftharpoons}} E\text{-}L\text{-}AMP + hv$$

where:

LH_2 = luciferin (substrate)
E = luciferase (enzyme)
ATP = adenosine triphosphate
AMP = adenosine monophosphate
PP = pyrophosphate
L = dehydroluciferin
hv = photon of light

Assumptions used in the assay include: firefly luciferin being specific for ATP; the reaction rate of being directly proportional to the concentration of ATP, all other reactants being in excess; emission of one photon of light per molecule of ATP hydrolyzed; and, ATP is not associated with non-living particulate material.

The detection of ATP has been made in a wide variety of natural habitats including seawater (Azam and Hobson 1977, Hamilton et al., 1968, Holm-Hansen 1969), fresh water (Paerl, et al., 1976), intertidal sands (Karl et al. , 1978), marine sediment (Bancroft et al., 1976, Ernest 1970, Karl and LaRock, 1975), and in multicellular marine organisms (Ansell 1977, Balch 1972, Sikora et al., 1977, Traganza and Graham 1977). (For a more complete bibliography on adenosine triphosphate in the marine environment see Jones, Hartwig and Hunt 1981).

SENSITIVITY

The lower limit of ATP detectablity is about 0.1 ugL^{-1} using crude firefly latern extract. However, operationally this limit is influenced by the amount of endogenous ATP in the enzyme preparation and the sensitivity of the photomultiplier tube. By addition of D-luciferin, sensitivity can be extended 1000 times (Karl and Holm-Hansen, 1976). The effect of endogenous ATP on sensitivity can be minimized by dilution and aging time.

ACCURACY AND PRECISION

Accuracy of the assay method can be determined through the use of internal standards. The accuracy varies greatly depending upon the form of the sample. Sediment samples have a lower recovery than filtered suspended particulates (Karl and LaRock, 1975). Maximum statistical variation for the extraction and assay of ATP from environmental samples has been reported as ± 10% at the 95% confidence level interval for samples containing 12 ng mL^{-1} (ca. 24 uM) (Strickland and Parsons, 1972).

LIMITATION

The emitted light is influenced by the presence of interfering cations and anions (trace metals and residual salts). The magnitude of the combined interference can be determined and corrected through dilutions and the use of internal standards and serial dilutions.

SPECIAL APPARATUS AND EQUIPMENT

1) 5 mL marked glass test tubes.
2) 0.2, 0.5, 1.0 mL automatic pipettes.
3) Sample injector.
4) ATP photometer.
5) Recorder.
6) Chloriometer vials, 20 x 40 mm.
7) Sand or water bath for extraction at 100° C.
8) Timer or watch.
9) Freezer or portable freezer unit for storage of samples.

The peak height emission of the firefly luciferin-ATP reaction is detected using a photometer and recorder. The peak height measurement is preferred over the integrated emission measurement due to the ease and speed of operation and high reproducibility (Karl and Holm-Hansen, 1978a). Comparisons between techniques by Karl and Holm-Hansen (1978a) indicate that "peak data was equal to, or better than, the integrated determination".

Several ATP photometers with or without accompanying sample injectors are available commercially. The ATP photometer consists of a photomultiplier tube which receives the light and produces a current proportional to the light intensity. The current is converted to a digital readout or a signal for an analog recorder. The photomultiplier tube must detect the initial light burst which is in the range of 560 to 580 nm. The recorder must be able to record the light peak which occurs within seconds of reaction initiation.

REAGENT PREPARATION

Reagents are prepared using acid-cleaned glassware. Care should be exercised in cleaning and storage of glassware and reagents to insure proper analytical results. Descriptions of reagent preparation with suggested storage is presented in this section.

1. Tris buffer

Dissolve 0.75 g Tris(hydroxymethyl)aminomethane (Sigma 7-9, Sigma Chemical Co., St. Louis, Mo.) in 300 mL distilled water to yield 0.02 M concentration. Adjust the pH to 7.7-7.8 with 20% hydrochloric acid. Autoclave at 15 psi for 15 minutes in 500 mL glass reagent bottles (Strickland and Parsons, 1972). Store solutions frozen (-20°) until needed. Use contents of each flask for only one batch of samples, then discard. Phosphate buffer has been used in recent field surveys (Karl and Craven 1980, Karl, Wirsen and Jannasch 1980). See Karl (1980) for more details on various extraction mediums.

2. Arsenate buffer

Commercially prepared arsenate buffer can be purchased with a concentration of 0.1 M and pH adjusted to 7.4 (Sigma Chemical Co.). This buffer can also be prepared from reagent grade sodium arsenate by adjusting the pH with 20% hydrochloric acid. Store the solution at room

temperature in glass.

3. Magnesium sulfate

Dissolve 4.81 g analytical grade anhydrous magnesium sulfate, $MgSO_4$, in 1000 mL distilled water to yield 0.04 M concentration. Store the solution in glass at room temperature.

4. Enzyme Preparation

Frozen Tris buffer and firefly lantern extract (FLE-50, Sigma Chemical Co.) are thawed to room temperature. Reconstitute 50 mg firefly lantern extract with 5.0 mL of Tris buffer, and allow solution to stand in the dark for 3 hours before adding 10 mL of arsenate buffer and 10 mL of magnesium sulfate buffer solutions to each 50 mg vial. If more than one vial of enzyme extract is required, combine the solutions. Allow diluted solution to stand for an additional hour before decanting the supernatant. The supernatant is used as the enzyme preparation for assaying samples.

Prior to preparation, FLE-50 is stored desiccated at -20°C. Dilution of the enzyme solution permits greater number of assays, decreases the concentration of inhibitory end-products such as dehydroluciferin, and reduces the level of endogenous ATP thereby decreasing background light emission and increasing assay sensitivity (Karl and Holm-Hansen, 1976).

FIELD PROCEDURE

There are four aspects to the collection of field samples for ATP determination: (1) collection of water samples; (2) filtration of the collected water samples; (3) extraction of the ATP from the microbial cells; and (4) storage of the extracted samples until laboratory assay.

1. Collection

Water samples taken with clean bottles or a pumping system are prefiltered through a 150-202 micrometer mesh screen to remove macroplankton, and transferred to acid-cleaned opaque containers and covered. Samples, once aboard the vessel, should be kept cool and treated as described below as soon as possible.

2. Filtration

A measured volume (500-1000 mL) is filtered through a membrane filter (Millipore HA, 0.45 micrometer pore size). Microfine glass fiber filters (Reeve Angel, 984-H) are also satisfactory. A gentle suction during filtration is recommended (not to exceed 1/3 atmosphere vacuum pressure). The filters should not be allowed to be vacuumed dry during filtration.

3. Extraction

Immediately (within seconds) following filtration, the filters are

transferred into test tubes containing 5 mL of boiling Tris buffer for 5 minutes. The time between the end of filtration and immersion into boiling Tris buffer is critical and should be minimized. The Tris buffer should be maintained at 100°C in a sand or water bath during extraction. The extraction process lyses the cells and inactivates the cellular ATPase, an enzyme which degrades ATP. In order to minimize evaporation, the Tris buffer should be allowed to stand in the sand or water bath just long enough to heat the Tris to 100°C prior to extracting the filter. For standard additions, an aliquot, 0.1 mL, of a known concentration is added to boiling Tris buffer in the field and extracted similar to samples.

4. Storage

After extraction, the samples are cooled to room temperature and stored frozen (at -20C) until the laboratory analysis can be performed. Samples kept frozen are stable for many months (Karl and Holm-Hansen, 1978b). [Note: for analytical ease it is desirable to restore the volume of extract to 5 mL with Tris buffer prior to freezing.]

ASSAY PROCEDURE

An aliquot (0.2 mL) of the sample is injected into 1.0 mL of enzyme solution with the sample injector and the kinetics are recorded as peaks on the strip chart recorder. Dispense 1.0 mL aliquots of the prepared enzyme solution into chloriometer vials. Disposable soda glass chloriometer vials, 20 mm x 40 mm, are used in the photometer chamber with a specially designed adapter of lucite which centers the vials on the photomultiplier tube. The vials chemiluminesce after exposure to fluorescent lights, but the light emission decays very rapidly and is significant for less than 5 seconds after being placed in the dark chamber. Place vial with enzyme in chamber.

Draw 0.2 mL of the sample for each assay into the automatic sample injector and position the injector above the photometer chamber. Open shutter to initiate assay. Record the background light emission and then inject the sample into the enzyme solution. The initial spike of the light emission reaction is recorded on the analog recorder.

Samples including Tris Blank and Filter Blank are run in triplicate at ambient temperature. A standard curve is run before and after the samples due to decay of the enzyme activity. At low concentrations, ensure that all glassware is properly cleaned as mentioned previously.

BLANK PREPARATION

1. Tris Blank Preparation

Tris buffer from the same batch used to prepare the enzyme solution and dilute the working standards is used to correct standards for influences of the Tris on the enzyme solution.

2. Filter Blank Preparation

A filter blank is prepared in the field with the same filters and Tris buffer used for field samples. Filter blanks are treated on site similar to samples except no water is filtered before extraction.

3. Field Tris blank

A Tris buffer blank is prepared in the field similar to filter blank. This blank is used as a check for contamination of Tris buffer, and is not used in the ATP calibration equation.

CALIBRATION

The stock standard and standard addition solution are prepared in advance. The working standards are prepared on the day of analysis from the frozen stock standard.

1. Stock Standard

Dissolve ca. 2 μM(~1.2 x 10^{-3}g) adenosine-5'-triphosphate disodium salt (vanadium free[1], Sigma Chemical Co.) in 1000 mL distilled water. Store frozen until needed in aliquots of 2-5 mL.

2. Working Standards

Serially dilute the frozen stock standard with Tris buffer to obtain a set of working standards in the range of 10^{-9} to 10^{-7} M (100, 50, 30, 10, 5, 3, 1, 0.5 ng mL^{-1}) (Karl and Holm-Hansen, 1978a). The following serial dilution is routinely used:

	Dilution	Final ATP Concentration (ng mL^{-1})
A	Undiluted	1000
B	0.5 mL (A) into 4.5 mL Tris.	100
C	1.0 mL (B) into 1.0 mL Tris.	50
D	0.4 mL (B) into 1.6 mL Tris.	30
E	0.5 mL (B) into 4.5 mL Tris.	10
F	1.0 mL (E) into 1.0 mL Tris.	5
G	0.4 mL (E) into 1.6 mL Tris.	3
H	0.5 mL (E) into 4.5 mL Tris.	1
I	1.0 mL (H) into 1.0 mL Tris.	0.5
J	4.0 mL Tris.	0

3. Standard Addition Solution

[1]Recent evidence indicates vanadium inhibits ATPase activity (see Cantley et al., 1977).

Serially dilute the stock standard with Tris buffer to obtain a standard addition solution of 10^{-9} moles ATP/0.1 mL (0.5 ng/0.1 mL). Store frozen until needed in aliquots of 2-5 mL.

DATA REDUCTION

A standard curve is plotted using peak height measurements of prepared standards with concentrations ranging from 0 (Tris buffer) to 100 ng mL^{-1}. From the standard curve, the ATP concentration can be extrapolated for the samples assayed. The peak height of samples measured from the base of enzyme background emission to the top of the initial peak are used to calculate concentration in extracted samples.

Peak heights measured to the nearest 0.5 mm are corrected for the scale used on the strip chart recorder. Peak heights of standards are plotted versus their known concentrations. Least square linear regression analysis derives an equation for the relationship of ATP (ng mL^{-1}) verse corrected peak height. Mean peak height values for Tris blank and filter blank are used to correct for influence of filter or differences between Tris batches. Sample concentration (S_i) is determined using the following equation:

$$S_i = [(a + bx_i) + b(TB - FB)]\ V_e/V_f\ * 1000$$

where:

S_i = i^{th} sample concentration in ngL^{-1}.
$a + bx$ = equation for standard curve using corrected peak heights.
a = y-intercept.
b = regression coefficient.
x_i = i^{th} corrected peak height measurement.
$\overline{TB}$ = mean Tris blank corrected peak height measurement.
$\overline{FB}$ = mean filter blank corrected peak height measurement.
V_e = volume of sample extract (5 mL).
V_f = volume of seawater filtered (500-1000 mL).

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