HANDBOOK OF METHODS IN

AQUATIC MICROBIAL ECOLOGY

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CHAPTER 54

Microbial RNA and DNA Synthesis Derived from the Assimilation of [2,^{3}H]-Adenine

David M. Karl

INTRODUCTION

The measurement of microbial growth in nature has presented, and continues to present, a challenge to experimental microbial ecologists. In microorganisms, nucleic acids are vital cellular constituents with well-known metabolic functions.[1] The observed correlations between rates of stable RNA and DNA synthesis and cell growth[2-4] are so universally applicable among unicellular microorganisms that they appear to be well suited for the analysis of complex communities such as one finds in nature. Consequently, measurements of total microbial RNA and DNA production should provide useful information regarding microbial growth and metabolism in nature.

Several different experimental methods have been described for measuring rates of nucleic acid synthesis of either the entire microbial community or isolated portions thereof. The various methods described rely upon the incorporation of either thymidine, adenine, or uridine as presumed measures of polynucleotide (DNA and RNA) biosynthesis. Although there is continuing debate on this issue, it can be stated without reservation that the mere incorporation of a given nucleic acid precursor into cold acid-insoluble cellular materials cannot be accepted as a measure of the *in situ* rates of RNA or DNA synthesis without a careful and comprehensive consideration of: (1) the community potential for assimilating the exogenous precursor; (2) the presence of intracellular and extracellular pools of structurally related compounds which dilute the specific radioactivity of the incorporated molecules; (3) a detailed analysis of the pathways and regulation of precursor metabolism including the balance between *de novo* synthesis and salvage pathways (i.e., utilization of exogenous pools); and (4) the specificity and extent of macromolecular labeling. These are fundamental concerns for the uptake and assimilation of all radiotracers and this situation is certainly not unique for ^{3}H-adenine assimilation.

A unique characteristic of the ^{3}H-adenine method is the ability to measure the specific radioactivity (nCi pmol^{-1}) of the immediate precursor pool of the adenine incorporated into nucleic acids (i.e., ATP for RNA synthesis and dATP [deoxyadenosine triphosphate] for DNA synthesis[5,6]). This measurement permits correction for isotope dilution when the added radiotracer mixes with exogenous pools of structurally related compounds before uptake and with endogenous pools after transport into the cells. If the specific activity of the precursor pool is not measured, then it is impossible to extrapolate incorporation measurements to

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estimates of nucleic acid synthesis with any degree of accuracy. Even comparisons of "relative" rates are probably not justified since differences in the measured rates of RNA and DNA labeling may result either from variations in the specific radioactivity of the precursor pool or from actual differences in the rates of nucleic acid synthesis.

An additional advantage of the ecological use of ^{3}H-adenine is the fact that (unlike commonly used nucleosides) it is incorporated into both RNA and DNA from intracellular precursor pools which are in isotopic equilibrium.[5,7] Consequently, it is possible to simultaneously measure the rates of both RNA and DNA synthesis (and the rate ratio) from a single incubation procedure. Under these conditions, habitats supporting biosynthesis or macromolecular turnover (RNA synthesis) in the absence of cell division (DNA synthesis) and other forms of uncoupled or transient growth can be readily identified.[8]

This chapter will focus primarily on the protocols used to measure RNA and DNA synthesis in naturally occurring communities of microorganisms. Detailed presentations of the biochemical and physiological principles, evaluation of the underlying assay assumptions, and numerous ecological applications of this technique are presented elsewhere[6,7,9,10] and will only be mentioned here as they apply to the conduct of the field protocol. The reader is encouraged to refer to the primary literature for additional details on the rationale, unique advantages, and limitations of the ^{3}H-adenine technique.

Most microorganisms possess the ability to utilize exogenous supplies of adenine, and certain other nucleic acid bases, as a supplement to *de novo* synthesis. The mechanisms and implications of these so-called "salvage pathways" have been reviewed elsewhere.[11] When adenine is transported into a cell, it is rapidly incorporated into a number of derivatives including ATP, dATP, ADP, and AMP, and eventually into the metabolically stable nucleic acids. It has been demonstrated that adenine uptake is directly coupled to nucleic acid synthesis so that the rate of entry of adenine into the cell (k_1, Figure 1) does not exceed the biosynthetic requirements for ATP and dATP (sum of $k_2 + k_3$). However, the actual quantity of adenine taken up by microbial cells is much less than the value $k_2 + k_3$ due to variable but substantial contributions of adenine from *de novo* synthesis and internal recycling of nucleic acid bases (RNA turnover).

When ^{3}H-adenine is added to a culture or seawater sample, it is diluted to an unknown extent by existing pools of adenine and adenine-containing compounds (e.g., ATP, ADP, AMP, and adenosine). This process decreases the specific radioactivity of the introduced radiotracer prior to transport into the cells. Since the salvage pathways generally cannot supply the total amount of adenine required by the cells, this process must be supplemented by *de novo* synthesis of adenine. This further decreases the specific activity of the introduced radiotracer after transport into the cells but before incorporation into nucleic acids. Consequently, before an absolute rate of RNA or DNA synthesis can be obtained, it is necessary to determine the specific radioactivity of the immediate nucleotide triphosphate precursor, as well as the total amount of radioactivity incorporated into the individual nucleic acid pools. Without knowledge of both precursor-specific activity (ATP in the case of ^{3}H-adenine assimilation) and the extent of endproduct labeling, rates of nucleic acid synthesis in seawater samples cannot be measured. This concern, of course, is not unique to ^{3}H-adenine experiments, but it is true for the use of all stable or radioisotopic tracers.

The uptake and incorporation of ^{3}H-adenine as a measure of total microbial (bacteria and unicellular algae) nucleic acid synthesis was first used in oceanographic field experiments by Karl.[12] Since that time, the method has been substantially improved[5,13-15] and adapted for use in marine sediments.[16,17] There are several testable assumptions implicit in the application of the ^{3}H-adenine method to the analysis of natural microbial assemblages, including: (1) all (or in practice, most) microorganisms assimilate exogenous adenine by a

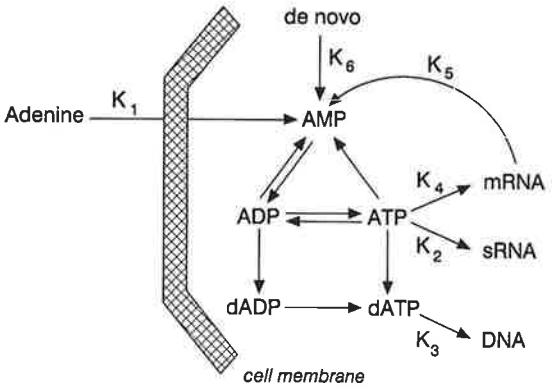


Figure 1. Schematic diagram depicting the flux of adenine through the intracellular adenine nucleotide pool (AMP, ADP, ATP, dADP, and dATP) and into the metabolically stable nucleic acid fractions (sRNA and DNA). The rate of removal of adenine for nucleic acid synthesis ($k_2 + k_3$) does not exceed the combined rate of salvage (k_1) and *de novo* synthesis (k_6). mRNA is an unstable intermediate and does not represent a net sink. The two alternative reductive pathways indicated for the formation of the DNA precursor pool (dATP) assure that radioisotopic equilibrium between ATP and dATP is maintained. (Adapted from Karl[5] and Winn and Karl.[6])

common pathway and exhibit a similar salvage response under *in situ* conditions; (2) addition of radioactive adenine does not affect the ATP cell quota, ATP turnover rate, or the rate of microbial RNA and DNA synthesis; and (3) there is no intracellular compartmentalization of microbial ATP pools or, if there is, such compartmentalization does not affect the accuracy of the measured rates. A thorough discussion of the validity of each of these assumptions has been presented elsewhere,[7] and will not be repeated here. The ^{3}H-adenine method has also been calibrated using chemostat and batch cultures of representative marine microorganisms[6] and with natural populations under field conditions.[18,19] The results show excellent agreement between the ^{3}H-adenine-derived rates of nucleic acid synthesis and the rates derived from independent measurements.

MATERIALS REQUIRED

Equipment

- Water sampling gear (Niskin bottles, or equivalent)
- Polycarbonate incubation bottles (0.1 to 4 l, depending upon design of labeling experiments)
- On-deck temperature- and light-controlled incubator or *in situ* array
- Filtration gear: PVC or stainless steel manifold (3- or 6-place), equipped with glass filter bases with stainless steel screens for 25-mm diameter filters and large volume funnels (100 to 200 ml capacity), and an adjustable vacuum pump with gauge (compressed N_2 or air can be used as an alternative to vacuum filtration), filters (24/25 mm diameter Whatman GF/F, Millipore HA/GS, or equivalent), filter forceps
- Block heater (Sybron Thermolyne Type #16500 Dri-bath, or equivalent) for ATP extraction, capable of heating extraction menstruum in test tubes to 100 ± 1°C (1 atm) and maintaining boiling temperatures throughout the extraction period
- Adjustable automatic pipettes (0.01 to 0.1 ml, 0.1 to 1 ml, and 1 to 5 ml capacities)
- Storage freezer (−20°C)
- 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 required. Instruments specifically designed for ATP analyses are marketed by several corporations including, but not limited to, E. I. DuPont, Turner Designs, LKB Instruments, Biospherical Instruments Corporation, Turner Designs and United Technologies. Whatever light-measuring device is selected, it is imperative to have either a strip chart recorder, integrator, or suitable computer-assisted data station to quantify light emission.

- Thin-layer chromatography (TLC) development tank (with glass cover)
- High intensity ultraviolet (UV) lamp
- Liquid scintillation counter
- Gyrotatory shaker table
- Refrigerated centrifuge with swinging-bucket rotor
- Vacuum evaporator (Savant Speed-vac concentrator or equivalent)
- Tissue grinder with Teflon pestle
- Water bath incubator (for 37°C and 95 to 100°C)

Supplies

- TLC plates, 20 × 20 cm, polyethyleneimine (PEI) impregnated cellulose (MN #300 PEI, Brinkmann Instrument Company, Westbury, NY)
- ATP extraction and assay glassware: test tubes for ATP extraction (approximately 15 mm diameter × 125 to 160 mm length); ATP assay vials (18 mm diameter × 40 mm height), size requirements may vary depending upon equipment/instruments used
- Disposable glass culture tubes (12 × 75 mm) and glass liquid scintillation counting (LSC) vials
- Pasteur pipettes

Solutions and Reagents

- Firefly lantern extract (Sigma Chemical Company, cat. #FLE-50)
- [2,^{3}H]-adenine radioisotopic tracer (specific radioactivity approximately 15 Ci mmol^{-1}; DuPont cat. #NET-350, or equivalent)
- Liquid scintillation counting fluid
- Sodium arsenate buffer (100 m*M*, pH 7.4)
- Tris(hydroxy)aminomethane-HCl buffer (20 m*M*, pH 7.75)
- ATP (5×10^{-4} *M*) sodium salt solutions in Tris buffer (20 m*M*, pH 7.7)
- Magnesium sulfate solution (40 m*M*) and magnesium chloride solution (0.7 *M*)
- Potassium phosphate solution (0.85 *M*, pH 3.4)
- RNA (baker's yeast; Sigma Chemical Company cat. #R-7125) and DNA (calf thymus; Sigma Chemical Company cat. #D-1501) solutions (5 mg ml^{-1})
- HCl (2 *M*), NaOH (1 *M*) and trichloroacetic acid (TCA; 100%, wt/vol)
- Ethanol (95%), reagent grade

PROCEDURES

Summary

A freshly collected water sample is incubated with ^{3}H-adenine for a predetermined period under *in situ* environmental conditions. A time course is preferred to single endpoint measurements, although endpoint measurements can also be used to estimate rates of nucleic acid synthesis. Following the incubation, subsamples are removed and extracted for ATP

and nucleic acids. The ATP is purified by one-dimensional TLC and radioassayed. Total ATP is measured by firefly bioluminescence. The mean ATP pool-specific radioactivity (nCi ^{3}H-ATP mol^{-1} total ATP) is then calculated from the chemical and radiochemical ATP data. RNA and DNA are also separated and purified and their total radioactivities measured. From the incorporation and precursor specific radioactivity data, the rates of RNA and DNA synthesis can be calculated and expressed as mass of adenine (or deoxyadenine) incorporated into RNA (or DNA) per unit volume per unit time. Biomass-specific rates of RNA and DNA synthesis can also be derived by dividing the measured rates by total microbial biomass (ATP $\times$ 250; see Karl[20]). Alternatively, relative RNA-to-DNA synthesis rate ratios can be determined directly from the incorporation data, thereby eliminating the necessity for ATP pool-specific radioactivity measurements. If time-course data are available, an independent calculation of the rates of RNA and DNA synthesis can be performed using the total radioactivity accumulated in RNA and DNA and the integral of the ATP-specific activity over the length of the time-course experiment[6,21] in order to obtain the most accurate estimate (see section on data from time-course experiments). The protocol presented here is focused on water column measurements. For sediment applications, refer to Craven and Karl[16] and Novitsky and Karl.[17]

Sample Collection, Incubation, and ATP Pool Extraction

1. Collect water sample for analysis using clean, noncontaminating PVC bottles, or equivalent. Try to minimize the time between sample collection and the beginning of the experiment if results are to be extrapolated to *in situ* rate processes. Keep water samples in ambient conditions (especially with regard to light and temperature regimes) during intermediate storage of samples in order to avoid carbon and energy flux transitions or perturbations caused by change in the environmental conditions. Remove subsamples of each water type for complementary chemical and microbiological measurements (e.g., total biomass, primary and secondary production, bacterial cell numbers, dissolved nutrients and gases, particulate matter composition, etc.).

2. Subsamples are placed into acid-cleaned, polycarbonate incubation bottles. The required volume is dependent upon microbial biomass. Typically for surface ocean samples, between 100 to 250 ml are used for each determination. Because time-course information on labeling kinetics is desirable, multiple (between 4 and 8) bottles may be required for each water sample. Alternatively, one large polycarbonate bottle (2 to 4 L) that can be conveniently subsampled with minimum disruption to the microbial communities (light or temperature shock, turbulence, etc.) can also be employed. Depending upon the design of the experiment, size-fractionated water samples may also be prepared.

3. The timed incubations begin with the addition of [2,^{3}H]-adenine to each sample to achieve a final activity of between 0.05 to 0.2 μCi ml^{-1}, depending upon the desired sensitivity. The incubation should extend for a period of greater than approximately 10% of the anticipated mean doubling time of the substrate-responsive population.[6] This approach will reduce potential problems caused by isotopic disequilibrium and kinetic compartmentalization which are especially important if endpoint measurements are made.[6,7] However, prolonged incubations (e.g., $>$3 to 4 times the mean population doubling time) should be avoided.

4. At each predetermined sampling time (or at the end of the incubation period for endpoint measurements), an aliquot is removed and filtered through a 25-mm diameter, microfine glass fiber filter (Whatman GF/F type) for the measurement of ^{3}H-RNA and ^{3}H-DNA. This filter is placed into a glass test tube and usually stored frozen for subsequent nucleic acid purification (see section on nucleic acid purification). A second aliquot is filtered

(also through a GF/F filter) and the filter is immediately immersed into boiling Tris (20 m*M*, pH 7.7) buffer for ATP extraction. The sample is extracted at 100°C for 5 min, and is then stored frozen for subsequent ATP purification (see section on ATP purification). For additional details on the ATP extraction technique refer to Chapter 41.

Nucleic Acid Purification

In this procedure, RNA and DNA (and protein) are separated by their differential susceptibility to alkaline hydrolysis using a modified Schmidt-Thannhauser procedure.[14,22]

1. Remove test tubes containing GF/F filters (see section on sample collection, step 4) from −20°C storage. Add 5 ml 5% (wt/vol) ice-cold (4°C) TCA and 1 mg each of reagent RNA and DNA to each tube. The latter are added to help catalyze the precipitation and subsequent quantitative isolation of the separate macromolecular fractions. Mix thoroughly and allow to extract, at 4°C, for at least 1 to 2 h.
2. Pulverize filters in a tissue grinder, or equivalent. Rinse the pestle and grinding vessel carefully with fresh 5% TCA. Combine sample and rinse volumes. Centrifuge (2500 × g, 5 min) at 4°C, and discard supernatant. (Note: a Pasteur pipette attached to a vacuum line is convenient for removal of supernatant).
3. "Wash" (i.e., add solvent, vortex vigorously, centrifuge at 2500 × g for 5 min and discard supernatant) the GF/F filter pellets three times with 5 to 8 ml each of 5% TCA and three times with 95% ethanol (ETOH), all at 4°C. Be careful not to lose any portion of the pellet. Evaporate the residual ETOH *in vacuo* or by gentle heating of the samples. RNA and DNA remain insoluble during these procedures.
4. Resuspend the pellets in exactly 2 ml 1 *M* NaOH. Cap the tubes and place into water bath at 37°C for 1 h. This will solubilize both RNA and DNA, but hydrolyze only the RNA.
5. The samples are transferred to an ice water bath (4°C) and allowed to chill for at least 15 min before acidification to a pH<1 using a mixture of HCl and TCA to yield a final TCA concentration of 5% (Note: we typically add 0.5 ml of a 2:1:1 mixture, by volume, of 9 *M* HCl:100% TCA:distilled water). Allow samples to stand at 4°C for an additional 15 min prior to centrifugation.
6. A quantitative portion (usually 0.5 ml) of the supernatant containing ^{3}H-RNA is transferred to a clean vial; the remainder of the supernatant is discarded. An appropriate scintillation cocktail is added to each vial and the samples are radioassayed for ^{3}H-RNA.
7. The pellet, containing ^{3}H-DNA, is washed as in step 3, but using only two rinses each of 5% TCA and 95% ETOH, all at 4°C, and residual liquid is evaporated. Finally, 2 ml of 5% TCA is added to each dried pellet and the samples are incubated at 95 to 100°C for 30 min. This procedure hydrolyzes DNA, but not protein.
8. Cool, add 2 ml of 1 *M* NaOH, mix thoroughly, and centrifuge.
9. Remove 0.5 ml of the supernatant and place into an LSC vial containing 0.5 ml of 2 *M* HCl to acidify the sample (Note: basic solutions result in high chemiluminescence background when in contact with certain LSC fluids). Add cocktail and radioassay for ^{3}H-DNA.
10. If desired (primarily for radiochemical mass balance purposes[13,14]) the ^{3}H-protein remaining in the pellet is hydrolyzed at 37°C for 18 h in 1 *M* NaOH, and counted as in the previous step.

ATP Purification

1. The aqueous ATP extracts are thawed at room temperature, mixed thoroughly, and centrifuged (1500 × g, 5 min) to remove particulate materials. Then 4 ml are removed, placed into a clean glass vial, and evaporated to dryness *in vacuo*.[14] Distilled water is added (100 to 200 μl) and the residue is redissolved. This procedure effects a 20- to 40-fold concentration of the ATP extract in preparation for TLC purification. Total ATP concentration in a diluted portion of the concentrated extract is determined by firefly bioluminescence (see Chapter 41).

2. Radiolabeled ATP (i.e., ^{3}H-ATP) is isolated by one-dimensional TLC using plastic backed (20 × 20 cm) sheets coated with PEI impregnated cellulose. Prior to use, the commercially available plates are soaked in a bath of 10% NaCl, air dried at room temperature and subjected to an ascending development with distilled water. These pretreated TLC plates are dried and stored at 4°C until needed.

3. In a clean 12 × 75 mm disposable test tube, mix 10 μl of concentrated ATP extract and 10 μl of a nonradioactive solution of ATP (5×10^{-4} *M*) and spot 10 μl of this mixture on to a predetermined location approximately 1 cm from the bottom edge of a PEI plate (i.e., "origin"). Approximately 8 to 10 lanes can be prepared per plate.

4. The plate is air dried, then washed twice (5 min each) by consecutive immersion into two 1-L distilled-water baths. This procedure separates unused precursor (^{3}H-adenine) from labeled adenine nucleotides.[14]

5. The plate is then air dried (alternatively, drying can be accelerated using an electric hair dryer), and developed in one dimension with potassium phosphate (0.85 *M*, pH 3.4) in a closed glass TLC tank.[23] When the solvent front has ascended to within 2 cm of the top edge, the plate is removed from the tank and air dried. Total separation time is approximately 2 to 3 h.

6. ATP (and ^{3}H-ATP) is visualized by irradiating the PEI plate with short-wavelength UV light and the spots are localized by circling lightly with a sharpened lead pencil. The spots are cut out with a pair of scissors, placed into a liquid scintillation counting vial containing 1 ml of $MgCl_2$ (0.7 *M*) and the ATP is eluted by shaking the samples for 1 h. This elution step is necessary to maximize liquid scintillation counting efficiency.

7. Liquid scintillation cocktail is added directly to each vial in preparation for ^{3}H counting. The counts per min^{-1} (cpm) are converted to disintegrations per min^{-1} (dpm) using standard techniques for quench correction. ATP pool-specific radioactivity (nCi $pmol^{-1}$) is calculated from the dpm in the isolated ATP spot (nCi) and ATP (pmol) in the concentrated extract after appropriate volume corrections have been applied. These data are used for the calculation of RNA and DNA synthesis rates (see section on data analysis).

Data Analysis

Data from Time-Course Experiments

The ^{3}H-adenine assimilation model (Figure 1) assumes that adenine flows through a single, well-mixed precursor pool and then into nucleic acids. If this representation is accurate, then the relationship between nucleic acid labeling and the specific activity of its direct precursor can be defined precisely as:[6]

$$d^*NA/dt = K_s\,(^*ATP/ATP) - K_b\,(^*NA/NA) \quad (1)$$

where *NA and NA are the radiolabel and chemical concentration of nucleic acid, respectively, K_s is the rate of synthesis of nucleic acid, K_b is the rate of breakdown of nucleic acid due to cell death, and * ATP and ATP are the radiochemical and chemical concentrations of the direct precursor (i.e., ATP). During short-term labeling experiments (i.e., less than the doubling time of the population), the specific activity of the endproducts (i.e., *NA/NA) will be much less than the specific activity of the precursor (.e., *ATP/ATP) so that the second term in the equation can be ignored. Equation 1 can then be simplified to:

$$d*NA/dt = K_s\ (*ATP/ATP), \text{ or} \tag{2}$$

$$*NA = K_s \int_0^t (*ATP/ATP) \tag{3}$$

and can be rearranged to solve for K_s, the rate of RNA or DNA synthesis:

$$K_s = *NA/\int_0^t (*ATP/ATP) \tag{4}$$

Consequently, the rate of either RNA (K_2; Figure 1) or DNA (K_3; Figure 1) synthesis can be calculated by dividing the total radioactivity accumulated into the respective nucleic acid pool (*RNA or *DNA) by the time-dependent integral of the ATP pool-specific radioactivity. This procedure has been shown to provide an accurate estimate of RNA and DNA synthesis rate for a variety of prokaryotic and eukaryotic microorganisms.[6]

Data from Endpoint Experiments

If time-course data are not available, the rates of RNA and DNA synthesis can be estimated by dividing the total radioactivity incorporated into the respectively RNA or DNA pool by the measured endpoint ATP pool specific radioactivity. This expression is precisely equal to the true rate of nucleic acid synthesis only when the specific activity of the precursor pool is constant with time and incorporation into macromolecules is linear with time. Because the ATP pool-specific activity is zero at the start of the experiment, this is never the case. However, because the intracellular adenine nucleotide pool turns over approximately 40 to 50 times per generation[24,25] (also see Chapter 55), the specific radioactivity of the ATP pool changes rapidly during the initial phases of the incubation period and achieves isotopic equilibrium. Consequently, the difference between the rate estimated by time-course and endpoint determinations decreases with time.[6]

Synthesis Rates and Extrapolations

RNA and DNA Synthesis. From data on ^{3}H-RNA, ^{3}H-DNA, and specific activity of the ^{3}H-ATP pool, rates of RNA and DNA synthesis can be calculated by dividing the total radioactivity incorporated into RNA or DNA (nCi L^{-1} of water sample per unit time) by the integral of the ATP pool specific radioactivity (nCi $pmol^{-1}$) during the appropriate incubation period (or if only endpoint data are available, divide by the endpoint ATP pool-specific radioactivity). This will provide nucleic acid synthesis rates in units of pmol A (or dA) incorporated into RNA (or DNA) L^{-1} h^{-1}.

Extrapolations. The majority of investigations concerned with the production, flux, and turnover of organic matter in natural ecosystems have relied upon carbon as the basic unit

of cell mass and growth. If the assumptions inherent in the application of nucleic acid precursors to ecological studies were determined to be valid under *in situ* conditions, the ^{3}H-adenine method as described would yield reliable estimates of the total rates of microbial RNA and DNA synthesis under natural environmental conditions. While one might argue that nucleic acid synthesis rate data, by themselves, provide useful information regarding the growth of microorganisms in nature, it is often necessary to extrapolate the DNA synthesis data to estimates of total microbial production (μg C produced per unit volume per unit time) for comparison with other ecological data. Of course, the precision and accuracy of the estimates are adversely affected by the common practice of extrapolation, since the "conversion factors" are, for the most part, highly variable.

Total microbial carbon production can be extrapolated from rates of DNA synthesis by converting pmol dA to pg DNA (by assuming dA is 25 mol% of total DNA) then to carbon production (by assuming DNA is 2% of total cell carbon). The rationale for these extrapolation factors is presented elsewhere.[5-7,26]

Specific growth rate can be determined from the above-referenced carbon production estimate and independent estimates of total microbial cell carbon based on chemical (particulate carbon, ATP) or direct microscopic methods and assuming: μ = (doubling day^{-1}) $\times$ 0.693 (Karl[5]). A more precise and accurate refinement of this general approach employs the measured DNA synthesis rates and total particulate DNA in the environment, corrected for the "nonreplicating" (e.g., detrital) fraction.[26]

NOTES AND COMMENTS

Any technique which requires the uptake and assimilation of a radioactive precursor, as for the method described in this chapter, is limited in that it can only detect the "substrate responsive" portion of the total microbial community. For ^{3}H-adenine uptake, this is known to include bacteria and unicellular algae, even when the substrate is added at nanomolar concentrations.[6,7,26] Nevertheless, the presence of nonresponsive organisms, whether bacteria, algae, protozoa or metazoa, could affect the accuracy of RNA and DNA synthesis measurements and extrapolations thereof. This is because the specific radioactivity of the ATP pool will be influenced by the presence of viable (ATP-containing) nonresponsive biomass. Fuhrman et al.[27] have challenged the ^{3}H-adenine technique on this basis; however, a detailed response placed their criticism into a proper quantitative perspective.[26]

The nucleic acid synthesis rates derived from the uptake and assimilation of ^{3}H-adenine represent mass-weighted mean estimates for the growing population. A method now exists to determine the standard deviation of the average ATP pool turnover rate.[28] With this additional information, it is possible to assess population heterogeneity. If there is a large variance in community growth rates, then pre- or postincubation size fractionation procedures can be employed in order to help ascertain the source of this population variability. Future applications using flow cytometry and cell sorting capabilities might also provide an opportunity to obtain population-specific estimates of nucleic acid synthesis.

It should be emphasized that extrapolation of nucleic acid synthesis data to estimates of carbon production assumes that marine microorganisms in nature exhibit "balanced growth", with all cellular components increasing at the same rate. As Eppley[29] points out, the concept of balanced growth is fundamental (but generally not explicitly stated) to the use of nutrient assimilation rates as measures of rate constants for growth. Karl[5] has already demonstrated that cell growth (as measured by RNA synthesis) and cell division (as measured by DNA synthesis) may be uncoupled in mixed assemblages of asynchronously growing marine

microorganisms over incubation periods as short as 1 h. By measuring both RNA and DNA synthesis, we may be in a better position to assess discontinuous or unbalanced growth[8] than with a less complete evaluation of growth processes.

Accurate measurements of microbial biomass, metabolic activity, productivity, and specific growth rate are essential for testing hypotheses in microbial ecology. Progress in our discipline is limited by methods. The use of ^{3}H-adenine to estimate rates of microbial nucleic acid synthesis in aquatic ecosystems is a relatively new and potentially useful approach for the study of marine microbial ecology. Methods employing other nucleic acid precursors, such as ^{3}H-thymidine, are discussed elsewhere in this book. In my view, nucleic acid synthesis measurements employing ^{3}H-adenine and ^{3}H-thymidine are not mutually exclusive methods in microbial ecology since each approach yields different information regarding microbial growth in nature. Unless evidence is presented to suggest otherwise, they should be considered complementary techniques. Finally, the potential significance of ecological information to be derived from estimates of *in situ* rates of RNA and DNA synthesis more than justifies the level of research effort that is required to ensure a proper interpretation and accurate extrapolation to estimates of microbial growth and production.

REFERENCES

1. Ingraham, J. L., Maaloe, O., and Neidhardt, F. C., *Growth of the Bacterial Cell,* Sinauer Associates, Sunderland, MA, 1983.
2. Kjeldgaard, N. O., Regulation of nucleic acid and protein formulation in bacteria, *Adv. Microbiol. Physiol.*, 1, 39, 1967.
3. Maaloe, O. and Kjeldgaard, N. O., *Control of Macromolecular Synthesis,* W. W. Benjamin, New York, 1966.
4. Nierlich, D. P., Regulation of bacterial growth, RNA, and protein synthesis, *Annu. Rev. Microbiol.*, 32, 393, 1978.
5. Karl, D. M., Simultaneous rates of ribonucleic acid and deoxyribonucleic acid syntheses for estimating growth and cell division of aquatic microbial communities, *Appl. Environ. Microbiol.*, 42, 802, 1981.
6. Winn, C. D. and Karl, D. M., Laboratory calibrations of the [^{3}H]adenine technique for measuring rates of RNA and DNA synthesis in marine microorganisms, *Appl. Environ. Microbiol.*, 47, 835, 1984.
7. Karl, D. M. and Winn, C. D., Adenine metabolism and nucleic acid synthesis: applications to microbiological oceanography, in *Heterotrophic Activity in the Sea,* Hobbie, J. E. and Williams, P. J. LeB., Eds., Plenum Publishing, 1984, 197.
8. Karl, D. M. and Novitsky, J. A., Dynamics of microbial growth in surface layers of a coastal marine sediment ecosystem, *Mar. Ecol. Prog. Ser.*, 50, 169, 1988.
9. Karl, D. M., Determination of in situ microbial biomass, viability, metabolism, and growth, in *Bacteria in Nature, Vol. 2,* Poindexter, J. S. and Leadbetter, E. R., Eds., Plenum Publishing, 1986, 85.
10. Karl, D. M. and Winn, C. D., Does adenine incorporation into nucleic acids measure total microbial production?: a response to comments by Fuhrman et al., *Limnol. Oceanogr.*, 31, 1384, 1986.
11. Hochstadt, J., The role of the membrane in the utilization of nucleic acid precursors, *CRC Crit. Rev. Biochem.*, 2, 259, 1974.
12. Karl, D. M., Measurement of microbial activity and growth in the ocean by rates of stable ribonucleic acid synthesis, *Appl. Environ. Microbiol.*, 38, 850, 1979.
13. Karl, D. M., Selected nucleic acid precursors in studies of aquatic microbial ecology, *Appl. Environ. Microbiol.*, 44, 891, 1982.

14. Karl, D. M., Winn, C. D., and Wong, D. C. L., RNA synthesis as a measure of microbial growth in aquatic environments. I. Evaluation, verification and organization of methods, *Mar. Biol.*, 64, 1, 1981.
15. Karl, D. M., Winn, C. D., and Wong, D. C. L., RNA synthesis as a measure of microbial growth in aquatic environments. II. Field applications, *Mar. Biol.*, 64, 13, 1981.
16. Craven, D. B. and Karl, D. M., Microbial RNA and DNA synthesis in marine sediments, *Mar. Biol.*, 83, 129, 1984.
17. Novitsky, J. A. and Karl, D. M., Characterization of microbial activity in the surface layers of a coastal sub-tropical sediment, *Mar. Ecol. Prog. Ser.*, 28, 49, 1986.
18. Karl, D. M. and Bossard, P., Measurement of microbial nucleic acid synthesis and specific growth rate by $^{32}PO_4$ and ^{3}H-adenine: a field comparison, *Appl. Environ. Microbiol.*, 50, 706, 1985.
19. Laws, E. A., Redalje, D. G., Haas, L. W., Bienfang, P. K., Eppley, R. W., Harrison, W. G., Karl, D. M., and Marra, J., High phytoplankton growth and production rates in oligotrophic Hawaiian coastal waters, *Limnol. Oceanogr.*, 29, 1161, 1984.
20. Karl, D. M., Cellular nucleotide measurements and applications in microbial ecology, *Microbiol. Rev.*, 44, 739, 1980.
21. Yamazaki, H. and Leung, K., Determination of the total rates of synthesis and degradation of RNA in bacterial cultures, *Can. J. Microbiol.*, 27, 168, 1981.
22. Munro, H. N. and Fleck, A., The determination of nucleic acids, in *Methods of Biochemical Analysis, Vol. 14*, Glick, D., Ed., Interscience Publishers, New York, 1966, 113.
23. Cashel, M., Lazzarini, R. A., and Kalbacher, B., An improved method for thin-layer chromatography of nucleotide mixtures containing ^{32}P-labeled orthophosphate, *J. Chromatogr.*, 40, 103, 1969.
24. Chapman, A. G. and Atkinson, D. E., Adenine nucleotide concentrations and turnover rates. Their correlation with biological activity in bacteria and yeast, *Adv. Microbiol. Physiol.*, 15, 253, 1977.
25. Karl, D. M., Jones, D. R., Novitsky, J. A., Winn, C. D., and Bossard, P., Specific growth rates of natural microbial communities measured by adenine nucleotide pool turnover, *J. Microbiol. Methods*, 6, 221, 1987.
26. Winn, C. D. and Karl, D. M., Diel nucleic acid synthesis and particulate DNA concentrations: Conflicts with division rate estimates by DNA accumulation, *Limnol. Oceanogr.*, 31, 637, 1986.
27. Fuhrman, J. A., Ducklow, H. W., Kirchman, D. L., Hudak, J., McManus, G. B., and Kramer, J., Does adenine incorporation into nucleic acids measure total microbial production?, *Limnol. Oceanogr.*, 31, 627, 1986.
28. Laws, E. A., Jones, D., and Karl, D. M., Method for assessing heterogeneity in turnover rates within microbial communities, *Appl. Environ. Microbiol.*, 52, 866, 1986.
29. Eppley, R. W., Relations between nutrient assimilation and growth in phytoplankton with a brief review of estimates of growth rate in the ocean, in Physiological bases of phytoplankton ecology, Platt, T., Ed., *Can. Bull. Fish. Aquat. Sci.*, 210, 1981, 251.