Validity of the Tritiated Thymidine Method for Estimating Bacterial Growth Rates: Measurement of Isotope Dilution During DNA Synthesis

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The rate of tritiated thymidine incorporation into DNA was used to estimate bacterial growth rates in aquatic environments. To be accurate, the calculation of growth rates has to include a factor for the dilution of isotope before incorporation. The validity of an isotope dilution analysis to determine this factor was verified in experiments reported here with cultures of a marine bacterium growing in a chemostat. Growth rates calculated from data on chemostat dilution rates and cell density agreed well with rates calculated by tritiated thymidine incorporation into DNA and isotope dilution analysis. With sufficiently high concentrations of exogenous thymidine, de novo synthesis of deoxoythymidine monophosphate was inhibited, thereby preventing the endogenous dilution of isotope. The thymidine technique was also shown to be useful for measuring growth rates of mixed suspensions of bacteria growing anaerobically. Thymidine was incorporated into the DNA of a range of marine pseudomonads that were investigated. Three species did not take up thymidine. The common marine cyanobacterium *Synechococcus* species did not incorporate thymidine into DNA.

In the past, techniques were not available for measuring growth rates of most heterogeneous bacterial communities in nature. Considerable advances have been made recently in the measurement of bacterial growth rates by determining the rate at which [methyl-3H]thymidine is incorporated into bacterial DNA (2, 3, 5, 6, 11, 12, 26, 27).

There are two principal pathways for the synthesis of thymine nucleotides. The first is the de novo pathway by which nucleotides are synthesized from precursor molecules such as carbamyl phosphate and aspartic acid. Ultimately, dUMP is methylated through the action of thymidylate synthetase (EC 2.1.1.45), thereby producing dTMP (22). The second is the salvage pathway, in which thymine is phosphorylated to form dTMP by the enzyme thymidine kinase (EC 2.7.1.21) (22).

With further phosphorylation, dTMP is converted to dTDP and then to dTTP. The enzyme DNA polymerase (EC 2.7.7.7) utilizes dTTP to incorporate thymine into DNA with the other three bases. Extracellular and intracellular deoxyribonucleotide pools and biosynthetic pathways can contribute thymine to DNA as well as dilute the radioactively labeled thymine that is incorporated into DNA from the supplied precursor (1H)thymidine.

The DNA polymerase is probably bound into a multienzyme complex together with the kinases needed to synthesize the deoxyribonucleotides (24, 25). Small pools of dTTP and other precursors in the complex are the immediate source of nucleotides for DNA synthesis. They turn over very rapidly and are functionally separate from the general cellular pools of the nucleoside triphosphates. Thus, it is impossible to measure the size of such pools and any change in specific radioactivity of an exogenous precursor by extracting the nucleotides. A technique known as isotope dilution analysis is available for measuring the change in the specific radioactivity of a precursor at the site of incorporation. This method was developed for use with mammalian cell cultures by ForSYD, Scott, and Sjostrom (9, 10, 35–38). We have used isotope dilution analysis in marine sediment and seawater to estimate bacterial growth rates (26, 27).

Because the technique has been criticized (13, 20), we report here results of studies with a chemostat, which show that the rate of tritiated thymidine incorporation into DNA gives a good estimate of growth rate and thus that isotope dilution analysis measures the dilution of radioactivity in dTTP. Furthermore, we show that de novo synthesis of dTMP from dUMP can be inhibited by high concentrations of thymidine, thus eliminating the need to carry out isotope dilution analysis with every sample.

An explanation of isotope dilution theory and methodology is given here (for a review, see references 9, 10, 26, 27, 36, 37; D. J. W. Moriarty, Adv. Aquat. Microbiol., in press). A fixed concentration of radioactively labeled thymidine and various concentrations of nonradioactive thymidine were incubated with growing bacteria. The DNA was extracted, and the total concentration of added thymidine was plotted against the reciprocal of the radioactivity in the DNA fraction (see Fig. 1). If there was no dilution of the isotope incorporated into DNA by any sources other than the unlabeled thymidine that was added, the plot should pass through zero. A negative intercept on the ordinate is an estimate of the amount of isotope dilution by other sources of thymine in DNA. It is not strictly a pool of thymidine but instead represents the influence of pools that diluted the tritiated thymidine before incorporation into DNA.

The isotope dilution method measures the dilution of labeled thymine in dTTP, the final precursor to DNA, in all sources of thymine, because the effect of added thymidine on the incorporation of radioactivity into DNA itself is measured. A necessary condition is that the rate-limiting step for the incorporation of thymine be the final one, i.e., the DNA polymerase step. The following explanation of the concepts of the isotope dilution methodology has been adapted from the work of Forsdyke and his colleagues (9, 10, 35–38). In the simplest model, the incorporation of a labeled precursor into a macromolecule may be represented as a
one-step transfer from a pool into a stable product; two assumptions were made.

First, the rate of labeling of the macromolecule should not be influenced by the concentration of the precursor. As indicated above, DNA synthesis is a complex process which depends on many factors and is linked ultimately to the supply of energy and nutrients to the cell. In a growing cell, with DNA synthesis initiated, the tight regulation of nucleotide biosynthetic pathways ensures that an adequate supply of precursors are available and that this supply of precursors does not limit or stimulate DNA synthesis.

Second, the rate of isotope transfer into the precursor pool should be constant; however, there need not be equilibrium between the exogenous and endogenous pools, which would occur only if there were a free two-way exchange between the two pools. In theory, the simple model for isotope dilution analysis requires that the final precursor pool be in chemical equilibrium with the pool of added radioactive precursor. This would be the case in an ideal reaction vessel in which there was, in effect, one pool and all chemical reactions were reversible. The actual situation is very complex, with transport across the cell wall occurring in only one direction and with many enzymatic reactions, particularly in multienzyme complexes, also occurring in one direction. The model is still valid in this situation, provided that the rate of isotope transfer through an enzymatic pathway and the rate of isotope mixing with unlabeled pools along the pathway remain constant. In addition, the rate-limiting step for isotope incorporation into a macromolecule must be the final one. Isotope dilution analysis measures dilution up to a rate-limiting step. If this step is at the replication fork, i.e., the DNA polymerase step, then the dilution of tritiated dTTP by other sources of dTTP in the pool at the replication fork is measurable.

Given these assumptions, a number of counts (n) should be incorporated into DNA in a given time from a precursor of given specific activity and should be a measure of the rate of synthesis if there is no dilution by a pool of similar compounds in the system. If there is a pool of similar compounds and its size happens to equal that of the added precursor (dilution pool, p = 1), the observed counts (x) in DNA will equal n/2. Thus, x = n/p (p + 1). If, in addition to the labeled precursor, a known quantity of unlabeled precursor is added to give a further dilution, y, then x = n/(p + y + 1). The added pool is y + 1. Upon rearrangement, the expression becomes x + 1 = (1/x)ln n - p. Thus, if the reciprocal of the observed counts (y) is plotted against the dilution (y + 1), a linear relationship is obtained with an intercept of −p, the dilution given by the pool in the system. In practice, it is easier to plot actual quantities of the precursor (Fig. 1), but it should be remembered that the “pool sizes” found are relative quantities and are used only to calculate the dilution of specific radioactivity of the precursor. For example, in the experiment without yeast extract (Fig. 1), 9.1 \times 10^5 dpm [1/0.11 \times 10^{-5}] of radioactivity was incorporated into DNA from 0.8 nmol of labeled thymidine. The specific activity of the added thymidine was 50 µCi/nmol. The intercept was −0.5 nmol. The specific activity of the final precursor was therefore 50 µCi/(0.5 + 0.8 nmol) in that sample. Another way to express this is as the extent of participation of labeled thymidine in DNA synthesis, which in the above example was 0.8/1.3 = 62%.

Because there has been some controversy about the use and specificity of thymidine to measure growth rates of heterotrophic bacteria in natural environments (20), we carried out experiments on the ability of some organisms to take up thymidine and incorporate it into DNA. Further justification for the use of thymidine is desirable, although it has been accepted as a means to measure heterotrophic bacterial production (2, 12, 26). Because some Pseudomonas species cannot incorporate thymidine into DNA, we have investigated this in a range of marine pseudomonads, in a marine cyanobacterium, and in some eukaryotic algae.

**MATERIALS AND METHODS**

DNA from herring sperm and crystalline thymidine were purchased from Sigma Chemical Co., St. Louis, Mo. Amer- sham Australia Pty. Ltd., Sydney; Australia, supplied aqueous solutions (containing 2% ethanol) of [methyl-3H]thymi- dine at a concentration of 1 mCi/ml and specific activity of 40 to 60 Ci/mmole. Purified and sterilized thymine-2,14C-DNA, extracted from Escherichia coli at 15 to 50 µCi/mg, was also purchased from Amersham, whose phase-combining sys- tem, PCS II, was used for liquid scintillation counting of radioactive samples.

**Microorganisms.** The marine pseudomonads and Synecho- cococcus species were kindly donated from the Australian Collection of Marine Microorganisms (Sir George Fisher Centre, James Cook University of North Queensland, Townsville) by J. L. Reichelt. The freshwater Pseudomonas species were supplied by K. C. Marshall, School of Microbi- ology, University of New South Wales, Sydney; three unicellular algal species were obtained from S. W. Jeffreys, Commonwealth Scientific and Industrial Research Organiza- tion Marine Laboratories, Cronulla, Australia. Pseudomo- nads and Synechococcus species were maintained in modi- fied luminous medium and MN medium, respectively, made
with 80% sterilized sea water (1, 42). Algae were grown on nutrient “F” medium (14).

The marine anaerobic bacterial culture was a mixture of organisms isolated from surface sediment in a sea grass bed (Halodule wrightii) in the northeastern Gulf of Mexico. Sediment was incubated under an atmosphere of 95% nitrogen (oxygen free)–5% carbon dioxide in a sterile nutrient medium (1% [wt/vol] glucose, 0.1% [wt/vol] yeast extract) for 2 days. Then fresh medium was inoculated with this suspension. Before and after assays with tritiated thymidine, microbial growth was monitored with a spectrophotometer (540 nm) to ensure that cells were in the log phase of growth during all experiments.

Ability of microorganisms to incorporate [3H]thymidine into DNA. Pseudomonas species were subcultured in nutrient medium containing (per liter) glucose (0.3 g), yeast extract (0.3 g), and peptone (0.6 g) in 80% sterile seawater. A portion (2 ml) of the suspension was sampled and incubated with 10 μCi (0.2 nmol) of tritiated thymidine. After 10 min, 2 ml of 0.6 M NaOH was added, and then DNA was extracted (26). Algal and cyanobacterial samples (2 ml) were incubated with 5 μCi of tritiated thymidine. After 8 min, 2 ml of 0.6 M NaOH was added, and DNA was extracted (26).

Time course assays and isotope dilution analysis were carried out with the mixed anaerobic cultures as follows. Tritiated thymidine (18 μCi; 0.4 nmol) was added to three culture tubes (20 ml), each fitted with a rubber septum in the cap through which syringe needles could be inserted. An additional 0.8, 1.0, and 1.2 nmol of unlabeled thymidine were added to the three tubes. The tubes were flushed with N2-CO2 gas mixture, and then 18 ml of the culture was added. Subsamples were removed at time intervals ranging from 1 to 40 min during purging with N2-CO2 gas mixture to maintain anaerobic conditions. The subsamples were injected into 10 ml of ice-cold trichloroacetic acid (TCA; 2% [wt/vol]) and filtered within 1 h.

Thymidine uptake kinetics. Batch cultures (13 ml) of the species Pseudomonas bathyecetes, P. marina, and Alteromonas undina (subcultured as above) were incubated with 30 μCi of tritiated thymidine. The population density of each species was similar at the time of the experiments; in optical density terms, A. undina was 0.87, P. marina was 0.84, and P. bathyecetes was 0.85. All were in the log phase. Every 30 s, 1 ml was filtered through a 25-mm cellulose acetate filter (pore size, 0.2 μm). The radioactivity in the filtrate was measured.

Chemostat. A chemostat (volume, 475 ml) was set up, containing a continuously mixed suspension of the marine bacterium A. undina. It was supplied at a constant rate (100 ml/h) with an aseptic nutrient medium containing (per liter) glucose (0.3 g), yeast extract (0.3 g), and peptone (0.6 g) in 80% seawater. The culture volume was kept constant; in some experiments, the yeast extract was omitted. The growth rate was calculated from data on nutrient flow rate, culture volume, and the number of cells per milliliter. Bacteria were counted by direct microscopy with a modification of the method of Zimmerman and Meyer-Reil (43) by using acridine orange staining (15) and with equipment described elsewhere (25).

Under steady-state growth conditions, the specific growth rate is equivalent to the dilution rate (30): specific growth rate = flow rate (milliliters per hour)/volume (milliliters); and bacterial growth rate = (flow rate [milliliters per hour]/volume [milliliters]) × number (cells per milliliter) = growth rate (cells per milliliter per hour).

A 15-ml suspension of A. undina (15 ml) from the chemostat was used as the sample. The time course was started by adding 300 μCi of tritiated thymidine, and two samples (2 ml each) were removed at each assay time. The incubation in one sample was stopped by the addition of 2 ml of 0.6 M NaOH, and the DNA was then extracted. In the second sample, the incubation was stopped by the addition of 2 ml of ice-cold 5% TCA, and then the samples were filtered and washed as described below.

Isotope dilution analysis. A. undina was grown rapidly in two chemostats, one with yeast extract (in the nutrient medium) and the other without. Samples were taken from each chemostat (0.4 and 1.0 ml), incubated with tritiated thymidine (10 μCi and 40 μCi, respectively), and diluted with increasing amounts of unlabeled thymidine. After 2 min, samples were immediately filtered and washed with cold 5% TCA.

The rate of DNA synthesis, and hence cell division, was calculated from the rate of thymidine incorporation as described by Moriarty and Pollard (26, 27), except that a conversion factor of 2 × 1010 cells per mol of thymidine incorporated was used (Moriarty, in press). The number of bacterial cells dividing per hour (dX/dt) equals the rate of DNA synthesis (molecules per hour): dX/dt = (5.6 × 109)(dpm incorporated into DNA)/(incubation time [min]/specific activity [Ci/mmol]). Specific growth rate (μ) = (dX/dt)/X, where X is the population density.

Sea water (containing particulate material from decaying sea grass leaves) was collected from an inshore sea grass bed (H. wrightii) in the northeastern Gulf of Mexico. Three isotope dilution experiments were set up in polypropylene bottles with 5, 20, and 50 nM of tritiated thymidine and increasing concentrations of unlabeled thymidine. The assays were started by adding 25-ml portions of the seawater at defined time intervals (generally 30 s apart). After a 20-min incubation with a radioisotope, each sample was immediately filtered. Filters were then washed five times with ice-cold 3% TCA and treated further as described below.

Extraction of radioactively labeled DNA. For some experiments (see Fig. 1 and 3), bacterial DNA was extracted in hot NaOH, cooled, and then precipitated in cold 5% TCA and increasing concentrations of unlabeled thymidine. The assays were started by adding 25-ml portions of the seawater at defined time intervals (generally 30 s apart). After a 20-min incubation with a radioisotope, each sample was immediately filtered. Filters were then washed five times with ice-cold 3% TCA and treated further as described below.

RESULTS

Tritiated thymidine uptake and incorporation into DNA. About 50% of the radioactively labeled thymidine (added to an Alteromonas batch culture) was removed from the extra-
cellular medium within 2.5 min (Fig. 2). Concomitant with rapid uptake was the initiation of the radioactive labeling of DNA (Fig. 3). Radioactivity was incorporated into DNA at a linear rate which was independent of the rate of uptake of tritiated thymidine (cf. Fig. 2 and 3). The rate of incorporation of the isotope into macromolecules that were insoluble in cold TCA was the same as the rate of incorporation in purified DNA (Fig. 3). Thus, DNA was the only macromolecule that was labeled by tritiated thymidine for at least 10 min in the chemostat culture.

**Growth rate measurement.** The growth rates of *A. undina* determined by isotope dilution analysis agreed very well with growth rates calculated by direct microscopy (Table 1). Agreement was good both in experiments in which there was dilution of the isotope as well as in those in which there was no dilution (Fig. 1). Therefore, the rate of labeled thymidine incorporation into DNA can be used to calculate accurately the rate of DNA synthesis.

**Patterns of isotope dilution in seawater.** The degree of isotope dilution in bacteria growing in seawater could be altered by starting with three different concentrations of radioactive thymidine (Fig. 4). At 50 nM, the plot was linear, with no indication of isotope dilution (zero intercept at the ordinate). At 5 nM, the plot could be described as linear below 35 nM total thymidine, with a negative intercept on the ordinate. Above 35 nM, the slope increased, which means that dilution had decreased. With 20 nM tritiated thymidine, data points for total thymidine concentrations greater than 35 nM fell on a line of best fit having an ordinate intercept of zero (implying no isotope dilution). Values for both of the duplicates of 20 nM tritiated thymidine were displaced to the right of the line, indicating that some dilution had occurred at this concentration. Because the seawater was not homogeneous but contained particulate detritus, variation was not unexpected; some points did not fit any pattern.

**TABLE 1.** Comparison of growth rates measured by isotope-dilution analysis and direct microscopy in aerobic chemostats (containing *A. undina*) and an anaerobic batch culture (heterogeneous bacterial population)

<table>
<thead>
<tr>
<th>Environment</th>
<th>Isotope dilution (%)</th>
<th>Specific growth rate/h by <strong>a</strong>:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Direct microscopy</td>
</tr>
<tr>
<td>Aerobic chemostats</td>
<td>0°</td>
<td>0.30 ± 0.04 (21)</td>
</tr>
<tr>
<td></td>
<td>0°°</td>
<td>0.34 ± 0.04 (21)</td>
</tr>
<tr>
<td></td>
<td>44°°</td>
<td>0.29 ± 0.08 (21)</td>
</tr>
<tr>
<td></td>
<td>44°°°</td>
<td>0.22 ± 0.04 (21)</td>
</tr>
<tr>
<td>Anaerobic batch culture</td>
<td>0</td>
<td>0.53 ± 0.1 (10)</td>
</tr>
</tbody>
</table>

* a Specific growth rate is expressed as the mean plus or minus standard error (n). See the text for details of this experiment.
* b Yeast extract present.
* c Yeast extract absent.
The bacterial growth rates calculated for these plots did not differ significantly (Table 2). The abrupt changes in the linearity (Fig. 4) at 35 nM were probably due to changes in the contribution of dTMP by the de novo pathways. In this system of rapidly growing bacteria on detritus in seawater, thymidine concentrations of greater than 35 nM were sufficient to maintain the level of dTTP needed for DNA synthesis, and thus de novo pathways were inhibited.

Ability of microorganisms to incorporate thymidine into DNA. Most of the bacteria studied here incorporated tritiated thymidine into DNA (Table 3). In only two species, *P. marina* and *P. bathycetes*, was DNA not labeled; because these species were unable to remove thymidine from the extracellular medium, it is likely that they lack transport enzymes for thymidine (Fig. 2). These organisms were growing during the experiment as indicated by the change in optical density.

There was no significant incorporation of tritiated thymidine into three species of microalgae, namely *Thalassiosira pseudonana*, *Isosyris galbana*, *Platymonas suecica*. Algal cultures were not axenic. The low levels of tritiated thymidine incorporated into DNA were probably due to bacterial growth. No thymidine was incorporated into the DNA of a marine *Synechococcus* species (Table 4).

**DISCUSSION**

**Regulation of de novo thymidine nucleotide synthesis.** Enzymes in the biosynthetic pathway for thymidine nucleotides and other nucleotides are probably organized in a multienzyme complex associated with DNA polymerase (24). Thymidylate synthetase, which methylates dUMP, is a key enzyme in the de novo synthesis of thymidine nucleotides (22). The supply of dUMP is regulated through the feedback inhibition by dTTP of enzymes in the pathways leading to dUMP (reviewed by Moriarty [in press]). The pool of dTTP used for control is functionally separate from the small, rapidly turning over pool used for DNA synthesis in the DNA polymerase complex (24), but the larger pool is possibly derived from that in the complex. An increase in the size of the cellular dTTP pool can quickly be followed by inhibition of de novo synthesis, leading to rapid depletion of the biosynthetic pool of dTTP. The cellular dTTP cannot penetrate the multienzyme complex and serve as a precursor for DNA synthesis (24). It is by this mechanism that excess thymidine can cause inhibition of de novo pathways and provide the sole source of dTTP for DNA synthesis. If the thymidine supply is insufficient to maintain dTTP at the level required for DNA synthesis, de novo synthesis is not fully inhibited; thus, dilution of the isotope would result. Kuebbing and Werner (23) have shown that when thymidine was added to growing mammalian cells, it was almost immediately incorporated into DNA at full specific activity, thereby blocking any further action of the de novo pathway. A similar mechanism probably operates in bacteria, as our results indicate with the chemostat when yeast extract was used. High concentrations of thymidine (ca. 1 μM) were used in the chemostat compared with natural seawater, because the cell density was very high and growing rapidly. The low concentrations used in seawater (ca. 10 nM) would have been sufficient for only a few seconds of DNA synthesis in the chemostat.

The discussion above illustrates a point that should be

**TABLE 2. Bacterial production rates in seawater at various concentrations of tritiated thymidine**

<table>
<thead>
<tr>
<th>Thymidine concn (nM)</th>
<th>Isotope dilution (%)</th>
<th>Bacterial production rate (10^7 cells/h per liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>76</td>
<td>2.9 ± 0.4</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>50</td>
<td>0</td>
<td>3.6 ± 0.4</td>
</tr>
</tbody>
</table>

*a* Bacterial density was 9.8 (±25) x 10^8 cells per liter; n = 10. Bacterial production rate is expressed as the mean plus or minus standard error. n = 7.

**TABLE 3. Survey of the ability of some marine pseudomonads to incorporate tritiated thymidine into DNA**

<table>
<thead>
<tr>
<th>Bacterial strain (Australian Collection of Marine Microorganisms no.)</th>
<th>Growth rate (ΔA_{540}/time)</th>
<th>Radioactivity incorporated (10^7 dpm/min per ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alcaligenes aquamarinus</em> (135)</td>
<td>0.08</td>
<td>0.06 (3)</td>
</tr>
<tr>
<td><em>A. cholinophagum</em> (822)</td>
<td>0.09</td>
<td>7.71 (3)</td>
</tr>
<tr>
<td><em>A. venustus</em> (168)</td>
<td>0.01</td>
<td>0.55 (2)</td>
</tr>
<tr>
<td><em>A. pacificus</em> (127)</td>
<td>0.07</td>
<td>17.07 (3)</td>
</tr>
<tr>
<td><em>Alteromonas communis</em> (157)</td>
<td>ND</td>
<td>12.92 (3)</td>
</tr>
<tr>
<td><em>A. haloplanktis</em> (129)</td>
<td>ND</td>
<td>85.52 (3)</td>
</tr>
<tr>
<td><em>A. macleodii</em> (827)</td>
<td>0.05</td>
<td>66.37 (3)</td>
</tr>
<tr>
<td><em>A. undina</em> (816)</td>
<td>0.12</td>
<td>146.40 (5)</td>
</tr>
<tr>
<td><em>A. putrefaciens</em> (530)</td>
<td>0.13</td>
<td>30.18 (3)</td>
</tr>
<tr>
<td><em>Pseudomonas bathycetes</em> (136)</td>
<td>0.24</td>
<td>0.003 (2)</td>
</tr>
<tr>
<td><em>P. doudoroffii</em> (167)</td>
<td>0.11</td>
<td>2.27 (3)</td>
</tr>
<tr>
<td><em>P. marina</em> (132)</td>
<td>0.08</td>
<td>0.01 (2)</td>
</tr>
<tr>
<td><em>P. nautica</em> (171)</td>
<td>0.09</td>
<td>0.90 (3)</td>
</tr>
<tr>
<td><em>P. nigrafaciens</em> (821)</td>
<td>0.20</td>
<td>3.17 (3)</td>
</tr>
<tr>
<td><em>Pseudomonas</em> species (freshwater organism)</td>
<td>0.05</td>
<td>0.20 (2)</td>
</tr>
</tbody>
</table>

*a* During the log phase of growth, each culture was incubated with 0.63 μM [methyl-¹H]thymidine for 10 min (*Pseudomonas*) sp. for 100 min. Background radioactivity has been subtracted. All cultures were in the log phase of growth during the experiments; a relative measure of growth during each experiment is given by the change in absorbance at 550 nm (ΔA_{540}/time). Radioactivity incorporated is expressed as the mean (n): ND, Not determined.
noted when applying the thymidine method in natural environments. The minimum concentration of thymidine that should be used will depend on the bacterial density and growth rate. Higher concentrations will be needed for higher densities and faster growth rates. Furthermore, the concentration chosen should be high enough to inhibit de novo synthesis and should eliminate the need to carry out isotope dilution analysis on every sample. Preliminary experiments with isotope dilution analysis would be needed to determine the minimum concentration of thymidine needed. Some workers have attempted to do this by increasing the amounts of radioactive thymidine added at the same specific activity and using the concentration at which an asymptote for incorporation into DNA is reached (2, 13). This method works, provided that there are no endogenous or exogenous pools of thymidine itself. A double-reciprocal plot is needed to show dilution with this technique (16). It is better and easier to use the isotope dilution analysis described previously (9, 10).

An example of the effect of tritiated thymidine concentrations on isotope dilution in a natural situation is given here (Fig. 4). At a high concentration (50 nM), de novo synthesis was inhibited; at the lowest concentration of tritiated thymidine (5 nM), however, insufficient dTTP was synthesized from thymidine, and so de novo synthesis of dTMP also occurred, thus diluting the isotope. At 20 nM tritiated thymidine, the thymidine concentration was not quite enough to supply all of the dTTP required, but above 40 nM total thymidine concentration was sufficient; thus, the middle plot could be extrapolated through point zero for the higher points only (Fig. 4). These experiments show that nonlinear isotope dilution plots will be obtained if the thymidine concentration is only a little less than that needed to supply all of the requirements for DNA synthesis. If it is much less, plots will be linear, at least over a small range of concentrations. This suggests that discrete concentrations of dTTP are needed to effect control of the allostERIC enzymes in the biosynthetic pathways.

Isotope dilution analysis has been criticized on the grounds that the rate of uptake into DNA was dependent on the concentration of substrate supplied (20). As the discussion above shows, the rate of DNA synthesis is independent of the concentration of thymidine supplied, because the de novo synthesis pathways can be turned on to make up any shortfall in supply. The processes of thymidine uptake (i.e., transport into the cell) and its incorporation into DNA are quite distinct processes and should not be confused.

**Isotope dilution and the rate-limiting step.** The multienzyme complex associated with DNA polymerase contains a small but rapidly turning over dTTP pool that is dedicated to DNA replication and is functionally separate from the main cellular dTTP pool (23). It is the in vivo specific activity of this pool that the isotope dilution analysis must measure. This analysis will only measure isotope dilution before the rate-limiting step in this pathway (38). If other steps, such as those involving thymidine transport enzymes or the thymidine kinase, become rate-limiting, any measure of bacterial growth will be seriously underestimated. Since this did not happen with the chemostat experiments, the rate-limiting step must have been at the level of DNA polymerase. The results reported here show that thymidine transport appears to be much faster than the rate of thymidine incorporation into DNA.

Under some circumstances, it is possible that thymidine kinase may become rate limiting for the incorporation of labeled thymidine into DNA. The end product of this biosynthetic pathway, dTTP, has been shown to be an inhibitor of thymidine kinase (17–19, 28, 29). Thymidine competes with dTTP and can reverse the inhibition if its concentration is high enough. In the presence of dTTP and low concentrations of thymidine, the $V_{max}$ of thymidine kinase is low, its $K_m$ is high, and it is rate limiting for thymidine incorporation (38). A probable example of this effect in studies on bacterial growth rates in sediments was observed by us in earlier work, although we did not correctly interpret it then (26). Biphase plots were obtained from isotope dilution experiments (Fig. 5) in which it seems likely that thymidine kinase was inhibited by dTTP originating from de novo synthesis of dUMP. This inhibition was not influenced by the labeled thymidine supplied because the concentration in the cell was likely to have been low. The actual concentration around the cells in these experiments was very low because the large amounts of sediment that were used adsorbed most of the thymidine. At higher concentrations of added thymidine, this inhibition was reversed, and the rate-limiting step moved to thymidine kinase to DNA polymerase. The effect of isotope dilution by thymidine nucleotide synthesis became apparent. Even after the reversal of thymidine kinase inhibition, the degree of participation by added thymidine in DNA synthesis was low; i.e., the concentration supplied was not sufficient to meet the needs of DNA synthesis. In later work, biphase plots were avoided because less sediment was used, thus, effective concentrations of thymidine were higher (27).

The inhibition of thymidine kinase and its reversal by extra thymidine may be seen in an isotope dilution analysis when biphase plots are obtained that tend to the right (see Fig. 5). If a plot bends to the left (see Fig. 4), it indicates that the contribution of de novo synthesis of dTMP is decreasing. No isotope dilution occurred when _Aeromonas_ species was grown in yeast extract nutrient medium (Fig. 3). Sufficient exogenous thymidine was probably available for DNA synthesis via the salvage pathway. Without yeast extract, however, the radioactive label was substantially diluted in DNA (44%), but this dilution was not due to extracellular or intracellular thymidine pools; otherwise, dilution would have been apparent in the experiment with yeast extract. We...
conclude, therefore, that the de novo biosynthetic pathway contributed to the pools of compounds that competed as thymine precursors for DNA synthesis when yeast extract was omitted.

We have established that correct estimates of growth rates were obtained by isotope dilution analysis in the presence and absence of yeast extract; therefore, thymidine kinase in both cases could not have been rate limiting. The dilution in the absence of yeast extract may be due to the activity of the catabolic enzyme thymidine phosphorylase. Escherichia coli lacking the enzyme thymidine phosphorylase readily incorporated 98% of exogenous thymidine into DNA (7). The yeast extract may contain either a phosphorylase inhibitor or sufficient deoxyribose to obviate the need for the degradation of thymidine; thus, enough exogenous thymidine may have been available to supply the requirement of dTTP for incorporation into DNA via the salvage pathway. Conversely, in the absence of yeast extract, phosphorylase may have competed with thymidine kinase for thymidine, thus decreasing the amount of thymidine available for DNA synthesis. De novo synthesis then contributed some of the dTTP needed as a DNA precursor, causing the dilution of tritium in DNA (Fig. 1).

Thymidine uptake by bacteria. Because there are reports of Pseudomonas species which cannot utilize thymidine (32), we investigated a range of marine pseudomonads and found that most incorporated thymidine into DNA (Table 3). It seems likely that most bacteria in the sea do utilize thymidine since good agreement has been found between other methods for estimating growth rates and the thymidine method (2, 13, 21). In the mixed culture of anaerobic bacteria from a sea grass bed, there was good agreement between growth rates determined by direct microscopy and the thymidine method (Table 1). Thus, thymidine is probably used by many common anaerobic bacteria. Those with specialized nutrient requirements, e.g., sulfate reducers, are probably not likely to be able to take up thymidine. The DNA in acetate-utilizing sulfate reducers could not be labeled with tritiated thymidine (unpublished observations). Because these bacteria do not grow efficiently, bacterial production in sediments would not be seriously underestimated with the thymidine method.

Specificity of the thymidine technique. One of the important advantages of using thymidine in studies on microbial ecology is that the growth rates of heterotrophic bacteria can be measured specifically (12, 26). This is because all cyanobacteria and eucaryotic algae and fungi that have been investigated lack thymidine kinase or at least have been shown not to incorporate thymidine directly into DNA. The work reported here with Synechococcus species and algae further supports this argument. Although protozoa contain thymidine kinase, their DNA is unlikely to be labeled in short-term experiments with low concentrations of thymidine (Moriarty, in press). Labeling of nuclear and cytoplasmic DNA in protozoa has been reported, but long time periods were needed (31, 34). A comment has been made that microbial ecology literature is "replete with reports of [H] thymidine incorporation into the DNA of eucaryotic algae, protozoa, yeasts, fungi and slime molds" (20). The first of the reports listed (4) makes no mention of thymidine. In other reports concerning algae and fungi, it is the inability of these organisms to incorporate thymidine into nuclear DNA that is discussed. Autoradiography was used to show that tritiated thymidine did label the chloroplast or cytoplasm of several different genera of these eucaryotes over a long time period (8, 33, 39–42). In every case, no significant labeling of the nuclei was reported. Indeed, Steffensen and Sheridan (39), using three genera of marine algae, Dictyota, Padina, and Bryopsis, found almost all of the radioactivity to be in the cytoplasm, reporting that "nuclei did not incorporate tritiated thymidine even though cells were dividing rapidly in the three genera examined." Sagan (33), in his study of Euglena species and [H]thymidine incorporation, reviewed some of the previous work in this field. In those reports in which the cytoplasm of microalgae was labeled with tritiated thymidine, he concluded that the investigators were observing chloroplast-related metabolism of exogenous thymidine over the long-term incubation periods.

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LITERATURE CITED


23. Kuebbing, D., and R. Werner. 1975. A model for compartmenta-