APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1979, p. 850-860
0099-2240/79/11-0850/11$02.00/0

Vol. 38, No. 5

Measurement of Microbial Activity and Growth in the Ocean by Rates of Stable Ribonucleic Acid Synthesis

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Received for publication 22 August 1979

A relatively simple and extremely sensitive technique for measuring rates of stable ribonucleic acid (RNA) synthesis was devised and applied to bacterial cultures and seawater samples. The procedure is based upon the uptake and incorporation of exogenous radiolabeled adenine into cellular RNA. To calculate absolute rates of synthesis, measurements of the specific radioactivity of the intracellular adenosine 5'-triphosphate pools (precursor to RNA) and of the total amount of radioactivity incorporated into stable cellular RNA per unit time are required. Since the rate of RNA synthesis is positively correlated with growth rate, measurements of RNA synthesis should be extremely useful for estimating and comparing the productivities of microbial assemblages in nature. Adenosine 5'-triphosphate, adenylate energy charge, and rates of stable RNA synthesis have been measured at a station located in the Columbian Basin of the Caribbean Sea. A subsurface peak in RNA synthesis (and therefore growth) was located within the dissolved oxygen minimum zone (450 m), suggesting in situ microbiological utilization of dissolved molecular oxygen. Calculations of the specific rates of RNA synthesis (i.e., RNA synthesis per unit of biomass) revealed that the mid-depth maximum corresponded to the highest specific rate of growth (420 pmol of adenine incorporated into RNA·day^{-1}) of all depths sampled, including the euphotic zone. The existence of an intermediate depth zone of active microbial growth may be an important site for nutrient regeneration and may serve as a source of reduced carbon for mesopelagic and deep sea environments.

There is little doubt among biological oceanographers that the heterotrophic microorganisms are central metabolic components of marine ecosystems. The extremely diverse biochemical activities of microorganisms in nature and the potential growth rates of isolated marine bacteria as measured in the laboratory, coupled with their small size and high surface-to-volume ratios, provide bacteria and other microorganisms with an enormous potential for biogeochemical transformations in nature. Recent reviews by Strickland (44), Pomeroy (35), and Sieburth (42) have elevated the marine microbes to a more significant ecological position. At the present time, however, very few unequivocal data exist on the in situ growth rates of microbial assemblages in the sea, primarily due to the lack of sensitive and reliable methodologies.

Ribonucleic acid (RNA) is a necessary precursor for protein synthesis and, therefore, cell growth. It is now well established that the rate of RNA synthesis in microorganisms is directly correlated with the rate of protein synthesis (13). The intracellular concentration of RNA is regulated in strict accord with the potential of the nutritional surroundings for supporting growth at a given rate (32). A direct quantitative relationship has been observed between the intracellular concentration of ribosomal RNA (rRNA) and the rate of protein synthesis and cell growth (12, 25, 30, 40), independent of the particular growth-limiting factor (i.e., carbon, nitrogen, vitamins, etc.) or the type of medium and set of growth conditions used to obtain the specific growth rates. The metabolically stable forms of RNA, rRNA, and transfer RNA (tRNA) account for approximately 90 to 95% of the total cellular RNA (23, 45), with the remaining few percent present as messenger RNA (mRNA). The rRNA content of bacteria can range up to 30 to 50% of the dry weight of the cells (23, 29), extrapolating virtually to 0% at infinitely slow growth rates (12).

The uptake and incorporation of exogenous radiolabeled nucleosides (e.g., uridine, thymidine, etc.) or amino acids (e.g., leucine) have been used extensively in laboratory and field investigations to estimate the rates of nucleic acid or protein synthesis, respectively (1, 4, 7, 24, 41, 47). The rate of labeling of these cellular macromolecules, however, cannot be assumed to be equal to the rate of synthesis, since the rate

will vary according to the specific transport mechanisms and dilution of the isotope by endogenous intracellular precursor pools before the incorporation into macromolecules. In addition, measurements of RNA synthesis are complicated by the metabolically unstable fraction of the RNA (i.e., mRNA) which is rapidly turned over ($t \sim 1$ s). Therefore, quantitative measurements of the rates of RNA synthesis also require determinations of the specific radioactivity of the intracellular pools which are the immediate precursors to RNA (i.e., the nucleotide triphosphate pools), as well as determinations of the amount of radioactivity incorporated into RNA (14). Observed variations in the rates of incorporation of uridine, thymidine, or leucine are generally taken to represent differences in metabolic activity or growth, but may in fact simply be the result of differences in the intracellular specific activities. This is especially true for environmental determinations where the exact (or even the approximate) organic chemical composition of the growth medium (i.e., seawater) is ill defined at best. Differences in natural substrate concentrations of uridine, thymidine, or leucine would tend to dilute the specific activity of the radiolabeled isotope, resulting in a lower intracellular specific activity of the precursor. This in turn would translate into an underestimate of the true metabolic activity or growth rate. It is therefore imperative, especially in ecological studies, to determine the specific radioactivity of the immediate intracellular precursor pool (i.e., the nucleotide triphosphates in the case of RNA synthesis) before any quantitative or even qualitative comparisons can be made between various rate calculations. This theoretical approach has been employed by several investigators to examine the rates of RNA synthesis in chicken fibroblasts (14), tobacco callus tissue (46), Chinese hamster cells (43), and sea urchin embryos (22), to name a few. To my knowledge, this approach has never been used or even suggested for use in environmental studies.

This report describes the theoretical principles, outlines the analytical procedures and possible limitations of these methods for measuring RNA synthesis in bacterial cultures and seawater samples, and discusses several specific advantages of this proposed experimental approach over previously published methodologies.

THEORETICAL CONSIDERATIONS

When [^{3}H]adenine is transported into cells, it is incorporated into a number of molecules including adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), and other purine derivatives. Therefore, before an absolute rate of RNA synthesis can be determined, it is imperative to calculate the specific radioactivity of the nucleotide triphosphate precursor pool, as well as the amount of radioactivity incorporated into RNA. The importance of determining the intracellular specific activity of the precursor pool cannot be overemphasized. Kemp and Sutton (21) have demonstrated that a 10-fold increase in the rate of incorporation of [^{3}H]leucine into protein of cultured tobacco pith was actually due to a 10-fold change in the intracellular specific activity of the precursor and not to an increase in the rate of protein synthesis. In subsequent experiments, they found that there may be differences of four orders of magnitude in the values of the specific activities for the intracellular versus exogenous pools of leucine. Likewise, Roller et al. (37) have shown that an increase in the rate of incorporation of [^{3}H]uridine into RNA of monkey CV-1 cells when treated with cyclic AMP was actually the result of a change in the specific activity of the precursor pool rather than a change in the rate of RNA synthesis. Therefore, it is essential, especially in environmental studies, to determine the specific radioactivity of the precursor pools before any comparisons can be made between individual microbial populations. This approach has never been followed in previous ecological studies with radioisotopic tracers.

The theory underlying the methods presented in this report is outlined in Fig. 1. Exogenous [^{3}H]adenine is transported across the membrane at rate k_1 and is converted into [^{3}H]AMP and subsequently into [^{3}H]ADP and [^{3}H]ATP, with the latter serving as the immediate precursor to RNA. Since the half-life of mRNA is very short, this pool rapidly equilibrates with the adenine nucleotide pools. Adenine is removed (as ATP) for the production of stable RNA (tRNA and rRNA) at rate k_2 (Fig. 1). Three basic assump-

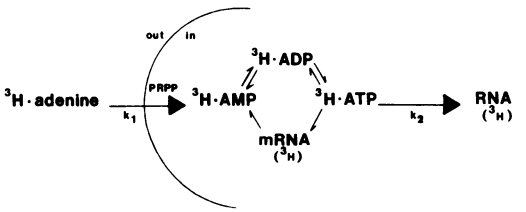

FIG. 1. *Schematic representation of the theory of the methods presented in this report for quantitative determinations of the rates of RNA synthesis in seawater samples. Symbols and abbreviations are defined in the text.*

tions are implicit in the application of these methods to the analysis of natural microbial assemblages: (i) all, or most, marine microorganisms can utilize exogenous adenine; (ii) there is no increase in the ATP (or total adenylate) pool upon the addition of exogenous adenine and no luxury consumption of adenine by the cells; and (iii) there is no intracellular compartmentation of the ATP pool.

The ability of microorganisms to utilize exogenous adenine supplies, in preference to de novo synthesis, has been investigated by several laboratories. A detailed review of the mechanisms and conditions of uptake of nucleic acid precursors by bacteria has recently been published (15). Purine bases are thought to be taken up by a group translocation process which has been shown to be specific for the transport and metabolism of purine bases. Purine nucleosides and nucleotides must first be cleaved to the corresponding base moiety before uptake (15). Moreover, it has been shown that purine biosynthesis is regulated by feedback inhibition in such a way that the addition of adenine to the medium greatly reduces the activity of de novo pathways in both microorganisms and animal cells (15). Competition for a common limiting supply of phosphoribosyl pyrophosphate between "scavenger" reactions and phosphoribosyl pyrophosphate aminotransferase appears to be at least part of the reason why exogenous nucleotide bases inhibit de novo biosynthesis. Since the biosynthesis of adenine is highly endergonic (i.e., requires at least 6 mol of ATP for de novo biosynthesis of 1 mol of AMP), the existence of these purine "salvage" pathways enables the organisms to conserve energy that might otherwise be required for purine biosynthesis. Recently Azam and Hodson (5) have reported a very rapid turnover of dissolved ATP in seawater samples and in 11 of 12 randomly selected marine bacterial isolates, thereby suggesting that adenine nucleotide (or more correctly, adenine) metabolism may be a general feature of natural microbial populations.

The second assumption regards luxury uptake of adenine by microbial cells, or subsequent adenine nucleotide pool expansion, or both. It has previously been shown that the activity of the enzyme, phosphoribosyl phosphotransferase, is regulated by the intracellular concentrations of the adenine nucleotides in such a way that it serves to regulate the flux of adenine into the cells (16). Furthermore, it has been demonstrated that purine uptake is directly linked to nucleic acid biosynthesis such that the rate of entry of adenine into the cells (k_1, Fig. 1) does not exceed the removal of adenine (k_2, Fig. 1) during the synthesis of stable RNA (8, 27, 31, 36). Another possibility that must be considered regarding environmental applications is the assimilation of adenine by "dying" cells as a "last effort" to stay alive. This possibility can be ruled out since adenine uptake is an energy-dependent mechanism requiring the "high-energy" compound phosphoribosyl pyrophosphate and the entire process appears to be under adenylate energy charge (EC) control (3). Therefore, any cells with EC ratios incompatible with cell growth (i.e., $EC \leq 0.80$) should not be capable of transporting adenine into the cells for respiration or for assimilation into macromolecules.

The final assumption concerns intracellular compartmentation of the adenine nucleotides and is more difficult to evaluate in conceptual terms. Compartmentation refers to the existence of metabolite pools within the cells that do not mix freely within the cytoplasm by the expected diffusion-controlled processes. Compartmentation may occur either through barrier (membrane) or kinetic processes. Kinetic compartmentation as described by Atkinson (2) results from spatial associations of enzymes such that transfer of a product from one reaction center to the active site of another occurs at a rate greater than predicted by diffusion alone. Although certain investigators have reported the existence of compartmentalized metabolite pools in bacterial cells (8), more recent studies have failed to detect them (33, 38, 39). Moreover, neither Brandhorst and Humphreys (6; sea urchin embryos), Emerson and Humphreys (14; chicken fibroblasts), nor Dinaur and La Marca (10; frog oocytes) were able to detect any interferences of compartmentation (if it occurred at all) on measurements of rates of RNA synthesis by labeled precursor methodologies. This does not mean to imply that separate ATP pools do not exist, but rather that if they do occur, they are in rapid equilibration with, and have specific radioactivities identical to, the actual RNA precursor pool. For our analysis of RNA synthesis, we have therefore assumed that the measured specific activity of the total ATP pool is equivalent to that of the RNA precursor pool. All experimental evidence is consistent with this assumption.

MATERIALS AND METHODS

Sample collection, medium composition, and culture conditions. The samples analyzed in this study included pure cultures of the marine bacterium *Serratia marinorubra* (ZoBell and Feltham) and seawater samples collected from the Caribbean Sea. *S. marinorubra* was grown in continuous culture on a medium consisting of 1.5 g of peptone, 0.05 g of yeast extract, and 25 mg of $FePO_4$ per liter in 80% seawater.

Under steady-state growth conditions, the growth rate (μ) was equivalent to the dilution rate [D = rate of input of fresh medium (milliliter·hour^{-1})/volume of culture (milliliters)], and was varied by using a peristaltic pump (Harvard Apparatus Co.). The culture vessel was continuously aerated, and all experiments were conducted at 26 ± 0.5°C. The bacterial cultures were sampled directly from the growth vessel with sterile glass pipettes.

Seawater samples were collected at a station in the Columbian Basin of the Caribbean Sea (10° 06.5'N; 77° 17.0'W) during the Amazon/Caribbean Phase IX expedition of the R/V *Alpha Helix* (June to July, 1977). Water samples (0 to 1,200 m) were obtained with 5-liter Niskin bottles (General Oceanics, Miami, Fla.), and the individual samples were processed immediately upon shipboard arrival.

Sample incubation with [^{3}H]adenine. A presentation of the methods used in this study for measuring RNA synthesis in bacterial cells and seawater samples is shown in Fig. 2 and 3, respectively. Duplicate subsamples of the bacterial cultures (500 μl) were pipetted into sterile culture tubes (12 by 75 mm) containing 5 μCi (in 50 μl of seawater) of [2-^{3}H]adenine (23 Ci·mmol^{-1}), and the samples were incubated for predetermined periods of time at 26 ± 0.5°C. The incubations were terminated with the addition of 500 μl of cold (4°C) 10% (wt/vol) trichloroacetic acid. Yeast RNA (1 mg) should be added to each sample to promote nucleic acid precipitation during the 4-h extraction period (4°C).

Seawater samples (250 ml) were added to acid-washed Pyrex glass bottles containing 30 μCi of [2-^{3}H]adenine (23 Ci·mmol^{-1}). The samples were incubated in the dark at approximate in situ temperatures (i.e., samples from 0 to 100 m were incubated at 25°C; those from 150 to 300 m were incubated at 15°C; and those from 350 to 700 m were incubated at 10°C) for 10 to 22 h. The uptake of adenine was determined to be linear over these time intervals. The incubations were terminated by filtering a 150-ml subsample of each bottle onto a microfine glass fiber filter (Reeve Angel, 984-H). The samples were stored frozen (−20°C) for shore-based determinations of the incorporation of [^{3}H]adenine into RNA. A second subsample (100 ml) from each bottle was filtered onto a 984-H glass fiber filter and immediately thereafter extracted in 5 ml of boiling tris(hydroxymethyl)-aminomethane (Tris) buffer (0.02 M, pH 7.7). The latter samples were used for determinations of the

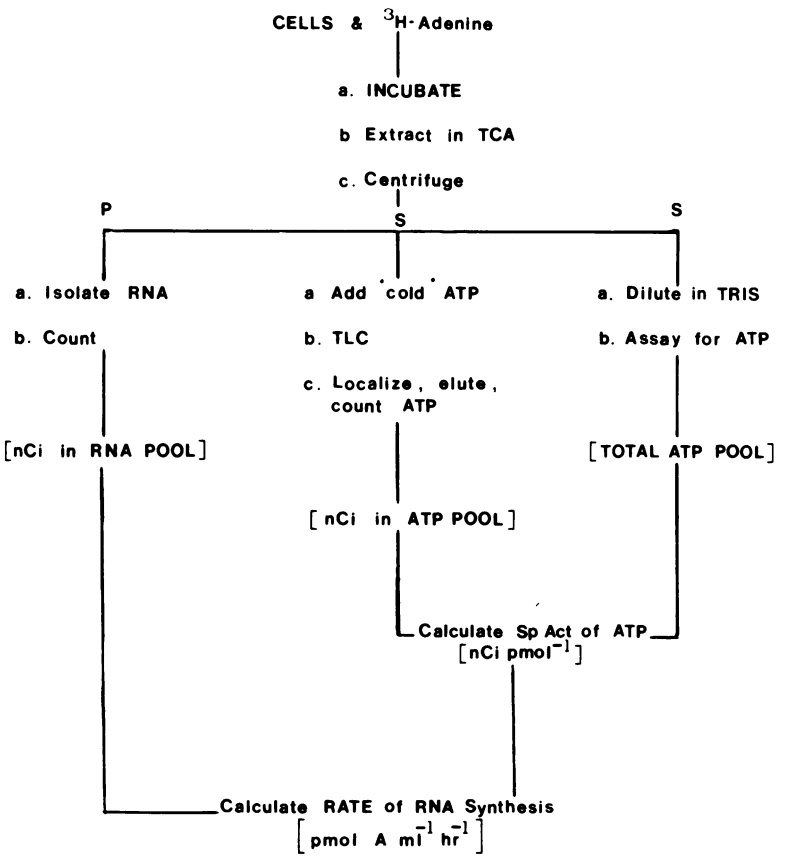

FIG. 2. *Flow diagram outlining the stepwise procedures for estimating rates of stable RNA synthesis in cultures of bacteria. TCA, Trichloroacetic acid; TLC, thin-layer chromatography.*

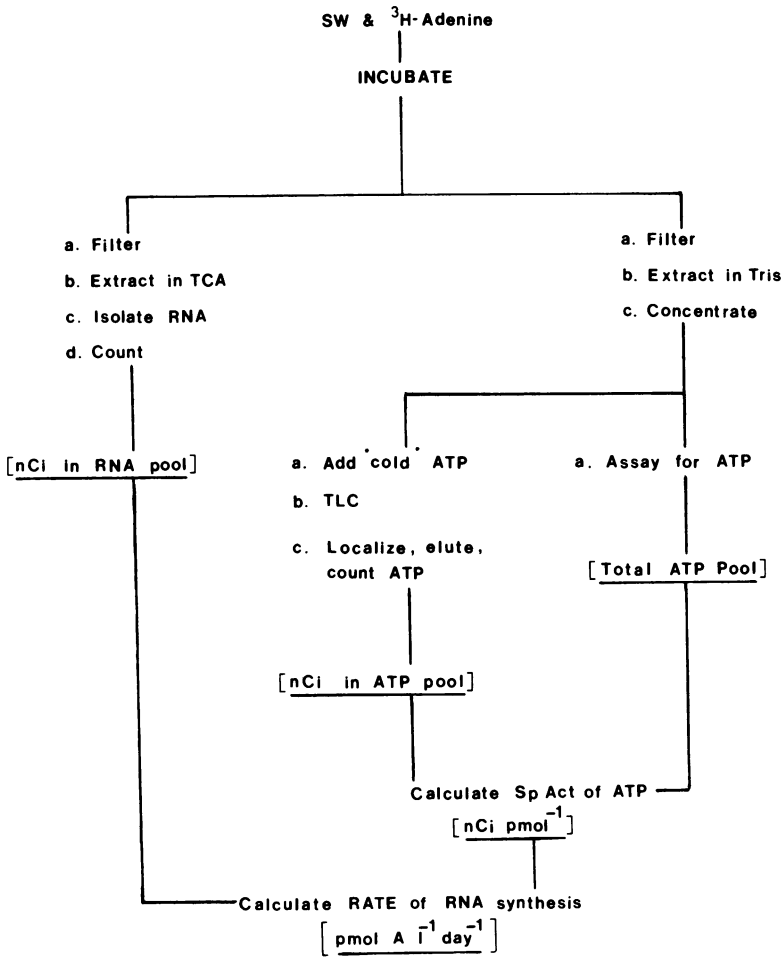

FIG. 3. *Flow diagram outlining the stepwise procedures for estimating rates of stable RNA synthesis in natural populations of marine microorganisms. TCA, Trichloroacetic acid; TLC, thin-layer chromatography.*

specific radioactivities of the ATP pools. Zero-time formaldehyde-killed controls (final concentration, 2%) were prepared and processed simultaneously.

Incorporation of [^{3}H]adenine into RNA. Total radioactivity incorporated into RNA was determined as the fraction of cold trichloroacetic acid-insoluble radioactivity which was solubilized by hot trichloroacetic acid (100°C). Although [^{3}H]adenine may be converted into [^{3}H]dATP and subsequently incorporated into deoxyribonucleic acid (DNA), this source of error is considered to be negligible unless the sample material is high in DNA (e.g., isolated nuclei). With natural microbial populations the amount of radioactivity incorporated into DNA has consistently been ≤5% of the activity in the RNA fraction. If desired, DNA and RNA may be separated after an alkaline hydrolysis and re-acidification (RNA soluble, DNA insoluble), but this procedure was not employed in the current study. It has, however, been incorporated into our subsequent investigations.

For the bacterial cultures, the acid-insoluble materials were isolated via centrifugation (2,500 × *g*, for 10 min), and 50 μl of the acid-soluble nucleotides was diluted into 5 ml of Tris buffer (0.02 M, pH 7.7) and stored frozen (−20°C) for subsequent determination of the specific radioactivities of the intracellular ATP pools. Trichloroacetic acid-insoluble materials were washed five times with 10 ml of cold (4°C) 5% trichloroacetic acid to minimize contamination from the excess [^{3}H]adenine. A portion (0.5 to 1.0 ml) of each supernatant was monitored for total radioactivity to determine the optimum number of rinses. The pellets were resuspended in exactly 1.5 ml of 5% trichloroacetic acid and heated to 100°C for 15 to 20 min. After cooling, the tubes were restored to 1.5 ml with distilled water and centrifuged (2,500 × *g*, 10 min), and a portion of the supernatant (0.2 to 1.0 ml) was added to a scintillation vial containing 10 ml of Aquasol (New England Nuclear Corp.). The radioactivity was determined with a Beckman liquid scintillation counter (Beckman LS 230). The counting efficiencies ranged from 22 to 35%, and quenching was measured and

corrected for by an external standard and quench curve.

The seawater samples were extracted in 5 ml of cold (4°C) 5% trichloroacetic acid for 4 h. Yeast RNA should also be added as described previously. The samples (including the filters) were transferred to a tissue-grinding tube, homogenized with a Teflon pestle, and quantitatively transferred to clean centrifuge tubes. The acid-insoluble materials were isolated, washed with trichloroacetic acid, and radioassayed as described above.

Determination of the radioactivity in the ATP pools. When adenine is incorporated into bacterial cells, it can be converted into a number of ribose and deoxyribose derivatives, including guanine ribonucleotides. The use of [2-^{3}H]adenine rather than the 2,8-^{3}H label eliminates the possibility of the formation of guanine ribonucleotides since the ^{3}H label is lost during this enzymatic interconversion. Nevertheless, the uptake and incorporation of [2-^{3}H]adenine result in the formation of a number of radiolabeled adenine derivatives, predominantly [^{3}H]AMP, [^{3}H]ADP, and [^{3}H]ATP. The labeled ATP, therefore, must be separated from the remainder of the nucleotides to accurately determine the specific radioactivity of the ATP pool.

For bacterial cultures, 4 ml of the Tris-buffered acid-soluble materials (from previous section) was added to a scintillation vial, evaporated to dryness at 40°C in vacuo, and reconstituted in 200 μl of distilled water. A 100-μl amount of this solution was diluted to 1 ml in Tris buffer (0.02 M, pH 7.7) and immediately frozen for subsequent determination of total ATP with the firefly bioluminescent reaction (20). A 50-μl amount of the concentrated material was added to a culture tube containing 10 μl of a 10^{-4} M solution of "cold" ATP and mixed thoroughly. A portion of each solution (10 to 30 μl) was spotted onto a polyethyleneimine-impregnated cellulose thin-layer chromatography plate (with plastic backing, Brinkmann Instrument Co.) and developed in one dimension with 0.85 M phosphate buffer (pH 3.4) as described by Cashel et al. (9). The exact volume applied to the thin layers was calculated by measuring the radioactivity in 10 μl of each of the extracts and then by measuring the total radioactivity remaining in each tube after spotting an unknown volume onto the polyethyleneimine plates (i.e., a difference measurement). The chromatograms were air dried at 25°C, and the ATP spots were visualized by using short-wavelength ultraviolet irradiation. After localization, the ATP spots were cut out with a pair of scissors, and the ATP was eluted from the cellulose plates by shaking the material with 1 ml of $MgCl_2$ (0.7 M) for 1 h. The elution step is necessary due to problems arising from β-absorption associated with the counting of ^{3}H on solid supports and in particular the discrepancies which have been noted between the counting efficiencies of nucleic acids and their nucleotide precursors (28). The recovery of ATP from the thin-layer plates was >95% as determined by the binding and firefly bioluminescent determination of an unlabeled ATP standard (10 ng·ml^{-1}). A portion of the eluate was added to 10 ml of Aquasol, or alternatively 10 ml of Aquasol may be added directly to the scintillation vial containing the $MgCl_2$, and the radioactivity was determined as described above.

For the seawater samples, 3 to 4 ml of the Tris-buffered cell extracts was evaporated to dryness, reconstituted in 200 μl of distilled water, diluted in Tris, applied to the polyethyleneimine thin layers, eluted, and radioassayed all as described above.

Measurement of ATP. To calculate the intracellular specific activity of the ATP pools, measurements must also be made of the total ATP concentrations. Quantitative ATP analyses were made with the firefly luciferase assay of ATP as described by Karl and Holm-Hansen (20).

Calculation of the intracellular specific radioactivities. Since the specific radioactivity value is merely a ratio of the radioactivity in the ATP pool to the total molar concentration and is therefore independent of concentration or dilution procedures, all determinations were expressed relative to the 200-μl (concentrated) samples. In addition, any hydrolysis or other loss of ATP during the preparation of the concentrated sample would not affect the specific activity or final rate calculations. The ATP was expressed in terms of picomoles of ATP·200 μl^{-1} (or per optical density) and the radioactivity was expressed as nanocuries. From these determinations, the specific radioactivity (nanocuries·picomole^{-1}) could be calculated. The rates of RNA synthesis were expressed as picomoles of adenine incorporated into RNA·liter^{-1} of seawater·day^{-1} (or for the bacterial cultures, picomoles of adenine incorporated into RNA·optical density^{-1}·minute^{-1}).

Measurement of adenylate energy charge. ADP and AMP concentrations and corresponding adenylate EC ratios were calculated for the seawater sample extracts by the methods described by Karl and Holm-Hansen (20).

RESULTS

A series of preliminary experiments was conducted with pure cultures of *S. marinorubra* to examine the kinetics of [^{3}H]adenine uptake and incorporation into ATP and RNA. Figure 4 presents the results of one set of experiments. The results indicated the following. (i) The addition of exogenous adenine does not significantly affect the size of the intracellular ATP pool (Fig. 4A) since the concentration of the ATP remained fairly constant (i.e., from 0.83 to 0.95 nmol·ml^{-1} of culture) throughout the first 15 min of the experiment. The cause of the decrease in the ATP pool beyond 30 min is most probably due to substrate (carbon) or oxygen limitation, since the cells were sampled from actively growing continuous cultures and were incubated as stationary (batch) cultures. In subsequent laboratory experiments of this nature, all incubations were limited to 10 min or less. (ii) Under the reaction conditions specified, the uptake of adenine and its incorporation into ATP were linear with time (Fig. 4B), demonstrating that adenine is taken up by *S. marinorubra* without any

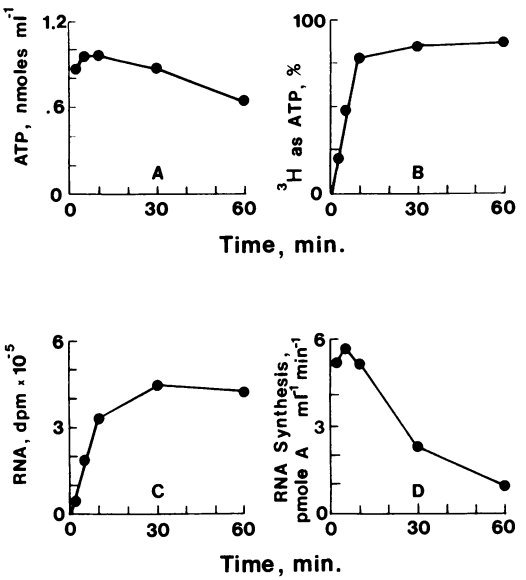

FIG. 4. *Kinetics of [³H]adenine uptake and incorporation into [³H]ATP and [³H]RNA in the marine bacterium S. marinorubra. The cells were sampled from a chemostat culture (μ = 11.1 h) and were incubated for predetermined periods of time as batch cultures. (A) The intracellular ATP levels expressed as nanomoles per milliliter of culture. (B) The percentage of the total acid-soluble radioactive pool recovered as [³H]ATP. (C) Amount of radioactivity contained in the acid-insoluble pool. (D) Rate of stable RNA synthesis expressed as picomoles of adenine incorporated into RNA per milliliter of culture medium per minute.*

appreciable lag. The conversion of [^{3}H]adenine into [^{3}H]ATP was rapid and complete (Fig. 4B), suggesting that exogenous adenine became limiting after approximately 10 min (i.e., 78% of the total acid-soluble radioactivity of the medium and cells was as [^{3}H]ATP). (iii) The conversion of [^{3}H]ATP into [^{3}H]RNA also occurred without a lag (Fig. 4C) and was linear within the first 10 min of incubation (until the exogenous [^{3}H]adenine was exhausted). Beyond 10 min the [^{3}H]RNA accumulated at a slower rate, reflecting the slowdown in growth presumably induced by substrate limitation. (iv) The rate of RNA synthesis was relatively constant over the first 10 min (i.e., 5.1 to 5.6 pmol of adenine incorporated into RNA·ml^{-1} of culture·min^{-1}) and decreased with the slowdown in growth (Fig. 4D).

Figure 5 presents the results that were obtained for the seawater samples collected in the Columbian Basin of the Caribbean Sea. The ATP concentrations decreased from 32 ng·liter^{-1} at 35 m to 4.4 ng of ATP·liter^{-1} at 200 m. These values are very low (~10%) when compared with the relatively eutrophic waters of the Southern California Bight (20) and serve to emphasize the oligotrophic nature of this region. Below 200 m, the ATP concentrations exhibited a general decrease with subsurface peaks at 250, 400, and 600 m. The ATP value at 1,200 m was 0.75 ng·liter^{-1} and is nearly identical to the concentrations that have been measured from comparable depths in the North Pacific Ocean off southern California (Karl, unpublished data) and elsewhere in oceans of the world. The most significant feature of the vertical distribution of the adenylate EC ratios is the discontinuity at a depth of approximately 500 m that separates the microorganisms into communities with relatively high EC ratios (0.72 to 0.83) above 500 m and relatively low EC ratios (0.56 to 0.70) below 500 m. This feature was also noted by Karl and Holm-Hansen (20) for Southern California Bight samples. These results suggest that a physiological or metabolic alteration occurs within the populations below 500 m, such that their potentials for cell growth are substantially reduced.

The maximum rate of RNA synthesis (2,500 pmol of adenine incorporated into RNA·liter^{-1}·day^{-1}) coincided with the water depth exhibiting the highest EC ratio (i.e., 70 m). Below 70 m, the measured rates of RNA synthesis rapidly decreased with increasing water depth to a minimum value of 40 pmol of adenine incorporated into RNA·liter^{-1}·day^{-1} at 250 m. At greater depths, two regions of elevated RNA synthesis were evident (albeit, 1-point maxima), one centered at 300 m and a more active zone centered at 450 m. Both subsurface peaks in RNA synthesis were well within the region of the water column where the adenylate EC ratios were relatively high (i.e., 0.72 to 0.83, Fig. 5). Below 450 m, the rates of stable RNA synthesis decreased to values less than 100 pmol of adenine incorporated into RNA·liter^{-1}·day^{-1}.

The concentration of dissolved O_2 decreased from 4.8 ml·liter^{-1} at 35 m to a minimum value of 2.7 ml·liter^{-1} at 450 m. With increasing water depth, the O_2 concentrations increased to 4.65 ml·liter^{-1} at 1,200 m. The depth interval displaying the minimum in dissolved O_2 concentration (450 m) corresponded to the same depth horizon as the midwater maximum in RNA synthesis (Fig. 5), suggesting a correlation between these two measured parameters.

The measured rates of stable RNA synthesis actually represent the combined activities of the total microbial assemblage. If the results are expressed as specific rates of RNA synthesis (i.e., RNA synthesis per unit of microbial biomass), the results may be used for more direct comparisons among the various populations of microorganisms. Figure 6 presents data for specific

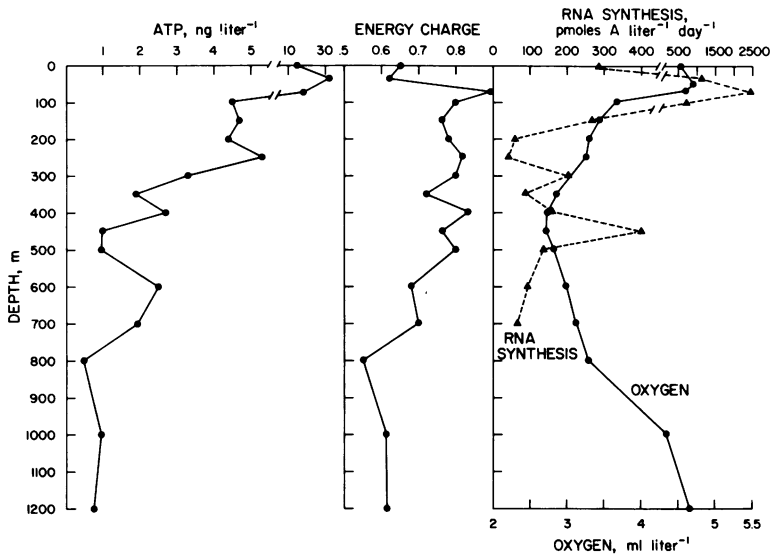

FIG. 5. *Vertical profiles of ATP, adenylate EC, dissolved molecular oxygen, and rates of stable RNA synthesis for water samples collected at a station located in the Columbian Basin of the Caribbean Sea. Station location was 10° 6.5'N, 77° 17.0'W.*

rates of stable RNA synthesis, assuming that ATP is a reliable indicator of total microbial biomass (17) and keeping in mind the difficulties and limitations in the application of a single C/ATP ratio (18). It is apparent from these data that the microbial assemblage at 450 m maintains a higher specific rate of stable RNA synthesis (and, therefore, specific growth) than any other portion of the water column, including the euphotic zone (Fig. 6).

DISCUSSION

Macromolecular synthesis (i.e., protein, DNA, and RNA synthesis) has not been used extensively to investigate the in situ growth states of microbial assemblages in the ocean. The observed correlations between the rates of stable RNA synthesis, protein synthesis, and cell growth (reviewed in reference 26) are so universally applicable that they lend themselves to the analysis of complex microbial populations such as one finds in nature. Since there is little, or no, turnover of rRNA in microorganisms at either fast or slow growth rates, it appears as though the formation of rRNA is geared in a precise and unique manner to the overall protein synthesis potential of a cell (29). All previous environmental applications have inferred rates of macromolecular synthesis from rates of incorporation of exogenous labeled materials into acid-insoluble cellular materials. Unfortunately, failure to calculate (and correct for) specific radioactivities of the intracellular precursor pools has pre-

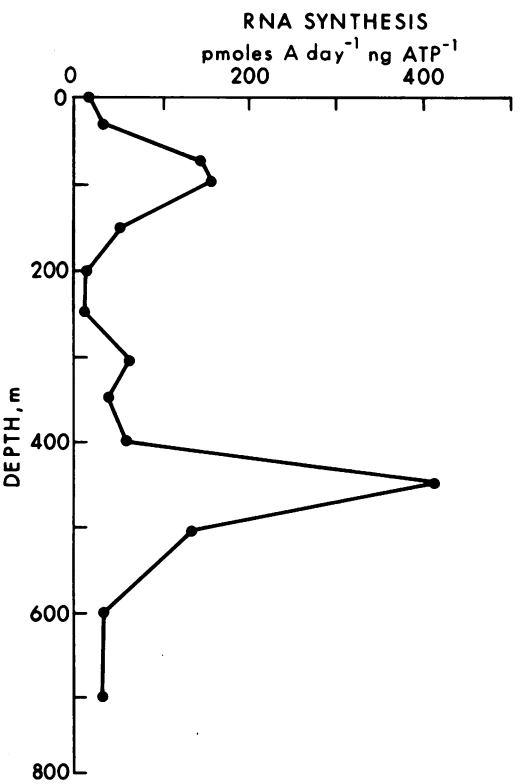

FIG. 6. *Vertical profile of specific rates of RNA synthesis, expressed as picomoles of adenine incorporated into RNA per day per nanogram of ATP biomass-carbon, for the water samples collected at the station described in the legend to Fig. 5.*

vented the reliable estimation of absolute rate measurements. Even for the determination of relative rates, failure to measure the intracellular specific radioactivities might lead to serious errors and gross misinterpretation of ecological data.

The present method for estimating microbial growth rates from direct quantitative measurements of the rates of stable RNA synthesis has several unique advantages over previously proposed methods, including: (i) most of the assumptions and criteria which must be satisfied (see theoretical assumptions section) have already been shown to hold true for the microorganisms thus far examined, and the entire theoretical basis of this technique is predicated upon sound principles of microbial physiology and growth; (ii) the determination of intracellular specific activities enables one to calculate absolute rate measurements and to directly compare rates from extremely different environments without the need for addressing the problems associated with the measurement of the concentrations of specific organic pools in natural waters; (iii) the technique is amenable to ecological and oceanographic investigations in that it allows for large numbers of samples to be processed simultaneously; and (iv) the technique is extremely sensitive owing to the availability of high-specific-activity [^{3}H]adenine and to the ultrasensitivity of the firefly bioluminescent assay of ATP.

Another unique aspect of this proposed method is the use of adenine to measure rates of RNA synthesis. In previous studies of RNA synthesis, uracil (or uridine) has been selected as the tracer of choice since this pyrimidine base is found exclusively in RNA. The same rationale has been invoked for the use of thymidine to estimate DNA synthesis. One problem with using pyrimidine bases as tracers results from the fact that there is no apparent pivotal point in their de novo biosynthesis that is comparable to the pathway of purine nucleotide biosynthesis. As a result, the addition of uridine results in the production of labeled uridine 5′-triphosphate, cytosine 5′-triphosphate, deoxycytosine 5′-triphosphate, and deoxyribosylthymine 5′-triphosphate, thereby labeling both DNA and RNA. Furthermore, the ratio of label entering RNA from uridine 5′-triphosphate versus cytosine 5′-triphosphate may vary radically (45). The currently proposed technique employs [^{3}H]adenine which specifically labels only the AMP of RNA. The ^{3}H label at the 2- position is lost during the conversion of adenine to other purine nucleotides or purine derivatives. Although the presently proposed thin-layer chromatography separation procedure does not resolve [^{3}H]dATP from [^{3}H]ATP, the contribution of dATP to the total ATP-specific radioactivity is less than 5% (Karl, unpublished data). Therefore, it is believed that the currently proposed method of adenine incorporation is much more accurate and specific for estimating rates of RNA synthesis than are the more commonly employed uridine methods.

The data presented in Fig. 5 represent the first direct evidence of elevated microbial growth rates within the dissolved O_2 minimum layer. Like many other ecological measurements, the RNA synthesis technique records the net response of the entire microbial community. Conceptually, all microorganisms are assumed to respond in an equivalent fashion to the addition of exogenous [^{3}H]adenine. Correspondingly, a mean intracellular specific activity is calculated, and from this, a mean rate of RNA synthesis is determined. In reality, the calculated intracellular specific activity is probably an underestimate of the true value since a certain proportion of the population may not be metabolically active or may not have the potential for cell growth. This fraction of cells would, however, contribute their cellular ATP to the determination of the mean specific activity calculation (i.e., nanocuries·picomole^{-1} of ATP). Microautoradiographic studies consistently reveal that approximately 20 to 40% of the total microbial population is inactive with respect to the uptake and assimilation of specific radiolabeled nutrients (A. F. Carlucci, personal communication). In this present study, we used adenylate EC to estimate the community potential for cell growth. In theory, cells with EC values less than ~0.8 are not energetically capable of cell growth. It is not known, however, whether an environmental value of 0.6 to 0.7 is representative of a senescent population of microorganisms, or whether it is the mean ratio derived from 50% actively growing cells (EC > 0.8) and 50% dead or dying cells (EC < 0.5). The observation that stable RNA synthesis continues even when the EC drops below 0.8 (Fig. 5) tends to support the latter hypothesis.

From the RNA synthesis data presented in Fig. 5, an order of magnitude calculation of the rate of microbial growth can be attempted. It is well established that the rRNA nucleotide composition of different taxonomic groups of microorganisms is nearly identical despite the observed variation in the genome base composition (32, 35). The average adenine mole percent value of the RNA from 12 diverse bacterial species was 25.3 ± 1.7 (34). Within the dissolved O_2 minimum region (450 m) the rate of RNA synthesis was calculated to be 400 pmol of adenine incorporated into RNA·liter^{-1}·day^{-1}. By con-

verting to grams (AMP = 347 pg·mol^{-1}) and correcting for the fact that adenine comprises ~25% of the stable RNA, a value of 555 × 10^{-9} g of RNA produced·liter^{-1}·day^{-1} is determined. If RNA ranges from 5 to 15% of the total dry weight of the cells in nature (a value selected on the basis of the assumed and reported growth rates of marine bacteria), the production rate of microbial cells would be on the order of 3.7 × 10^{-6} to 11 × 10^{-6} g (dry weight) of cellular material·liter^{-1}·day^{-1}. From the ATP data, and by assuming a bacterial C/ATP value of ~500 (19) and a value of 50% for the ratio of carbon to total cellular dry weight (11), a value of 10^{-6} g·liter^{-1} is calculated for the total standing stock of microbial cells. From the production and biomass data a mean turnover, or generation time, of 2.2 to 6.5 h is derived, assuming steady-state conditions (i.e., d biomass/dt = 0).

In conclusion, a new experimental approach to the study of microbial growth in nature has been devised, and the various physiological assumptions, analytical considerations, and limitations of the method have been discussed. Although the environmental data presented in this report should be taken as preliminary, they demonstrate the sensitivity and potential usefulness of this assay procedure for marine microbiological investigations. Additional laboratory and field experiments are currently underway to further investigate the occurrence, kinetics, and specific mechanisms of adenine uptake, to evaluate the effects of nongrowing cells on the RNA rate calculations, and to optimize the analytical application of this technique for the analysis of microbial assemblages in seawater and elsewhere in nature.

ACKNOWLEDGMENTS

I thank P. A. LaRock, R. Dennis, and S. King for their assistance in obtaining and analyzing the Caribbean Sea water samples, A. F. Carlucci, O. Holm-Hansen, C. Winn, and D. Wong for helpful comments and constructive criticism, and K. Paulo for musical inspiration. The enthusiasm and help supplied by the scientists and crew of the R/V *Alpha Helix* (Caribbean-Amazon leg III, A. Fleminger, chief scientist) contributed to the success of this project.

This project was supported in part by National Science Foundation grants OCE 77-09936 (awarded to P. A. LaRock) and OCE 78-25446 (awarded to D. M. Karl).

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