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Underestimation of DNA Synthesis by [^{3}H]Thymidine Incorporation in Marine Bacteria

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A direct comparison of [^{3}H]thymidine incorporation with DNA synthesis was made by using an exponentially growing estuarine bacterial isolate and the naturally occurring bacterial populations in a eutrophic subtropical estuary and in oligotrophic offshore waters. Simultaneous measurements of [^{3}H]thymidine incorporation into DNA, fluorometrically determined DNA content, and direct counts were made over time. DNA synthesis estimated from thymidine incorporation values was compared with fluorometrically determined changes in DNA content. Even after isotope dilution, nonspecific macromolecular labeling, and efficiency of DNA recovery were accounted for, [^{3}H]thymidine incorporation consistently underestimated DNA synthesized by six- to eightfold. These results indicate that although the relationship of [^{3}H]thymidine incorporation to DNA synthesis appears consistent, there are significant sources of thymine bases incorporated into DNA which cannot be accounted for by standard [^{3}H]thymidine incorporation and isotope dilution assays.

[^{3}H]thymidine incorporation has become the most widely used means by which bacterial activity and productivity are estimated in water column (8, 9, 11, 18, 25) and sediment (5, 31) samples. The accuracy of the technique, however, is contingent on four factors and assumptions: (i) the need to assess and correct for isotope dilution; (ii) the need to determine the amount of radioactive label incorporated specifically into DNA; (iii) the assumption that all bacteria in the sample are capable of incorporating thymidine; and (iv) the empirical relationship between thymidine incorporation and DNA synthesis (13). Previous studies have examined isotope dilution (2, 3, 12, 17, 18, 25) and nonspecific macromolecular labeling (9, 12, 29, 31; K. Carmen, F. C. Dobbs, and J. B. Guckert, Limnol. Oceanogr., in press). However, there has not yet been an absolute standard nonisotopic determination of bacterial DNA synthesis under laboratory and environmental conditions with which results from [^{3}H]thymidine incorporation could be compared. In this study, we present the first comparison of bacterial DNA synthesis as estimated by [^{3}H]thymidine incorporation and as measured directly by a nonisotopic means.

The culture organism used was *Vibrio proteolyticus*, which we have previously shown to incorporate [^{3}H]thymidine into DNA (10). Overnight cultures were diluted 1:50 into 300 ml of fresh growth medium (ASWJP-PY [19]) and grown at room temperature for 3 h on a gyratory shaker at 100 rpm. Six 25-ml samples were removed to sterile 125-ml polycarbonate flasks for analysis of isotope dilution (25). To all flasks, [*methyl*-^{3}H]thymidine (60 to 80 Ci/mmol; ICN Pharmaceuticals Inc., Irvine, Calif.) was added at 2.0 μCi/ml. Unlabeled thymidine was added such that total thymidine concentrations ranged from 1 to 30 μM. To the remaining cell solution, thymidine was added at 2.0 μCi/ml and a final concentration of 10 μM. Subsamples were taken immediately and at 20-min intervals up to a total of 2 h for measurements of thymidine incorporation, direct counts, and cellular DNA. Triplicate 2.0-ml samples for thymidine incorporation were added to test tubes containing 0.2 ml of 100% trichloroacetic acid, mixed, and placed on ice. After a minimum of 1 h, the contents were filtered onto 0.2-μm-pore-size polycarbonate filters (Nuclepore Corp., Pleasanton, Calif.), and each was washed with 5 ml of ice-cold 5% trichloroacetic acid. Radioactivity was determined by liquid scintillation counting. Acid-base hydrolysis of macromolecules was not required for determination of nonspecific labeling since this organism incorporates [^{3}H]thymidine only into DNA (10; W. Jeffrey, unpublished data). Subsamples for direct counts were fixed and diluted in filtered (0.2-μm-pore-size filter) ASWJP containing 1.8% Formalin. Direct counts were later determined by epifluorescence microscopy (26). Cellular DNA was determined fluorometrically with Hoechst dye 33258 by the method of Paul and Myers (24). This technique involves extracting DNA by sonication, removing cell debris and filter particles by centrifugation, and reacting the clarified extract with Hoechst dye 33258. DNA concentration is determined against known standards.

For calibration of thymidine incorporation with DNA synthesis in natural waters, samples were taken from Bayboro Harbor, a eutrophic embayment of Tampa Bay in St. Petersburg, Fla. (11, 12, 22), and oligotrophic offshore waters of the southeast Gulf of Mexico (26°40.81′ N, 83°58.33′ W).

Environmental water samples were gently filtered (<0.2-atm [1 atm = 101.29 kPa] vacuum) through a 1-μm-pore-size Nuclepore filter to remove the majority of bacteriovores (1). The sample was then incubated at room temperature for 2 to 4 h on a gyratory shaker at 100 rpm. Isotope dilution (25) was then determined in a series of 25-ml subsamples with thymidine added at concentrations ranging from 2.5 to 30 nM. [^{3}H]thymidine was added at 35 nM to the remaining sample, and subsamples were immediately taken for measurements of thymidine incorporation, direct counts, and cellular DNA. Replicate subsamples were subjected to acid-base hydrolysis as described previously (28) to determine the amount of nonspecific macromolecular labeling during [^{3}H]thymidine incorporation. Sampling intervals were extended to 2 h (Bayboro Harbor) or 4 h (offshore waters) to ensure significant changes in the measured parameters.

Subsamples for DNA were filtered onto 0.22-μm-pore-size membrane filters (type GS; Millipore Corp., Bedford, Mass.)

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TABLE 1. Comparison of thymidine incorporation with DNA synthesis for *V. proteolyticus*

Time interval (min)	ΔDNA			No. of cells/mol of thymidine incorporated[a] (10^{17})
	Measured[b] (ng/ml)	Calculated[c] (ng/ml)	Measured/calculated[d]	
0–20	650	38	17.1	2.24
20–40	294	46	6.4	2.83
40–60	218	45	4.8	4.29
60–80	368	79	4.6	2.23
80–100	702	76	9.2	5.67
100–120	482	80	6.0	5.10

[a] Average of all values = $3.73 \times 10^{17} \pm 1.50 \times 10^{17}$.
[b] Determined by direct fluorometric measurements of DNA.
[c] Calculated from thymidine incorporation, using 624 g/mol as the average molecular weight of a base pair (15) and 50% A · T base-pair composition. Values have been corrected for isotope dilution.
[d] Average of all values = 8.0 ± 4.7. Average of values excluding outlier from 0 to 20 min = 6.2 ± 1.8.

and stored frozen in 3 ml of SSC (0.15 M NaCl plus 0.015 M sodium citrate) (21) for later analysis (24). Direct counts of subsamples were fixed in 1.8% Formalin for later epifluorescence microscopy determinations (26).

The efficiency of acid-base hydrolysis of DNA was determined by adding either [^{3}H]thymidine-labeled *V. proteolyticus* cells (which label only DNA [10]) or 0.1 μCi of high-specific-activity end-labeled λ [^{3}H]DNA per ml (16) to water samples and immediately subjecting 2-ml subsamples to acid-base hydrolysis (28). Total DNA was determined by adding a 2-ml sample to 0.2 ml of 100% trichloroacetic acid, chilling the sample, and collecting the precipitate by filtration. This value was taken as 100% of the added radioactivity. Percent recovery was calculated after determining the amount of radioactivity lost during warm-base hydrolysis and the amount of radioactivity remaining after hot-acid extraction.

The results from the DNA calibration experiment using *V. proteolyticus* are presented in Table 1. The DNA values are the amount of DNA increase during the 20-min time interval. The calculated DNA value was determined by using 624 g/mol as the average molecular weight of a base pair (15) and a 50% A · T base-pair composition. The value was corrected for isotope dilution by determining the amount of total dilution, which in the case of the exponentially growing *V. proteolyticus* in ASWJP-PY was equivalent to 5.6 μM. Thymidine incorporation rates were then calculated by using 15.6 μM as the total thymidine concentration. The corrected moles of thymidine incorporated was then multiplied by 1,248 g of DNA per mol (two times the base-pair molecular weight) to get estimated DNA synthesis.

The ratios of measured to calculated DNA values indicated a significant underestimation of DNA synthesis as determined by thymidine incorporation during the 2-h duration of the experiment. The values ranged from 17.1- to 4.6-fold and averaged an 8-fold underestimation of DNA synthesis as determined by thymidine incorporation (Table 1).

Similar results were observed during experiments comparing DNA synthesis with [^{3}H]thymidine incorporation using environmental samples (Table 2). In Bayboro Harbor waters, [^{3}H]thymidine incorporation underestimated DNA synthesis by an average factor of 5.8. In offshore waters, DNA synthesis was underestimated by approximately eightfold (Table 2). The values were corrected for isotope dilution (less than or equivalent to 3 nM thymidine for these samples), nonspecific macromolecular labeling, and efficiency of acid-base hydrolysis. Determined extraction efficiency values ranged from 60 to 100%, but the majority were between 80 and 85%. Therefore, we used a conservative extraction efficiency of 80%.

The data presented here indicate that thymidine incorporation significantly and consistently underestimates DNA synthesis in three widely different aquatic environments. The amount by which [^{3}H]thymidine incorporation underestimates DNA synthesis is not significantly different regardless of whether an exponentially growing culture (Table 1), a eutrophic estuarine sample (Bayboro Harbor, Table 2), or oligotrophic offshore waters (Table 2) are examined. Further evidence of the underestimations is indicated by values for DNA content per cell (Table 2). The measured DNA-per-cell values presented here are slightly higher than those reported by others (8, 20, 23), but these differences may be due to the higher growth rate of these cells caused by stimulation from filtration and incubation. Actively growing cells will have more than one copy of the genome since DNA is synthesized before cell division (7). In contrast, the DNA-per-cell values derived from [^{3}H]thymidine incorporation and cell counts were extremely low (Table 2), approximately seven to nine times lower than published values (8, 20, 23).

It is unlikely that the assumptions and corrections used to calculate DNA synthesis are the reasons for the large discrepancy between measured values and those derived from [^{3}H]thymidine incorporation. Using an average molecular weight for a base pair of 624 g/mol (15) is appropriate on the basis of DNA structure and is commonly done to estimate DNA content in a variety of procedures (15). The percent efficiency of DNA hydrolysis is conservative. Using 100% efficiency would actually increase the ratio of measured to predicted DNA. Assuming 50% A · T base-pair composition also seems logical for a heterogeneous population. Any variations would be slight and certainly could not account for the differences in measured versus calculated values.

Our data are independent of the percentage of active cells in each sample. We were measuring the increase in DNA per volume of liquid over time, which is presumably attributable

TABLE 2. Comparison of thymidine incorporation with DNA synthesis for environmental bacteria

Location	ΔDNA			DNA (fg)/cell		No. of cells/mol of thymidine incorporated (10^{18})
	Measured[a] (ng/ml)	Calculated (ng/ml)	Measured/calculated[b]	Measured	Calculated	
Bayboro	8.00	1.35	5.9	14.0	0.51	0.6
	1.62	0.28	5.8	8.7	0.80	0.9
Offshore	0.765	0.096	8.0	5.3	0.64	4.3

[a] Determined during a 2-h time interval for Bayboro Harbor and a 4-h interval for offshore waters.
[b] Average value = 6.6 ± 1.2. Values have been corrected for isotope dilution, nonspecific macromolecular labeling, and efficiency of acid-base hydrolysis of DNA.

to the active cells (the same active cells that are incorporating [^{3}H]thymidine). Inactive cells do not synthesize DNA and did not contribute to or detract from the results. We must assume that the vast majority of active bacteria in our samples were incorporating thymidine. The limited available information (8; W. H. Jeffrey and J. H. Paul, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, N79, p. 257) appears to support this assumption.

We present values for cells synthesized per mole of thymidine incorporated (Table 2), since this has become the most common means by which thymidine incorporation is correlated with cell growth (2, 4, 27). As is apparent from the data in Tables 1 and 2, there is a wide range in values. The values for *V. proteolyticus* and Bayboro Harbor are quite similar yet much lower than the value for the offshore waters. The reason for this difference is not immediately clear, although the faster growth rates and larger cellular DNA contents for these samples contribute to these results. The greater numbers of cells synthesized per mole of thymidine incorporated for the offshore sample indicate a slower growth rate and lower DNA content per cell. However, all of these values fall within the range reported by other researchers (2, 4, 27). The large variance in values presented here indicates that this type of factor for converting thymidine incorporation to productivity may not be consistent in all environments or for all growth rates. In contrast, the ratio of measured to calculated DNA synthesized appears constant.

Presumably, thymine bases incorporated into DNA may arise from two biosynthetic pathways. The salvage pathway incorporates exogenous thymidine into dTMP, which is twice more phosphorylated (to dTTP) before incorporation into DNA (14). The de novo synthesis pathway uses intracellular constituents to eventually synthesize dTMP from dUMP via the thymidylate synthetase reaction (14). By determining the amount of label incorporated into DNA and isotope dilution, all of the thymine bases should be accounted for. The dramatic and consistent underestimation of DNA synthesis by [^{3}H]thymidine incorporation in three widely different environments indicates that there are other significant sources of thymine bases incorporated into DNA for which we cannot account.

The apparent explanation for these results is that isotope dilution analysis (2, 3, 12, 17, 18, 25) is not appropriate for use with in situ [^{3}H]thymidine incorporation assays. The isotope dilution method (17, 18, 25) is based on a technique originally designed for use with mammalian cell cultures (6, 30). Several studies have examined its use in environmental samples (2, 3, 12, 17, 18, 25), and the method appears to function much as it does when applied to mammalian tissue cells. These previous studies have demonstrated that isotope dilution analysis accounts for some intra- and extracellular dilution of the isotope, although the amount for environmental samples was rarely more than the equivalent of a few nanomolar thymidine. In previous studies, there was no means to determine whether all dilution was measured. By directly measuring the amount of DNA synthesized in our samples with the Hoechst dye 33258 technique (24), we calculated the number of thymine bases incorporated into DNA. We consistently found substantial sources of thymine bases that could not be accounted for by isotope dilution analysis. Our results agree very closely with those of Fuhrman and Azam (8), who elected to determine dilution by comparing [^{3}H]thymidine incorporation with labeled phosphorus incorporation into DNA. They reported a three- to sevenfold underestimation of DNA synthesis on the basis of [^{3}H]thymidine incorporation compared with phosphorus incorporation.

Although [^{3}H]thymidine incorporation has its limitations, it is still the simplest and most rapid means by which bacterial activity can be examined in most environments. Our intent is to suggest caution as to how the results obtained by this technique may be interpreted and applied. Bacterial activity, DNA synthesis, and heterotrophic production are three different processes. Although [^{3}H]thymidine incorporation may be used to estimate all three of these processes, the assumptions required to use [^{3}H]thymidine for each are different. It is the responsibility of the researcher to show that the thymidine method is valid for a particular application. Relative bacterial activity between similar samples may easily be compared by using gross [^{3}H] thymidine incorporation values. In this case, nonspecific labeling and isotope dilution become less important. However, when attempts are made to extrapolate [^{3}H]thymidine incorporation to DNA synthesis or heterotrophic production values, nonspecific labeling, isotope dilution, and other factors become very important. Our data and those of Fuhrman and Azam (8) indicate that estimates of DNA synthesis may be obtained by multiplying [^{3}H]thymidine-predicted values by 6 to 8. Heterotrophic productivity values may be derived from cells produced per mole of thymidine incorporation without first estimating DNA synthesis values. We stress, however, that the increasing body of literature indicates that no single conversion factor holds for all environments at all times. Most recent evidence indicates that cell volume may be more closely related to [^{3}H]thymidine incorporation-derived productivity estimates than are cell numbers (4). Again, it is the responsibility of the researcher to determine that the conversion factors used are valid for the application in question.

There is much to be learned about the enzymatic pathways associated with thymidine incorporation for environmental bacteria. The prevalence of nonspecific macromolecular labeling (9, 12, 29, 31; K. Carmen et al., in press) and the apparently significant source(s) of thymine bases incorporated into DNA that cannot be accounted for by standard methods further illustrate the need to scrutinize thymidine incorporation as a means of estimating bacterial DNA synthesis and productivity.

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