Purification of DNA for Bacterial Productivity Estimates

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[methyl-\textsuperscript{3}H]thymidine-labeled DNA from natural populations of aquatic bacteria was completely separated from RNA and protein by hydroxylapatite chromatography. The procedure was validated by monitoring increases in Escherichia coli cell count, A\textsubscript{550}, DNA concentration, and thymidine incorporation into DNA isolated by the proposed technique. The procedure can be used in the field and does not rely on the use of acid-base hydrolysis or volatile organic solvents.

The use of [methyl-\textsuperscript{3}H]thymidine by Fuhrman and Azam (7) to measure bacterial production in aquatic habitats has stimulated research in microbial ecology. The thymidine technique has been shown to be specific for heterotrophic bacteria; all cyanobacteria, eucaryotic algae, and fungi that have been investigated lack thymidine kinase or at least have been shown not to incorporate thymidine directly into DNA (17). The analytical procedures of Fuhrman and Azam (7) have been used by others (3, 11, 20) and subsequently modified (6, 21). Although these procedures may seem to give reasonable estimates of bacterial productivity, they have three major drawbacks: (i) the imprecision of the factor which converts rates of thymidine incorporation into rates of bacterial production (2, 10); (ii) the unknown percentage of the microbial population capable of incorporating extracellular thymidine (17, 18); and (iii) the need to assume what fraction of the cold trichloroacetic acid (TCA)-insoluble material is DNA (7, 11). The method of extraction and purification of DNA labeled with [methyl-\textsuperscript{3}H]thymidine has been advocated by many researchers (5, 15, 21–23). Often, this process has been performed by the acid-base hydrolysis procedure originally used for aquatic bacterial production estimation (7). The procedure has been slightly modified by others (11, 19), and even the nucleases DNase and RNase have been used to confirm the identities of the three fractions obtained (19). The acid-base hydrolysis of the TCA-insoluble fraction has recently been shown to be inadequate for quantifying labeled DNA in the presence of other labeled macromolecules (22). However, that paper did not present sufficient data to confirm that the radioactivity of the DNA fraction is influenced by the unknown nature of the possible RNA fraction. The need for DNA purification has been indicated, and three alternative procedures to the acid-base hydrolysis scheme have been proposed (23, 26, 27). Witzel and Graf (27) ruptured \textsuperscript{3}H-labeled microorganisms by grinding them with sea sand at a low temperature, centrifuging the sample, and then using hydroxylapatite columns to isolate the nucleic acids from protein and other radiolabeled contaminants. The RNA was eluted with 0.14 M phosphate buffer, and the DNA was eluted with 0.24 M phosphate buffer. Under these elution conditions, the RNA and DNA overlap and are not quantitatively separated (K.-P. Witzel, personal communication). In addition, Marmur (12) states that cells grown in alumina or glass powder yield a DNA of lower molecular weight than the DNA released by enzyme or detergent lysis. Servais et al. (23) lysed tritiated thymidine-labeled bacteria with detergent and then incubated a sample with DNase to selectively hydrolyze the DNA. The macromolecules from both the DNase-treated and untreated solutions were precipitated with TCA, and the DNA was determined by the difference in radioactivity between the treated and untreated solutions. Wicks and Robarts (26) advocate washing the TCA-precipitated macromolecules (after RNA has been removed by alkaline hydrolysis) with phenol-chloroform to remove the protein, with an additional 80% ethanol wash to remove lipids and unincorporated thymidine. In this paper, we report a modification of the hydroxylapatite procedure (27) and compare it with TCA precipitation of macromolecules (7) and the phenol-chloroform wash technique (26).

MATERIALS AND METHODS

Tritiated thymidine ([methyl-\textsuperscript{3}H]thymidine; 3.26 TBq-mmol\textsuperscript{-1}) in 2% ethanol was purchased from Amersham Corp., Arlington Heights, Ill., and stored at 5°C. This specific activity was used for all water samples, except where noted. Water samples (2 liters each) were collected from the surface of Hamilton Harbor, Ontario, Canada. Subsamples (10 ml each) were incubated with 10 nM \textsuperscript{3}H thymidine for 20 min. The incubation was terminated by adding 1 mM unlabeled thymidine and by placing the incubation flasks on ice (10). Three procedures were compared: (i) the procedure of Fuhrman and Azam using ice-cold TCA-insoluble material (7); (ii) the phenol-chloroform procedure of Wicks and Robarts (26); and (iii) the proposed hydroxylapatite procedure. Six replicates of water samples were processed for each procedure, and controls (1 mM unlabeled thymidine added to 10 ml of Hamilton Harbor water, then 10 nM \textsuperscript{3}Hthymidine, and treated as the comparative samples) and DNA recoveries were run in triplicate and duplicate, respectively. In a separate experiment, the phenol-chloroform procedure was compared with the hydroxylapatite method with water obtained from six glass laboratory microcosms (capacity, 11 liters). These microcosms had various levels of dissolved oxygen (range, 3.6 to 20 mg-liter\textsuperscript{-1}) and chlorophyll \textit{a} (range, 1 to 66 \mu g-liter\textsuperscript{-1}) (unpublished data).

For the hydroxylapatite procedure, the water samples were filtered on polycarbonate filters (pore size, 0.2 \mu m; Nuclepore Corp., Pleasanton, Calif.) and immediately placed in a liquid scintillation glass vial containing 0.5 ml of lysing reagent. This reagent contained 4 mg of egg white lysozyme (grade 1, L 6876; Sigma Chemical Co., St. Louis,
Mo.) ml⁻¹ in 10 mM Tris buffer (pH 7.0)–100 mM NaCl–10 mM EDTA–100 μg of carrier DNA (salmon testes DNA, type III, D 1626; Sigma). The vial was capped and frozen for at least 12 h (field samples can be stored at this point). At the end of this time, the vial was thawed rapidly at 60°C for 3 min, and then 1.5 ml of 0.24 M MUP (0.24 M sodium phosphate buffer [pH 6.8] containing 8 M urea) was added. The contents of the vial were sonicated for 15 s (16) with a Sonic dismembrator (model 150; Artrek Systems Corp., Farmingdale, N.Y.) with a 12-mm probe at 90 W (60% power setting). Purification of the DNA was performed by the method of Adriaenssens et al. (1) on hydroxylapatite columns (product 44257; British Drug House). Hydroxylapatite was suspended in 0.014 M phosphate buffer (pH 6.8) and heated for 15 min at 85°C. A small column was made with a 23-cm Pasteur pipette. Glass wool was used to retain the hydroxylapatite, which was packed to a depth of 6 cm. Being careful not to let the column go dry, we pumped 0.24 M MUP over the column, using a peristaltic pump or onto several columns at one time with a vacuum manifold. Five to ten milliliters was pumped through the column before the sample was added. The sample was allowed to pass through the hydroxylapatite column by gravity flow. Twenty milliliters of 0.24 M MUP was passed through the column; this amount is usually sufficient to wash out all non-DNA compounds (including protein and RNA), as indicated by monitoring radioactivity in the effluent. The hydroxylapatite was rinsed with 10 ml of 0.014 M sodium phosphate buffer (pH 6.8) to wash out the urea. DNA was eluted from the hydroxylapatite column with 0.48 M sodium phosphate buffer. The 0.48 M sodium phosphate buffer fraction was carefully collected, and the volume was measured (and usually found to be 10 to 11 ml). A 2-ml sample was placed in a glass scintillation vial, 150 μl of concentrated HCl was added, and the sample was heated for 30 min at 95°C. The sample was cooled, 10 ml of ACS II (Amersham) was added, and the radioactivity was counted with a model 4430 liquid scintillation counter (Packard Instrument Co., Inc., Down- ville, Md.). The disintegrations per minute were automatically determined by the external standard procedure. The total radioactivity of the 0.48 M sodium phosphate buffer fraction was taken to be the amount of [³H]thymidine incorporated into the DNA in the original 10 ml of lake water.

Recovery of DNA from the hydroxylapatite column was determined by adding either [thymine-methyl-³H]DNA (specific activity, 632.7 MBq·mg⁻¹; Du Pont, NEN Research Products, Boston Mass.) or [thymine-methyl-¹⁴C]DNA (specific activity, 1.44 MBq·mg⁻¹; Amersham). Because of the high molar specific activity of [¹⁴C]DNA, which increases the rate of radiation decomposition, the specific activity was lowered by adding 10 ng of Escherichia coli DNA (type VIII, D-2001; Sigma) dissolved in 4 ml of 0.05 M borate buffer (pH 8.0). Each 50-μl sample of labeled DNA contained approximately 10,000 dpm of [¹⁴C]DNA and 100 μg of unlabeled DNA. Recovery of [¹⁴C]DNA dissolved in 0.5 M NaOH was also tested. The lysing reagent efficacy was tested with liquid cultures of E. coli ATCC 11775. Five milliliters of culture was filtered and treated as above. The released DNA was quantified fluorometrically by the method of Bruck et al. (4) with E. coli DNA as the standard. A 0.5-ml sample was combined with 4.5 ml of Tris buffer (100 mM NaCl, 10 mM EDTA, 10 mM Tris hydrochloride [pH 7.0]) containing 20 ng of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) ml⁻¹.

### DNA Purification with Hydroxylapatite

<table>
<thead>
<tr>
<th>Run</th>
<th>Eluent buffer</th>
<th>Volume collected (ml)</th>
<th>[³H]DNA recovered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run 1</td>
<td>0.24 M MUP</td>
<td>12.5</td>
<td>5.33*</td>
</tr>
<tr>
<td></td>
<td>0.014 M sodium phosphate</td>
<td>7.3</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>0.48 M sodium phosphate</td>
<td>10.5</td>
<td>93.13</td>
</tr>
<tr>
<td></td>
<td>Additional 2 ml</td>
<td>2.0</td>
<td>1.41</td>
</tr>
<tr>
<td>Run 2</td>
<td>0.24 M MUP</td>
<td>12.6</td>
<td>5.67*</td>
</tr>
<tr>
<td></td>
<td>0.014 M sodium phosphate</td>
<td>7.5</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>0.48 M sodium phosphate</td>
<td>10.5</td>
<td>93.82</td>
</tr>
<tr>
<td></td>
<td>Additional 2 ml</td>
<td>2.0</td>
<td>0.37</td>
</tr>
</tbody>
</table>

* Radioactivity in a 2-ml sample from collected volume counted by liquid scintillation.

### RESULTS AND DISCUSSION

**Cell disruption and DNA recovery.** The lysing technique (lysozyme, freeze-thaw, and sonication) that we used to disrupt the microorganisms in aquatic samples quantitatively extracted DNA from E. coli. We obtained a value of $1.48 \times 10^{-14}$ ± $0.11 \times 10^{-14}$ g of DNA cell⁻¹ (mean ± standard deviation). This value is similar to previously published values for log-phase cultures of E. coli B and B/r, which are $1.37 \times 10^{-14}$ and $1.82 \times 10^{-14}$ g of DNA cell⁻¹, respectively (24). A value of $0.90 \times 10^{-14}$ g of DNA cell⁻¹ has also been reported for the B/r strain (28). We felt that an organism with a known DNA content, such as E. coli, should be used for checking the lysing reagent efficacy. Often, a detergent is used to lyse aquatic bacteria (8, 16); sonication alone has also proven to be an effective lysing technique (13). The detergent sodium dodecyl sulfate was not used in the present study because it quenched the DAPI-DNA fluorescence in the DNA analysis (unpublished data). Assuming that E. coli is an adequate test organism, this result verifies that the lysing technique is accurate for rupturing gram-negative cell walls. Although we did not specifically test for the lysis of gram-positive bacteria, many are also susceptible to lysozyme action (14). Since gram-negative bacteria are the predominant bacteria found in most freshwater ecosystems (9), we feel that our lysing reagent lyses the majority of the bacterial cells found in the aquatic environment. Even short exposure (30 min) to TCA is assumed to cause cell lysis, but microscopic examination of the cells has not confirmed this assumption (19). Extracting the filtered cells with NaOH (26) seemed an ideal procedure to lyse cells. However, when [³H]DNA was dissolved in 0.25 N NaOH, neutralized, put in MUP buffer, and run through the hydroxylapatite procedure, a recovery rate of only 60% was obtained. The probable explanation is that when DNA is exposed to a pH of at least 12 or to a pH lower than 3, single-stranded molecules with half the molecular weight of native DNA are produced (25). Single-stranded DNA does not adhere to hydroxylapatite as strongly as double-stranded DNA does (see below).

**Recovery of tritiated DNA from hydroxylapatite columns.** The radioactivity in the various buffers used to adsorb and elute [³H]DNA from a hydroxylapatite column is shown in Table 1. The majority of the radioactivity (93.4%) was eluted with 0.48 M sodium phosphate buffer, as expected. The initial 0.24 M MUP buffer carried 5.5% of the radioactivity through the column. The technical information of the manufacturer states that a 3% single-stranded DNA component is present in the DNA at the time of shipment. Single-
stranded DNA does not have the same affinity for hydroxylapatite as native double-stranded DNA (14) and therefore was eluted with the initial buffer. Protein and RNA are also completely eluted in this fraction (1, 14).

**Comparison of TCA, phenol-chloroform, and hydroxylapatite.** Three of the commonly employed procedures were tested for thymidine incorporation into DNA, DNA recovery, radioactivity of controls, and reproducibility. Table 2 shows that the cold-TCA precipitate contained the highest radioactivity in the sample as well as in the control, the lowest sample coefficient of variation, and 91.8% recovery of DNA. However, several researchers (22, 23) have previously shown and we have also demonstrated (unpublished results) that macromolecules (protein, RNA, and perhaps lipids) other than DNA are labeled in the presence of tritiated thymidine. The radioactivity retained by TCA precipitation would therefore be expected to be greater than that retained by methods which selectively recover only DNA. The acid-base hydrolysis procedure recommended by Fuhrman and Azam (7) was not used in this study because of its inaccuracy in differentiating DNA, RNA, and protein (19). The phenol-chloroform procedure (26) recovered 76.8% of the added tritiated DNA but had highly variable control values (coefficient of variation, 89%) as well as variable sample values (coefficient of variation, 29.1%). Other disadvantages of the phenol-chloroform procedure with our samples were very low filtration rates at the TCA precipitation step and often at the phenol-chloroform wash step and the obnoxious smell of the phenol-chloroform reagent. The proposed hydroxylapatite procedure recovered 90.7% of the tritiated DNA and had very low consistent control values and reasonable reproducibilities of sample values (coefficient of variation, 11%). For stable liquid scintillation counting of the radioactivity in all procedures, we found it necessary to acid-hydrolyze the DNA before addition of the fluor. During the routinely monitoring of our laboratory microcosms, we measured the recovery of DNA by the phenol-chloroform procedure and our hydroxylapatite method. Table 3 shows

### Table 2. Radioactivity of TCA precipitate, phenol-chloroform-washed filter, and hydroxylapatite-isolated DNA

<table>
<thead>
<tr>
<th>Source</th>
<th>Radioactivity (dpm/10-ml sample) of DNA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>TCA precipitate</th>
<th>Phenol-chloroform-washed filter</th>
<th>Hydroxylapatite adsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamilton Harbor sample</td>
<td>38,726 (4.0)</td>
<td>27,205 (29.1)</td>
<td>29,557 (11.0)</td>
<td></td>
</tr>
<tr>
<td>Control (n = 3)</td>
<td>2,954 (58.2)</td>
<td>2,123 (89.0)</td>
<td>500 (2.0)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Percentages of DNA recovered were 91.8, 76.8, and 90.7, respectively.

<sup>b</sup> Values in parentheses are coefficients of variation.

<sup>c</sup> Samples corrected for control radioactivity and percent DNA recovery.

### Table 3. Radioactivity of DNA fractions from water samples in laboratory microcosms

<table>
<thead>
<tr>
<th>Microcosm</th>
<th>Sample&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Radioactivity (dpm/5 ml) of DNA</th>
<th>Controls</th>
<th>DNA recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phenol-chloroform</td>
<td>phenol-chloroform</td>
<td>Hydroxyl-apatite</td>
<td>Hydroxyl-apatite</td>
</tr>
<tr>
<td></td>
<td>Hydroxyl-apatite</td>
<td>Hydroxyl-apatite</td>
<td>Hydroxyl-apatite</td>
<td>Hydroxyl-apatite</td>
</tr>
<tr>
<td>1</td>
<td>20,118</td>
<td>21,892</td>
<td>1,094</td>
<td>388</td>
</tr>
<tr>
<td>2</td>
<td>6,397</td>
<td>4,794</td>
<td>960</td>
<td>486</td>
</tr>
<tr>
<td>3</td>
<td>2,479</td>
<td>2,565</td>
<td>1,165</td>
<td>244</td>
</tr>
<tr>
<td>4</td>
<td>13,212</td>
<td>14,047</td>
<td>1,511</td>
<td>280</td>
</tr>
<tr>
<td>5</td>
<td>9,907</td>
<td>9,577</td>
<td>1,165</td>
<td>282</td>
</tr>
<tr>
<td>6</td>
<td>2,705</td>
<td>4,944</td>
<td>1,094</td>
<td>594</td>
</tr>
</tbody>
</table>

<sup>a</sup> Samples corrected for control radioactivity and percent DNA recovery.

![Graph](http://aem.asm.org/Downloadedfrom)

**FIG. 1.** Increase in A<sub>590</sub> (△), DNA concentration (○), [<sup>3</sup>H]thymidine incorporation (*), and bacterial cell numbers (□) in a batch culture of E. coli versus time. Ln, Log n.
the low recovery of DNA (average, 64.3%) and high control values (average, 1,165 dpm) for the phenol-chloroform procedure. The hydroxylapatite method showed an average of 96.2% DNA recovery and a control value of 379 dpm. The corrected sample radioactivities from each microcosm are similar for microcosms 1, 3, 4, and 5 but deviate for microcosms 2 and 6. Although these values are based on only single determinations for sample activity, control, and DNA recovery, they illustrate the variability in the phenol-chloroform procedure and the relative consistency of the hydroxylapatite method.

E. coli growth experiment. Figure 1 illustrates the logarithmic growth phase of an E. coli culture. Bacterial growth was monitored by increases in optical density, cell count, DNA concentration, and incorporation of tritiated thymidine into DNA isolated on hydroxylapatite columns. The slopes of the lines generated from plotting log n versus time indicate a close correlation among all variables except increases in cell number. Recently, Coveney and Wetzel (5) have shown that changes in biovolume are more important than cell number changes in determining the conversion factors relating thymidine incorporation to bacterial growth.

The isolation of DNA labeled by tritiated thymidine on hydroxylapatite columns is an efficient technique which removes protein and RNA contaminants. The lysis of the microorganisms seems to be complete when freeze-thawing, lysis enzyme, and sonication are used. Further research to check other suitable lysing techniques (such as incorporation of a detergent) may be warranted. The released native DNA is not subjected to harsh bases, acids, or organic solvents, and therefore the hydroxylapatite procedure is readily adapted to field use. Radioactivity in controls, consisting of tritiated thymidine, is consistently low and recoveries of radioactive DNA are high, but DNA recovery experiments are recommended for each new environment.

LITERATURE CITED