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Spatial and Temporal Variations in Bacterial Macromolecule Labeling with [*methyl*-^{3}H]Thymidine in a Hypertrophic Lake†

RICHARD D. ROBARTS,[1]* RICHARD J. WICKS,[2] AND LYNNE M. SEPHTON[1]

National Institute for Water Research,[1] and National Chemical Research Laboratory,[2] Council for Scientific and Industrial Research, Pretoria 0001, South Africa

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The incorporation of [*methyl*-^{3}H]thymidine into three macromolecular fractions, designated as DNA, RNA, and protein, by bacteria from Hartbeespoort Dam, South Africa, was measured over 1 year by acid-base hydrolysis procedures. Samples were collected at 10 m, which was at least 5 m beneath the euphotic zone. On four occasions, samples were concurrently collected at the surface. Approximately 80% of the label was incorporated into bacterial DNA in surface samples. At 10 m, total incorporation of label into bacterial macromolecules was correlated to bacterial utilization of glucose ($r = 0.913$, $n = 13$, $P < 0.001$). The labeling of DNA, which ranged between 0 and 78% of total macromolecule incorporation, was inversely related to glucose uptake ($r = -0.823$), total thymidine incorporation ($r = -0.737$), and euphotic zone algal production ($r = -0.732$, $n = 13$, $P < 0.005$). With decreased DNA labeling, increasing proportions of label were found in the RNA fraction and proteins. Enzymatic digestion followed by chromatographic separation of macromolecule fragments indicated that DNA and proteins were labeled while RNA was not. The RNA fraction may represent labeled lipids or other macromolecules or both. The data demonstrated a close coupling between phytoplankton production and heterotrophic bacterial activity in this hypertrophic lake but also confirmed the need for the routine extraction and purification of DNA during [*methyl*-^{3}H]thymidine studies of aquatic bacterial production.

The incorporation of [*methyl*-^{3}H]thymidine into DNA as a measure of aquatic bacterial production (6) is being widely used in marine and freshwater microbial ecology studies. The imprecision of converting thymidine incorporation rates into bacterial production rates is a major weakness of the method (6, 10). Many aspects of this problem have been examined (2, 7, 14, 15). One aspect, the possible lack of macromolecular specificity by thymidine labeling, has received some attention, but conclusions in the literature remain uncertain. By using acid-base hydrolysis, it is possible to determine how much of the tritiated thymidine is incorporated into DNA, RNA, and protein fractions. Fuhrman and Azam (6) reported that of the cold 10% trichloroacetic acid (TCA)-insoluble material, which included DNA, RNA, and proteins, 80 to 95% was labeled DNA. Further work by Fuhrman and Azam (7) generally supported these findings.

The application of the full acid-base hydrolysis procedure to separation of the different macromolecular fractions is time-consuming and is not easily adaptable to field use. Fuhrman and Azam (7) proposed that only the 10% TCA-insoluble material, containing DNA, RNA, and proteins, need be measured, since labeled DNA constituted the bulk of this material. Subsequent studies by various investigators (1–3, 10, 11, 15) were done by this approach, following an initial examination of the DNA content of the TCA-insoluble material; this content was then assumed to be representative for a particular microbial population. However, Riemann et al. (16) reported that the DNA content of the TCA-insoluble material in a Danish lake ranged between 18 and 38% over a diel cycle, while similar low values were found for five additional lakes. Lovell and Konopka (10) found only 28% labeled DNA in the insoluble material of anaerobic hypolimnetic samples compared with 80% in epilimnetic and metalimnetic samples. Similarly, Hanson and Lowery (8) reported greater label incorporation into protein for marine samples at 500- to 2,000-m depths. Riemann et al. (16) and Findlay et al. (5) recommended that to avoid this potential error, labeled DNA should regularly be extracted and purified in labeled thymidine studies of aquatic bacterial production.

Although some diel and depth variations in the percentage of labeled DNA in TCA-insoluble material from thymidine bacterial production studies have been recorded, the variations that may occur annually in an aquatic system are unknown. In this paper, we report the temporal and spatial variations of the tritiated thymidine labeling of bacterial macromolecules in relation to phytoplankton production in a hypertrophic lake.

MATERIALS AND METHODS

Temporal and spatial measurements. Water samples were collected monthly from approximately a 10-m depth in hypertrophic Hartbeespoort Dam, South Africa (21). This depth was chosen because it was always in the aerobic epilimnion and because the concentrations of the dominant autotroph, *Microcystis aeruginosa*, were usually low (21). Bern (4) reported that *Microcystis* spp. did not utilize thymidine at low concentrations. On four occasions toward the end of the study, water samples were collected concurrently from the surface.

Water samples (5 liters) were collected in ethanol-washed plastic containers. Samples were transported to the laboratory within 1 h. In the laboratory, 10-ml portions were dispensed into 30-ml sterile McCartney bottles. For each experiment, there were three sets of three experimental and two control bottles. Controls were prepared by the addition of 0.1 ml of Lugol iodine solution.

* Corresponding author.

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Working solutions (ca. 100 μCi ml^{-1}) of [*methyl*-^{3}H] thymidine (79 Ci mmol^{-1}; Radiochemical Centre, Amersham, England) were made daily with membrane-filtered (pore size, 0.2 μm) sterile distilled water. Additions of approximately 130 μl were made to each bottle to give a final thymidine concentration of 20 nM. Plots of increasing thymidine concentration against total uptake (cold-TCA precipitate, i.e., DNA, RNA, and protein) indicated that incorporation rates could increase up to 20 nM and that severe reductions in incorporation could occur at higher concentrations. At 20 nM, total uptake was linear for at least 30 to 60 min. In the work reported here, the samples were incubated in the dark for 30 min at room temperature (ca. 21°C).

After incubation, the following acid-base hydrolysis procedures, essentially as described by Riemann and Søndergaard (17), were carried out. One set of samples for the determination of DNA, RNA, and protein was chilled in ice before equal volumes of ice-cold 10% TCA were added. After 15 min, the precipitate was collected on 0.2-μm-pore-size cellulose nitrate membrane 47-mm-diameter filters (Sartorius GmbH). The filters were washed with 5 ml of ice-cold 5% TCA. Following filtration, the nonfiltering margin of each filter, plus 4% was removed with a circular cutter. This procedure reduced control values and stabilized variation between replicates to usually less than 10%. The cut filters were then placed in 10 ml of Filter-count (Packard Instrument B.V.), which completely dissolved the filters. Tritium incorporation was determined with a liquid scintillation counter, and counts were corrected for quench with an external standard. Assuming even precipitate distribution on the filters, the counting program automatically increased the counts by 4% for the portion of the filtering surface excised.

A second set of samples for DNA and protein determinations were extracted with 0.5 N NaOH (final concentration) at 60°C for 1 h and then chilled and acidified with TCA (1 g ml^{-1}, 1.4 ml per 5-ml water sample). Proteins were extracted from the third set of samples with equal volumes of 40% TCA at 95 to 100°C for 30 min. The samples were then chilled on ice. The precipitates from both these procedures were collected on membrane filters, and the radioactivity was determined as described above. Labeled DNA was calculated by subtracting the radioactivity in the hot-TCA precipitate from that in the hot-alkali precipitate. RNA labeling was calculated by subtracting the radioactivity of the hot-alkali precipitate from that of the cold-TCA precipitate. The relative labeling of macromolecules is presented as a percentage of the label appearing in the cold-TCA precipitate.

On three occasions, time course measurements of bacterial macromolecule labeling in water samples from 10 m were carried out by using the procedures described above.

The recovery of DNA from Hartbeespoort Dam water samples by acid-base hydrolysis was checked by using labeled DNA. DNA (type III salmon sperm DNA; Sigma Chemical Co., St. Louis, Mo.) was 5′ end labeled with ^{32}P by the exchange reaction technique (12). [^{32}P]DNA (0.27 μg) was added to triplicate sets of water samples and immediately processed through the acid-base hydrolysis procedures described above. Additional sets of triplicate samples were also run but instead of washing the precipitates collected on membrane filters with cold 5% TCA, we washed the filters with 5 ml of cold 80% ethanol. This was done to check the effect of this washing procedure on the DNA content immobilized on membrane filters (see Table 4).

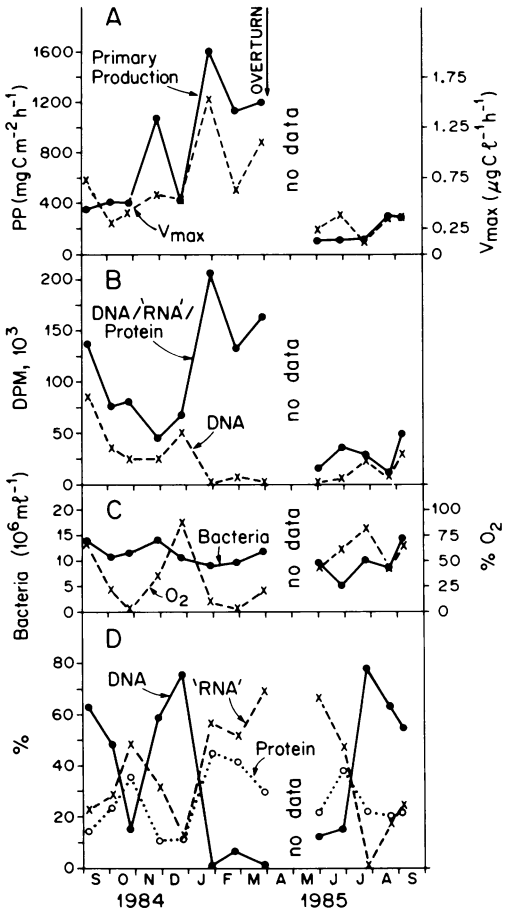

FIG. 1. (A) Annual cycle of euphotic-zone primary production (PP) and the maximum rate of glucose uptake (V_{max}) at 10 m; (B) amount of [^{3}H]thymidine incorporated into bacterial macromolecules (cold-TCA precipitate) and DNA at 10 m; (C) annual cycle of bacterial numbers and percent O_2 saturation at 10 m; (D) percent composition of [^{3}H]thymidine incorporated into bacterial DNA, RNA, and protein fractions at 10 m in Hartbeespoort Dam, South Africa, as determined by acid-base hydrolysis.

Total bacterial counts for each sample were made with an epifluorescence microscope and DAPI (4′6-diamidino-2-phenylindole) stain (19).

As part of an ongoing study of Hartbeespoort Dam, a number of other measurements relevant to the data reported here were made within 1 to 2 days of the measurements of thymidine incorporation: dissolved oxygen (by the azide modification of the Winkler technique), ^{14}C primary production (18), and the uptake of [^{14}C]glucose by heterotrophic bacteria (20).

Bivariate data were analyzed by a best-fit curve-fitting program.

Enzymatic digestion and chromatographic studies. Filters containing cold-TCA precipitate (i.e., DNA, RNA, and protein fractions) were washed with 5 ml of ice-cold 0.01 M $KHCO_3$ to remove excess TCA and stored at −20°C until required. Additional filters containing cold-TCA precipitate were washed with 5 ml of ice-cold 0.01 M $KHCO_3$, 80% (vol/vol) ethanol, or chloroform-methanol, (2:1, vol/vol). Triplicate washed filters were then cut to remove the nonfiltering margin, and radioactivity was counted.

RNase A (50 Kunitz units mg^{-1}), DNase I (grade I; 3,000

TABLE 1. Correlation matrix of aquatic bacterial DNA labeling with [^{3}H]thymidine at 10 m and various biological and chemical parameters in Hartbeespoort Dam

Parameter	V_{max} (μg of C l^{-1} h^{-1})	Primary production (mg of C m^{-2} h^{-1})	% O_2	Bacteria (10^6 ml^{-1})	TCA precipitate (dpm)	DNA (dpm)	DNA (%)
DNA (%)	−0.823[a]	−0.732[a]	0.736[a]	0.301	−0.737[a]	0.967*	
DNA (dpm)	−0.762[a]	−0.628[a]	0.508	0.471	−0.585[a]		
TCA precipitate (dpm)	0.913[a]	0.786[a]	−0.492	0.325			
Bacteria (10^6 ml^{-1})	0.170	0.392	−0.077				
% O_2	−0.412	−0.620[a]					
Primary production (mg of C m^{-2} h^{-1})	0.865[a]						
V_{max} (μg of C l^{-1} h^{-1})							

[a] $r \geq 0.553$, $P \leq 0.05$, $n = 13$.

Kunitz units mg^{-1}), and proteinase K (20 U mg^{-1}; nuclease free) were purchased from Boehringer GmbH, Mannheim, Federal Republic of Germany. The lyophilized enzyme preparations were made 1 mg ml^{-1} in deionized water. Contaminating DNase activity in the RNase was inactivated by boiling the RNase solution for 10 min. RNase, DNase, and proteinase digestions were carried out in the following buffer solutions, respectively: 20 mM morpholinepropanesulfonic acid (pH 7.5), 20 mM morpholinepropanesulfonic acid–5 mM $MgCl_2$–0.5 mM $CaCl_2$ (pH 7.5), and 20 mM Tris (pH 8.0). Duplicate $KHCO_3$-washed filters were incubated with 2.5 ml of buffer containing 10 μl (10 μg) of enzyme at 37°C for 30 min with shaking. In addition, the DNase incubation solution contained 2 μl of human placental RNase inhibitor (25,000 U ml^{-1}; Radiochemical Centre, Amersham, England) to inhibit contaminating RNase activity. Controls, without enzyme, were run in duplicate. After incubation, the solution was centrifuged at 15,000 rpm for 1 min in an Eppendorf bench centrifuge (model 5414 S) to remove particulate material. Labeled oligonucleotides or short peptides resulting from enzymatic digestion were separated from larger fragments of macromolecules by high-performance gel filtration chromatography on an Ultropac TSK-G2000SW column (7.5 by 600 mm; LKB-Produkten AB, Bromma, Sweden). The column was equilibrated at a flow rate of 1 ml min^{-1} with 800 mM NaCl–1 mM trisodium citrate (pH 7.0) and run isocratically with the same buffer. Labeled oligonucleotides and short peptides eluted 20 to 35 min after injection. Lumax hydrophilic scintillant (Lumac, The Netherlands) was mixed with eluants to determine radioactive content.

RESULTS

Integral primary production for the euphotic zone (1.3 to 4.8 m) of Hartbeespoort Dam ranged between 111.0 and 1,605.4 mg of C m^{-2} h^{-1} (Fig. 1A). The maximum rate of bacterial utilization of glucose (V_{max}) at 10 m ranged between 0.150 and 1.544 μg of C l^{-1} h^{-1} (Fig. 1A). V_{max} was correlated with primary production (Table 1).

The seasonal variation of the incorporation of labeled thymidine (as disintegrations per minute) into DNA, RNA, and proteins (cold-TCA precipitate) in water samples from 10 m reached a peak in midsummer (January) (Fig. 1B). Label incorporated into DNA showed a similar trend, except between December and March (Fig. 1B). Total label incorporation was correlated to primary production and V_{max}, while DNA labeling was inversely related to these two parameters (Table 1).

Bacterial numbers at 10 m ranged between 5.22×10^6 and 14.83×10^6 ml^{-1}, while the percent saturation of oxygen ranged between 1 and 86% (Fig. 1C). Bacterial numbers were not correlated with any of the other measured parameters (Table 1). The percent saturation of oxygen was inversely related to primary production but was not correlated with V_{max}, cold-TCA precipitate, percent DNA, or number of bacteria (Table 1). The inverse relationship between percent O_2 saturation and primary production was expected. The euphotic zone did not extend to 10 m, and so O_2 saturation at this depth was a function of downward mixing processes, while production was positively correlated to water column stability (18).

Figure 1D shows the annual variation in the percent composition of labeled thymidine in the TCA precipitate. The percentage of the label in DNA ranged between nondetectable in January 1985 to 78% in July 1985 (midwinter). Label in the RNA fraction ranged between nondetectable in July and 68.9% in March, while that in the protein fraction ranged between 10.4% in November and 43.7% in January. The relative composition of DNA in the TCA precipitate was inversely related to primary production, V_{max}, and total label incorporation into macromolecules but was positively related to oxygen saturation (Table 1). The relative contribution of the RNA fraction in the TCA precipitate was correlated to V_{max} ($r = 0.586$, $P = <0.05$) and inversely related ($r = -0.681$, $P = 0.01$) to oxygen saturation, while the relative contribution of protein was inversely related ($r = -0.667$, $P = <0.02$) to oxygen saturation.

Table 2 compares the relative labeling of the bacterial DNA, RNA, and protein fractions in water samples collected concurrently from Hartbeespoort Dam at the surface and at a 10-m depth. The labeling of DNA in the surface samples was relatively constant and represented about 80%

TABLE 2. Percentage of macromolecules labeled with tritiated thymidine that were extracted by acid-base hydrolysis from bacterial populations taken from two depths in Hartbeespoort Dam

Date (day-mo-yr)	Depth (m)	% DNA	% RNA	% Protein	10^6 bacteria ml^{-1}
26-06-85	0	81.2	0	22.7	5.17
	10	15.2	47.1	37.7	5.22
27-07-85	0	78.9	1.4	19.8	10.16
	10	78.0	0.0	23.8	10.38
19-08-85	0	91.3	8.7	0.0	9.69
	10	62.5	17.1	20.4	8.86
04-09-85	0	83.3	8.0	8.7	13.4
	10	54.7	24.3	21.0	14.6

TABLE 3. Time-course incorporation of [*methyl*-^{3}H]thymidine into macromolecules of bacteria from Hartbeespoort Dam as determined by acid-base hydrolysis

Date (day-mo-yr)	Fraction	% Incorporation[a] at time (min):					
		5	10	15	20	30	60
24-10-85	DNA	29.5	39.1		2.8	1.2	3.2
	RNA	32.0	18.3		52.1	40.8	39.8
	Protein	38.6	42.6		45.2	58.0	57.1
19-12-85	DNA	8.6				9.6	
	RNA	44.2				45.7	
	Protein	47.2				44.7	
01-05-86	DNA	1.9		0.0		13.8	
	RNA	98.1		19.4		32.7	
	Protein	0.0		80.6		53.5	

[a] Data are given as percentages relative to total incorporation in cold-TCA precipitates.

of the total label incorporated by bacteria. This contrasted markedly with the data from 10 m (Fig. 1D; Table 2).

The results of three time course experiments of [^{3}H] thymidine labeling of bacteria are presented in Table 3. In the first experiment, the relative labeling of DNA increased for 10 min and then rapidly decreased. In the second, DNA represented a constant proportion for up to 30 min, while in the third, the relative labeling of DNA increased with time.

The cold-TCA treatment recovered about 75% of ^{32}P-labeled DNA added to Hartbeespoort Dam water samples (Table 4). The hot-NaOH procedure and the hot-TCA treatment recovered 68% and hydrolyzed 96% of the labeled DNA, respectively. Washing of precipitated DNA with cold 80% ethanol did not remove it from the cellulose nitrate filters.

A mass balance of ^{3}H in the cold-TCA extraction precipitate of bacteria labeled with thymidine is given in Table 5. The $KHCO_3$ buffer used to neutralize the TCA before enzymatic digestion removed 11% of the label. We attempted to remove TCA from the filters without losing label by using cold 80% ethanol and a chloroform-methanol mixture. Both of these solvents removed ca. 37% of the label from the filters. The results of the enzymatic digestion and subsequent chromatographic separation of macromolecule fragments indicated that RNA was not labeled and that four times more label was incorporated into DNA than into protein. Of the total radioactivity in the cold-TCA precipitate, 22% remained unaccounted for.

DISCUSSION

Several studies of bacterial production that involve the use of tritiated thymidine in freshwater systems found significant amounts of label incorporation in the RNA fraction (9, 10, 16, 23). However, Moriarty (13) stated that tritiated thymidine is incorporated only significantly into DNA and not into RNA and that results to the contrary would be due to imperfect extraction and separation of macromolecules. Much of the label taken up by bacterial cells could be lost by catabolism to thymine and then by loss of the ^{3}H-methyl group during further degradation of thymine (13a). If the ^{3}H-methyl group enters the general pool of metabolites in the cell, the label may eventually be distributed by indirect routes into all cell components, including RNA and DNA (13a). Moriarty further noted that such nonspecific labeling of DNA is insignificant compared with direct incorporation in short-term (10 to 30 min) experiments. Riemann (15) used DNase and RNase to determine the macromolecular composition of the different fractions from acid-base hydrolysis procedures and found that the [^{3}H]RNA fraction was a very small (<7%) component of the labeled macromolecules. Our data for Hartbeespoort Dam (Table 5) confirm the insignificance of [^{3}H]RNA in the cold-TCA precipitate. A repeat of this experiment 4 months later indicated that <3% of the cold-TCA precipitate was RNase active.

A varying proportion of the thymidine taken up by bacteria was incorporated into proteins and the RNA fraction (Fig. 1D). The percent labeling of both the RNA fraction ($r = -0.954$, $n = 13$, $P < 0.001$) and the proteins ($r = -0.813$, $n = 13$, $P < 0.001$) was inversely related to the relative labeling of DNA. In the time course experiments, the relative labeling of these three fractions was not constant, even though the accumulation of label in the precipitates from the three extraction procedures was linear for at least 30 min (data not

TABLE 4. Recovery efficiency of [^{32}P]DNA from Hartbeespoort Dam water samples by acid-base hydrolysis and effect of washing precipitates with cold 80% ethanol instead of cold 5% TCA

Treatment	Washing solvent	dpm remaining on filters[a]	% Recovery
Cold TCA	5% TCA	19,300	72
	80% ethanol	21,000	78
Hot NaOH	5% TCA	18,600	69
	80% ethanol	18,200	67
Hot TCA	5% TCA	945	4

[a] Results are means of triplicate samples. Total [^{32}P]DNA added to water samples was 27,000 dpm or 0.3 μg of DNA.

TABLE 5. ^{3}H mass balance for thymidine-labeled bacteria and effect of enzymes and solvent washes on cold-TCA precipitates immobilized on cellulose nitrate membrane filters

Treatment	dpm removed from filter[a]	% of ^{3}H in cold-TCA precipitate
$KHCO_3$	44,300	11
80% ethanol or chloroform-methanol	149,000 or 145,000[b]	37 or 36[b]
RNase	0	0
DNase	96,200	24
Proteinase	23,700	6

[a] All results are means of duplicate samples. Total ^{3}H in cold-TCA precipitate, 403,100 dpm.

[b] Second number refers to chloroform-methanol treatment.

shown). These data are similar to those given by Riemann et al. (16) for DNA. Hot NaOH recovered only 68% of the [^{32}P]DNA added to water samples (Table 4). The mean DNA recovery of 75% with cold TCA was similar to that reported by Findlay et al. (5). The efficiency with which these techniques extract DNA from bacterial cells is not shown by these data. Furthermore, the data in Table 5 show that in addition to the labeling of DNA and proteins, there was a major labeling of ethanol- and chloroform–methanol-soluble compounds. Chloroform-methanol is a solvent recommended for the removal of lipids from cell extracts (22). The radioactivity removed from the filters with cold 80% ethanol was identical to that removed with chloroform-methanol, and we assumed that both solvents remove labeled lipids. Ethanol did not remove precipitated DNA (Table 4). Of the label contained in the cold-TCA precipitate, 22% remained unaccounted for. This may have represented incomplete removal from the filter of DNA and proteins by enzymatic digestion or represented one or more unidentified labeled macromolecules. It may be concluded that macromolecules other than DNA and proteins may be labeled in short incubation experiments with [*methyl*-^{3}H]thymidine, that the acid-base hydrolysis procedure is not adequate for quantifying labeled DNA in the presence of other labeled macromolecules, and that the fraction previously referred to as RNA may contain labeled lipids and other macromolecules.

The data in Fig. 1 and Table 1 show a close coupling between the autotrophic production within the euphotic zone (1.3 to 4.8 m) and the heterotrophic bacterial activity at 10 m in Hartbeespoort Dam. There were no correlations between bacterial numbers and measurements of autotrophic and heterotrophic activity, since total microscopic counts do not distinguish between physiological types or active and inactive bacteria. Both measures of heterotrophic activity (glucose and total thymidine uptake) were strongly correlated to each other and to euphotic-zone primary production (Table 1). Furthermore, both the amount of label incorporated into DNA and its relative composition in the cold-TCA precipitate of macromolecules were inversely related to primary production and both measures of bacterial activity. As bacterial activity increased in response to increased phytoplankton production, owing presumably to increased substrate supply, larger amounts of labeled thymidine were incorporated into lipids and other macromolecules (Fig. 1A and D). The converse occurred with decreasing bacterial activity.

The strong statistical correlations just noted suggest that the changes in DNA and protein labeling were real even if not quantitatively correct owing to the problems (discussed above) associated with the acid-base hydrolysis procedure. The bacterial population at 10 m appeared to be shifting between states of balanced and unbalanced growth with changes in the availability of substrates from the euphotic zone. The surface bacterial populations, on the other hand, seemed to be in a constant state of balanced growth, as indicated by the low level of labeling of proteins and other macromolecules. These changes may be related to the spatial differences between 10-m and surface bacterial populations and the photosynthesizing phytoplankton. The close proximity to metabolically active algae may ensure a continuous, if not constant, supply of nutrients for heterotrophic bacteria in the upper water column.

In conclusion, the tritiated labeling experiments of Hartbeespoort Dam bacteria have shown that the label will not necessarily be predominantly incorporated into DNA. The relative labeling of DNA was inversely proportional to bacterial activity (glucose and thymidine uptake), with varying proportions of label appearing in proteins, lipids, and other macromolecules. Moriarty (13a) concluded that the relative labeling of bacterial macromolecules with thymidine varies between water bodies depending upon factors such as temperature, microbial composition, and nutrient availability. Variations in the relative labeling of bacterial macromolecules from Hartbeespoort Dam occurred both temporally and spatially, which in addition to the demonstrated relationship to changes in algal production, may also have been related to changes in bacterial population composition. This requires further experimental confirmation, but the relative labeling of DNA and proteins potentially offers a means of assessing the metabolic state of heterogeneous aquatic bacterial populations. The Hartbeespoort Dam data confirm the need for the extraction and purification of DNA labeled with tritiated thymidine during aquatic bacterial production studies, a point made previously (5, 9, 13, 13a, 16, 23) but seldom heeded.

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