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Selected Nucleic Acid Precursors in Studies of Aquatic Microbial Ecology

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The use of radiolabeled nucleosides and nucleic acid bases to estimate the rates of RNA and DNA synthesis in naturally occurring microbial assemblages requires numerous assumptions, several of which are evaluated herein. Comparative time series analyses of the uptake and incorporation, labeling specificity, and extent of catabolism of [2-^{3}H]adenine, [*methyl*-^{3}H]thymidine, and [5-^{3}H]uridine were performed with pure bacterial and algal cultures, as well as with environmental samples. [^{3}H]thymidine yielded the most variable results, especially with regard to the extent of nonspecific macromolecular labeling. The pathways of [^{3}H]thymidine and [^{3}H]adenine metabolism were further evaluated by isotope dilution methods and by comparing incorporation patterns of thymidine labeled at different sites of the molecule. The advantages, uncertainties, and limitations of the use of radiolabeled nucleic acid precursors in studies of aquatic microbial ecology are discussed and a prospectus for future studies presented.

The uptake and incorporation of radiolabeled nucleosides and nucleic acid bases provide a meaningful experimental approach for the study of the metabolism and in situ growth kinetics of microorganisms in nature. The various methods described rely upon the incorporation of either thymidine (1, 3, 8, 9, 21, 29, 31–33), adenine (12–15), or uridine (1, 18) as presumed measures of polynucleotide (DNA and RNA) synthesis. Although there is continuing debate on this issue, it can be stated without reservation that the mere incorporation of nucleic acid precursors into cold acid-insoluble cellular materials cannot be accepted as a measure of the in situ rates of RNA or DNA synthesis without a careful and comprehensive consideration of: (i) the community potential for assimilating the exogenous precursors, (ii) the existence of intracellular and extracellular pools of structurally related compounds which dilute the specific radioactivity of the incorporated molecules, (iii) a detailed analysis of the pathways and regulation of precursor metabolism including the balance between de novo synthesis and salvage pathways (i.e., utilization of exogenous pools), and (iv) the specificity and extent of macromolecular labeling. If microbial ecologists intend to use sophisticated physiological or biochemical approaches for studies of microorganisms in natural environments, the methods must be applied with the same rigor as they would be in their parent disciplines lest they lose the significance of their intended use.

At present there are at least three separate approaches available for quantitative measurements of RNA and DNA synthesis. However, this field is currently in a period of rapid expansion and I am aware of several additional methods which are also being considered. Consequently, I consider it a valuable exercise to critically evaluate the data which are currently available so as to better focus our future research efforts. Karl and co-workers (12–15) have discussed the theoretical principles and have systematically evaluated the validity of the numerous assumptions inherent in the use of [^{3}H]adenine for estimating simultaneous rates of microbial RNA and DNA synthesis. As currently employed, this method can be used to measure total microbial (bacterial and unicellular algal) production. However, if used in carefully selected environments (e.g., anoxic sediments, deep ocean, etc.) or in conjunction with size fractionation procedures (2), the [^{3}H]adenine approach may be used to assess predominantly bacterial rates of growth (RNA) and cell division (DNA). There are still, however, several assumptions that need to be carefully evaluated with cultures and field samples. Fuhrman and Azam (8, 9) have proposed the use of [^{3}H]thymidine to measure specifically bacterial DNA synthesis in mixed microbial plankton assemblages. Bacterial production estimates in their procedure are determined from direct measurements of the incorporation of [^{3}H]thymidine followed by an extrapolation based upon several, as yet,

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untested assumptions. Two important considerations regarding this approach are the inability to evaluate, and correct for, the variable dilution of the introduced [^{3}H]thymidine by the combined effects of existing substrate pools and de novo synthesis and the validity of the assumption regarding the specificity of labeling exclusively bacterial DNA. More recently, Moriarty and Pollard (21) have proposed the use of an isotope dilution technique for estimating the dilution of the introduced [^{3}H]thymidine before its incorporation into DNA. Although this method was originally applied to sediments, there is no a priori reason why it could not be used for water samples or with other precursors as well (i.e., adenine). Despite this substantial improvement in the [^{3}H]thymidine method, extrapolation to bacterial production is still dependent upon several untested assumptions, one of which is the specificity of labeling only bacterial DNA.

It is clear from the lively debate and numerous discussions of the shortcomings of each method that there is no single approach that is universally accepted or acceptable. Intercalibrations are difficult since each method purports to measure a slightly different aspect of microbial growth or cell division; hence, there is no basis for expecting that the independent methods might yield comparable ecological information. Furthermore, since there is no independent or absolute standard for microbial production in nature, it is virtually impossible to assess the reliability of production estimates derived from each individual experimental approach. Nevertheless, the well-established correlations between rates of nucleic acid syntheses and cell growth and division in microorganisms (reviewed in 17, 19, 23) have provided the physiological data base and impetus for conducting studies of nucleic acid precursor metabolism among naturally occurring microbial assemblages. This present report provides experimental data and discussion of several important theoretical assumptions which are implicit in the application of nucleic acid precursors as tools in experimental microbial ecology. More specifically, the criteria which should be used for the selection of the most appropriate precursor are evaluated and discussed.

MATERIALS AND METHODS

Sample collection, medium composition, and conditions of incubation. Bacterial cultures and water samples from several marine and freshwater ecosystems were used in this study. The marine bacterium, *Serratia marinorubra* (ZoBell and Feltham), was grown in batch culture on C-P medium (4) consisting of 1.5 g of peptone, 50 mg of yeast extract, and 25 mg of $FePO_4$ per liter of 80% seawater. The cultures were maintained at 24 ± 1°C on a gyrotatory shaker table (125 to 150 rpm). All experiments were performed with cells in the early to mid-log phase of growth (optical density at 520 nm < 0.1). Freshwater samples were collected from Krauss Pond on the University of Hawaii at Manoa campus. Seawater samples were collected from several representative coastal and oceanic stations. Surface seawater from Ala Moana Beach, Oahu, Hawaii was collected manually in an acid-washed polyethylene bottle at a station approximately 0.5 km offshore. The water was transported to the laboratory (approximately 15 min) and filtered (90-μm mesh Nitex) before use. Oceanic samples were collected at two stations, one located in the eastern North Pacific Ocean (35° 47.8'N; 123° 39.8'W) and the second approximately 100 km south of Hawaii (18° 43.8'N; 156° 50.4'W) during research cruises aboard the R/V *Cayuse* (August to September 1980) and R/V *Thomas G. Thompson* (February 1981), respectively. The water samples were collected in ethanol (70%)-rinsed Niskin bottles and were filtered (90-μm mesh Nitex) before use. The samples were incubated either on deck in temperature-controlled incubators (surface samples) or in situ (all others), depending upon the nature of the experiment. The concentrations of [2-^{3}H]adenine (15 Ci mmol^{-1}), [2-^{14}C]thymidine (55 mCi mmol^{-1}), [*methyl*-^{3}H]thymidine (80.1 Ci mmol^{-1})or [5-^{3}H]uridine (26.7 Ci mmol^{-1}) varied from 0.1 to 100 μCi ml^{-1} depending upon the specific experiment. All were from New England Nuclear Corp., Boston, Mass.

Sample analyses. (i) Acid-insoluble materials. Sample incubations were terminated by the addition of an equal volume of cold (4°C) 10% (wt/vol) trichloroacetic acid (TCA) to the bacterial cultures or by filtration onto Whatman GF/F filters and extraction in 5 ml of cold 5% TCA (freshwater and marine samples). One milligram each of nonradioactive RNA, DNA, and protein (as bovine serum albumin) was added to catalyze the precipitation and aid in the quantitative separations and recoveries of the individual "operationally defined" classes of ^{3}H-labeled macromolecules. Comparative studies conducted with and without the supplemental materials indicated that the additions yielded significantly greater recoveries (up to 38%) of [^{3}H]RNA, [^{3}H]DNA, and ^{3}H-labeled protein. All samples were allowed to extract for at least 1 h at 4°C.

For the purposes of this study, I have operationally defined four separate classes of cold acid-insoluble macromolecules: RNA, DNA, RNA + DNA, and protein. Schematic representations of two independent separation procedures and their resultant chemical fractions are presented in Fig. 1. For RNA, DNA, and protein separations (Fig. 1, scheme A) the samples were washed three times with 5% TCA and twice with 95% ethanol (all at 4°C) before evaporation in vacuo. The insoluble materials were hydrolyzed in 1 N NaOH for 1 h at 37°C by using the modified Schmidt-Thannhauser procedure (14, 22). Quantitative separation of RNA and DNA is based upon differential susceptibility to alkaline hydrolysis, DNA remaining in a form precipitable upon acidification. The pellet containing DNA and protein was washed twice with 5% TCA, twice with 95% ethanol (all at 4°C), evaporated to dryness in vacuo, and extracted in hot 5% TCA (100°C, 30 min). This step hydrolyzes the DNA, leaving the protein insoluble. The pellet was again washed and evaporated to dryness before a final extraction in 1 NaOH at 37°C for 18 h to solubilize the protein.

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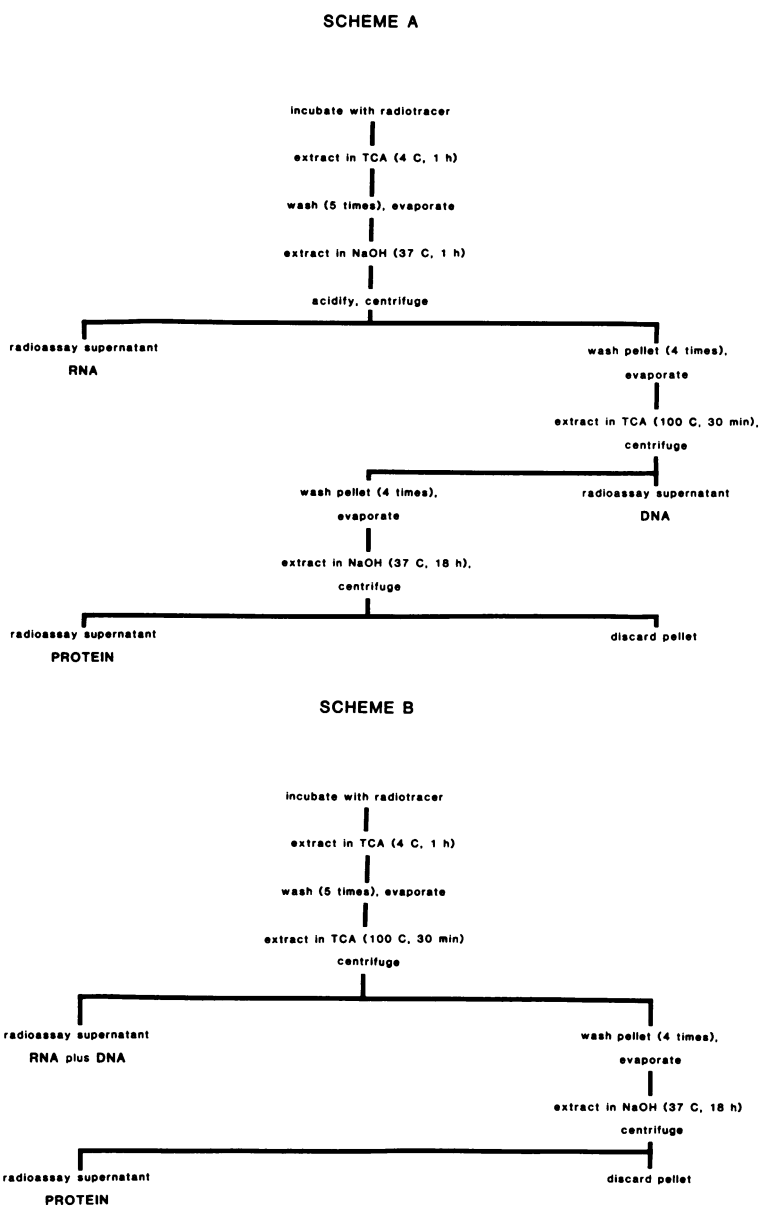


FIG. 1. Two independent procedures for the extraction, separation and measurement of radioactive RNA, DNA, and protein from cultures and environmental samples.

Since a small but variable percentage of the total cellular protein is irreversibly hydrolyzed during the primary alkaline hydrolysis step of the Schmidt-Thannhauser procedure (22), a second procedure was occasionally used for the quantitative separation of nucleic acids from protein (Fig. 1, scheme B). The major disadvantage of this procedure was its inability to resolve RNA from DNA. It should be mentioned at this point that there are no simple methods available for routine quantitative separations of individual macromolecules. The justification for the methods currently in use have been presented elsewhere (13, 14). Nevertheless, the data presented herein are at least internally consistent such that differences in labeling specificity between individual samples are believed to reflect variations in the metabolism of the radioactive precursors and are not analytical artifacts.

(ii) Radiochemical inventories. In addition to measuring the uptake and assimilation of the radiolabeled precursors into the various classes of acid-insoluble cellular macromolecules, an investigation was undertaken to separate and quantify the extracellular radio-

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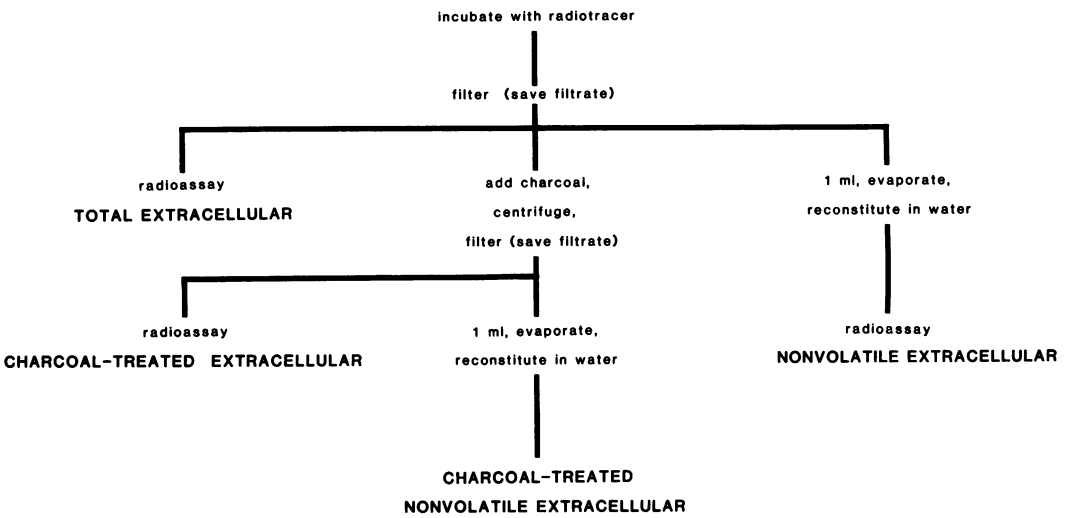


FIG. 2. Stepwise procedure for the fractionation of total cell-free radioactivity into separate chemical classes.

activity. Figure 2 summarizes the individual procedures used to fractionate the labeled materials found outside the cells. An activated charcoal procedure (14) was used to separate the nucleic acid precursors (and structurally related molecules) from their dissimilation by-products, and low-temperature vacuum evaporation was used to separate the ^3H-labeled organics from ^3H_2O (Fig. 2).

RESULTS AND DISCUSSION

Kinetics and specificity of macromolecular labeling. The reliability of radiotracer experiments designed to estimate the rates of nucleic acid (RNA, DNA, or both) synthesis requires a careful assessment of the mechanism(s) and regulation of uptake, the pattern(s) of intermediary metabolism, and the extent and specificity of macromolecular labeling. Figure 3 presents the results of an experiment which monitored the production of [^{3}H]RNA, [^{3}H]DNA, and ^3H_2O with time, for a Krauss Pond microbial community incubated with [^{3}H]adenine or [^{3}H]thymidine. These data indicate that the distribution of radioactivity varies with the precursor used: for [^{3}H]adenine, [^{3}H]RNA > ^3H_2O > [^{3}H]DNA (approximately 59:39:2%), whereas for [^{3}H]thymidine, ^3H_2O > [^{3}H]RNA > [^{3}H]DNA (approximately 86:11:3%).

Table 1 summarizes a few of the many specificity-of-labeling experiments conducted with seawater samples. In all samples analyzed, incubation with either [^{3}H]adenine or [^{3}H]thymidine resulted in the production of both [^{3}H]RNA and [^{3}H]DNA. This is not unexpected for [^{3}H]adenine (12–14) but is expected for [^{3}H]thymidine, especially since several previous investigators have implied that [^{3}H]thymidine predominantly, or exclusively, labels cellular DNA (3, 8, 33). Since the techniques used in this study (Fig. 1, scheme A) do not provide absolute separations of RNA, DNA, and protein (see reference 22) and since the extensive labeling of "RNA" in environmental samples was unexpected, an additional experiment was conducted to add credibility to these results. In this second experiment, RNA was estimated by difference between RNA + DNA and DNA; the former was determined by using a technique designed to eliminate any possibility of partial protein hydrolysis (see Fig. 1, scheme B), and the latter was determined by using the standard DNA procedure (see Fig. 1, scheme A). A summary of the results is presented in Table 2. These data confirm the labeling of cellular RNA, and demonstrate that the activity previously measured in the "RNA" fraction was not simply the result of partial and irreversible protein hydrolysis during the NaOH digestion step of scheme A (Fig. 1).

Table 3 summarizes the results of an experiment designed to further evaluate the nature of the metabolic pathways involved in the observed nonspecific labeling by exogenous [^{3}H]thymidine. It should be mentioned that this experiment was performed with equal initial radioactivities (not equal molar additions) so the data for the individual classes of macromolecules have been normalized to total acid insoluble incorporation and presented accordingly. It is apparent from this summary that a greater percentage of thymidine-derived radioactivity is deposited as DNA with the 2-^{14}C precursor than with the *methyl*-^{3}H isotope. Moreover, the proportion of label incorporated into cellular RNA is greatly reduced when 2-^{14}C is used, indicating that transmethylation of the *methyl*-^{3}H moiety is

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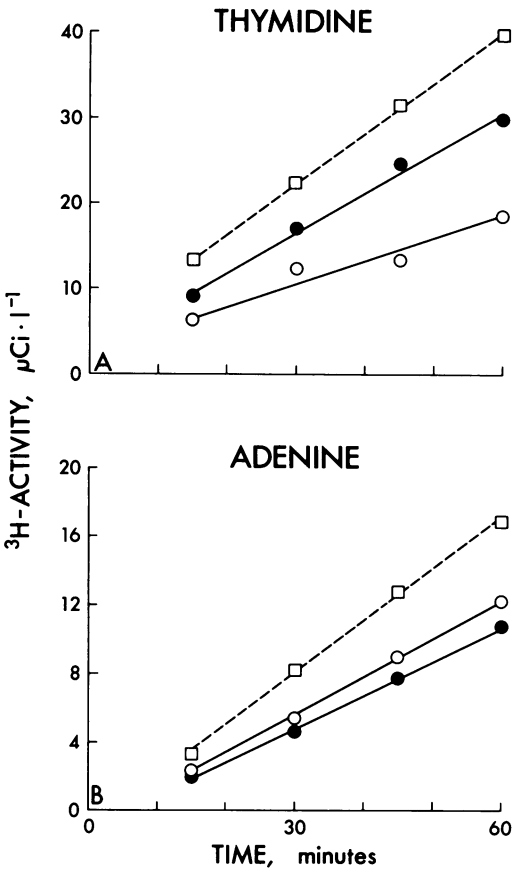


FIG. 3. Time series incorporation of [*methyl*-^{3}H]thymidine (A) and [2-^{3}H]adenine (B) into RNA, DNA, and H_2O for a water sample collected from Krauss Pond. Total radioactivity added to each sample was 100 μCi liter^{-1}, which was equivalent to an addition of 6.67 nmol of adenine and 1.25 nmol of thymidine. For (A): □, RNA (×10); ●, H_2O (×1); ○, DNA (×20). For (B): □, RNA (×1); ○, DNA (×20); ●, H_2O (×1).

at least in part responsible for the nonspecific labeling patterns. Labeled protein, on the other hand, must arise through catabolism of the pyrimidine ring since a greater percentage of the 2-^{14}C label is incorporated. Although these differences may be due, in part, to differences in the concentrations of thymidine added (note differences in specific radioactivity, Table 3), they do serve to demonstrate the complexity of the pathways which exist for the assimilation of certain nucleotide derivatives and emphasize the necessity for a comprehensive characterization of the label in the cells to achieve a complete understanding of precursor metabolism.

Effects of isotope dilution and estimation of precursor specific radioactivity. There is no apparent disagreement among the various investigators that measurement of the specific radioactivity (e.g., nCi pmol^{-1}) of the immediate precursor pool (i.e., ATP and dATP for adenine; dTTP for thymidine; UTP for uridine) is absolutely essential for accurate estimates of the rates of nucleic acid syntheses (8, 12, 21, 33). The only disagreement is in the methods used to estimate the specific activity of the cellular nucleotide triphosphate pools. In addition, there is an obvious need for the rigorous separation and purification of the presumed labeled materials (DNA or RNA). Without this necessary information on precursor-product labeling, uptake data alone are insufficient to calculate the mass flux of materials into stable nucleic acid pools.

Tables 4 through 6 summarize some of the representative data on the effects of initial [^{3}H]thymidine or [^{3}H]adenine concentrations on the uptake and extent of nucleic acid labeling. As the concentration of [^{3}H]thymidine added to the sample was increased (Table 4), a variable response was observed among the individual samples analyzed. Surprisingly, the total amount of [^{3}H]thymidine incorporated into ^{3}H-nucleic acids (DNA + RNA) was sometimes greater when incubated with 1.25 pmol added per liter (100 μCi of [^{3}H]thymidine liter^{-1}) than it was with the addition of 2.5 pmol (e.g., 100- and 150-m samples at the Tropical North Pacific Station; Table 4); however, in general the response conformed to the data of Fuhrman and Azam (8). It has been suggested that since the effect of [^{3}H]thymidine isotope dilution is small (8), the specific radioactivity of the dTMP incorporated into DNA (i.e., nCi pmol^{-1}) must be identical to that of the added radiotracer. This conclusion ignores the contributions of de novo synthesis and internal cycling of nucleic acid base moieties, which might represent the greatest source of isotope dilution (14). The only way to accurately assess the combined effects of extracellular and intracellular dilution of the specific radioactivity of the introduced precursor is through direct measurements of the specific radioactivity of the immediate nucleotide triphosphate precursor pool (12–15, 21).

Tables 5 and 6 present summaries of two [^{3}H]adenine isotope dilution experiments, one with a pure culture of *S. marinorubra* and the other with a sample of Krauss Pond. In both cases, data are presented for incorporation of [^{3}H]adenine into RNA, the magnitude of the precursor specific radioactivity, and the estimated rates of RNA synthesis. As expected, the total amount of radioactivity deposited in RNA increased with increasing [^{3}H]adenine in the medium. The important thing to note is that the mean specific radioactivity (or extent of label-

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TABLE 1. Comparison of the metabolism of [2-^{3}H]adenine and [*methyl*-^{3}H]thymidine in several marine microbial communities

Station	Isotope[a]	Incubation time (h) or depth (m) of sample	Activity (nCi liter^{-1})			DNA/RNA (×100)
			RNA	DNA	^3H_2O	
1[b]	Adenine	1	197.2	13.7	580	6.9
		4	1,119	93.3	1,820	8.3
		8	2,819	154.6	3,230	5.5
	Thymidine	1	52.8	23.4	0	44
		4	132.1	74.5	700	56
		8	512.8	254.3	990	50
2[c]	Adenine	12	2,550 ± 3.4%	412.3 ± 5.5%	5,680	16.2 ± 6.6%
		24	5,250 ± 1.2%	802.5 ± 8.3%	11,340	15.3 ± 8.0%
	Thymidine	12	285.9 ± 21%	350.6 ± 10.9%	13,820	125 ± 14.3%
		24	466.9 ± 2%	514.7 ± 3.1%	27,620	110 ± 4%
3[d]	Adenine	10 m	225.6	21.6		9.6
		50 m	97.4	8.6		8.8
		100 m	77.8	5.7		7.3
		150 m	40.7	9.3		22.9
	Thymidine	10 m	11.7	3.1		26.5
		50 m	11.4	2.8		24.6
		100 m	12.7	3.2		25.2
		150 m	9.9	7.2		72.7

[a] Isotopes were added to an initial activity of 100 μCi liter^{-1}, which corresponded to additions of 6.67 nmol (adenine) and 1.25 nmol (thymidine) per liter.
[b] 35° 47.8'N, 123° 39.8'W, 30 August 1980, 1 m depth.
[c] 35° 47.8'N, 123° 39.8'W, 6 September 1980, 1 m depth. Mean ± 1 standard deviation for all entries except ^3H_2O.
[d] 18° 43.8'N, 156° 50.4'W, February 1981, various depths. All station 3 entries were 6-h in situ incubations. ^3H_2O was not measured at station 3.

ing) of the ATP pools also increased in a constant proportion such that the final calculated rate of RNA synthesis was independent of the initial concentration of [^{3}H]adenine (Tables 5 and 6).

TABLE 2. Confirmation of [*methyl*-^{3}H]thymidine incorporation into RNA of a Krauss Pond microbial community by a procedure to distinguish [^{3}H]RNA from ^{3}H-protein

Incubation period (min)	Activity (nCi ml^{-1})			DNA/RNA (×100)
	Measured DNA + RNA	Measured DNA	Estimated RNA[a]	
30	3.85	1.59	2.26	99.5
	3.73	2.19	1.54	
60	10.46	5.50	4.96	121
	8.12	4.66	3.46	

[a] Estimated by difference, RNA = (DNA + RNA) − DNA; see Fig. 1.

Since the final calculated rate of RNA synthesis for the mixed microbial assemblage (i.e., bacteria plus microalgae; Table 6) is unaffected by a 100-fold increase in adenine, it does not appear as though competition for adenine is a problem, otherwise one might have expected to observe an apparent increase in the total community rate of RNA synthesis as [^{3}H]adenine was made available to a greater proportion of the population.

Furthermore, one might make the argument that since the effect of isotope dilution with the Krauss Pond sample was small, the specific activity of the incorporated adenine must have been equal to the introduced precursor specific activity (8). This is clearly not the case, as the measured specific radioactivities of the ATP pool never exceeded 15% of the value of the [^{3}H]adenine originally added (2 nCi pmol^{-1} versus 15 nCi pmol^{-1}; Table 6). If similar processes are occurring during the assimilation and incorporation of [^{3}H]thymidine, then the calculated

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TABLE 3. Comparison of incorporation of [*methyl*-^{3}H]thymidine and [2-^{14}C]thymidine into RNA, DNA, and protein of a microbial community sampled from Krauss Pond

Thymidine label	Incubation time (min)	% Total acid insoluble material		
		RNA	DNA	Protein
[*methyl*-^{3}H][a]	5	60.8	26.6	12.6
	15	56.9	32.0	11.1
	30	53.5	33.8	12.6
	60	54.0	35.4	10.6
	120	52.4	33.6	14.0
	240	52.7	35.8	11.5
[2-^{14}C][b]	5	7.7	61.5	30.8
	15	17.9	53.6	28.5
	30	7.4	55.6	37.0
	60	9.4	53.1	37.5
	120	10.8	55.0	34.2
	240	11.4	51.0	37.6

[a] Added to a final radioactivity of 0.1 μCi ml^{-1}, which is equivalent to an addition of 1.25 pmol of thymidine ml^{-1}.

[b] Added to a final radioactivity of 0.1 μCi ml^{-1}, which is equivalent to an addition of 1.82 nmol of thymidine ml^{-1}.

rates of DNA synthesis might be expected to underestimate the actual rates by a factor of up to an order of magnitude. This might help explain the discrepancies between the realistic and conservative estimates of DNA synthesis recently calculated by Fuhrman and Azam (9).

As an alternative to the direct measurement of the specific radioactivity of the nucleotide precursor pool, Moriarty and Pollard (21) have suggested an approach based on the magnitude of change (or dilution) of the activity of [^{3}H]thymidine incorporated into DNA upon the addition of nonradioactive thymidine to the samples. Using this approach, they have detected the presence of substantial pools (up to 10 nmol g^{-1} of sediment [wet weight]) of exogenous thymidine (or other precursors to dTMP) which resulted in a proportionate dilution of the specific radioactivity of the introduced precursor before incorporation into cellular DNA. However, this method of isotope dilution is not without limitation or criticism. For example, on several occasions the authors observed bi- or multiphasic plots of 1/disintegrations per minute incorporated into DNA versus thymidine added, which obviously complicates the interpretations of isotope dilution experiments. Furthermore, the theory of isotope dilution upon which this approach is predicated suffers from general criticisms, elaborated previously (34), regarding the validity of the assumption that the velocity of substrate utilization is completely independent of the concentration of substrate added. In addition, this experimental method represents a substantial investment in terms of time and effort (i.e., requires a minimum of seven separate analyses per sample, per time point) (see reference 21). Of course, this latter criticism (i.e., time investment) is not really appropriate unless accurate estimates of precursor specific radioactivity can be determined by simpler techniques.

Radiochemical inventories. Time series experiments were conducted with a culture of *S. marinorubra* and samples collected from Krauss Pond and Ala Moana Beach to examine the kinetics and patterns of incorporation of [^{3}H]adenine, [^{3}H]thymidine, and [^{3}H]uridine. The results of this comparative survey are summarized in Tables 7 through 9. It is apparent that the rates of uptake and incorporation, the degree of catabolism (as assessed by the ratio of ^3H_2O production to total acid-insoluble materials), and specificity of macromolecular labeling vary substantially among the individual precursors for a given sample and among the various samples for a given precursor. Furthermore, [^{3}H]thymidine stands out in terms of the variability of incorporation of radioactivity among the individual samples analyzed. When *S. marinorubra* cultures were incubated with [^{3}H]thymidine, no detectable ^3H_2O was produced and >88% of the incorporated radioactivity was recovered as [^{3}H]DNA (Table 7). However, when environmental samples were incubated with [^{3}H]thymidine, large amounts of ^3H_2O were produced, indicating substantial catabolism of the [^{3}H]thymidine precursor, and significant labeling of RNA and protein was observed (Tables 8 and 9).

The pathways and regulation of purine and pyrimidine biosyntheses, metabolic interconversions, and catabolism in microorganisms have been known for at least two decades (reviewed in 11). The degree of specificity in labeling cellular macromolecules and the relative kinet-

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TABLE 4. Effects of [*methyl*-^{3}H]thymidine concentrations on the incorporation of radioactivity into RNA and DNA of representative marine and freshwater microbial communities

Community	Sampling depth (m)	Thymidine added		Activity (nCi ml^{-1})		DNA/RNA (×100)
		μCi ml^{-1}	pmol ml^{-1}	RNA	DNA	
Krauss Pond (September 1980)[a]	1	0.1	1.25	2.52	0.68	26.9
		0.5	6.25	5.54	2.41	43.5
		1	12.5	6.87	3.09	44.9
		5	62.6	8.08	4.99	61.8
		10	125	9.05	6.25	69.1
Krauss Pond (April 1981)[a]	1	0.08	1.0	4.68	2.52	53.8
		0.2	2.5	8.99	3.42	38.0
		0.4	5.0	12.53	3.85	30.7
		0.8	10	17.74	4.66	26.3
		2.0	25	15.48	3.44	22.2
		4.0	50	15.49	3.83	24.7
Tropical North Pacific Ocean (February 1981)[b]	10	0.1	1.25	11.7	3.1	26.5
		0.2	2.5	8.4	6.8	80.9
	50	0.1	1.25	11.4	2.8	24.6
		0.2	2.5	14.1	3.8	26.9
	100	0.1	1.25	12.7	3.2	25.2
		0.2	2.5	8.0	2.2	27.5
	150	0.1	1.25	9.9	7.2	72.7
		0.2	2.5	4.1	4.6	112.2
Ala Moana Beach (May 1981)[c]		0.1	1.25	2.22	7.27	327
		0.2	2.5	3.86	9.48	246
		0.5	6.25	4.56	10.93	240
		1	12.5	7.04	12.19	173
		5	62.5	8.41	17.29	206

[a] A 1-h incubation under in situ conditions.
[b] A 6-h incubation under in situ conditions.
[c] A 5-h incubation under in situ conditions.

ics of precursor-derived tritium incorporation into the individual classes of compounds can be used to evaluate the pathways and intermediary metabolism of the exogenous nucleic acid bases and nucleosides under in situ conditions. The data for [^{3}H]adenine conform to the present model for its assimilation by bacterial cultures and aquatic microbial communities with primary labeling of RNA and DNA (via ATP and dATP, respectively), variable labeling of protein (presumably via ATP required for histidine biosynthesis), and the production of ^3H_2O (12–15). The assimilation of exogenous [^{3}H]uridine by marine and freshwater communities proceeded with the

TABLE 5. Effects of [^{3}H]adenine concentration on the measured rates of RNA synthesis in *S. marinorubra*

Adenine added		RNA produced (nCi ml^{-1})[a]	ATP pool sp. act. (nCi pmol^{-1})[a]	Rate of RNA synthesis (pmol adenine ml^{-1} min^{-1})
μCi ml^{-1}	pmol ml^{-1}			
0.1	6.7	25	0.030	166.7
0.5	33.5	167.3	0.123	272.0
1	67	321.2	0.326	197.1
5	335	953.4	0.983	194.0
10	667	1,049	1.060	197.9

[a] Measured after a 5-min incubation at 25°C.

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TABLE 6. Effects of [^{3}H]adenine concentration on the measured rates of RNA synthesis in Krauss Pond

Adenine added		RNA produced (nCi ml^{-1})[a]	ATP pool sp. act. (nCi pmol^{-1})[a]	Rate of RNA synthesis (pmol adenine ml^{-1} h^{-1})
μCi ml^{-1}	pmol ml^{-1}			
0.1	6.7	12.8	0.312	42.2
		13.8	0.319	
0.5	33.5	35.0	0.697	47.4
		34.9	0.782	
1	67	44.5	1.136	36.2
		46.9	1.418	
5	335	70.5	1.987	35.4
		66.1	1.877	
10	667	74.0	1.928	37.9
		74.9	2.000	

[a] Measured after a 1-h incubation under in situ conditions.

majority (>90%) of the label being deposited as [^{3}H]RNA, with lesser amounts in [^{3}H]DNA and ^{3}H-protein. Labeling of both RNA and DNA is consistent with the results of Dennis (6) and Karlstrom and Larsson (16) for *Escherichia coli* cultures. ^3H_2O was produced to a greater extent (i.e., when normalized to total acid-insoluble materials) in natural microbial assemblages than in *S. marinorubra* cultures, suggesting a greater degree of catabolism. The labeling patterns observed with [^{3}H]thymidine in *S. marinorubra* cultures (i.e., >90% of label in [^{3}H]DNA production) suggest active salvage pathway metabolism with little or no [^{3}H]thymidine catabolism. However, the extensive data obtained for [^{3}H]thymidine assimilation by natural microbial communities (Fig. 3; Tables 1 through 4 and 7 through 9) bear little resemblance to the pure culture results. The distribution of radioactivity with environmental samples generally yielded ^3H_2O > [^{3}H]RNA > ^{3}H-labeled protein ≥ [^{3}H]DNA, but the results varied substantially among individual samples. Furthermore, there were significant differences in the rates at which tritium was incorporated into the individual macromolecules (e.g., DNA, RNA, protein) of a given sample, suggesting that the biosynthetic pathways and their regulation were uncoupled and independent, even over short incubation periods. Clearly, the pathways of [^{3}H]thymidine metabolism are dependent upon the microbial community in question such that a priori judgments concerning the metabolic pathways and specificity of labeling may be misleading.

Response of the microbial community to added precursor. Implicit in the application of nucleic acid precursor metabolism as a means of assessing microbial community growth in nature is the assumption that all microorganisms (or a unique subset of the total community; e.g., all bacteria) have the ability to utilize the exogenous materials in preference to de novo synthesis. This assumption has been evaluated and confirmed for the use of [^{3}H]adenine with bacterial and algal cultures (14, 15; D. Karl, unpublished results) (Tables 5 and 6). However, one substantial criticism of the [^{3}H]adenine approach as currently employed is the implicit assumption that all cellular ATP pools are in isotopic equilibrium. At present, a biomass–activity-weighted mean ATP pool specific activity is measured, and from this value and the total amount of [^{3}H]RNA and [^{3}H]DNA produced a mean community RNA or DNA rate is calculated. If the variance among the individual cellular ATP pool specific activities is large, the variance in the estimated rates will likewise be large. This possibility has not yet been systematically evaluated, but it is known that laboratory cultures of algae yield ATP pool specific radioactivities which are indistinguishable from those measured in bacteria under identical laboratory conditions, implying that the two diverse groups of microorganisms have the ability to salvage a similar percentage of their required nucleic acid precursors from the environment. In addition, the mean ATP pool specific radioactivities measured for different size fractions of naturally occurring microbial communities support the notion that diverse groups of microorganisms have similar salvage capabilities under in situ conditions (15).

Although [^{3}H]thymidine has been assumed to be assimilated predominantly or exclusively by bacteria (8, 9, 21, 33), the literature is replete with reports of [^{3}H]thymidine incorporation into the DNA of eucaryotic algae, protozoa, yeasts, fungi, and slime molds (5, 7, 20, 24–28, 30), even at nanomolar concentrations or less (C. Winn and D. Karl, unpublished data for the marine alga *Pavlova lutheri*). Among the procaryotes, the ability to assimilate exogenous [^{3}H]thymidine is not a universal trait. Fuhrman and Azam (9) have recently demonstrated by autoradiographic techniques that only between 34 and 50% of the recognizable bacteria in coastal seawater assimilate [^{3}H]thymidine. The presumed absence of thymidine kinase is insufficient to argue against possible exogenous [^{3}H]thymidine incorporation and in fact, it is this absence which may have contributed to the nonspecificity of [^{3}H]thymidine incorporation by natural microbial communities, relative to the *S. marinorubra* control cultures (Table 7 through 9).

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TABLE 7. Time series radioactivity inventory for the metabolism of [2-^{3}H]adenine, [*methyl*-^{3}H]thymidine, and [5-^{3}H]uridine by *S. marinorubra*

Isotope[a]	Time (min)	Extracellular activity (nCi ml^{-1})[b]			Intracellular activity[b]			
		Total	Nonvolatile	^3H_2O	Total (nCi ml^{-1})	% In: RNA	DNA	Protein
Adenine	0	4,609	4,446	0	0			
	1	4,400	4,040	197	99	97.9	2.1	0
	2.5	3,825	3,629	403	355	96.1	3.6	0.3
	5	3,104	2,567	580	763	93.9	5.8	0.3
	7.5	2,701	1,975	738	1,065	92	7.8	0.2
	10	2,365	1,550	846	1,154	88.4	11.3	0.3
	15	2,031	1,284	893	1,334	84.2	15.5	0.3
	20	1,986	1,105	943	1,285	76.9	22.8	0.3
	30	1,896	988	934	1,224	68.9	30.7	0.4
Thymidine	0	4,758	4,589	0	0			
	1	4,922	4,751	3	7	11.0	89.0	0
	2.5	4,732	4,723	6	38	11.2	88.5	0.3
	5	4,723	4,609	5	90	5.3	93.9	0.8
	7.5	4,551	4,484	5	139	4.5	95.2	0.3
	10	4,443	4,418	9	219	4.4	95.1	0.5
	15	4,232	4,254	4	300	4.7	94.3	0.8
	20	4,136	4,167	12	394	7.8	91.5	0.7
	30	3,885	3,903	17	469	5.0	94.1	0.8
Uridine	0	4,211	4,138	0	0			
	1	3,835	3,811	15	98	99.8	0.1	0.1
	2.5	3,065	3,007	35	363	99.1	0.9	0
	5	2,226	2,212	65	731	98.2	1.7	0.1
	7.5	1,820	1,767	105	969	97	2.9	0.1
	10	1,376	1,244	149	1,068	95.4	4.4	0.2
	15	1,142	942	200	1,029	92.1	7.7	0.1
	20	1,124	850	286	1,053	89.0	10.9	0.1
	30	1,279	881	409	1,015	82.5	17.3	0.3

[a] All isotopes were added at approximately 4 to 5 μCi ml^{-1}, which corresponded to additions of 306 pmol (adenine), 59 pmol (thymidine), and 158 pmol (uridine).
[b] Refers to operational definitions given in Fig. 1 and 2.

TABLE 8. Time series radioactivity inventory for the metabolism of [2-^{3}H]adenine, [*methyl*-^{3}H]thymidine, and [5-^{3}H]uridine by a microbial community from Krauss Pond

Isotope[a]	Time (min)	Extracellular activity (nCi ml^{-1})[b]			Intracellular activity[b]			
		Total	Nonvolatile	^3H_2O	Total (nCi ml^{-1})	% In: RNA	DNA	Protein
Adenine	0	384	368	0	0			
	10	376	350	13.1	13.1	91.9	5.3	2.8
	30	339	297	38.6	31.6	86.6	8.8	4.6
	60	293	217	71.2	66.0	87.3	7.8	4.9
	90	245	148	100.5	95.5	88.4	5.7	5.8
	300	184	57	127.9	165.0	85.5	7.8	6.7
Thymidine	0	385	385	0	0			
	10	381	377	6.1	4.0	83.0	3.5	13.4
	30	371	338	26	10.3	87.2	3.9	8.9
	60	351	282	67.5	18.5	84.2	5.3	10.5
	90	340	214	119.1	26.9	81.7	5.6	12.7
	300	313	75	233.5	33.7	78.9	3.9	17.3
Uridine	0	342	344	0	0			
	10	340	338	2.2	15.4	97.4	0.7	1.8
	30	309	303	9.1	34.4	97.6	0.6	1.7
	60	363	250	22.3	66.8	97.9	0.7	1.4
	90	222	186	41.5	97.3	97.7	0.9	1.5
	300	131	27	100.6	141.4	94.5	1.6	3.9

[a] All isotopes were added at approximately 350 nCi ml^{-1}, which corresponded to additions of 26 pmol (adenine), 4.8 pmol (thymidine), and 13.1 pmol (uridine).
[b] Refers to operational definitions given in Fig. 1 and 2.

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TABLE 9. Time series radioactivity inventory for the metabolism of [2-^{3}H]adenine, [*methyl*-^{3}H]thymidine, and [5-^{3}H]uridine by a coastal marine microbial community

Isotope[a]	Time (min)	Extracellular activity (nCi ml^{-1})[b]			Intracellular activity[b]			
		Total	Nonvolatile	^3H_2O	Total (nCi ml^{-1})	% In:		
						RNA	DNA	Protein
Adenine	0	409	403	0	0			
	30	408	400	0.5	0.88	87.3	9.1	3.6
	60	406	399	0.9	2.04	85.2	8.8	6
	120	396	391	3.2	5.69	83.1	10.4	6.5
	240	394	376	4.9	12.0	76.4	15.7	7.9
Thymidine	0	390	375	0	0			
	30	390	374	0.6	0.32	0	87.2	12.8
	60	381	373	2.4	0.81	1.6	80.7	17.6
	120	385	370	4.3	3.27	18.4	68.7	12.9
	240	359	336	20.2	12.54	30.6	55.5	13.9
Uridine	0	436	422	0	0			
	30	435	425	0.3	0.77	97	0	3
	60	436	422	2	2.14	98	0	2
	120	—[c]	—[c]	—[c]	5.90	93.6	4.5	1.9
	240	420	394	8.3	15.80	88.4	9.9	1.7

[a] All isotopes were added at approximately 400 nCi ml^{-1}, which corresponded to additions of 27.3 pmol (adenine), 4.9 pmol (thymidine), and 16.7 pmol (uridine).

[b] Refers to the operational definitions given in Fig. 1 and 2.

[c] The 120-min uridine sample (extracellular) was lost in processing.

Grivell and Jackson (10) have noted that microorganisms lacking thymidine kinase incorporate exogenous radiolabeled thymidine with less specificity than organisms possessing the preferred salvage pathways. This suggestion is supported by preliminary experiments performed in our laboratory with *P. lutheri*, which exhibits equivalent labeling of both RNA and DNA after short-term (<1 h) incubations with [^{3}H]thymidine (C. Winn and D. Karl, unpublished data). These data once again emphasize the necessity for establishing accurate precursor-product (e.g., NTP [or dNTP]-RNA [or DNA]) relationships when estimating rates of nucleic acid metabolism in natural microbial assemblages.

In conclusion, this study has attempted to further evaluate the proposed use of nucleic acid precursors in microbial ecology by examining a few of the numerous assumptions inherent in this approach. The results presented and discussed herein are unique for [2-^{3}H]adenine, [*methyl*-^{3}H]thymidine, and [5-^{3}H]uridine, and it should be emphasized that both the qualitative and quantitative patterns of incorporation may be affected by the specific site of labeling. Most of the inadequacies and limitations cited in this study apply to the uptake and assimilation of all radiolabeled nucleic acid precursors, at least until there has been a concerted effort to evaluate critically each of the numerous assumptions inherent with their use. Even when unequivocal data on the rates of RNA and DNA synthesis in natural ecosystems are available, we will still need to evaluate the validity of the numerous assumptions used to extrapolate these measured rates to microbial growth rate and production estimates which are generally desired for comparison with other field data. However, the potential significance of the ecological information to be derived from estimates of in situ rates of nucleic acid syntheses more than justifies the level of research effort that is required to ensure a proper interpretation of the experimental results obtained in field studies.

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