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# Consequences of Thymidine Catabolism for Estimates of Bacterial Production: An Example from a Coastal Marine Sediment

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# Consequences of thymidine catabolism for estimates of bacterial production: An example from a coastal marine sediment

*Abstract*—Radioactively labeled thymidine (TdR) has been used extensively to measure bacterial production in aquatic environments, but critical assumptions of the TdR technique often have gone untested. In this study of a coastal marine sediment, the metabolic fate of *methyl* [³H]TdR and *methyl* [¹⁴C]TdR was at variance with the assumptions necessary for determining bacterial production. Only 2% of incorporated radioactivity was recovered in the DNA fraction of TCA-insoluble material following time-course incubations of 1–300 min. At least the methyl group of TdR was extensively catabolized, as shown by copious production of ¹⁴CO₂. The temporal patterns of ³H : ¹⁴C ratios in macromolecular fractions indicated that products of catabolism were recycled into the DNA fraction. The accuracy of the TdR technique depends in large part on the degree to which such catabolism occurs.

Estimation of bacterial production in aquatic environments is of interest to microbial ecologists, organic geochemists, limnologists, and oceanographers, among others. There has been a recent proliferation of such estimates, accompanied by controversy regarding the technique of choice (e.g. Hagström et al. 1979; Karl 1986; Moriarty 1986; Fuhrman et al. 1986; Karl and Winn 1986). Many investigators use radiolabeled precursors of nucleic acids and, for example, incorporation of radioactivity into deoxyribonucleic acid (DNA) as a measure of bacterial growth. Undoubtedly the most popular precursor has been the nucleoside thymidine (thymidine deoxyribose, hereafter TdR); *methyl* [³H]TdR is the most frequently used form. The expectation has been

that TdR in natural samples is incorporated specifically and directly into DNA of heterotrophic bacteria over incubation times ranging from minutes to hours (Fuhrman and Azam 1980, 1982; Moriarty and Pollard 1981; Pollard and Moriarty 1984).

Several assumptions are associated with estimating bacterial production in nature with the TdR technique (Brock 1971; Karl 1986; Moriarty 1986). Incorporation of label must be linear with time and due only to bacterial uptake of TdR. Radioactivity in DNA must result from direct incorporation of labeled TdR, not from recycling of degradation products of catabolized TdR. If production is estimated by radioassaying unfractionated, acid-insoluble material, one must assume that radioactivity is not present in acid-insoluble macromolecules other than DNA. Violation of any of these assumptions would decrease the accuracy of the production estimate.

Previous studies indicate that radioactivity from labeled TdR may be incorporated into TCA-insoluble macromolecules other than DNA (i.e. RNA, protein, and possibly lipids) (e.g. Riemann et al. 1982; Findlay et al. 1984; Robarts et al. 1986; Hollibaugh 1988); this phenomenon has been referred to as "nonspecific labeling" (Karl 1982; Moriarty 1986). We became interested in the degree and extent of nonspecific labeling while investigating the utility of *methyl* [³H]TdR as a tracer of bacterial grazing by meiofauna in marine sediment (Dobbs et al. in prep.). We observed that after a 5-h incubation more ³H was incorporated into nonpolar lipids than into DNA. These observations formed the impetus for the present study, in which we conducted a more thorough investigation of the fate of labeled TdR in a coastal marine sediment. We used a mixture of *methyl* [³H]TdR and *methyl* [¹⁴C]TdR to determine the extent and temporal pattern of catabolism of TdR and nonspecific labeling of macromolecules. This dual-label approach also provided evidence that radioactivity appeared in DNA and other macromolecules via recycling of label from catabolized TdR, even after very brief incubations.

On 22 June 1987, we hand-collected 27 cores of intertidal marine sediment using 2.1-cm-i.d., 10-cm-long, plastic core liners.

Sediment at the study site (500 m west of the F.S.U. Marine Laboratory; 29°55.00″N, 84°30.36″W) is a medium-fine quartz sand. A distinct transition from light- to dark-colored sediment indicated that approximately the top 0.5 cm of sediment was oxidized. Silt-clay and organic content of the sand were each <1% by weight. About 2 cm of water overlay the sediment at collection. Cores were placed in an insulated container filled with seawater (to maintain ambient temperature) and transported to the laboratory. Incubations were started within 2 h of collection.

Radioisotopes were used shortly after purchase (Research Products Int. Corp.). Immediately before use, radiolabels were dried under a stream of nitrogen and reconstituted in filtered (0.2 $\mu$m) seawater. After reconstitution, total $\mu$Ci ml$^{-1}$ were determined separately for [³H]TdR and [¹⁴C]TdR. The isotopes were then mixed and total $\mu$Ci ml$^{-1}$ for ³H and ¹⁴C were determined simultaneously with a dual-label program (discussed below). A mixture of 9.02 $\mu$Ci of *methyl* [³H]TdR (43 Ci mmol$^{-1}$) and 1.97 $\mu$Ci of *methyl* [¹⁴C]TdR (50 mCi mmol$^{-1}$) was injected horizontally 2 mm below the sediment surface of the cores (Findlay et al. 1985) with a 50-$\mu$l syringe (Hamilton Co.). The ratio of radioactivity ($\mu$Ci ³H : $\mu$Ci ¹⁴C) added to cores was 4.58. Cores were incubated for 1, 10, 30, 60, 180, and 300 min after injection of isotopes. Cores were incubated in the dark to prevent photosynthetic fixation of any potential respired ¹⁴CO$_2$. (Dark fixation of ¹⁴CO$_2$ in the top 1 cm of this sediment is negligible: Carman pers. obs.; therefore fixation of respired ¹⁴CO$_2$ by chemoautotrophic bacteria was assumed to be unimportant.) Three replicate cores were used for each incubation period.

At the end of an incubation period, the water overlying the sediment was removed, its volume measured (Fig.1), and a 4-ml subsample was taken to determine its ¹⁴CO$_2$ content (Hobbie and Crawford 1969; Iverson et al. 1976). Because only ¹⁴CO$_2$ that had diffused from sediments into the overlying water was measured, ¹⁴CO$_2$ data should be considered as a minimum and delayed estimate of respired TdR. Next, the top 1 cm of sediment was extruded, homoge-

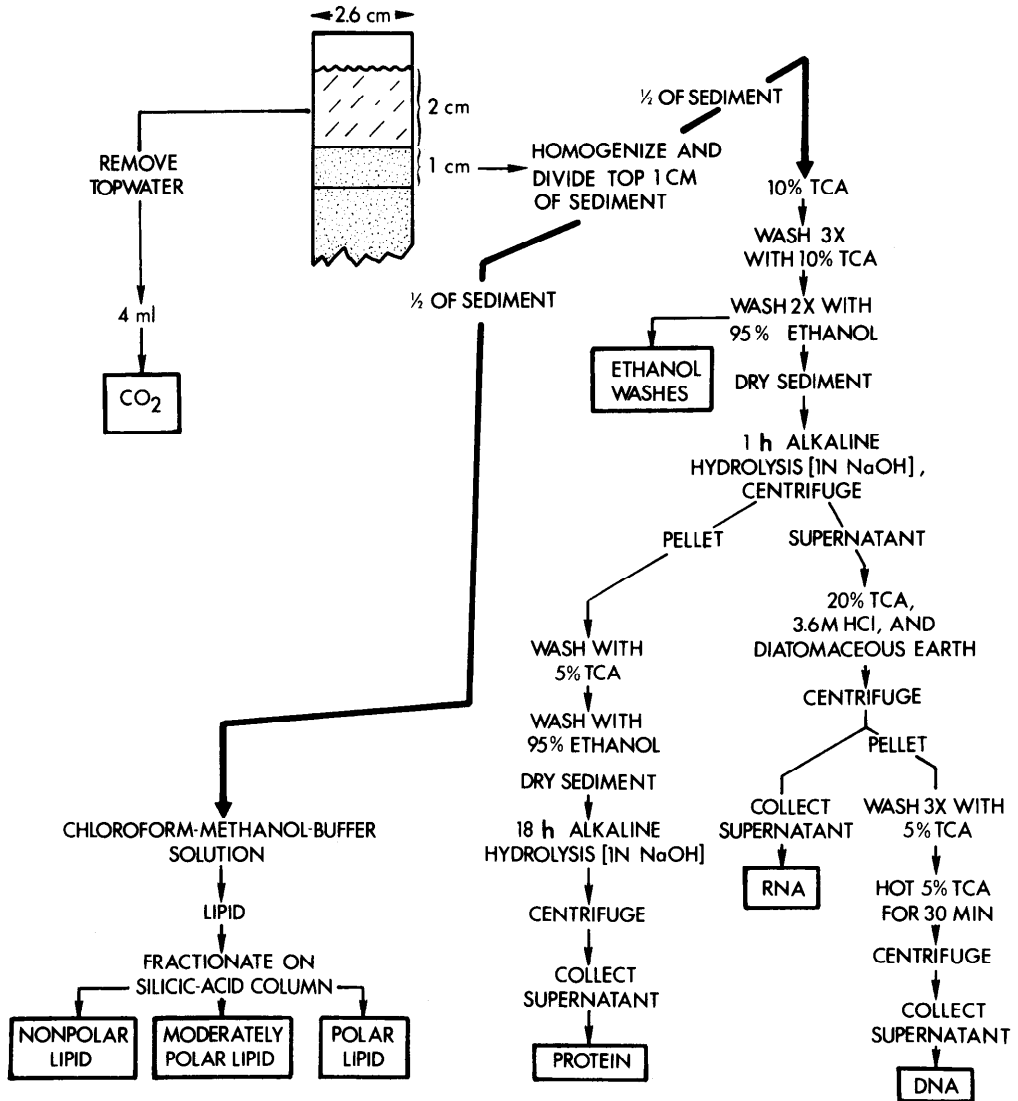Fig. 1. Procedure used to analyze sediment cores injected with *methyl* [³H]TdR and *methyl* [¹⁴C]TdR. Components that were radioassayed are enclosed in boxes. Further details are provided in the text.

nized, and divided approximately in half. Half of the sediment was placed in ice-cold 10% TCA. DNA and RNA fractions were extracted from acid-insoluble material according to the type 2 procedure of Craven and Karl (1984) for noncarbonate sands. The Craven and Karl procedure includes two ethanol washes of TCA-insoluble material before separation of macromolecular fractions. These ethanol washes were also radioassayed.

After removing RNA and DNA fractions, the protein fraction was separated from the

sediment pellet with a modification for sediments of Karl's (1982) method for filters. Specifically, the pellet was washed twice with ice-cold 5% TCA and twice with ice-cold 95% ethanol, then dried. Ten milliliters of 1 N NaOH were added to the pellet, which was held for 18 h in a water-bath shaker at 37°C. The material was vortexed several times during hydrolysis. Afterward, the material was centrifuged for 10 min at 1,800 × *g* and the supernatant radioassayed.

The other half of the sediment was placed in a chloroform-methanol solution, and lip-

Table 1. Control experiments. Radioactivity recovered in macromolecular fractions from formaldehyde-killed sediments incubated for 1 and 300 min, and extraction controls (*see text for details*). EtOH wash—ethanol wash. Values represent the mean (SD) of three replicates. Data are expressed as dpm gdw$^{-1}$.

| | Formaldehyde killed (min incubated) | | | | Extraction control | |
|---|---|---|---|---|---|---|
| | 1 | | 300 | | | |
| | $^3$H | $^{14}$C | $^3$H | $^{14}$C | $^3$H | $^{14}$C |
| **Fractions of TCA-insoluble material** | | | | | | |
| DNA | 0* | 0 | 0 | 0 | 0 | 0 |
| RNA | 366(276) | 60(33) | 474(124) | 86(28) | 683(69) | 97(15) |
| Protein | 113(178) | 2(20) | 177(115) | 23(6) | 0 | 0 |
| EtOH wash | 615(527) | 131(118) | 676(112) | 174(32) | 2,238(594) | 495(137) |
| **Lipids** | | | | | | |
| Nonpolar | 7(7) | 0 | 4(2) | 0 | 34(12) | 7(4) |
| Moderately polar | 5,822(6,363) | 1,273(1,438) | 4,130(3,829) | 910(865) | 42,669(6,758) | 9,449(1,481) |
| Polar | 229(205) | 48(63) | 225(175) | 49(37) | 1,447(235) | 316(110) |
| CO$_2$ | — | 294(83) | — | 302(140) | — | — |

\* Raw dpm (i.e. unadjusted for subsampling and weight of sediment) <10 were considered 0 (SD not calculated).

ids were extracted according to the method of White et al. (1979). Bulk lipids were separated into nonpolar (neutral), moderately polar (glyco-), and polar (phospho-) lipid fractions (Guckert et al. 1985) with prepacked, 3-ml, 500-mg silicic-acid columns (Thomas Scientific).

Radioactivity was quantified with a dual-label program on an LKB Wallac liquid-scintillation counter (model 1217). Background counts were subtracted from sample counts before counts per minute (cpm) were converted to disintegrations per minute (dpm) by the method of external standards-channels ratio. Ethanol washes of TCA-insoluble material and lipid fractions were bleached with light to reduce color quenching and evaporated under a stream of nitrogen before adding 10 ml of Handifluor (Mallinckrodt). Handifluor was also used as a scintillation cocktail for the radioassay of $^{14}$CO$_2$. Protein was radioassayed by adding 10 ml of Hionicfluor (Packard Instr. Co.) to 1 ml of protein extract. DNA and RNA were radioassayed by adding 10 ml of Picofluor (Packard Instr. Co.) to 1 ml of extract.

The reliability of the dual-label program was tested with a mixture of $^3$H and $^{14}$C ($^3$H:$^{14}$C ratio = 4.58) at dilutions that encompassed the dpm measured in experimental samples. The dual-label program yielded ratios accurate to within 6% of expected ratios (4.58) at levels comparable to radioactivity present in most of our samples (i.e. 400–40,000 dpm of $^3$H). At levels near

the practical detection limit, $^{14}$C was over-estimated by ~40%. Thus, at the lowest levels of radioactivity in macromolecular fractions (i.e. the DNA and protein fractions at 1 and 10 min), the amount of $^{14}$C present was overestimated. No attempt was made to correct for this bias. As discussed below, however, overestimation of the amount of $^{14}$C at brief incubation times makes our interpretation of the data conservative.

Two types of control experiments determined the importance of nonbiologically incorporated radioactivity. In one experiment, long-term (300 min) and short-term (1 min) incubations were performed on formaldehyde-killed samples to determine if adsorption of TdR to sediments contributed to the radioactivity recovered in macromolecular fractions over time. The overlying water and top 1 cm of sediment from six cores were removed and placed in glass jars. Two milliliters of 37% formaldehyde (stock solution) and radiolabels (same quantity as experimental cores) were added to each jar. Sediment and overlying water were assayed as described above. Radioactivity in macromolecular fractions did not change appreciably from 1 to 300 min (Table 1), indicating that adsorption of TdR to sediment was not responsible for the increasing amounts of radioactivity recovered in macromolecular fractions from unpoisoned sediments.

A separate experiment was performed to determine the amount of radioactivity that
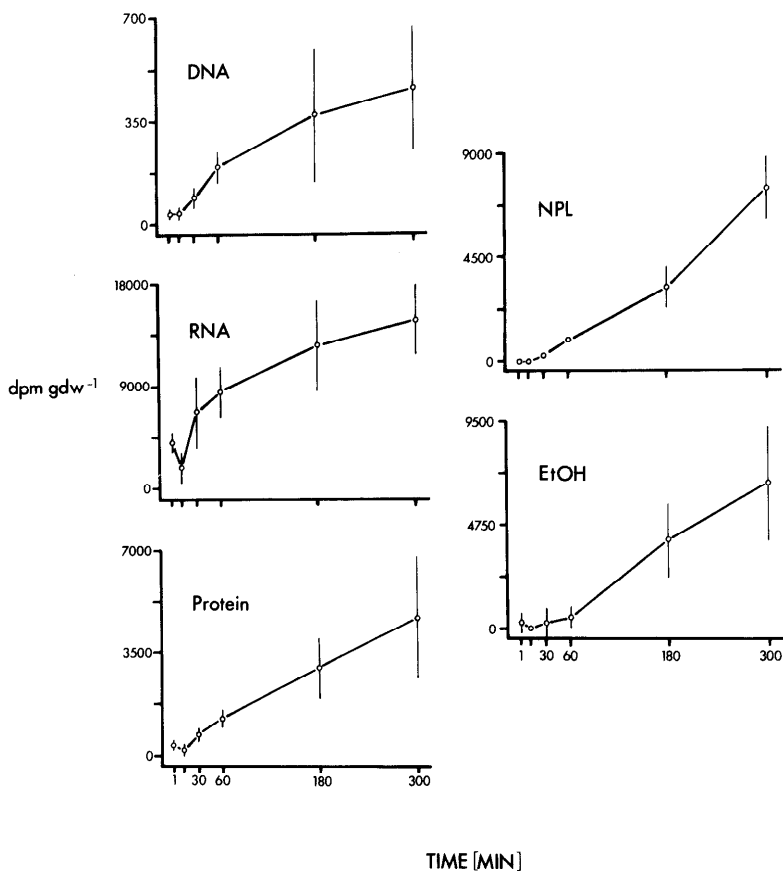
TIME [MIN]

Fig. 2. Temporal patterns of incorporation of ³H (from [³H]TdR) into fractions of TCA-insoluble material and the nonpolar-lipid fraction. Radioactivity in extraction controls (Table 1) has been subtracted. Values represent means ($n = 3$) ±1 SD. NPL—nonpolar-lipid fraction; EtOH—ethanol washes of TCA-insoluble material.

appeared in macromolecular fractions simply as the result of the extraction procedure (i.e. without the possible confounding influence of formaldehyde). We refer to this experiment as the "extraction control." From each of three cores, the top 1 cm of sediment was divided in half, and radiolabeled TdR was added immediately after sediment was placed in TCA or chloroform-methanol. Macromolecular fractions were separated as above. The design of the extraction control precluded the assay of $^{14}CO_2$ in overlying water.

Data from sediment extractions were normalized to sediment weight and expressed as dpm per gram of dry weight of sediment (dpm gdw⁻¹). Mean dpm gdw⁻¹ in extraction controls (Table 1) was subtracted from dpm gdw⁻¹ in experimental incubations.

With two exceptions, radioactivity recovered in molecular fractions from unpoisoned-sediment incubations (Figs. 2 and 3) was much higher than radioactivity in extraction controls (Table 1). The exceptions were moderately polar and polar-lipid fractions, in which counts were as high in extraction controls (Table 1) as in experimental incubations (data not shown). These fractions will not be considered further.

Data from sediment incubations were compared with those obtained from a laboratory culture of an aerobic, heterotrophic bacterial isolate from the study site. By using cells in log-phase growth (*see below*), the expectation was that nearly all radioactivity should have been incorporated into DNA. A dual-label mixture of TdR (17.13 μCi *methyl* [³H]TdR and 3.54 μCi *methyl*
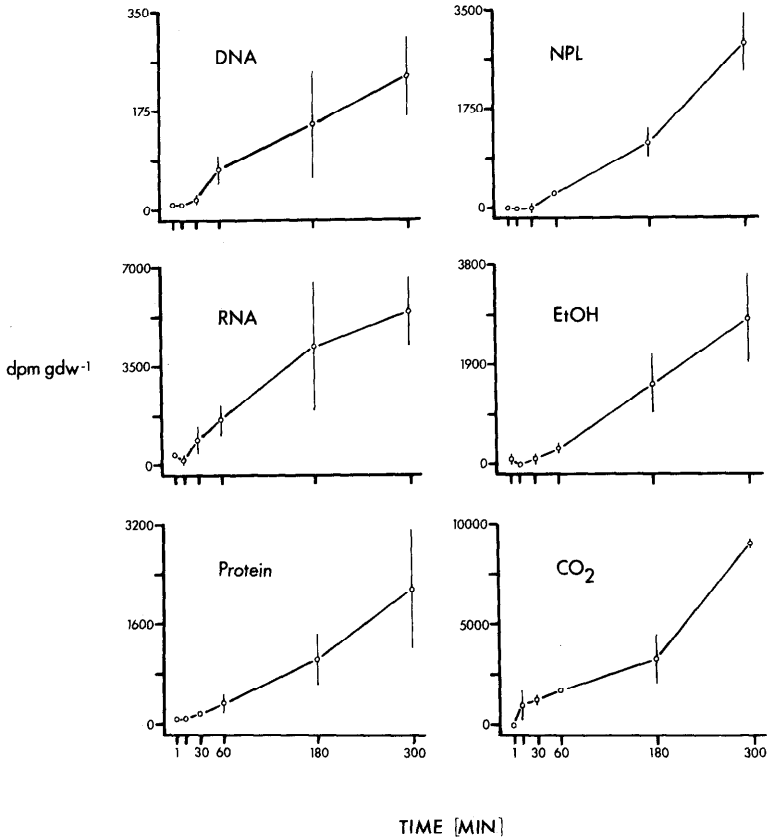
Fig. 3.   Temporal patterns of incorporation of $^{14}C$ (from [$^{14}C$] TdR) into fractions of TCA-insoluble material, the nonpolar-lipid fraction, and $CO_2$. Format as in Fig. 2, except the 300-min value for $CO_2$ is based on only two replicates. $CO_2-CO_2$ in overlying water.

[$^{14}C$]TdR) was added to a 30-ml culture in early log-phase growth ($\sim2.5 \times 10^7$ cells ml$^{-1}$). The incubation was ended 2 h later ($\sim4.0 \times 10^7$ cells ml$^{-1}$), and nine 3-ml sub-samples were removed to test tubes. Cells were pelletized ($1,800 \times g$, 10 min) and washed three times with 2.5% NaCl solution. Diatomaceous earth (0.25 g) was added to three pellets, autoclaved sediment (2.0 g) from the study site was added to three pellets, and three pellets received no additions. Macromolecular fractions of TCA-insoluble material were then separated with the same procedure that was applied to sediment samples.

Results of the incubations of unpoisoned sediments are presented in Figs. 2 and 3. Data from 10-min incubations tended to deviate from otherwise consistent temporal patterns of incorporation. The anomaly in dpm gdw$^{-1}$ was reflected in relative incor-

poration and, to a lesser degree, in $^3H$ : $^{14}C$ ratios. We have no explanation for the anomalous 10-min data. When discussing temporal patterns, it is with the implicit caveat that these data may be exceptional.

Tritium (Fig. 2) and $^{14}C$ (Fig. 3) radioactivity in DNA, RNA, and protein fractions increased steadily with time. Relative to DNA, RNA, and protein fractions, incorporation of radioactivity into the nonpolar-lipid fraction lagged over the first three incubation periods, but increased markedly from 60 to 300 min. A similar pattern occurred in the ethanol washes of TCA-insoluble material. Quantitatively, dpm in the nonpolar-lipid fraction were similar to dpm in ethanol washes (Figs. 2 and 3). $^{14}CO_2$ was barely detectable after 1 min, $12\pm7$ (mean$\pm$SD) dpm gdw$^{-1}$, but rapidly increased thereafter (Fig. 3).

Of the $^3H$ accounted for in the DNA,

RNA, and protein fractions, 2% or less was present in the DNA fraction (Fig. 4) over the range of incubation times. From 1 to 300 min, the proportion of ^{3}H in the protein fraction increased from 8 to 23% and decreased from 91 to 75% in the RNA fraction. The proportions of ^{14}C in components of TCA-insoluble material were qualitatively and nearly quantitatively identical to those observed for [3]H. Relative to incorporation into DNA, RNA, and protein fractions, the amount of ^{14}C appearing as [14]CO$_2$ steadily increased with time and accounted for most (58%) of the radioactivity after 300 min (Fig. 4).

The low percentage of radioactivity recovered in the DNA fraction could have been due, in part, to the high (micromolar) concentrations of TdR that we used. Hollibaugh (1988) found that, in general, percent incorporation of label into the DNA fraction of estuarine bacterioplankton was an inverse function of *methyl* [^{3}H]TdR concentration. Karl (1982), however, found greater incorporation of 2-[^{14}C]TdR (at micromolar concentrations) than *methyl* [^{3}H]TdR (at nanomolar concentrations) into the DNA fraction of freshwater bacterioplankton. Fallon and Newell (1986) found that the incorporation rate of TdR was nonsaturable over a wide range of TdR concentrations ($10^1$–$10^5$ nM). Moriarty and Pollard (1981) advocated the use of high concentrations of TdR to stop de novo synthesis of TdR. Micromolar concentrations of TdR were used in our experiment with the bacterial isolate (Table 2), and about 80% of the radioactivity in TCA-insoluble material was recovered in the DNA fraction. Thus, although TdR concentration may influence the manner in which TdR is used by natural assemblages of bacteria, even the direction of the relationship is unclear.

Another factor that could have influenced these results is the procedure used for separating macromolecular fractions. Moriarty (1986) referred to Munro and Fleck (1966) in contending that cold-acid precipitation followed by hot-alkaline hydrolysis degrades DNA, with the result that radioactivity recovered in the RNA fraction is partially from degraded DNA. However, Munro and Fleck clearly stated that DNA is degraded if acid-insoluble material is rinsed
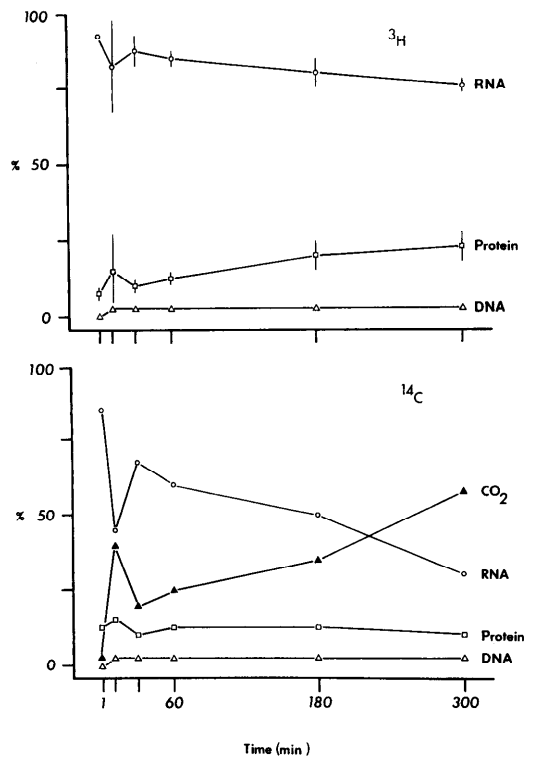


Fig. 4. Percent radioactivity in molecular fractions incubated with [^{3}H]TdR and [^{14}C]TdR. Total radioactivity equals the sum of radioactivity in all components shown. Values represent means (*n* = 3, except for 300-min data for ^{14}C, where *n* = 2). Error bars on ^{3}H data are ±1 SD; error bars for DNA are less than the width of the symbol. For clarity, SD is not shown for ^{14}C data, but is of the same magnitude as for [3]H.

with hot ethanol or the alkaline hydrolysis is carried out at 100°C. In our study, ice-cold ethanol was used and the hydrolysis was performed at 37°C. Moreover, the general procedure described by Craven and Karl (1984) is consistent with the method recommended by Munro and Fleck for fractionating nucleic acids. When we used Craven and Karl's procedure to separate nucleic-acid fractions from a bacterial culture in log-phase growth, we obtained results consistent with theoretical expectations. That is, most (80%) of the incorporated radioactivity was recovered in the DNA fraction (Table 2). Further, recovery of radioactivity in the DNA fraction was not diminished by the presence of sediment. Karl (1982) found that the general procedure used in the present study yielded internally consistent results, but the propor-

Table 2. Experiment to determine the pattern of labeling in macromolecular fractions of TCA-insoluble material from a bacterial culture incubated with *methyl* [³H]Tdr and *methyl* [¹⁴C]TdR, and the effect of sediment or diatomaceous earth on the extraction procedure. Three different treatments, each consisting of three replicates, were compared: bacteria only (B), bacteria and autoclaved sediment (B+AS), bacteria and diatomaceous earth (B+DE).

| | ³H | | | | ¹⁴C | | | |
|---|---|---|---|---|---|---|---|---|
| | DNA | RNA | Protein | EtOH wash | DNA | RNA | Protein | EtOH wash |
| Average (SD) dpm per 3 ml of culture in fractions of TCA-insoluble material | | | | | | | | |
| B | 11,047 | 2,407 | 543 | 4,830 | 2,415 | 460 | 220 | 979 |
| | (1,432) | (245) | (573) | (611) | (305) | (104) | (157) | (66) |
| B+AS | 15,186 | 1,473 | 1,917 | 991 | 3,340 | 270 | 403 | 178 |
| | (1,061) | (255) | (427) | (88) | (228) | (35) | (38) | (36) |
| B+DE | 12,742 | 1,860 | 1,437 | 2,254 | 2,760 | 383 | 410 | 567 |
| | (521) | (191) | (265) | (968) | (176) | (60) | (105) | (83) |
| Average (SD) percent of combined dpm | | | | | | | | |
| B | 78.9 | 17.2 | 3.8 | — | 78.2 | 14.7 | 7.1 | — |
| | (4.6) | (1.1) | (3.8) | — | (4.6) | (1.6) | (4.7) | — |
| B+AS | 81.8 | 7.9 | 10.3 | — | 83.2 | 6.7 | 10.1 | — |
| | (1.9) | (0.8) | (2.1) | — | (0.4) | (0.6) | (1.0) | — |
| B+DE | 79.4 | 11.6 | 8.9 | — | 77.7 | 10.8 | 11.5 | — |
| | (1.8) | (1.5) | (1.6) | — | (3.8) | (1.9) | (2.7) | — |

tion of label from TdR recovered in the DNA fraction varied according to the environment (56–87% in coastal water; 89–95% in bacterial culture; 4–6% in a freshwater pond) and with location of the label on the TdR molecule. Therefore, although it is recognized that no fractionation procedure is 100% efficient, there is no evidence that our results are artifacts of the extraction procedure used.

A considerable amount of radioactivity was recovered in the ethanol washes of TCA-insoluble material from control experiments (Table 1), the bacterial isolate (Table 2), and incubations of unpoisoned marine sediments (Figs. 2 and 3). Radioactivity in the ethanol washes from unpoisoned sediments was at least partially due to biological incorporation, as evidenced by the increasing recovery of radioactivity over time. Although ethanol washes of acid-insoluble material are recommended by some investigators (Munro and Fleck 1966; Kennell 1967; Craven and Karl 1984), they are not included in the TdR technique most commonly used (i.e. Fuhrman and Azam 1980, 1982). Ethanol washes were originally included in the fractionation procedure to remove phospholipids from TCA-insoluble material (Munro and Fleck 1966). Robarts et al. (1986) concluded that radioactivity in

the ethanol rinses of cold-TCA precipitates was due to incorporation into lipids, but later decided that this radioactivity represented unincorporated TdR (Wicks and Robarts 1987). Our data suggest that ethanol washes remove two pools of radioactivity: unincorporated, possibly adsorbed thymidine that is not removed by washes with cold TCA and biologically incorporated radioactivity equivalent to radioactivity recovered in the nonpolar-lipid fraction.

Our results underscore the importance of isolating the DNA fraction from other macromolecular fractions (as a minimum requirement) when estimating bacterial productivity of natural systems with the TdR technique (e.g. Karl 1982, 1986; Robarts et al. 1986; Servais et al. 1987; Hollibaugh 1988). In water-column studies, it is codified methodology (Parsons et al. 1984) to regard the radioactivity in the cold-TCA-insoluble fraction as being incorporated exclusively into DNA. Had we done so in this study of sediments, we would have overestimated incorporation of radioactivity into the DNA fraction by a factor of 50–100.

Use of TdR to measure bacterial growth rates in nature was pioneered by Brock (1971), who carefully stipulated the technique's assumption (p. 50) "that there is nothing intrinsically different about the way
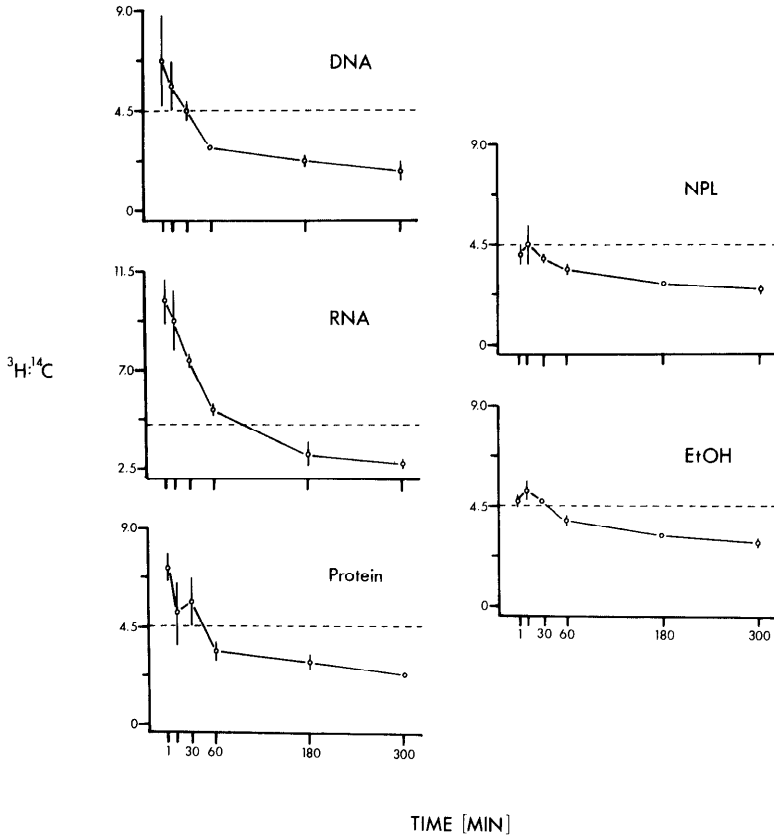
TIME [MIN]

Fig. 5. Temporal pattern of $^3$H:$^{14}$C ratios in macromolecular fractions and the ethanol washes of TCA-insoluble material, and the nonpolar-lipid fraction. Values are means ($n = 3$) ±1 SD. Dashed lines designate the $^3$H:$^{14}$C ratio of TdR that was added to sediment, i.e. 4.58. NPL—nonpolar-lipid fraction; EtOH—ethanol washes of TCA-insoluble material.

cultures and natural samples incorporate tritiated thymidine." Clearly our results attest to a vast difference in the metabolic fate of TdR supplied to bacteria in culture (Table 2) vs. bacteria in marine sediments (Fig. 4). Recent reports (Fallon and Newell 1986; Servais et al. 1987) have suggested that, under nutrient-limiting conditions, TdR is more likely to be used as a carbon and energy source than as a precursor of DNA. Most bacteria in sedimentary systems are not growing (Novitsky 1987; Carman pers. obs.), a circumstance that could be the result of nutrient limitation. We suggest, therefore, that the high degree of catabolism and nonspecific labeling observed in this study may have been due to the use of TdR as a source of carbon and energy by a nutrient-limited assemblage of sedimentary bacteria.

Inspection of $^3$H:$^{14}$C ratios in macro-molecular fractions (Fig. 5) provides insight as to how radioactivity was incorporated. If macromolecules had been labeled predominantly by proton transfer, $^3$H:$^{14}$C ratios would have consistently been above 4.58 (the ratio at which radioactivity was added). If macromolecules had been labeled primarily by transmethylation, the $^3$H:$^{14}$C ratio would not have changed with time. Similarly, if DNA had been labeled only by direct incorporation of TdR, the $^3$H:$^{14}$C ratio in the DNA fraction should have been consistently around 4.58. None of these patterns was observed. Instead, at incubation times of 1 and 10 min, the $^3$H:$^{14}$C ratio was above 4.58 in DNA, RNA, and protein fractions. In all fractions, the ratio decreased with time to well below 4.58 at incubation times of 180 and 300 min.

From these observations, and the result

that at least the methyl group of TdR was extensively catabolized, we propose an explanation of the $^3$H : $^{14}$C ratio of incorporation into macromolecular fractions: Catabolism of *methyl* [$^3$H]TdR results in the loss of $^3$H, chiefly as $^3$H$_2$O, which has a low specific activity when mixed with the cell's internal pool of water. On the other hand, in addition to $^{14}$CO$_2$, $^{14}$C-labeled organic products are produced as *methyl* [$^{14}$C]TdR is catabolized. If $^3$H$_2$O is released earlier in the catabolic process than $^{14}$C organics, however, macromolecules would be enriched in $^3$H after brief incubations as the result of proton transfer. Over longer incubations, $^{14}$C-organic molecules would be incorporated into macromolecules via anabolic pathways. In short, carbon from catabolized TdR is more likely to be conserved and recycled over time than is hydrogen. Thus, recycling of products of TdR catabolism would result in macromolecules with $^3$H : $^{14}$C ratios that decrease with time.

The $^3$H : $^{14}$C ratio in each macromolecular fraction did decrease with time, but the rate of decrease and absolute values of ratios differed among the various fractions (Fig. 5). These differences may reflect different rates of macromolecular synthesis and turnover or different mechanisms of incorporation. For example, $^3$H : $^{14}$C ratios in the RNA fraction were relatively high at short incubation times and rapidly decreased thereafter. From the above argument on recycling of labels, the pattern of ratios observed in the RNA fraction would be expected for macromolecules with a relatively high rate of synthesis and turnover.

In contrast to the RNA fraction, $^3$H : $^{14}$C ratios in the nonpolar-lipid fraction were near the "expected" value of 4.58 over incubation times from 1 to 30 min (Fig. 5). The ratios then decreased from 60 to 300 min. This pattern, in conjunction with the lag in incorporation over brief incubation times (Figs. 2 and 3), suggests that radioactivity in the nonpolar-lipid fraction may have been due to secondary incorporation by eucaryotes (i.e. grazing). Unlike the macromolecules DNA, RNA, and protein, relatively little nonpolar lipid is produced by bacteria (Gehron and White 1982), but it is an important storage product of eucaryotes (Benson et al. 1973). Secondary incorporation into nonpolar lipids should result in further enrichment of $^{14}$C relative to $^3$H as bacterial biomass is catabolized and resynthesized by eucaryotes. Further, the absolute values and temporal patterns of $^3$H : $^{14}$C ratios were similar in ethanol washes and the nonpolar-lipid fraction. These observations support the earlier suggestion that biologically incorporated radioactivity recovered in ethanol washes was of the same macromolecular origin as that recovered in the nonpolar-lipid fraction.

We emphasize that a time-course study was necessary to interpret the manner in which labels were recycled. For example, if only a 30-min incubation had been performed, it would have appeared that $^3$H and $^{14}$C were incorporated into DNA in the proportion they were added. The $^3$H : $^{14}$C ratio at any given incubation time represents the transient integration of a dynamic process. The temporal pattern of $^3$H : $^{14}$C ratios in the DNA fraction (Fig. 5) indicates that radioactivity was incorporated by recycling of labels. We suggest therefore that labeling of DNA occurred, at least partially, from recycling of products of TdR catabolism and de novo synthesis of purines and pyrimidines.

Quantitative determination of macromolecular synthesis with radiotracer techniques requires that the in situ specific activity of the labeled precursor to the macromolecule of interest is known (Karl 1986; Moriarty 1986). If radioactivity appears in DNA by de novo synthesis of purines and pyrimidines, however, the biochemical pathways of incorporation into DNA cannot be determined reliably and thus knowledge of the specific activity of dTTP is of little value.

In conclusion, we emphasize that our data emanate from studies of a coastal marine sediment, and it would be premature to generalize to other environments. Although nonspecific labeling by TdR has been reported from various aquatic environments, our observation of 2% incorporation into the DNA fraction is low in comparison to most other reports (*but see* Servais et al. 1987; Hollibaugh 1988). This result may be

due partially to the relatively high concentrations of TdR that we used or imperfect separation of macromolecular fractions; it may also be due to the diverse physiologies of sedimentary bacteria. Our results suggest that nonspecific labeling in the environment examined is symptomatic of catabolism, which is a prerequisite for recycling of labels into all macromolecules, including DNA. Catabolism of TdR will adversely affect the accuracy of TdR-based estimates of bacterial production, even if DNA is isolated from other macromolecules.

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# Dark production of $H_2O_2$ in the Sargasso Sea

*Abstract*—Hydrogen peroxide is introduced into the marine environment by wet and dry deposition and is produced there by photochemical reactions. Dark production, particularly by the biota, has not been demonstrated in situ. To investigate dark $H_2O_2$ production, we adapted a method of measuring $H_2O_2$ for use in the field. The method uses the enzyme-catalyzed oxidation by $H_2O_2$ of hydroxyphenylpropionic acid to a fluorescent dimer; its detection limit is about 3 nM. Results of a field study in the Sargasso Sea (32°N, 62°W) confirmed several general characteristics of $H_2O_2$ distributions—a surface maximum and a diel cycle in surface waters. Data from the Sargasso Sea also demonstrated that dark, probably biological, production was occurring at significant rates at depths of 40–60 m.

Hydrogen peroxide occurs in surface ocean water at a concentration of about 10–

200 nM (Van Baalen and Marler 1966; Zika 1984). As an important intermediate in redox processes involving oxygen, this reactive species may profoundly influence the chemistry and biological activity of the oceans, particularly through its interaction with transition metals and organic compounds. The interaction of $H_2O_2$ with the biota may also occur directly, for example, through a recently demonstrated activity as a chemotactic agent (Haeder 1984; Nultsch and Kumar 1984). Elucidation of the role of $H_2O_2$ in marine systems, however, still requires a better understanding of its distribution and dynamics.

Hydrogen peroxide in the marine environment can be introduced via wet and dry deposition (Zika et al. 1982; Thompson and Zafiriou 1983) and can originate from photochemical reactions (Cooper and Zika 1983). It can also be produced by the biota. We have recently reported on the production of $H_2O_2$ by a marine coccolithophorid in the dark (Palenik et al. 1987). In order to investigate the importance of biological sources of $H_2O_2$ in the marine environment, we adapted a method of measuring $H_2O_2$