APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1990, p. 1245–1254
0099-2240/90/051245-10$02.00/0
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Vol. 56, No. 5

Catabolism of Tritiated Thymidine by Aquatic Microbial Communities and Incorporation of Tritium into RNA and Protein†

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Received 25 October 1989/Accepted 5 February 1990

The incorporation of tritiated thymidine by five microbial ecosystems and the distribution of tritium into DNA, RNA, and protein were determined. All microbial assemblages tested exhibited significant labeling of RNA and protein (i.e., nonspecific labeling), as determined by differential acid-base hydrolysis. Nonspecific labeling was greatest in sediment samples, for which ≥95% of the tritium was recovered with the RNA and protein fractions. The percentage of tritium recovered in the DNA fraction ranged from 15 to 38% of the total labeled macromolecules recovered. Nonspecific labeling was independent of both incubation time and thymidine concentration over very wide ranges. Four different RNA hydrolysis reagents (KOH, NaOH, piperidine, and enzymes) solubilized tritium from cold trichloroacetic acid precipitates. High-pressure liquid chromatography separation of piperidine hydrolysates followed by measurement of isolated monophosphates confirmed the labeling of RNA and indicated that tritium was recovered primarily in CMP and AMP residues. We also evaluated the specificity of [2-^{3}H]adenine incorporation into adenylate residues in both RNA and DNA in parallel with the [^{3}H]thymidine experiments and compared the degree of nonspecific labeling by [^{3}H]adenine with that derived from [^{3}H]thymidine. Rapid catabolism of tritiated thymidine was evaluated by determining the disappearance of tritiated thymidine from the incubation medium and the appearance of degradation products by high-pressure liquid chromatography separation of the cell-free medium. Degradation product formation, including that of both volatile and nonvolatile compounds, was much greater than the rate of incorporation of tritium into stable macromolecules. The standard degradation pathway for thymidine coupled with utilization of Krebs cycle intermediates for the biosynthesis of amino acids, purines, and pyrimidines readily accounts for the observed nonspecific labeling in environmental samples.

The use of [*methyl*-^{3}H]thymidine (^{3}H-TdR) to estimate bacterial growth rates in nature was introduced by Brock (4). His autoradiographic procedure was elegant but highly specialized and, therefore, had only limited application in aquatic microbial ecology. Fuhrman and Azam (15) later introduced a more generalized ^{3}H-TdR procedure and explicitly stated the assumptions necessary to determine bacterial production in oceanic samples. Although ^{3}H-TdR has now been used in diverse environments by numerous researchers for nearly a decade (Table 1), these investigations have neither developed a uniform method (2, 39, 41, 49, 54) nor received universal acceptance (24; D. M. Karl and A. M. Brittain, submitted for publication).

The original expectation (15), that a theoretically derived factor could be used to convert the radioactivity in a trichloroacetic acid (TCA) precipitate of a ^{3}H-TdR-labeled microbial assemblage to heterotrophic bacterial carbon production, has not been realized. As the method is now most commonly used, an empirical conversion factor is derived by comparing ^{3}H-TdR labeling to increases in bacterial cell numbers or total biovolume after sample dilution with particle-free (filtered; pore size, 0.2 μm) water or selective filtration treatments (8, 16, 27, 34, 43). A range in excess of 100-fold has been reported for these empirical conversion factors (8, 27, 34). Two parameters which greatly influence the conversion factor are the degree of isotope dilution by exogenous and endogenous pools of thymidine and the percentage of the total TCA-insoluble radioactivity that is contained in DNA. Fuhrman and Azam (15) originally assumed that ^{3}H-TdR is incorporated almost exclusively into DNA, although they subsequently determined that 5 to 20% of the TCA-insoluble radioactivity was not in the DNA pool (16, 21). Since that time, the incorporation of tritium from ^{3}H-TdR into macromolecules other than DNA has been widely reported (Table 1), and every study that determined the labeling of individual macromolecular pools has observed some nonspecific labeling.

The nonspecific labeling results obtained to date have been, in part, contradictory due to the lack of a uniform method for the isolation and purification of individual macromolecular pools. For example, the filter precipitation method as used by Fuhrman and Azam (15, 16) and many others has been recently reported to be potentially flawed due to absorption of unincorporated ^{3}H-TdR to the TCA precipitate (45). This absorbed thymidine is removed by rinsing with a lipophilic solvent, such as cold ethanol (EtOH), but remains bound to the filter in the presence of acid. The exact method used to isolate the separate macromolecular pools is, therefore, crucial to an understanding of thymidine catabolism. Fuhrman and Azam (15) cited Luria (30) for their method, but then departed significantly from the Luria method by retaining the precipitated macromolecules on filters, as opposed to centrifugation, and by deleting the crucial lipophilic solvent rinse (30). Moriarty (32) used a NaOH extraction technique that precludes the recovery of RNA. Karl (23) used the acid-base differential hydrolysis procedure, originally developed by Schmidt and Thannhauser (48). This latter method uses centrifugation and extensive washing with cold EtOH (see Materials and Methods), and while it is tedious, it has been used successfully by biochemists for decades.

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TABLE 1. Ubiquity of nonspecific ^{3}H-TdR labeling for a variety of marine and freshwater habitats

Sample type and location	Distribution of tritium (%) in acid-insoluble materials			Extraction method used[a]	Reference
	DNA	RNA	Protein		
Marine					
Offshore (California)	80	NR[b]	NR	F/A	15
Offshore (British Columbia)	82	NR	NR	F/A	21
Offshore (Hawaii)					
Surface	21	79	NR	Karl	24
Deep (700 m)	53	47	NR	Karl	24
California Bight	65–85	NR	NR	F/A	16
Drake Passage (Antarctica)	20–90	NR	NR	F/A	18
Baltic Sea	80	NR	NR	F/A	1
North Sea	66–82	NR	NR	ENZ	49
Delaware Bay	0–30[c]	NR	30–100	F/A	26
Charlotte Harbor (Florida)	50	30	20	F/A	40
Coastal zone (Belgium)	55	20	25	ENZ	49
Coastal zone (Hawaii)	68	32	NR	Karl	24
Bayboro Harbor (Florida)	25	50	25	F/A	22
Scripps Pier (California)	29–80	NR	20–70	F/A	51
Mediterranean Sea					
Surface	21–83	NR	NR	ENZ	49
Deep (700 m)	43–65	NR	NR	ENZ	49
Fresh water					
Lakes (Denmark)	5–25	28–49	14–49	F/A	43
Lake (Indiana)	80	NR	NR	F/A	29
Lake Michigan	40–60	NR	NR	F/A	47
Lake (South Africa)	15–91	1–47	0–37	F/A	46
Lake (Denmark)	15–75	15–72	0–40	F/A	52
Reservoir (South Africa)	33–103	NR	NR	Other	54
Meuse River (Belgium)	36–38	15–28	41–43	ENZ	49
Lake Arlington (Texas)	54	NR	NR	F/A	6
Lawrence Lake (Michigan)	16–85[c]	NR	NR	F/A	8
Lake Oglethorpe (Georgia)	15–30	23–46	30	F/A	31
Lake Vechten (Netherlands)	0–63[d]	NR	NR	F/A	3
Krauss Pond (Hawaii)	36	53	11	Karl	24
Sediments					
San Francisco Bay (California)	0–35	34–67	20–59	F/A	20
Beach sand (Hawaii)	5.8	59.9	NR	Karl	36
Sediment trap materials (Atlantic Ocean)	16	63	21	Karl	10

[a] F/A, Fuhrman and Azam (16); Karl, Karl (24); ENZ, enzymatic; other, alternate method.
[b] NR, Not reported.
[c] Probably includes RNA.
[d] Also reported "negative" (<0%) values.

During the course of this investigation of nonspecific labeling of natural microbial communities by ^{3}H-TdR, we used methods for the complete hydrolysis of RNA, followed by high-pressure liquid chromatography (HPLC) to separate, isolate, and quantify the amount of tritium in each RNA monomer pool. The production and incorporation of degradation products of ^{3}H-TdR by natural populations of bacteria were also examined. These experiments were designed to determine whether thymidine is used as more than a precursor to DNA, and our results, presented below, suggest that it is.

MATERIALS AND METHODS

Sample collection. Several microbial ecosystems were examined for nonspecific labeling. Routine experiments utilized a mixed algal and bacterial community from Krauss Pond, a 0.5-m-deep eutrophic fish pond on the University of Hawaii campus. Samples were collected by hand into 250-ml Nalgene containers, and incubation experiments began within minutes. Kahana Bay seawater and sediment samples were collected from the mouth of a shallow estuary on the windward shore of Oahu, Hawaii. The water samples were collected from the sea surface by using a 1-m^2 Nitex mesh screen sampler (17) and from 10 cm below the surface and 10 cm off the bottom by using a polycarbonate sampling bottle. Total water depth was approximately 1 m. Sediment samples were obtained by coring with a 10-cm-diameter Plexiglas tube to a minimum depth of 15 cm. The complete core with overlying seawater was transported back to the laboratory. The interface was removed by aspiration (35), and the consolidated sediment was extruded 1 cm at a time, placed into a beaker, and manually homogenized with a spatula before use. Samples from the North Pacific Gyre were collected during the VERTEX Sea-3 research cruise to 33°N and 139°W aboard the research vessel *Wecoma* (26 January 1987 to 10 February 1987). Water samples were collected by using 14-liter Niskin bottles attached to a CTD-rosette sampler. Sediment traps were used to collect sinking particulate matter. For these experiments we used the live trap solution and deployment configuration previously described

by Karl and Knauer (25). A set of replicated, closed ecosystems (14) was established by using a sediment sample collected from Kaneohe Bay on the windward shore of Oahu, Hawaii. The bulk sediment, primarily coarse-grained sand and coral rubble, was first thoroughly homogenized in a blender (for 2 min) with overlying seawater (60 g of wet sediment plus 600 ml of seawater). This sediment-seawater slurry was then used as the inoculum for the closed ecosystem culture experiments. A 50-μl portion of the slurry was added to 8 ml of bicarbonate-amended ASN-III medium (37, 44) contained in a series of 20-ml Vacutainer tubes. The systems were sealed tightly and incubated for 2 months in an environmental chamber at 200 microeinsteins m^{-2} s^{-1} and 22°C. Two hundred eighty replicate closed ecosystems were prepared at time zero, and triplicate samples were sacrificed for each determination.

Incubation and extraction of labeled macromolecules. The radioisotopes ^{3}H-TdR (70.4 Ci $mmol^{-1}$) and [^{3}H]adenine (^{3}H-Ade) (16 Ci $mmol^{-1}$) were diluted to 100-μCi ml^{-1} stock solutions and stored as recommended by the manufacturer (Dupont, NEN Research Products). The radiochemical purity of both ^{3}H-TdR and ^{3}H-Ade was monitored by HPLC, and ^{3}H-TdR was occasionally repurified (see below). Tritiated precursors were generally added at 1 μCi ml^{-1} (14.2 nM TdR) for water samples and 5 μCi ml^{-1} of slurry (71 nM TdR) for sediment samples. Typically, our time course experiments were conducted on time scales from tens of minutes to several hours, except for the open-ocean VERTEX experiments, which lasted up to 30 hours. Labeling experiments in the laboratory were performed at 22°C in Erlenmeyer flasks attached to a gyrotatory shaker table (~25 rpm). VERTEX samples, in Nalgene polycarbonate bottles, were incubated on the deck of the ship in a surface-seawater-cooled incubator (10°C). All incubations were terminated by being filtered through either Whatman glass fiber filters (GF/F type) or Millipore mixed acetate filters (GS type). The filters were either processed immediately or stored frozen (−20°C) for subsequent processing. Selected experiments utilized degraded ^{3}H-TdR, which was produced by freezing the stock solutions for up to 6 months. This procedure is known to cause autoradiolysis of ^{3}H-TdR (11) and was done to preclude objections based on the suggestion that only impure ^{3}H-TdR labels RNA. Other experiments were conducted as isotope dilution experiments (33) by the addition of unlabeled TdR to parallel incubations.

The method of Karl (23) was used to process the filters in order to quantify the magnitude of nonspecific labeling and as a basis for comparison of the other hydrolysis procedures. The particulate materials collected on the GF/F filters were initially extracted for a period of at least 1 h (but no longer than 12 h) in Vacutainer tubes (16 by 125 mm) with 4 ml of 5% (wt/vol) TCA at 4°C to precipitate the macromolecules. Nonradioactive DNA and RNA (1 mg each) were added to enhance precipitation and to allow for the posthydrolysis detection and quantification of the RNA and DNA monomers. The filters were then homogenized at 4°C with a tissue grinder and rinsed with an additional 4 ml of cold TCA. The homogenates were centrifuged (2,500 × g, 8 min), and the supernatants, containing acid-soluble materials, were discarded. The samples were washed (i.e., suspended and centrifuged as described above) once more with cold TCA and three times with 8 ml of cold EtOH (95%, 4°C). At this point, one set of samples was processed by the standard method of Karl (23), which entailed, in brief, NaOH extraction (1 N, 37°C, 1 h) to hydrolyze RNA, hot TCA extraction (5% TCA, 30 min, 100°C) to hydrolyze DNA, and a final NaOH extraction (1 N, 37°C, 18 h) to hydrolyze proteins. Each hydrolysis step was followed by reprecipitation and centrifugation of the residual nonreactive macromolecules, removal of a subsample of the supernatant containing the hydrolysis products for liquid scintillation counting, and washing of the pellet with three rinses of cold EtOH, as described above, before the next extraction step.

Parallel samples for HPLC analyses were concentrated onto Millipore GS filters, and cold acetone (4°C, 100%) was substituted for the last two ethanol rinses in order to dissolve and remove the cellulose acetate filter materials. RNA was completely hydrolyzed to monomers by using 10% redistilled piperidine (19); neither NaOH nor KOH was effective for complete RNA hydrolysis to monomers (A. M. Brittain, M. S. thesis, University of Hawaii, 1989). The macromolecules from each acetone precipitate were transferred into 5-ml breakneck ampoules by using two 0.5-ml rinses of 10% redistilled piperidine, and the ampoules were sealed with an oxypropane flame. The samples were hydrolyzed at 100°C for 90 min. The hydrolysates were simultaneously neutralized and concentrated by drying them at 60°C with a stream of CO_2 gas.

In one series of experiments, KOH hydrolysis (1 N, 37°C, 1 and 108 h) and RNase A hydrolysis (6 h, 37°C) (13) were compared with both NaOH hydrolysis and piperidine hydrolysis. With selected samples, the DNA remaining after piperidine treatment was hydrolyzed with 88% formic acid (180°C, 40 min) in sealed ampoules to yield nucleosides and bases (9) and subsequently evaporated to dryness under a stream of warm (60°C) N_2 gas.

Before HPLC analysis, the dried piperidine hydrolysates were redissolved in 1 ml of phosphate buffer (0.05 M KH_2PO_4, pH 3.0) and eluted isocratically into individual nucleotide monophosphate fractions by using a strong anion exchanger column (Alltech Spherisorb, 5-μm packing, 25 by 0.46 cm). This HPLC separation procedure will be hereafter referred to as method A. The column eluent was collected automatically by using a fraction collector (Gilson, model no. 201) either directly into Nalgene film tubes for scintillation counting or into glass test tubes for further chemical analysis.

The formic acid hydrolysate was rehydrated in HPLC buffer 1 (0.01 M potassium acetate, pH 5.0) and separated on a reverse-phase C-18 column (RP-18 Spheri-5', Brownlee Labs). The nucleosides and bases were eluted with a linear gradient that started with 95% buffer no. 1 and 5% buffer no. 2 (acetonitrile and water [25:75]) and ended, after 16 min, with 50% buffer no. 1 and 50% buffer no. 2 (9). This procedure will be hereafter referred to as HPLC method B. The column eluent was also collected for scintillation counting as described above.

The rate of disappearance of ^{3}H-TdR (80 nCi ml^{-1}) from the incubation medium was determined in a Krauss Pond incorporation experiment. The loss of ^{3}H-TdR was measured at preselected time intervals by separation of a GF/F filtrate subsample using HPLC method B and collection of the TdR peak for liquid scintillation counting. The HPLC system void volume (the fraction that eluted between 0 and 3 min) was collected to determine the content of ^3H-H_2O. A volatile fraction was determined by evaporating a subsample of the filtrate at 60°C under N_2. The radioactivity of the residue was determined and subtracted from the total radioactivity in the filtrate to give a volatile fraction. The labeling of DNA, RNA, and protein by ^{3}H-TdR was determined by the standard hydrolysis method (as described above) over the same time points.

TABLE 2. Incorporation of ^{3}H-TdR and ^{3}H-Ade into acid-insoluble macromolecules by samples collected from a variety of aquatic ecosystems

Sample type [date taken (mo/yr)]	Precursor added[a]	Acid-insoluble radioactivity recovered (nCi ml^{-1})	Distribution of radioactivity (%)[b]		
			RNA	DNA	Protein
Krauss Pond (7/86)	Ade	16	82	11	3.8
	Tdr	1.5	92	4.7	3.3
Krauss Pond (1/84)	Ade	10.4	73	25	1.2
	Tdr	1.3	83	6.8	11
Krauss Pond (6/88)	Ade	3.2	67	18	15
	Tdr	0.17	75	12	13
Closed ecosystem	Ade	0.6	84	11	4.9
	Tdr	0.042	86	3.8	10
Closed ecosystem	Ade	1.0	89	6.8	4.8
	Tdr	0.09	89	7.5	3.7
Kaneohe Bay sediment (10/87)	Ade	86	59	26	15
	Tdr	16.5	92	4.7	3.3
Kaneohe Bay sediment (11/87)	Ade	134	61	23	16
	Tdr	19	90	4.9	4.8

[a] Precursors (*methyl*-^{3}H-TdR and 2-^{3}H-Ade) were added at concentrations of 14.2 and 67 nM, respectively, for all incubations. Incubation times ranged from 1.5 to 4 h, but the distribution of radioactivity was generally independent of incubation time (see text).

[b] Distribution into acid-insoluble macromolecular fraction was determined by differential acid-base hydrolysis (see text).

^{3}H-TdR purity. The purity of ^{3}H-TdR was determined by using HPLC method B. A ^{3}H-TdR stock solution that had been allowed to undergo autoradiolysis was separated, and the activity of the TdR fraction was compared with the activity of the total injection. This procedure was also used to purify ^{3}H-TdR by injecting several millicuries of ^{3}H-TdR and collecting the eluted TdR peak.

RESULTS

Ubiquity of nonspecific labeling. In every natural microbial assemblage that we examined, radiolabeled RNA and protein were produced during incubation with ^{3}H-TdR (Tables 2 through 6, Fig. 1). The highest percentage of incorporation of ^{3}H-TdR into DNA (31.9%) occurred in the photic zone at the VERTEX sample site (Table 4), and the lowest percentage (2.1%) was observed in the Kahana Bay water-sediment interface sample (Table 3). Our field results can be generalized into four categories on the basis of the specificity of DNA labeling: (i) oligotrophic euphotic zone (25 to 30%), (ii) oligotrophic aphotic zone (18 to 26%) decreasing with increasing water depth, (iii) eutrophic waters (5 to 15%), and (iv) sediment samples (<5%).

TABLE 3. Incorporation of ^{3}H-TdR and ^{3}H-Ade into acid-insoluble macromolecules by water and sediment samples collected from Kahana Bay

Sample type and location (depth [cm])	Precursor added[a]	Acid-insoluble radioactivity recovered (nCi)[b]	Distribution of radioactivity (%)[c]		
			RNA	DNA	Protein
Air-water interface	Ade	41.3	76	21	3.2
	Tdr	0.41	34	7.8	58
Seawater (10)	Ade	15.3	79	19	2.2
	Tdr	0.73	39	12	50
Seawater (90)	Ade	5.6	61	35	4.3
	Tdr	0.76	30	13	57
Water-sediment interface	Ade	76.8	79	19	1.8
	Tdr	9.9	89	2.5	8.2
Sediment (0–2)	Ade	52.9	85	12	2.9
	Tdr	5.2	88	4.2	7.8

[a] See Table 2 for details. All incubations were for 1 h.

[b] Total nanocuries recovered were determined on a per-milliliter basis for water samples and a per-gram basis for the water-sediment interface and sediment samples.

[c] Distribution into acid-insoluble macromolecular fraction was determined by differential acid-base hydrolysis (see text).

TABLE 4. Incorporation of ^{3}H-TdR into macromolecules as a function of depth for a variety of samples collected at a station in the North Pacific Ocean

Sample type and depth (m)	Acid-insoluble radioactivity recovered (nCi liter^{-1})	Distribution of radioactivity (%)[a]		
		RNA	DNA	Protein
Water column[b]				
1	41.9	41.1	27.2	31.5
20	58.3	48.9	27.4	23.6
50	52.7	37.5	31.0	31.6
150	15.1	39.5	31.9	28.6
200	9.2	69.5	18.5	12.1
250	6.9	59.3	27.2	13.6
300	4.9	66.0	26.0	8.1
350	3.9	71.9	17.5	10.6
400	2.8	73.5	14.6	12.0
Sediment trap[c]				
100	110	62.9	22.9	14.1
250	49.2	46.7	28.0	25.3
400	70.4	48.4	17.8	33.8

[a] Distribution into acid-insoluble macromolecule fraction was determined by differential acid-base hydrolysis (see text). Results are averages of duplicate samples.

[b] Water samples were incubated with 100 μCi of ^{3}H-TdR liter^{-1} (1.42 nM) for 30 h.

[c] Sediment trap samples were incubated with 1 mCi of ^{3}H-TdR liter^{-1} (14.2 nM) for 13 h.

TABLE 5. Incorporation of ^{3}H-TdR into acid-insoluble macromolecules in water samples collected from the North Pacific Ocean

Depth (m)	Incubation time (h)	Acid-insoluble radioactivity recovered (nCi liter^{-1})	Distribution of radioactivity (%)[a]		
			RNA	DNA	Protein
1	1	6	17	29	54
	3	28	14	10	76
	6	130	29	11	60
	11	587	21	17	61
	24	601	17	24	59
150	1	2.5	72	18	10
	3	3.1	75	13	13
	6	8.9	70	16	14
	12	24.3	73	22	5
	24	24.4	60	21	18
400	1	0.9	70	22	8
	3	1.4	81	14	5
	6	2.4	86	9	5
	12	2.6	71	18	11
	24	4.9	68	20	12

[a] Distribution into acid-insoluble macromolecular fraction was determined by differential acid-base hydrolysis (see text). Results are averages of duplicate samples.

TABLE 6. Incorporation of ^{3}H-TdR into Kaneohe Bay sediment at increasing concentrations of thymidine

Sp act (Ci mmol^{-1})[a]	Thymidine concn (nM)	Total ^{3}H-TdR uptake (pmol g^{-1})[b]	Distribution of radioactivity (%)[c]		
			RNA	DNA	Protein
70.4	1.4	0.2	51	2.7	46
70.4	7.1	1.3	52	2.5	46
70.4	14.2	2.1	43	3.4	53
16.6	30	3.9	55	3.4	42
5.0	100	8.1	50	3.0	46
1.0	500	18.6	54	4.6	41
0.5	1,000	21.7	52	3.1	45
0.25	2,000	26.1	60	2.6	37

[a] Unlabeled thymidine was added to dilute the specific activity of ^{3}H-TdR.
[b] Uptake was calculated by using added specific activity and total activity in macromolecules.
[c] Distribution of acid-insoluble macromolecules was determined by differential acid-base hydrolysis (see text).

The bulk of the incorporated radioactivity for most incubations was recovered in the RNA fraction, except in the case of the VERTEX program near-surface samples (Table 4) and the Kahana Bay water samples (Table 3), in which protein was the predominant end product. The degree of nonspecific labeling was dependent on neither incubation time (Table 5) nor concentration of TdR (Table 6). The patterns of nonspecific labeling were established within 10 min and are, consequently, not related to recycling during extended incubation periods.

The labeling patterns of RNA, DNA, and protein pools from ^{3}H-Ade incorporations were generally similar, but not identical, to the labeling patterns of ^{3}H-TdR incorporation for parallel samples (Tables 2 and 3, Fig. 1). The total radioactivity recovered in the ^{3}H-Ade incorporations was approximately 10 times greater than the label recovered in parallel ^{3}H-TdR incorporations. The water samples from Kahana Bay showed divergent RNA and protein patterns, with protein being more heavily labeled by ^{3}H-TdR, while ^{3}H-Ade labeled RNA to a greater extent (Table 3).

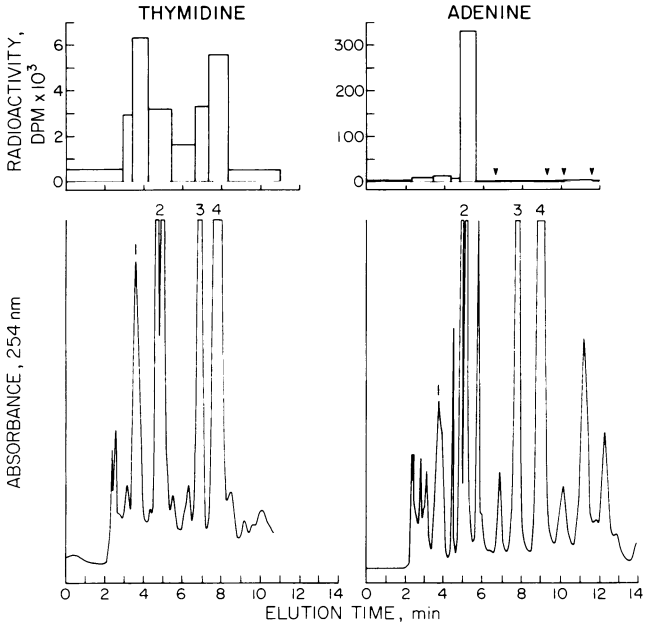


FIG. 1. HPLC traces of piperidine hydrolysates of a Kaneohe Bay sediment sample incubated with ^{3}H-TdR and ^{3}H-Ade. The numbers on the chromatograms indicate the positions of the four major RNA hydrolysis products: 1, CMP; 2, AMP (double peak); 3, UMP; 4, GMP. The bar graphs above each HPLC trace indicate the amount of radioactivity in each of the fractions that were collected. The small arrowheads on the adenine graph indicate the beginnings and ends of fractions which are not otherwise apparent from the data.

TABLE 7. Comparison of the yields of ^{3}H-TdR- and ^{3}H-Ade-labeled RNA from a Krauss Pond community after four different hydrolysis procedures

Hydrolysis conditions			RNA radioactivity recovered [nCi (% of control)] with:	
Reagent (concn)	Temp (°C)	Time (h)	^{3}H-Ade	^{3}H-TdR
NaOH (1 N)	37	1	1,477 (100)	85 (100)
NaOH (1 N)	37	18	1,330 (90)	65 (77)
KOH (1 N)	37	1	668 (45)	67 (79)
KOH (1 N)	37	18	540 (37)	74 (87)
Piperidine (10%)	100	1.5	1,090 (74)	91 (107)
RNase I (9 U)	37	6	341 (23)	19 (22)

Four RNA hydrolysis protocols were compared after ^{3}H-TdR and ^{3}H-Ade incubations into parallel Krauss Pond water samples (Table 7). All of the methods used released substantial radioactive RNA, with the piperidine hydrolysis procedure yielding values that were comparable to those obtained with the standard 1-h NaOH hydrolysis. The RNase A released <25% of activity of the chemical hydrolysis procedures.

Monomers responsible for labeling of RNA. HPLC separation and liquid scintillation counting of the isolated fractions after piperidine hydrolysis showed that CMP and AMP were labeled (Table 8) during ^{3}H-TdR incorporation of a Krauss Pond water sample. An identical pattern of ribonucleotide monophosphate labeling was observed for all Krauss Pond

TABLE 8. Identification of monomers responsible for labeling of RNA during incorporation of ^{3}H-TdR[a]

Elution time (min)	Peak identification	A_{260}[b]	Total radioactivity collected (dpm)[c]	Monophosphate concn (μg) by HPLC[d]
0–4.6		0	42	
4.6–5.8		0.06	204	
5.8–7.2		0.02	812	
7.2–9.3	CMP	0.15	1,327	2.8
9.3–10.3		0.50	318	
10.3–12.8	AMP	0.22	1,632	4.0
12.8–13.5		0.03	32	
13.5–15.0		0.01	53	
15.0–16.2		0.01	29	
16.2–18.4	UMP	0.14	88	3.0
18.4–19.8		0.05	42	
19.8–23.3	GMP	0.39	5	5.3
23.3–27.0		0.03	54	

[a] Incubation of Krauss Pond sample with 1 μCi of ^{3}H-TdR ml^{-1} (14.2 nM) for 2 h; hydrolysis by piperidine was followed by HPLC separation (see text for details).
[b] A_{260} was determined on elution fraction in a 1-cm path microcell.
[c] Total radioactivity collected from 0 to 27 min was 4,638 dpm, which is 35.4% recovery.
[d] Monophosphates were quantified by peak area multiplied by a conversion factor determined with standards for each of the presumptive monophosphate peaks. The total amount of monophosphates recovered was 15.1 μg, which is 43% recovery.

TABLE 9. Identification of monomers responsible for labeling DNA during incorporation of ^{3}H-TdR[a] into a Krauss Pond water sample as determined by formic acid hydrolysis and HPLC[b]

Elution time (min)	Peak identification	Total radioactivity (dpm)[c]	% of total radioactivity
0–2.0		8	0.4
2.0–4.2		130	6.3
4.2–6.0	Cytosine	101	4.9
6.0–7.3		27	1.3
7.3–8.8	Guanine	262	12.8
8.8–10.1	Thymine	494	24.1
10.1–11.1		90	4.4
11.1–12.9	Thymidine	360	17.6
12.9–14.3	Adenine	46	2.2
14.3–16.5	Polymers	288	14.0
16.5–18.2	Polymers	245	11.9

[a] Incorporation was done with 1 μCi of ^{3}H-TdR ml^{-1} (14.2 nM) for 2 h.
[b] Hydrolysis was done with formic acid at 180°C for 40 min. HPLC was done by method B, and elution fractions were collected as indicated (see text for details).
[c] Total radioactivity recovered was 2,051 dpm, which was 63% of the radioactivity injected.

TABLE 10. Identification of monomers responsible for labeling DNA during incorporation of ^{3}H-Ade[a] into a Krauss Pond water sample as determined by formic acid hydrolysis and HPLC[b]

Elution time (min)	Peak identification	Total radioactivity (dpm)[c]	% of total radioactivity
0–2.0		30	1.6
2.0–3.2		52	2.8
3.2–4.7		68	3.6
4.7–5.3	Cytosine	0	0.0
5.3–6.2	Cytidine	10	0.5
6.2–7.3		13	0.7
7.3–8.6	Guanine	16	0.9
8.6–10.0	Thymine	39	2.1
10.0–11.0		16	0.9
11.0–11.7	Thymidine	2	0.1
11.7–13.1	Adenine	1,623	86.8
13.1–14.9		145	7.8

[a] Incorporation was done with 1 μCi of ^{3}H-Ade ml^{-1} (62.5 nM) for 2 h.
[b] Hydrolysis was done with formic acid at 180°C for 40 min. HPLC was done by method B, and elution fractions were collected as indicated (see text for details).
[c] Total radioactivity recovered was 1,869 dpm, which was 63% of the radioactivity injected.

water samples incubated with ^{3}H-TdR. The same pattern was suggested for the VERTEX samples, but the generally low level of ^{3}H-TdR labeling for these oligotrophic samples resulted in less-definitive results (data not presented). The Kaneohe Bay sediment samples also showed labeling of AMP and CMP, with GMP and, to a lesser extent, UMP also being labeled (Fig. 1).

A chromatographic analysis of the piperidine hydrolysates from Krauss Pond water samples that had incorporated ^{3}H-Ade yielded only labeled AMP. After HPLC separation, over 85% of the tritium recovered was in the AMP fraction (Fig. 1). The Kaneohe Bay sediment samples also incorporated the tritium from ^{3}H-Ade into RNA, with ≥90% of the recovered tritium in the AMP fraction (data not presented). The remaining nonadenine tritium was distributed throughout the elution profile and was not recovered as any specific labeled monomer.

Monomers responsible for DNA labeling. The formic acid hydrolysis procedure resulted in a mixture of nucleosides and bases, and these were separated by using HPLC method B. The extreme hydrolysis conditions (180°C) apparently caused an exchange of tritium between the radiolabeled DNA monomers and the solvent. Consequently, we obtained low yields of tritium after the hydrolysates were dried and then redissolved in buffer. By using this method, however, it was determined that the tritium from ^{3}H-TdR was incorporated into the TdR residue of DNA, but with minor labeling of cytosine (Table 9). The tritium from ^{3}H-Ade was incorporated into DNA exclusively as dAMP (Table 10).

Disappearance of ^{3}H-TdR from the incubation medium. The rate of uptake of ^{3}H-TdR from a Krauss Pond water sample was much greater than the rate of incorporation of ^{3}H-TdR into macromolecules (Table 11). The production of volatile metabolites was very rapid and closely followed the disappearance of ^{3}H-TdR. The nonspecific labeling patterns were already established after 10 min, and the relative labeling changed very little thereafter. After the 2-h incubation, volatile metabolites accounted for 77% of the added label, macromolecules accounted for 12%, and unutilized ^{3}H-TdR accounted for less than 8% (Table 11). The presumptive ^3H-H_2O accounted for 58% of the volatile metabolites after 2 h.

DISCUSSION

The catabolism of TdR by natural assemblages of microorganisms should not be, in itself, a controversial concept. The fact that tritium derived from ^{3}H-TdR can be recycled at a high enough specific activity to label other macromolecules has not, conversely, been universally accepted (32; D. J. W. Moriarty and P. C. Pollard, submitted for publication). Although many investigators have reported radiolabeling of protein and in some cases of RNA after exposure of a variety of microbial communities to ^{3}H-TdR (Table 1), none have offered an adequate explanation. This nonspecific labeling has been referred to as being "inconsistent with our current understanding of TdR incorporation pathways" (10, 20) or, simply, "impossible" (32). The data presented above demonstrate that the labeling is real, and a brief review of thymine degradation will show that nonspecific labeling is not only possible but is fully expected.

The macromolecular labeling results based on ^{3}H-TdR

TABLE 11. Mass balance radiochemical inventory for a time course incubation of Krauss Pond water with ^{3}H-TdR

Incubation time (h)	Amt (nCi ml^{-1}) of:						
	Filtrate ^{3}H[a]	Volatile ^{3}H[b]	Wash ^{3}H[c]	^{3}H-TdR[d]	Macromolecules[e]		
					RNA	DNA	Protein
0	77.7	0.8	ND[f]	77.0			
0.2	78.4	6.6	8.0	64.8	1.50	0.28	0.20
0.5	73.8	14.2	14.2	30.2	2.60	0.71	0.51
1.0	70.0	27.7	22.7	15.6	4.60	1.76	0.65
2.0	71.3	60.3	35.4	6.3	6.33	1.84	1.17
4.0	70.2	56.5	37.4	3.3	2.96	0.95	0.40
8.0	73.0	65.9	37.3	3.5	2.26	0.47	0.37

[a] Tritium that passes through a 0.22-μm-pore-size filter.
[b] Tritium that is lost after filtrate is dried at 60°C.
[c] Tritium that is eluted within 3 min of injection of a sample of filtrate analyzed by HPLC method B (see text for details).
[d] Tritium recovered in the thymidine fraction after analysis of the filtrate by HPLC method B (see text for details).
[e] Total macromolecules recovered as RNA, DNA, and protein after differential hydrolysis of filterable material (see text for details).
[f] ND, Not determined.

uptake for a variety of aquatic habitats revealed different patterns of nonspecific labeling (Tables 2 through 4). These results are probably caused by differences in community metabolism and substrate availability among the different microbial assemblages. For example, the depth profile in oligotrophic seawater (Table 4) revealed a decrease in DNA labeling, in relation to RNA and protein labeling, with depth. This corresponds to the general decrease in bacterial numbers and specific growth rates and the presumed decrease in the availability of growth substrates which occur with increasing water depth. The Kahana Bay samples displayed distinct and profound changes in nonspecific labeling patterns on centimeter space scales (Table 3). The microorganisms collected from the air-seawater interface of Kahana Bay labeled RNA during ^{3}H-TdR incorporation to a much greater extent than did the bulk water sample microbiota collected at a depth of just 10 cm beneath the interface. Further changes in the pattern of nonspecific labeling were observed for the Kahana Bay seawater-sediment interface microbial assemblage and within the sediment column, where a much greater degree of nonspecific labeling was observed. The metabolism of ^{3}H-TdR by Kaneohe Bay sediment samples also labeled RNA and protein to a much greater extent than DNA. Compared with planktonic habitats, the patterns of nonspecific labeling observed in sediment samples, in general, correspond to the concept of a more substrate-limited environment. These data are consistent with the hypothesis that the degree of nonspecific labeling during ^{3}H-TdR incorporation may be related to bacterial growth rate and substrate availability. The Krauss Pond and closed ecosystem samples are more difficult to fit into a growth rate-substrate availability model, as the relative substrate levels in these ecosystems are not known.

The comparison of the four hydrolysis procedures showed that the labeling of RNA is not an artifact of a particular hydrolysis technique (Table 7), as suggested by Moriarty and Pollard (submitted). The lower recovery of labeled RNA with RNase A could be the result of denaturation of the RNase to proteases initially present or released during the extraction of the sample; the requirement of complete cell lysis of the mixed assemblage of microorganisms to ensure exposure of the cellular RNA; or the presence of interfering ions, trace metals, or clay particles. Nevertheless, the fact that labeled material in our experiments is released by RNase treatment confirms the labeling of RNA by ^{3}H-TdR.

The isolation of the individual monomers of RNA after piperidine hydrolysis and the determination of the radioactivity of each monomer inarguably demonstrates the labeling of RNA by ^{3}H-TdR. The HPLC methods used in this study clearly separated TdR and TdR monophosphate from the other nucleosides and nucleotides. No TdR or TdR monophosphate was recovered in the RNA fractions, and no peak of radioactivity eluted at the TdR or TdR monophosphate retention times. Thus, the hydrolysis of the RNA was specific; monomers of DNA were not recovered in the RNA fraction. AMP and CMP (Table 8) were clearly labeled and carried the bulk of the radioactivity that was recovered from the Krauss Pond samples. The specificity of AMP and CMP labeling in RNA for Krauss Pond indicates that the base portion of the nucleotide, and not the ribose sugar moiety, is labeled. If the sugar had been labeled, all of the nucleotides would be uniformly labeled. The preferential labeling of AMP and CMP is not as strong in the Kaneohe Bay samples (Fig. 1), as some [^{3}H]GMP and [^{3}H]UMP was also recovered. These data show that the recycling of tritium from ^{3}H-TdR can occur at a high enough specific activity to significantly label RNA.

If selected RNA bases are labeled during ^{3}H-TdR incorporation, it follows that their deoxyribonucleotide derivatives also probably contribute to the total labeling of the DNA fraction. If the ribonucleotide and deoxyribonucleotide pools are in isotopic equilibrium (23), then the degree of participation of labeled AMP (or CMP) into RNA should be the same as that of dAMP (or dCMP) into DNA. The rate of incorporation of labeled dAMP or dCMP into DNA should be equivalent to the rate of incorporation of AMP or CMP into RNA multiplied by the DNA synthesis rate/RNA synthesis rate ratio for the particular population incorporating ^{3}H-TdR. The recovered labeled RNA/labeled DNA ratios in Krauss Pond ^{3}H-TdR incorporations varied from 12 to 18 (Table 2), while the reported ratios of RNA synthesis rate to DNA synthesis rate varied from 1 to 15 (23). These ratios imply that the labeling of DNA by recycled tritium from ^{3}H-TdR could occur at significant levels.

In contrast to the patterns observed for ^{3}H-TdR, the labeling of RNA and DNA during ^{3}H-Ade incorporations showed a high level of specificity. Only labeled AMP or adenine was recovered (Table 10, Fig. 1), demonstrating that no significant recycling of the tritium label from ^{3}H-Ade occurred. This is interesting because protein does become labeled (Tables 2 and 3) (24, 27). Apparently, the labeling of protein via ^{3}H-Ade is the result of a specific, rather than a nonspecific, metabolic pathway. Histidine biosynthesis, for example, requires ATP as a precursor and preserves the 2 position of the purine ring along with the associated tritium atom.

Catabolism of ^{3}H-TdR is a ubiquitous feature of natural microbial assemblages. However, it is important to distinguish between true catabolism and the research results described above. In an incorporation assay with tritiated organic substrates, only the labeled protons can be monitored, not the carbon skeletons to which they are attached. Nevertheless, when the tritium atoms of *methyl*-^{3}H-TdR are followed through the standard thymine degradation pathway and then through the biosynthesis of purine and pyrimidine ribonucleotides, the labeling of RNA during ^{3}H-TdR incorporations can be seen as a natural and predictable consequence of bacterial metabolism.

The degradation of thymine results in the labeling of two carbons with associated tritium in oxaloacetate (7, 53). Oxaloacetate can either be completely oxidized to CO_2 catabolically or it can be used in biosynthetic pathways to produce amino acids, purines, pyrimidines, and even sugars through gluconeogenesis via pyruvate (53). This can explain the labeling of protein during ^{3}H-TdR incorporations, as any amino acid synthesized from oxaloacetate, pyruvate, 3-phosphoglycerate, or phosphoenolpyruvate could potentially be labeled. Thus, the standard catabolic pathway would be able to conserve the tritium label to a point where it could enter the biosynthetic pathways for many constituents in the cell.

The specific pathways to the formation of tritiated pyrimidines and purines are now part of standard textbook biochemistry (Fig. 2) (53). For pyrimidines, the hydrogen on the 5-position carbon is the potential site for tritium labeling of cytosine or uracil. This could theoretically allow the production of labeled CMP or UMP at the 5-position carbon. Thus, the production of labeled CMP should involve the production of labeled UMP (38).

For purines, the two methyl groups at positions 2 and 8 are the only carbon atoms that carry hydrogen atoms (and,

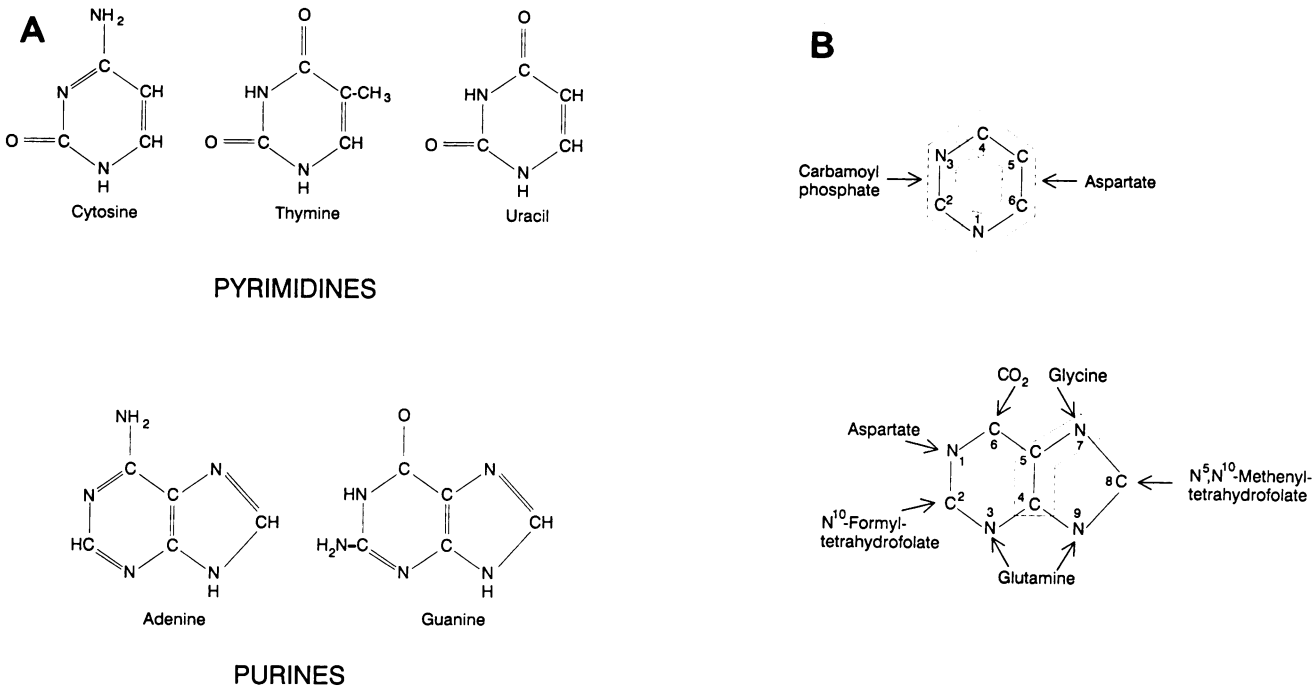


FIG. 2. (A) Structures of purine and pyrimidine nucleic acid bases. (B) Sources of C and N atoms in the de novo biosynthesis of purine and pyrimidine bases.

potentially tritium). These methyl groups are derived from tetrahydrofolate, which could have been ultimately derived from the original (labeled) methyl group of TdR. Well-understood biochemical pathways could, therefore, result in the labeling of all four RNA constituents and many amino acids. Any other biomolecule whose synthesis involves either pyruvate or tetrahydrofolate is potentially a recipient of tritium along these pathways.

The results of this study indicate that AMP and CMP are labeled preferentially over GMP and UMP. This preferential labeling may reflect the actual in vivo patterns or may simply be a result forced by our experimental protocols. For example, the labeling patterns that we observed could have resulted from the relative stabilities of the various labeled protons to pH and temperature during sample hydrolysis. On the other hand, what we call preferential labeling may be an accurate reflection of the requirements of a particular microbial assemblage for precursors necessary to balance de novo synthesis and salvage pathway sources.

Both the NaOH and the piperidine hydrolysis procedures release all the RNA but, in addition, the piperidine hydrolysis conditions could also cause an appreciable exchange of tritium from the nucleotides with water (12, 50). The differences between the total ^3H recoveries of these two methods (Table 8) then might be explained by this isotopic exchange, as ^3H-H_2O would be lost when the piperidine is evaporated. The observed 25% difference attributed to ^3H-H_2O loss would be equivalent to the labeling of UMP, GMP, and one-half of the AMP. On the basis of these assumptions and arguments, the ratio of labeled nucleotides could not have been equimolar, and the preponderance of CMP and AMP must reflect, if not exactly, a preferential labeling phenomenon. The pathways described above predict that the nonspecific labeling of protein by ^3H-TdR could be due to labeling by all amino acids except the glutamate family and, possibly, histidine. The actual labeling pattern will reflect the availability of salvage sources and the biosynthetic requirements of the population.

It may be of interest to estimate the total uptake of ^3H-TdR relative to DNA synthesis requirements. The actual rate of ^3H-TdR uptake, as opposed to rate of incorporation, was measured and found to be much greater than the incorporation rate into macromolecules (Table 11). This observation by itself is indirect evidence for TdR catabolism. The production rate of ^3H-H_2O was not as rapid as the consumption of ^3H-TdR. The maximum amount of labeled macromolecules was recovered after 2 h, while the ^3H-TdR was reduced to less than 5% of its original concentration at that time. The presence of a labeled, nonvolatile product derived from ^3H-TdR and released rapidly into the medium is apparent from our results (Table 11). The amount of label retained in the cells as macromolecules was never greater than 10% of the total label added. It appears that TdR is rapidly catabolized in this ecosystem and that the production of ^3H-H_2O implies that some organisms use TdR or its breakdown products as a growth substrate. The labeled macromolecular products are also eventually catabolized, as seen by the decrease in the amount of label found in the macromolecular fractions after 2 h.

What can we conclude about the use of ^3H-TdR for the determination of bacterial growth rates in nature? The problem of nonspecific labeling has been known for some time (5, 20, 24) (Table 1), and the proponents of the use of ^3H-TdR now routinely determine the label actually present in DNA by various techniques of DNA isolation (16, 42, 43). The isolation of DNA, however, may not be adequate, because some of the tritium in the DNA fraction may also be derived from the indirect incorporation of ^3H-TdR as a result of ^3H-TdR catabolism and DNA labeling via dAMP or dCMP. The assumption that ^3H-TdR in nanomolar amounts is a nonperturbing tracer of DNA synthesis is not supported by this research. The rapid and efficient catabolism of ^3H-TdR,

probably for the synthesis of carbon skeletons and as a substrate for energy production, at higher rates than its incorporation into DNA indicates that the addition of ^{3}H-TdR could be stimulating the growth of the subpopulation that is capable of assimilation. This effect is probably more important in substrate-limited environments, such as mesopelagic and sedimentary habitats. If our interpretation is valid, these processes would violate the underlying assumption of the isotope dilution technique (28) and would invalidate the use of TdR in most oligotrophic habitats.

ACKNOWLEDGMENTS

We thank F. Roberts and B. Adams for constructive criticisms of an earlier version of the manuscript and L. Wong for assistance in the preparation of the text and figures.

This research was initiated by A.M.B. under the tutelage of the late Claire Folsome and was supported, in part, by a training grant awarded to A.M.B. by the National Aeronautics and Space Administration (NGT-50291) and by National Science Foundation grants OCE86-00462 and OCE83-51751 awarded to D.M.K.

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