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RNA Synthesis as a Measure of Microbial Growth in Aquatic Environments. I. Evaluation, Verification and Optimization of Methods

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Abstract

Measurement of rates of microbial (bacteria and unicellular algae) ribonucleic acid (RNA) synthesis in environmental samples by radioactive tracers requires several assumptions that we test and evaluate herein. After addition of ^{3}H-adenine to water samples from eutrophic freshwater or pelagic and oceanic ecosystems, microorganisms assimilate it without (1) any luxury uptake or (2) any expansion of intracellular adenine nucleotide pools. The calculated rate of stable RNA synthesis tends to be overestimated during the initial time periods of labeling (i.e., less than 30 s for cultures of *Serratia marinorubra*, and less than 20 min for most environmental samples) due to kinetic compartmentalization of the nucleic acid precursor (ATP) pool. Time-series analyses are recommended because the effects of compartmentalization decrease with increasing incubation. The kinetics of ^{3}H-adenine uptake and aspects of its subsequent assimilation, intermediary metabolism and breakdown are described in bacteria and natural assemblages of microorganisms. Mass-balance radioactivity inventories greatly facilitate interpretation of nucleic acid precursor assimilation. Finally, we demonstrate that independent measurements of precursor (ATP) specific radioactivity are required for any elucidation of rates of stable RNA synthesis (and thus microbial growth) in nature.

Introduction

Ecologists have attempted to measure the growth of microorganisms in nature by applying a variety of methods, all with varying degrees of success. Recently, nucleic acid biosynthesis has been used to measure microbial growth *in situ* (Brock, 1967; Thomas *et al.*, 1974; Straat *et al.*, 1977; Tobin and Anthony, 1978; Karl, 1979a; LaRock *et al.*, 1979; Fuhrman and Azam, 1980). All but one of the studies (Karl, 1979a) have inferred rates of synthesis from incorporation of exogenous radiolabeled bases or nucleosides into cellular nucleic acids, but have not corrected for the specific radioactivities of the various intracellular precursors of RNA and DNA; it is reasonable to assume that this omission has resulted in substantial quantitative errors.

Karl (1979a) discussed the radioactive labeling of ATP and RNA by addition of ^{3}H-adenine to seawater samples for measuring *in situ* microbial growth. Three assumptions must be made if the method is to succeed. First, all or most marine microorganisms must transport and assimilate exogenous ^{3}H-adenine. Second, addition of exogenous ^{3}H-adenine must not result in expansion of the ATP pool, luxury uptake of adenine, or stimulation of the rate of RNA synthesis *in situ*. Third, there should be no intracellular compartmentalization of ATP, which is the immediate precursor to adenine in RNA. To these prerequisites should be added the existence of methods for accurate measurements of ATP, the specific activity of the ATP pool (i.e., nCi pmol^{-1}) and the amount of radioactivity incorporated into cellular RNA.

Accurate interpretation of radiotracer experiments with nucleic acid precursors obviously requires the construction of kinetic models which are based upon complete and explicit experimental data. This includes a careful evaluation of the pathway(s) of precursor metabolism (including a consideration of all intermediates and end-products) and a thorough understanding of the response of the ecosystem to the precursor introduced to ensure proper ecological interpretation of the radiotracer data. The present paper reports the results of laboratory and field experiments designed to evaluate the validity of the stable RNA synthesis technique (Karl, 1979a) as a probe for evaluating the growth rates of microorganisms in nature. Table 1 summarizes the sequence of measurements, rationale and conditions necessary for the application of this technique to the study of microbial assemblages in nature.

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Table 1. Sequence of measurements, rationale and assumptions for application of the environmental microbial RNA synthesis technique in nature

Objective or rationale	Conditions for ecological application	Experiments in present paper to evaluate conditions
Step 1		
Incubate water sample with ^{3}H-adenine		
(a) Microorganisms assimilate certain nucleic acid bases if present in the medium	(a) All (or most) microorganisms assimilate adenine by a common and predictable pathway	(a) Uptake and assimilation of adenine by marine microorganisms
(b) Salvage pathways for adenine, and regulation thereof, are well known in bacteria	(b) A uniform metabolic response is required if the technique is to be used for estimating community growth	
Step 2		
Extract and measure total microbial ATP; isolate and radioassay ^{3}H-ATP; calculate mean community ATP pool-specific radioactivity (nCi pmol^{-1})		
(a) ^{3}H-adenine enters microbial cells and is rapidly converted into ^{3}H-ATP and other adenine derivatives	(a) Rate of adenine uptake does not exceed rate of removal from ATP pool during nucleic acid synthesis	(a) Effects of ^{3}H-adenine addition on total cellular ATP concentrations
(b) Precursor (ATP pool) specific radioactivity is required to calculate rate of RNA synthesis	(b) Addition of adenine does not affect ATP cell quota or adenine nucleotide turnover rate	(b) Effects of vacuum filtration
	(c) Measurement of ATP pool-specific radioactivity is free from artifacts and analytical interferences	(c) Mass balance inventories of radioactivity
		(d) Pulse-chase experiment
Step 3		
Isolate and radioassay ^{3}H-RNA; calculate rate of RNA synthesis from total incorporation and precursor (ATP pool)-specific radioactivity data		
(a) Measures of total ^{3}H-RNA (nCi l^{-1}) produced and mean ATP pool-specific radioactivity (nCi pmol^{-1}) enable calculation of total rate of RNA synthesis (pmol adenine incorporated into RNA l^{-1} h^{-1})	(a) There is no compartmentalization of cellular ATP pools. Compartmentalized pools, if they exist, may affect the flow of ^{3}H-adenine into ^{3}H-RNA	(a) Mass balance inventories of radioactivity
		(b) Kinetics of ^{3}H-ATP, ^{3}H-RNA, ^3H_2O production and rate of RNA synthesis
(b) Rate of RNA synthesis provides a measure of mean community growth rate	(b) There is no luxury uptake of adenine by the cells	(c) Effects of ^{3}H-adenine concentrations
	(c) There is no stimulation of RNA synthesis upon addition of adenine to the medium	(d) Pulse-chase experiment

Materials and Methods

Collection of Samples

Coastal seawater samples from Kewalo Basin, Kaneohe Bay and Waikiki Beach, Oahu, Hawaii, were hand-collected in acid-washed polyethylene bottles. Oceanic samples from Ke-ahole Point, Hawaii and from a station in the Northeast Pacific Ocean off Point Sur, California (36°06.6′N; 122°46.5′W) were collected in EtOH-rinsed 8 liter Niskin® bottles (General Oceanics, Miami, Florida). Seawater samples for the isolation of marine bacteria were manually collected in sterile, evacuated, glass Vacutainer® tubes (B-D, No. 4710). All seawater samples were prefiltered through a 150 μm Nitex® mesh prior to analysis to remove large zooplankton.

For several experiments we collected samples from a eutrophic freshwater pond located on the University of Hawaii at Manoa campus; throughout most of the year Krauss Pond was dominated by the alga *Chlorella* sp., and contained a high concentration of particulate organic detritus. Acridine-orange epifluorescence microscopy (Hobbie *et al.*, 1977) revealed that bacteria were frequently associated with particles >5 μm. Additional freshwater samples were obtained from aquaculture ponds located on Oahu, Hawaii. All freshwater samples were prefiltered through a 90 μm Nitex® mesh prior to analysis.

Pure Culture Studies

Serratia marinorubra (ZoBell and Feltham) was grown in either batch or continuous culture at 25° ± 1 C° on a medium consisting of 1.5 g of peptone, 50 mg yeast extract and 25 mg $FePO_4$ l^{-1} in 80% seawater (100% = 35‰S). For selected experiments, the composition of the medium was altered to include glucose (1.0 g l^{-1}) or to provide variations in the concentration of yeast extract and, in certain cases, defined levels of adenine or guanine.

Uptake and Assimilation of Adenine by Marine Macroorganisms

Assimilation of exogenous adenine into macromolecules was studied by two experimental approaches. In the first, selected seawater samples were streaked onto nutrient agar plates (Marine agar, ZoBell No. 2216, Difco) enriched with 1 μCi of [2-^{3}H] adenine (15 to 25 Ci mmol^{-1}, New England Nuclear Corporation, Boston, Massachusetts) ml^{-1}. After colonies had developed (1 to 3 d, 25 °C), the cells were carefully removed from the agar surface, added to 10 ml Aquasol® (New England Nuclear Corporation) and radioassayed. Control swabs of the clean agar surface contained negligible radioactivity (<1% of the mean level found in selected colonies). If colonies contained less than 25 times the radioactivity of the controls, the isolates were subcultured onto fresh agar plates containing ^{3}H-adenine and the fresh colonies were processed for ^{3}H-RNA to confirm (or refute) the assimilative capacity of that particular isolate. This approach allows a large number of heterotrophic marine bacteria to be screened for their ability to assimilate exogenous adenine.

^{3}H-Adenine Autoradiography

Water samples from Krauss Pond were incubated with either ^{3}H-adenine (0.5 μCi ml^{-1}) or ^{3}H-amino acid mixture (0.5 μCi ml^{-1}, 125 mCi mg^{-1} Amersham Radiochemicals, Arlington Heights, Illinois) under *in situ* conditions for time periods ranging from 20 min to 4 h. The incubations were terminated by the addition of buffered formalin to 20 ml sub-samples. Samples were prepared for microautoradiography following the methods of Meyer-Reil (1978), with minor modifications. We found acridine orange to be unsatisfactory in these experiments due to de-staining during development and fixation of the autoradiograms. Substitution of 4', 6-diamidino-2-phenylindole (DAPI, Accurate Chemical Co., Hicksville, New York) for acridine orange produced superior results. The proportion of labeled cells was estimated by epifluorescence microscopy (Hobbie *et al.*, 1977).

Stepwise Procedure for Measuring Rates of RNA Synthesis

The procedures used in this study represent a minor modification of the methods previously described by Karl (1979a). Incorporated changes have resulted from experiments designed to optimize reaction conditions for the isolation, recovery, purification and measurement of RNA (and DNA) and the specific activity of the ATP pool.

Bacterial cultures or environmental samples were incubated in acid-washed glass bottles containing ^{3}H-adenine at an activity of between 0.05 and 0.5 μCi ml^{-1} (generally 0.1 μCi ml^{-1}). For selected experiments, the activity was increased to 100 μCi ml^{-1}. Stock solutions (100 μCi ml^{-1}) of the radiotracer were prepared by dilution with filter-sterilized distilled water or seawater, and were stored frozen (−20 °C) in 5 ml aliquots. Under the conditions specified, there was observed a gradual decomposition (i.e., as monitored by the production of ^3H_2O) of no more than 5% per month. Aqueous solutions of ^{3}H-adenine should not be autoclaved, since the production of ^3H_2O via exchange reactions is accelerated under these conditions. We observed a loss of 30% of the adenine label during one autoclave cycle which is consistent with data published by Evans *et al.* (1970).

The incubations were terminated by simultaneous filtrations and extractions for ATP and nucleic acids. Sub-samples for ATP were filtered onto microfine glass-fiber filters (Reeve Angel, 984-H) and extracted in 5 ml of boiling phosphate buffer (60 m*M*, pH 7.4). After an extraction period of 5 min, the nucleotide extracts were stored frozen (−20 °C). ATP concentrations and specific activity of the ATP pool were stable for at least 1 month.

Total radioactivity incorporated into stable RNA was measured by placing a second sub-sample into 5 ml of ice-cold trichloroacetic acid (TCA, 5% wt/vol), to which 1 mg each of non-radioactive RNA and DNA (from stock solutions of 5 mg ml^{-1}) were then added. We found that the addition of cold RNA and DNA was necessary to promote the precipitation of low concentrations of radiolabeled nucleic acids in the TCA extracts. Samples were extracted at 4 °C for a minimum of 1 h (not to exceed a few hours). In certain situations where RNA samples could not be processed immediately (e.g. remote field sampling), the filters were frozen. Freezer storage of the filters for periods up to 2 wk resulted in no significant loss of radioactivity from the RNA and DNA fractions.

For selected experiments we used Millipore® (GS, 0.22 μm) filters for the collection of particulate materials. In these instances, the filters were dissolved by initial extraction in acetone (see Holm-Hansen *et al.*, 1968) followed by the addition of 25 mg of celite (Sigma Chemical Co., St. Louis, Missouri) as a centrifugation aid. Nuclepore® filters proved inconvenient, primarily because they are not easily homogenized or dissolved.

To establish meaningful inventories of the added radioactivity, both the intracellular and extracellular environments must be monitored. During the uptake and assimilation of ^{3}H-adenine, radiolabeled water (i.e., ^3H_2O) is produced under most experimental conditions. To monitor and quantify ^3H_2O, 25 mg of activated charcoal (Sigma Chemical Co.) was added to 1 ml of a 0.22 μm filtered sample, incubated for 5 min, centrifuged and the radioactivity of the supernatant measured. All adenine derivatives are removed from solution by the charcoal adsorption procedure. We verified that the radioactivity remaining in solution was ^3H_2O by its loss to the atmosphere upon evaporation. The technique just described is applicable to freshwater as well as marine samples.

The removal of ^{3}H-adenine from solution was also monitored in several experiments. A 3 ml portion of the filtrate was evaporated to dryness and re-constituted in 200 µl of H_2O. The concentrated solution was mixed 1:1 with 10^{-4} *M* adenine and spotted onto a thin-layer chromatographic plate. ^{3}H-adenine was separated from the remainder of the radiolabeled metabolites by either polyethyleneimine (PEI)-impregnated (Brinkman Instruments, Inc., Westbury, New York) or standard cellulose plates, using 0.85 *M* phosphate (pH 3.4) and 1.0 *M* ammonium acetate (pH 7.0) as the respective developing buffers. The procedure described was not applicable to seawater samples due to the accumulation of salts during the evaporation step. It is possible to separate adenine from the dissolved salts using an activated charcoal adsorption-elution procedure (Hodson *et al.*, 1976), but due to problems arising from incomplete recovery of adenine from charcoal and additional difficulties related to deamination of adenine (Ireland and Mills, 1966), we did not pursue these measurements with seawater samples.

Isolation of ATP from other intracellular adenine derivatives was done according to Karl (1979a), with only the following minor modification. For most environmental extracts, the amount of unreacted precursor (^{3}H-adenine) carried over with the filter (50 to 100 µl total volume) greatly exceeds the total amount of radioactivity in the ATP pool. A portion of the ^{3}H-adenine (2 to 5%) co-migrates with ATP under our experimental conditions, resulting in an overestimation of ^{3}H-ATP. Although proper controls will, in theory, correct for this analytical interference, we found that variations in the concentration of contaminating adenine made this correction unreliable. Fortunately, ^{3}H-adenine and ^{3}H-ATP can be separated on the basis of their differential elution characteristics from PEI-impregnated cellulose plates (Swedes *et al.*, 1979). After 10 µl of a mixture containing equal volumes of concentrated nucleotide extract (Karl, 1979a) and 5×10^{-4} *M* non-radioactive ATP was spotted onto a PEI plate, the plate was air-dried before undergoing two consecutive 15-min washes in volumes of 1 liter distilled water. Subsequent treatment of the TLC plates has been previously described by Karl (1979a). The distilled water washes eliminated > 98% of the ^{3}H-adenine from the sample and did not significantly affect the ATP concentrations (recovery 96 to 103%) as evaluated by binding and elution characteristics of radiolabeled adenine and unlabeled ATP standards.

In order to calculate the specific radioactivity of the ATP pool, total ATP was measured in the concentrated nucleotide extracts. ATP concentrations were measured using the firefly bioluminescence assay described by Karl and Holm-Hansen (1978). ATP standards must be prepared in the same ionic composition as the concentrated samples, since any errors in the measurement of ATP will affect the specific activity calculation and, therefore, the rate of RNA synthesis. The specific radioactivity (ratio of ^{3}H-ATP to total ATP concentration) was calculated from the chromatographic purification of radioactive ATP (nCi) and the bioluminescence assay of total ATP (pmol), and was expressed as nCi pmol^{-1} ATP.

Total radioactivity incorporated into RNA was taken as that fraction of the cold TCA-insoluble material which remained soluble following acidification of a NaOH hydrolysate (Munro and Fleck, 1966). Following the minimum 1 h extraction period in 5% TCA (4 °C), the filters were homogenized and the acid-insoluble materials collected by centrifugation (1 500 *g*, 10 min) were washed successively with 3 volumes of 5% TCA (8 ml each) and 2 volumes of 95% ethanol (EtOH). This washing procedure removed > 98% of the contaminating ^{3}H-adenine and radiolabeled acid-soluble metabolites from the sample. No loss of either ^{3}H-RNA or ^{3}H-DNA was detected during the washing procedure. Following removal of the final supernatant, the residual EtOH contained within the pellet (150 to 300 µl) was removed by immersing the samples into a boiling water bath. The entire evaporation step required less than 2 to 3 min. Removal of the excess liquid was necessary for the accurate assessment of reaction volumes. Exactly 2 ml of 1 *N* NaOH was added to each dried sample and the tubes were placed into a 37 °C water bath for a minimum of 1 h. The kinetics of radiolabeled nucleic acid (RNA and DNA) solubilization were measured experimentally using representative environmental samples. Following hydrolysis, the samples were transferred to an ice bath for 15 min before acidification to a pH < 1 with a mixture of HCl/TCA, to yield a final TCA concentration of 5% in the hydrolysis mixture. The acidified samples were allowed to stand for 10 to 15 min prior to centrifugation. The supernatants were radioassayed for ^{3}H-RNA. In selected experiments the pellets were rinsed twice with 5% TCA, and twice with 95% EtOH prior to a second evaporation step. Finally, 2 ml of 5% TCA was added to the dried pellets and the samples placed into a boiling water bath for 30 min. After cooling, the tubes were centrifuged and the supernatants radioassayed for ^{3}H-DNA.

All samples were radioassayed using standard liquid scintillation counting (LSC) procedures (Searle Delta 300) with Aquasol® (New England Nuclear) as the LSC cocktail. Quench corrections utilized either channels ratio or external standards, depending upon the nature of the sample and the total counts/min. We encountered a serious discrepancy between the counting efficiencies of nucleic acids and their nucleotide precursors. Table 2 summarizes the effects of incorporation of ^{3}H-adenine into macromolecules on the efficiency of LSC counting. As ^{3}H-adenine is assimilated into ^{3}H-nucleic acids, the total radioactivity per unit volume of the *Serratia marinorubra* culture appears to decrease; however, when the individual components are isolated, hydrolyzed and summed, total radioactivity is conservative, as expected. The apparent discrepancy is the result of self-adsorption of the low energy β emission of the ^{3}H atoms by the various cellular macromolecules, and is neither detected, nor corrected for, by measurements of quench. If subsamples of the culture are hydrolyzed for 1 h in 1 *N*

Table 2. *Serratia marinorubra*. Effects of ^{3}H-adenine incorporation into macromolecules on liquid scintillation counting efficiency, using batch cultures

Time sampled	Total dis/min in 1 ml of sample	% of initial dis/min	Sum of dis/min in 1 ml cell-free medium+ATP+RNA+DNA	% of initial dis/min
0	7.50×10^6	100	7.50×10^6	100
15 s	7.46×10^6	99.5	7.60×10^6	101
30 s	7.52×10^6	100	7.46×10^6	99.5
1 min	7.43×10^6	99.1	7.39×10^6	98.5
2 min	7.27×10^6	97	7.40×10^6	98.7
5 min	6.89×10^6	91.9	7.39×10^6	98.5
10 min	6.51×10^6	86.8	7.67×10^6	102
30 min	5.68×10^6	75.7	7.35×10^6	98
60 min	5.38×10^6	71.7	7.51×10^6	100

NaOH at 37°C prior to LSC-counting, 100% of the radioactivity is recovered at all time periods. Our results are consistent with previously observed counting discrepancies (Meyer and Keller, 1972; Karl, 1979a), and serve to emphasize the importance of maintaining a total inventory of the radioactivity during a particular experiment.

From the estimates of the total radioactive RNA produced (nCi RNA) and the specific activity of the precursor ATP pool (nCi pmol^{-1} ATP), the rate of stable RNA synthesis was calculated as pmol adenine incorporated into stable RNA l^{-1} h^{-1}. For selected experiments, the rates of RNA synthesis were normalized to total ATP or A_T [total adenylate concentration (ATP + ADP + AMP = A_T)] as measures of biomass (i.e., pmol adenine incorporated into RNA h^{-1} biomass^{-1}) in order to compare specific rates of RNA synthesis among different microbial communities.

Pulse-Chase Experiment

A pulse-chase experiment measured the relative rates of labeling and metabolic turnover of the cellular ATP and RNA pools under environmental conditions. A water sample from Krauss Pond was incubated for 20 min with ^{3}H-adenine (0.3 μCi ml^{-1}), after which time the sample was divided into two equal portions and non-radioactive adenine (10^{-4} *M*) was added to one. The samples were continuously monitored for ATP specific activity, ^{3}H-RNA and ^3H_2O.

Results and Discussion

Uptake and Assimilation of Adenine by Marine Microorganisms

Our cursory screening of marine bacteria for the ability to transport and assimilate exogenous ^{3}H-adenine into cellular macromolecules indicated that 49 out of 50 morphologically distinct, randomly selected marine bacterial isolates examined possess this capability. Our results may be biased, since the spread-plate method used for our primary isolation undoubtedly resulted in unknown and variable selection pressures. We cannot be certain that we have evaluated a truly representative portion of the total marine heterotrophic population. However, our results do indicate that the ability to assimilate exogenous adenine appears to be a common trait among the bacteria which can be isolated from the neritic and oceanic portions of the Pacific Ocean.

Semi-quantitative microautoradiography corroborated the enrichment-culture data. The proportion of the total heterotrophic microbial population assimilating exogenous ^{3}H-adenine in various freshwater and marine environments was always equal to, or greater than, the proportion of cells assimilating a ^{3}H-amino acid mixture. Although we never observed 100% labeling, we have no way to insure that all cells enumerated by fluorescence microscopy were alive. Data presented below (based on mean community ATP pool-specific radioactivity measurements) support the conclusion that most living microorganisms assimilate exogenous adenine under *in situ* conditions. The almost universal ability of bacteria and unicellular algae to transport adenine is consistent with the proposed ecological role and metabolic advantages of a functional purine salvage pathway (Karl, 1979a). Furthermore, the kinetics of uptake and assimilation of ^{3}H-adenine into both ^{3}H-ATP and ^{3}H-RNA indicate that the microbial populations most likely utilize these salvage pathways under *in situ* environmental conditions.

Effects of ^{3}H-Adenine Addition on Total Intracellular ATP Concentrations

An experiment was conducted to evaluate the effects of ^{3}H-adenine additions on the community ATP levels of natural microbial assemblages. A water sample from Krauss Pond was incubated with ^{3}H-adenine (0.1 μCi ml^{-1}) for 2 h, during which time the ATP levels of the sample were periodically monitored. Concurrently, the total amount of radioactivity incorporated into cellular RNA was measured. After 2 h, the sample was divided

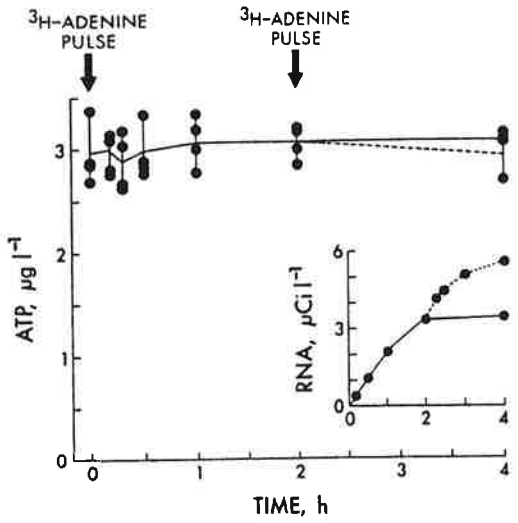


Fig. 1. Effect of ^{3}H-adenine addition on total intracellular ATP concentrations of a Krauss Pond sample (eutrophic freshwater pond, Hawaii) incubated for 4 h. Inset shows rate of ^{3}H-adenine incorporation into RNA with time, for the same sample. A pulse of ^{3}H-adenine equivalent to 0.1 µCi ml^{-1} was added at zero time and a second pulse was added to one-half of the sample after 2 h, as indicated by dashed lines

into two equal portions and a second pulse of ^{3}H-adenine was added to one sub-sample. Following a second 2 h incubation, the total ATP in each sample was measured. The results are presented in Fig. 1.

Between 0 and 2 h there was no significant change in the total community ATP pool despite a linear increase in ^{3}H-RNA. The second pulse of ^{3}H-adenine at 2 h likewise had no effect on cellular ATP levels. These data indicate that exogenous ^{3}H-adenine enters the cells, is processed through the intracellular ATP pools and is deposited, in part, as ^{3}H-RNA without any detectable effects on the total community ATP concentrations. Therefore, it appears unlikely that adenine is assimilated at rates exceeding the biosynthetic requirement for nucleic acid production. Even when added in excess of the maximum assimilative capacity (i.e., at saturating levels) there is no detectable luxury uptake of adenine or expansion of ATP pools (see pulse-chase experimental data). Identical results were observed with neritic and oceanic seawater samples, as well as batch and chemostat cultures of *Serratia marinorubra*. Apparently the pathways of adenine transport and assimilation are regulated in such a way that the flux of adenine into microbial cells (as AMP) is balanced by the removal of adenine (as ATP) during the synthesis of macromolecules.

Effects of Vacuum Filtration

It has recently been demonstrated that vacuum filtration may have an effect on total ATP concentration and on adenylate energy charge ratios of certain environmental samples (Karl and Holm-Hansen, 1978; Karl, 1980). An experiment was conducted to evaluate possible effects of vacuum filtration stress on the determination of the specific activity of the ATP pool and estimated rates of RNA synthesis of natural communities of microorganisms. A sample from Krauss Pond was incubated with ^{3}H-adenine and was extracted either by direct injection into boiling phosphate buffer or by concentration onto glass-fiber filters prior to extraction. This experiment required the use of a eutrophic freshwater ecosystem and was conducted in full awareness of the potential artifacts of direct injection techniques (Karl, 1979b). The results are presented in Table 3.

Consistent with previous reports (Sutcliffe *et al.*, 1976; Jones and Simon, 1977; Karl and Holm-Hansen, 1978; Riemann, 1978; Karl, 1980), vacuum filtration resulted in a decrease of total ATP, when normalized to a standard volume. We found that the effect increased

Table 3. Effects of filtration on ATP, specific activity of ATP pool, and rates of RNA synthesis for a eutrophic freshwater sample collected from Krauss Pond

Sample treatment and volume extracted (ml)	ATP (pmol ml^{-1})	Specific activity (nCi pmol ATP)	Rate of RNA synthesis (pmol Ade RNA l^{-1} min^{-1})
Direct injection			
0.5	27.8	0.43	606
1.0	24.3	0.44	600
2.0	25.5	0.53	498
	—	—	—
X(±SD)	25.9(±1.78)	0.47(±0.055)	568(±61)
Vacuum filtration			
0.5	20.1	0.51	513
1.0	19.7	0.53	499
2.0	20.7	0.46	568
5.0	18.5	0.48	552
10.0	17.5	0.45	581
	—	—	—
X(±SD)	19.3(±1.29)	0.49(±0.034)	543(±35)

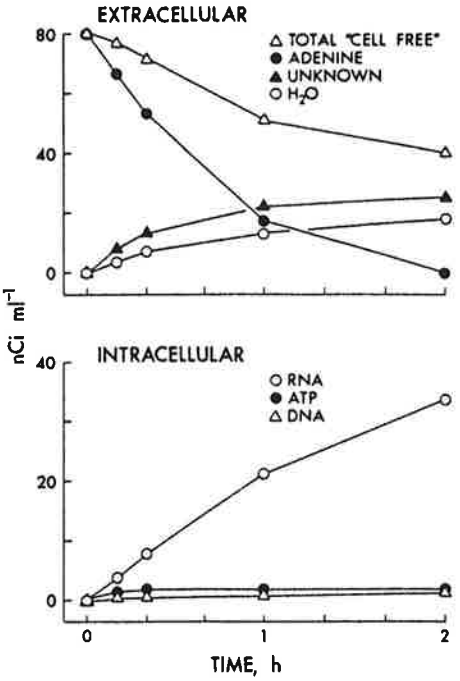


Fig. 2. Radioactivity measured in various extracellular and intracellular fractions with time, of a Krauss Pond water sample. Summation of radioactivity of all fractions shows favorable mass balance with time-zero radioactivity (see text for further discussion)

with increasing volume filtered, to an extreme of 65% recovery with 10 ml of pond water. By comparison, neither the specific activity of the ATP pool nor the calculated rate of RNA synthesis were affected, indicating that the proportion of radiolabeled ATP to total ATP is independent of filtration effects. Since the determination of the rate of RNA synthesis is also independent of the total ATP concentration recovered (it is affected only by the specific radioactivity), it is likewise unaffected by the filtration effect. However, the filtration problem will affect calculated ATP (biomass) specific rates of RNA synthesis (Karl, 1979a). Since it has previously been demonstrated that A_T (i.e., [ATP] + [ADP] + [AMP]) is conservative even though the concentration of ATP decreases as a function of volume filtered (Karl and Holm-Hansen, 1978), it may be advisable to normalize rate measurements to A_T rather than ATP when comparing the relative growth rates of various microbial assemblages.

Mass Balance Inventories of Radioactivity During ^{3}H-Adenine Uptake and Incorporation

To understand the pathways involved in the assimilation of exogenous ^{3}H-adenine, and to ensure complete recoveries of all radiolabeled metabolites, a time-series experiment was conducted to establish a radioactivity mass balance inventory. A water sample from Krauss Pond was incubated with 80 nCi of ^{3}H-adenine ml^{-1} and sub-samples were removed following pre-determined incubations (10 min to 2 h) and analyzed for various extracellular and intracellular compounds. The results are presented in Fig. 2.

Radiolabeled adenine is rapidly taken up by natural microbial communities without any detectable lag, and is converted into a variety of extracellular and intracellular metabolites. A portion of the assimilated ^{3}H-label is released back into the environment as ^3H_2O and as several, yet unidentified, organic compounds (Fig. 2). The ^3H_2O production is due, in part, to the intracellular conversion of adenine nucleotides to guanine nucleotides (label at Position 2 of adenine is lost as ^3H_2O) and, in part, to the relatively slow but measurable turnover of cellular RNA (see below: "Pulse-Chase Experiment"). Other possible sources of ^3H_2O include the catabolism of adenine (via xanthine), biosynthesis of histidine, cell death and lysis, and release of labeled intermediates. Although the ^{3}H at Position 2 of the purine ring of adenine is not readily exchanged in aqueous solutions, we cannot rule out the possibility of enzyme-catalyzed exchange with intracellular H_2O. For example, the contribution of the Position 2-3 portion of the purine ring of ATP for *de novo* synthesis of histidine is expected to yield radiolabeled protein. However, no significant labeling was detected, due perhaps to the enzymatic mobilization of ^{3}H at Position 2 during biosynthesis.

We should emphasize that ^3H_2O was produced in all environmental samples as well as in carbon-limited *Serratia marinorubra* chemostat cultures grown on C-P medium (Carlucci and Pramer, 1957), but was notably absent from carbon-limited *S. marinorubra* cultures grown on glucose-peptone-NH_4Cl. When ^3H_2O was detected, the rate of production was always proportional to the removal of ^{3}H-adenine from solution. With extended incubation periods (up to 72 h), $>$ 90% of the radioactivity in the extracellular environment appeared as ^3H_2O, probably due to metabolic turnover of cellular RNA (see below: "Pulse-Chase Experiment").

The labeled non-adenine organics (i.e., unknown compounds, Fig. 2) could be separated from the ^{3}H-adenine by PEI cellulose thin-layer chromatography. All of the radioactivity in the unknown fraction was removed from solution by the addition of activated charcoal. If isolated, concentrated, and added back into samples of natural microbial populations or *Serratia marinorubra* cultures, the labeled organics were readily assimilated into ^{3}H-RNA, ^{3}H-DNA and ^3H_2O. We believe that these compounds are intermediates in adenine dissimilation (i.e., hypoxanthine or inosine derivatives) or perhaps even adenine nucleotides (ATP, ADP, AMP) resulting from the steady-state processes of RNA turnover, excretion, cell death and lysis.

During the transport of ^{3}H-adenine into cells, the adenine nucleotides become rapidly labeled, with the majority of the activity ($>$85%) residing in the ATP pool. After the intracellular precursor pools are labeled to constant specific activities, there is a net transfer of

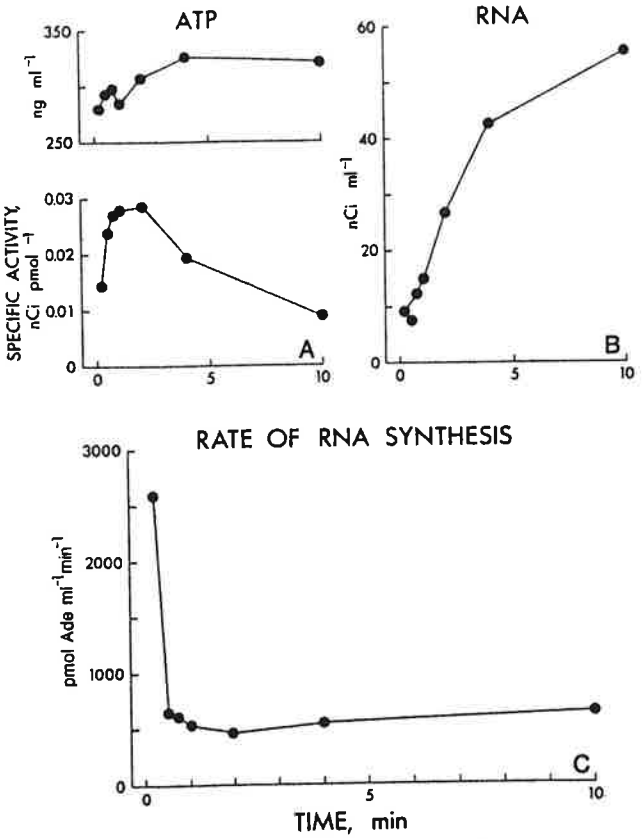


Fig. 3. *Serratia marinorubra*. Kinetics of ^{3}H-ATP, ^{3}H-RNA production and rate of RNA synthesis for a batch culture. (A) Specific radioactivity of ATP pool and total intracellular ATP concentrations with time; (B) incorporation of ^{3}H-adenine into RNA with time; (C) rate of RNA synthesis with time

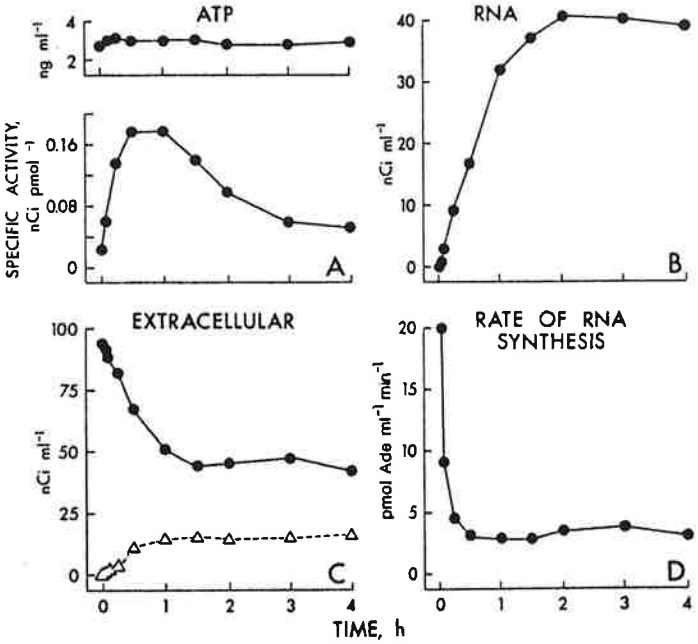


Fig. 4. Kinetics of ^{3}H-ATP, ^{3}H-RNA and ^3H_2O production and rate of RNA synthesis for a Krauss Pond freshwater microbial population. (A) Specific activity of ATP pool and total intracellular ATP concentrations during 4 h incubation; (B) rate of labeling of RNA with time; (C) ^{3}H-adenine uptake by cells with time (circles) and ^3H_2O production with time (triangles); (D) rate of RNA synthesis during 4 h incubation

extracellular ^{3}H-adenine into ^{3}H-RNA with an efficiency of 35 to 40%. The initial incorporation of the ^{3}H-label into ^{3}H-ATP and ^{3}H-RNA is linear with time and occurs without any detectable lag. Greater than 95% of the total intracellular inventory at all time periods can be accounted for by the summation of radioactivity in ^{3}H-ATP, ^{3}H-RNA and ^{3}H-DNA, and throughout the course of all experiments the total radioactivity of all fractions measured (intracellular and extracellular) was within 5% of the initial radioactivity (Fig. 2 and Table 2). These data indicate that we have not omitted, overestimated or underestimated any quantitatively significant labeled intermediates or products and also add credibility to our extraction and purification procedures.

Kinetics of ^{3}H-ATP, ^{3}H-RNA, ^3H_2O Production and Rate of RNA Synthesis

Figs. 3 and 4 present the results of time-series experiments of a culture of *Serratia marinorubra*, and a freshwater environmental sample, respectively. ^{3}H-adenine (0.1 μCi ml^{-1}) is completely utilized after a 10 min incubation of a mid-log phase bacterial culture of *S. marinorubra* (5×10^7 cells ml^{-1}, doubling time, t_d = 40 min), compared to 90 min for eutrophic freshwater samples. Subsequent experiments with coastal seawater samples required 10 to 12 h to achieve 90% removal, and for oligotrophic seawaters less than 5% of the exogenous ^{3}H-adenine was assimilated during a 10 h incubation. The rate of ^{3}H-adenine removal, however, is also dependent upon its dilution, since the rate of removal decreases upon the addition of cold adenine or yeast extract (which contains AMP and other adenine derivatives) to bacterial cultures or environmental samples. If time-course experiments are not conducted, it is essential to measure the amount of ^{3}H-adenine remaining in solution to ensure that ^{3}H-adenine was present in the medium for the duration of the incubation.

The data presented serve to emphasize two points:

First, the ATP pools of *Serratia marinorubra* cultures and natural microbial communities are rapidly labeled to a maximum specific activity in 1 to 10 min for *S. marinorubra* (Fig. 3A) and 30 min for Krauss Pond (Fig. 4A); the specific activity decreases after ^{3}H-adenine is exhausted from the extracellular environment. How-

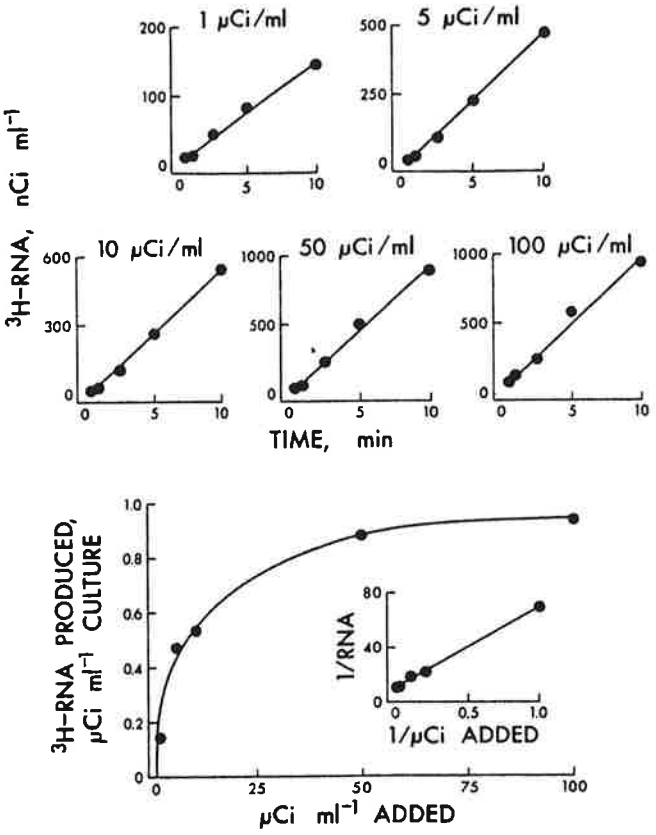


Fig. 5. *Serratia marinorubra*. Effects of ^{3}H-adenine concentrations on the kinetics of labeling of RNA of a mid-log phase culture. Incorporation of ^{3}H-adenine into RNA is linear for all concentrations tested (upper graphs). Lower graph shows that saturation is approached with > 50 μCi ml^{-1} ^{3}H-adenine; (inset is a Lineweaver-Burke plot indicating maximum rate of incorporation at ~ 100 μCi ml^{-1} ^{3}H-adenine

ever, the rate at which radioactivity is removed from the ATP pool is slower than the initial rate of labeling, a consistent feature which will be discussed in more detail below. The maximum specific activity attained is dependent upon the extracellular specific activity of the precursor pool and upon the extent of *de novo* synthesis of adenine, which dilutes the specific activity from within the cell. As discussed previously, the addition of ^{3}H-adenine did not affect the steady-state ATP pools of either *S. marinorubra* or natural assemblages of microorganisms, even though adenine was processed through the cellular adenine nucleotide pools.

The second interesting feature is the apparent existence of separate pools of non-equilibrated intracellular adenine nucleotides. Frequently we observed initial RNA rate measurements that were anomalously high compared to those calculated for longer incubation periods (Figs. 3 and 4). We believe that these elevated values are the result of compartmentalized ATP pools. Our conclusion is that RNA (and DNA) are preferentially synthesized from kinetically compartmentalized ATP pools located near the periphery of the cell. Varrichio (1972) has shown that the specific activity of the membrane-associated RNA fraction in *Escherichia coli* was approximately 3 to 4 times higher than the RNA of the cytoplasm following a 1 min pulse of ^{3}H-uracil. With longer labeling periods, the effect of this compartmentalization became less pronounced. Furthermore, Mueller and Bremer (1968) have indicated that the cytosol may have a viscosity 600 times that of H_2O, so it is not surprising that diffusion can act to separate or impede the mixing of intracellular precursor pools. Of course, our extraction procedures yield mean ATP specific activities, since we have no mechanism for maintaining the integrity of these spatially separated metabolic pools (if they do exist). Additional experiments have been conducted to verify the presence of these compartmentalized pools during the early time periods of pulse-labeled experiments. A comparison of the rates of labeling of the total cellular ATP and AMP pools (i.e., increase in specific activity) has revealed that during the initial time periods the average ATP pool is labeled faster and to a higher specific activity than the AMP pool. Since AMP is the precursor for ATP, this result is impossible, unless separate intracellular nucleotide pools exist. Beyond 0.5 min for *Serratia marinorubra* cultures and 20 min for Krauss Pond, the calculated specific activities approach the real specific activities as isotopic equilibrium is achieved among the kinetically compartmentalized pools. The effects of ATP pool compartmentalization, both kinetic and barrier (membrane), on the calculated rates of RNA synthesis may be expected to vary due to the ratio of prokaryotes to eukaryotes in each sample. In this regard, time-series analyses are essential.

Effects of ^{3}H-Adenine Concentration on Kinetics of Labeling and Calculation of RNA Synthesis

Several experiments evaluated the effects of adenine concentration on the patterns of labeling cellular metabolites and macromolecules. In the first experiment, the relative rates of labeling of the ATP and RNA pools were monitored following the addition of increasing concentrations of radiolabeled adenine (1.0 to 100 μCi ml^{-1}) to a mid-log-phase *Serratia marinorubra* culture (Fig. 5). The kinetics of labeling were similar over the entire concentration range (64.5 to 6 450 pmol ml^{-1}). As expected, the rate of incorporation of exogenous ^{3}H-adenine into both ^{3}H-ATP and ^{3}H-RNA (i.e., nCi min^{-1}) increased in direct proportion to the amount of ^{3}H-adenine added to the medium. Consequently, the calculated rates of RNA synthesis were not significantly different despite the large differences in intracellular precursor and product radioactivities. Similar results were obtained with natural microbial communities. Failure to measure and correct for intracellular precursor specific activity will lead to serious quantitative errors in attempts to measure RNA or DNA synthesis merely from the uptake and incorporation of radiolabeled precursors. Previous investigators have assumed that the specific radioactivities of the extracellular and intra-

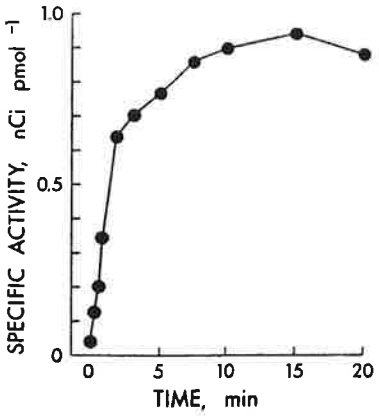


Fig. 6. *Serratia marinorubra*. Kinetics of ATP pool labeling under saturating concentrations of ^{3}H-adenine (100 μCi ml^{-1}) for a mid-log phase culture

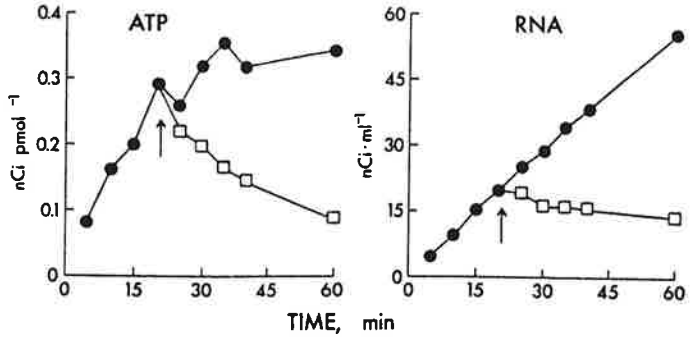
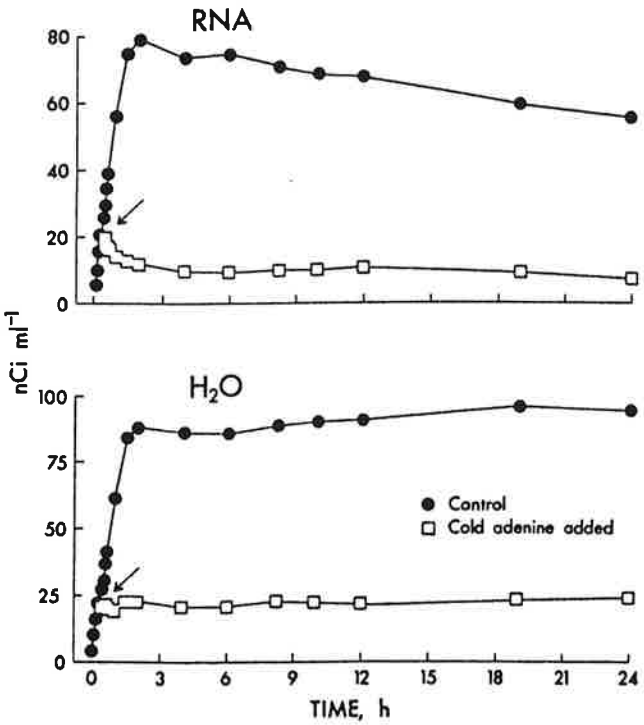


Fig. 7. Kinetics of labeling of ATP pool and RNA after an initial pulse of 0.3 μCi ml^{-1} ^{3}H-adenine for a Krauss Pond freshwater population. Arrow indicates point at which 10^{-4} *M* non-radioactive adenine was added to one subsample to chase-out ^{3}H-label



cellular precursors were identical under all experimental conditions. However, we have demonstrated that the specific activity of the immediate precursor varies as a function of the concentration of added ^{3}H-adenine.

A second set of experiments was conducted to examine the patterns of ATP pool labeling under saturating concentrations of ^{3}H-adenine (100 μCi ml^{-1}); the results are presented in Fig. 6. The specific activity did not increase above 1 nCi pmol^{-1}, despite the fact that the specific radioactivity of the saturating precursor was 15.5 nCi pmol^{-1}. Therefore, even when ^{3}H-adenine is present at saturating levels in this bacterial culture, *de novo* synthesis and/or internal recycling of nucleic acid bases (and therefore dilution of specific activity of the transported ^{3}H-adenine molecules) must represent an important source (>75%) of adenine for the cells (Fig. 6). These data again emphasize the crucial necessity for independent measurements of precursor (ATP pool) specific activity if accurate rates of RNA synthesis are to be obtained.

Pulse-Chase Experiment

Upon the addition of cold adenine to the sample, incorporation of ^{3}H-adenine into ^{3}H-ATP, ^{3}H-RNA and ^3H_2O effectively ceased (Figs. 7 and 8). This confirms one of the original assumptions of our technique regarding the absence of luxury adenine uptake (and intracellular storage) by microbial cells. If luxury uptake had occurred, the intracellular radioactivity in the ATP pool would be chased out at an accelerated rate with the addition of 10^{-4} *M* adenine. It is apparent, however, that the ^{3}H-label initially (0.25 min after addition) leaves the ATP pool at a rate that is identical to the rate at which the pool became labeled after the original pulse ($\sim 10^{-9}$ *M*) of ^{3}H-adenine. This indicates a constant turnover of the ATP pool during this period of the experiment, despite an extremely large addition of adenine to the medium. Our results again indicate that the uptake of adenine into microbial cells is limited by the net removal of nucleic acid precursors from the intracellular pools and that the free exchange of adenine across the membrane is probably negligible. Beyond 25 to 30 min, ^{3}H-ATP was diluted from the intracellular pool at a decelerated rate. Our data suggest that degradation products of stable ^{3}H-RNA (i.e., ^{3}H-AMP) re-enter the adenine nucleotide pools and eventually establish isotopic equilibrium with the precursor ATP pool. This hypothesis is supported by the gradual decrease in total ^{3}H-RNA which occurs following the addition of cold adenine, and is consistent with published models of nucleic acid metabolism (Hurlbert and Hardy, 1972; Rutherford *et al.*, 1974).

◀ **Fig. 8.** Long-term kinetics of ^{3}H-RNA and ^3H_2O production after an initial pulse of 0.3 μCi ml^{-1} ^{3}H-adenine for Krauss Pond freshwater sample. Effects of non-radioactive adenine addition (10^{-4} *M*, as indicated by arrow) on ^{3}H-RNA and ^3H_2O production are also shown

Our results from the pulse-chase experiment indicate that there is a small, albeit measurable, turnover of "stable" RNA in natural assemblages of microorganisms. During extended periods of incubation (24 h) the ^{3}H-RNA decreased at a rate of approximately 3.9% h^{-1} (Fig. 8). A portion of the radioactivity derived from the breakdown of RNA is recycled through the adenine nucleotide pools and re-incorporated into new RNA, as verified by measuring the production of these two classes of radiolabeled compounds after the ^{3}H-RNA of a population from Krauss Pond had been labeled, washed and re-suspended in freshly filtered sterilized pond water (data not presented). The activity of purine salvage pathways accounts for the slow removal of radioactivity from the ATP pool (Fig. 7), and for the constant rates of RNA synthesis measured following exhaustion of ^{3}H-adenine from the extracellular environment (Fig. 4).

The observed turnover of nascent rRNA in natural microbial communities may be related to growth rate, community turnover, or may also be indicative of transitions from periods of growth to dormancy. It is well documented that turnover of so-called stable RNA does occur under laboratory conditions of balanced growth, but only at very slow growth rates (Norris and Koch, 1972; Pedersen, 1976; Gausing, 1977). The degradation of rRNA during starvation conditions is also well known (Dawes and Ribbons, 1965) but could have occurred only if actively growing cells assimilated the precursor initially and then entered a period dominated by endogenous metabolism (starvation) during the time course of our incubation. Dormant, resting, starved or otherwise debilitated cells incapable of net RNA accumulation, are not assayed by our technique (Karl, 1979a).

In summary, the data presented here strengthen the validity of the ^{3}H-adenine technique for measuring rates of stable RNA synthesis in aquatic environments. Each experiment described evaluated a critical assumption or condition which must be satisfied for the application (and proper interpretation) of data obtained on mixed assemblages of microorganisms (see Table 1). The well-established correlations between RNA synthesis and cell growth are so universally applicable that this method should have numerous applications in experimental microbial ecology.

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Literature Cited

Brock, T. D.: Bacterial growth rate in the sea: direct analysis by thymidine autoradiography. Science, N.Y. *155*, 81–83 (1967)

Carlucci, A. F. and D. Pramer: Factors influencing the plate method for determining abundance of bacteria in seawater. Proc. Soc. exp. Biol. Med. *96*, 392–394 (1957)

Dawes, E. A. and D. W. Ribbons: Studies on the endogenous metabolism of *Escherichia coli*. Biochem. J. *95*, 332–343 (1965)

Evans, E. A., H. C. Sheppard and J. C. Turner: Validity of tritium tracers. Stability of tritium atoms in purines, pyrimidines, nucleosides and nucleotides. J. labelled Compounds Radiopharm. (Wiley, Chichester) *6*, 76–87 (1970)

Fuhrman, J. A. and F. Azam: Bacterioplankton secondary production estimates for coastal waters of British Columbia, Antarctica, and California. Appl. envirl Microbiol. *39*, 1085–1095 (1980)

Gausing, K.: Regulation of ribosome production in *Escherichia coli*: synthesis and stability of ribosomal RNA and of ribosomal protein messenger RNA at different growth rates. J. molec. Biol. *115*, 335–354 (1977)

Hobbie, J. E., R. J. Daley and S. Jasper: Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. envirl Microbiol. *33*, 1225–1228 (1977)

Hodson, R. E., O. Holm-Hansen and F. Azam: Improved methodology for ATP determination in marine environments. Mar. Biol. *34*, 143–149 (1976)

Holm-Hansen, O., W. H. Sutcliffe, Jr. and J. Sharp: Measurement of deoxyribonucleic acid in the ocean and its ecological significance. Limnol. Oceanogr. *13*, 507–514 (1968)

Hurlbert, R. B. and D. Hardy: Turnover of HnRNA and Poly A in rat liver nuclei. Fedn. Proc. Fedn Am. Socs. exp. Biol. (Abstr.) *31*, p.428 (1972)

Ireland, D. M. and D. C. B. Mills: Detection and determination of adenosine diphosphates and related substances in plasma. Biochem. J. *99*, 283–296 (1966)

Jones, J. G. and B. M. Simon: Increased sensitivity in the measurement of ATP in freshwater samples with a comment on the adverse effect of membrane filtration. Freshwat. Biol. *7*, 253–260 (1977)

Karl, D. M.: Measurement of microbial activity and growth in the ocean by rates of stable ribonucleic acid synthesis. Appl. envirl Microbiol. *38*, 850–860 (1979a)

Karl, D. M.: Comments on a paper by J. G. Jones and B. M. Simon, "Increased sensitivity in the measurement of ATP in freshwater samples with a comment on the adverse effect of membrane filtration." Freshwat. Biol. *9*, 281–284 (1979b)

Karl, D. M.: Cellular nucleotide measurements and applications in microbial ecology. Microbiol. Rev. (formerly Bact. Rev.) *44*, 739–796 (1980)

Karl, D. M. and O. Holm-Hansen: Methodology and measurement of adenylate energy charge ratios in environmental samples. Mar. Biol. *48*, 185–197 (1978)

LaRock, P. A., R. D. Lauer, J. R. Schwarz, K. Watanabe and D. A. Wiesenburg: Microbial biomass and activity distribution in an anoxic hypersaline basin. Appl. envirl Microbiol. *37*, 466–470 (1979)

Meyer, R. R. and S. J. Keller: DNA polymerase activity: factors affecting counting efficiency of radioactive DNA on filter paper discs. Analyt. Biochem. *46*, 332–337 (1972)

Meyer-Reil, L.-A.: Autoradiography and epifluoresence microscopy combined for the determination of number and spectrum of actively metabolizing bacteria in natural waters. Appl. envirl Microbiol. *36*, 506–512 (1978)

Mueller, K. and H. Bremer: Rate of synthesis of messenger ribonucleic acid in *Escherichia coli*. J. molec. Biol. *38*, 329–353 (1968)

Munro, H. N. and A. Fleck: The determination of nucleic acids. *In*: Methods of biochemical analysis, Vol. 14. pp 113–176. Ed. by D. Glick. New York, N.Y.: Interscience Publishers 1966

Norris, T. E. and A. L. Koch: Effect of growth rate on the relative rates of synthesis of messenger, ribosomal and transfer RNA in *Escherichia coli*. J. molec. Biol. *64*, 633–649 (1972)

Pedersen, S.: Stability of nascent ribosomal RNA in *Escherichia coli*. *In*: Control of ribosome synthesis, pp 345–355. Ed. by N. Kjeldgaard and O. Maaløe. New York, N.Y.: Academic Press 1976

Riemann, B.: Differentiation between heterotrophic and photosynthetic plankton by size fractionation, glucose uptake, ATP and chlorophyll content. Oikos *31*, 358–367 (1978)

Rutherford, C. L., W. Y. Kong, D. Park and B. E. Wright: Precursor-product relationships between nucleotides and RNA during differentiation in *Dictyostelium discoideum*. J. gen. Microbiol. *84*, 173–187 (1974)

Straat, P. A., R. Wolochow, L. Dimmick and M. A. Chatiguy: Evidence for incorporation of thymidine into deoxyribonucleic acid in airborne bacterial cells. Appl. envirl Microbiol. *34*, 292–296 (1977)

Sutcliffe, W. H. Jr., E. A. Orr and O. Holm-Hansen: Difficulties with ATP measurements in inshore waters. Limnol. Oceanogr. *21*, 145–149 (1976)

Swedes, J. S., M. E. Dial and C. S. McLaughlin: Regulation of protein synthesis during energy limitation of *Saccharomyces cerevisiae*. J. Bact. *138*, 162–170 (1979)

Thomas, D. R., J. A. Richardson and R. J. Dicker: The incorporation of tritiated thymidine into DNA as a measure of the activity of soil micro-organisms. Soil Biol. Biochem. *6*, 293–296 (1974)

Tobin, R. S. and D. H. J. Anthony: Tritiated thymidine incorporation as a measure of microbial activity in lake sediments. Limnol. Oceanogr. *23*, 161–165 (1978)

Varrichio, R.: "Compartmentalization" of *Escherichia coli* ribosomes and ribonucleic acid. J. Bact. *109*, 1284–1294 (1972)

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