Consequences of Accounting for Isotopic Dilution in Thymidine Incorporation Assays

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Received 29 December 1987/Accepted 13 April 1988

Rates of thymidine incorporation into DNA were corrected for isotopic dilution by internal nucleotide pools and were compared with rates obtained from uncorrected data. Differences as large as 109% were observed between corrected and uncorrected estimates of thymidine incorporation. The degree of underestimation varied seasonally and, to a lesser extent, spatially.

Measures of the rate of incorporation of exogenously supplied radiolabeled thymidine into DNA have become accepted as estimates of bacterial growth rates. Theoretical aspects of the procedure have been widely discussed (4, 7, 9, 13), and experimental protocols vary. Variations in experimental protocols revolve around the concentration of exogenously supplied thymidine and the measurement of dilution of radiolabeled thymidine by intracellular pools of deoxyribonucleotides (2, 7-9, 11).

The concentration of exogenously supplied thymidine should be sufficient to saturate uptake systems and ensure a stable uptake rate. This is usually accomplished by adding thymidine at below 20 nM (1, 2, 9, 11). However, a constant uptake rate does not ensure that a sufficient supply of thymidine precursors (as dTTP) will be available to maintain a given rate of DNA synthesis. To meet the demands of DNA synthesis, dTTP precursors may have to be synthesized de novo by cells and used to supplement precursors obtained by salvage uptake (7).

De novo synthesis of precursors may dilute the specific activity of exogenously supplied thymidine during incorporation into DNA (7). The level of dilution will depend on the concentration of exogenously supplied thymidine, uptake rate, and then-current demands of DNA synthesis (7). The major consequence of not accounting for dilution of the specific activity of the label is an underestimate of thymidine incorporation into DNA and, therefore, an underestimate of bacterial secondary production. Apart from the diel study of Bell (1), there is no assessment for field data of the impact of the dilution of the specific activity of labeled thymidine on estimates of thymidine incorporation into DNA. This paper compares rates of thymidine incorporation into DNA derived from data which are corrected and uncorrected for isotopic dilution.

Samples were collected from Lake Arlington (32°42'30"N, 97°12'30"W) (3, 5) 16 times between July 1985 and July 1986. Samples were collected at midday with a 2-liter VanDorn bottle from a site located over the deepest portion of the lake. Sampling depths corresponded to 93, 58, 37, 18, and 4% of nonreflected surface irradiance. The annual average depths in meters corresponding to these light levels were (mean ± standard error) 0.02 ± 0.01 (93%), 0.23 ± 0.04 (58%), 0.65 ± 0.06 (37%), 1.19 ± 0.10 (18%), and 2.20 ± 0.19 (4%). Samples were placed in opaque polyethylene bottles and transported in iced coolers to laboratory facilities, where analytical procedures were initiated within 1 h of sample collection. Surface and subsurface irradiances were measured with a spherical sensor photometer (Li-Cor Corp.). Water temperature was measured at 1-m intervals with a submersible thermistor (Applied Research Austin) and ranged from 9°C in January to 32°C in August. The water temperature was uniform throughout the photic zone during each sampling period and was above 25°C from June to late September.

Isotopic-dilution analysis was used to estimate the dilution of added isotope by intracellular deoxyribonucleotide pools (8). All subsamples (20 ml) received 10 nM labeled thymidine (70 to 90 Ci mmol⁻¹; ICN Pharmaceuticals, Inc.). Unlabeled thymidine (Sigma Chemical Co.) was added to additional subsamples to create a range in final thymidine concentrations of 10 to 50 nM. Incubations were for 1 h in the dark at in situ temperatures. Thymidine uptake was calculated from disintegrations per minute accumulated at 10 nM additions or from disintegrations per minute (at 10 nM) predicted from linear regression analysis. Preliminary experiments indicated that thymidine uptake systems saturated at approximately 10 nM and that the uptake was linear for at least 2 h. Incubations were terminated by the addition of 2 ml of 4% sodium azide in 37% formaldehyde. A duplicate set of samples from the 35% light level was used as a control for all light levels and killed prior to the addition of thymidine.

The degree to which exogenously supplied thymidine participated in DNA synthesis (degree of participation [DP]) was calculated as DP = \([RI(C + P)/SA] \times 100\), where \(R\) was the amount (in microcuries) of radioactivity added per sample, \(C\) was the amount (in nanomoles) of labeled thymidine yielding \(R\), \(P\) was the amount (in nanomoles) of the "pool" nucleotides diluting \(C\), and \(SA\) was the specific activity (in microcuries per millimole) of the exogenously supplied thymidine. The "pool" size was the negative \(x\) intercept from plots of reciprocal uptake (y axis) against thymidine concentration (x axis) (see reference 8 for a detailed description of this method and references 6, 10, and 12 for criticisms).

Labeled macromolecules were extracted from cells by using cold trichloroacetic acid (4) and collected on 0.2-µm-pore-size cellulose nitrate filters (25 mm; Schleicher & Schuell, Inc.). Filters were dissolved by shaking overnight at room temperature in 10 ml of 3a70B scintillation cocktail.

The percentages of label appearing in DNA, RNA, and protein were determined by acid-base hydrolysis (11). The amount of label appearing in DNA was determined by subtraction. An average of 53.9 ± 4.7% (n = 5; experiments conducted August through December) of added label was found in DNA, and all uptake data were corrected with this percentage.
Isotopic dilution plots were usually linear, but occasional deviations from linearity occurred at 40 nM (right shift as described in reference 8). The degree to which labeled thymidine participated in DNA synthesis (DP) varied temporally and spatially and appeared to be affected by temperature (analysis of variance [ANOVA], \( P < 0.003; \) Fig. 1). As temperature increased during the year, the DP decreased (Pearson’s \( r = -0.47, n = 85, \) and \( P < 0.001)\). During the winter months the DP was high, typically above 80\%, whereas during the summer months the DP was typically below 60\%. Spatial variations in DP occurred mainly during the summer months. The DP tended to be high in near-surface waters and low in near-metalimnetic waters. Because of differences in winter-summer depth distributions, the DP was not significantly affected by depth (annual data set, ANOVA, \( P > 0.1\)); however, the DP was negatively correlated to depth \((r = -0.23, n = 80, P < 0.03)\).

The temporal and spatial variations in the DP suggest that during the summer, bacteria in the lower part of the photic zone were growing rapidly enough to force de novo synthesis of 40 to 50\% of the precursors required to support DNA synthesis. Winter growth rates were apparently slow enough to permit salvage pathways to supply essentially all of the precursors required for DNA synthesis.

If the specific activity of labeled thymidine obtained by salvage pathways is diluted by de novo synthesis of precursors and this dilution is not accounted for, then rates of thymidine incorporation into DNA should be underestimated. The degree of underestimation should be dependent on the DP of labeled thymidine in DNA synthesis. If growth rates are slow and salvage pathways can meet the requirements of DNA synthesis, then slight differences between uptake rates calculated by isotopic dilution and uptake rates uncorrected for isotopic dilution should be expected. However, if growth rates are rapid and the demand for thymidine cannot be met by salvage pathways alone, then the discrepancy between isotopic-dilution-corrected uptake and uptake rates uncorrected for isotopic dilution should increase.

To test this, thymidine uptake rates corrected for isotopic dilution were compared with uptake rates uncorrected for isotopic dilution, under the assumption that increases in thymidine uptake rates correspond to increases in growth rates (Fig. 2). Both approaches yielded similar seasonal distributions in uptake, with relatively high incorporation rates during the summer and low rates during the winter. Despite similar distributions, the data sets were significantly different when compared on the basis of analysis type (corrected to uncorrected, ANOVA, \( P < 0.0001)\), time of sampling (ANOVA, \( P < 0.0001)\), or interaction of analysis type and time of sampling (ANOVA, \( P < 0.0002)\). It is apparent (Fig. 2) that accounting for isotopic dilution did little to alter incorporation estimates during the colder months of the year (November through February), when independent measures of activity indicate a relatively inactive assemblage (acetate and glutamate uptake, either as total or cell specific; J. G. Hubbard, M.S. thesis, The University of Texas at Arlington, Arlington, 1986). Except for the data for January, uptake rates corrected for isotopic dilution were not statistically different from uncorrected data (by the paired \( t \) test, minimum \( n \) for each comparison was 5, and \( P > 0.05)\). However, during other months, when heterotrophic activity is normally high (acetate and glutamate uptake, either as total or cell specific; Hubbard, M.S. thesis), the difference in incorporation rates became pronounced. Thymidine incorporation rates calculated from isotopic dilution analysis were significantly higher than rates calculated from uncorrected data (by the paired \( t \) test, minimum \( n \) for each comparison was 5, and \( P < 0.05)\), and in some cases, isotopic-dilution-corrected uptake rates were double the uncorrected uptake rates. While substantially different in magnitude, the means calculated for each form of analysis for June and July were only marginally different statistically \((P = 0.06 \) for both months). This resulted from high variability brought about by depth variation in the DP (Fig. 1).

It is clear from these data that accounting for the dilution of exogenously supplied thymidine by de novo synthesis of precursors has an impact on estimates of radiolabel incor-
poration into DNA. Accounting for isotopic dilution by de novo synthesis of precursors yields significantly higher estimates of thymidine incorporation into DNA than when uncorrected data are used but does little to change seasonal patterns of uptake. In light of criticisms of the isotopic dilution procedure (6, 10, 12), it remains to be determined, through further experimentation and discussion, if such a protocol should be routinely applied.

LITERATURE CITED