A repressible alkaline phosphatase has been isolated from the extreme bacterial thermophile, *Thermus aquaticus*, and has been purified to homogeneity as judged by disc acrylamide electrophoresis and sodium dodecyl sulfate electrophoresis. Upon investigation, the purified enzyme was shown to hydrolyze certain phosphodiesterases in addition to a wide variety of phosphomonoesters. The diesters included bis-p-nitrophenyl phosphate and thymidine 3'-monophosphate-5'-nitrophenyl ester. The temperature optimum for the diesterase activity was 80-85° at pH 7.2. Orthophosphate competitively inhibited both activities. Nucleotides such as AMP, ADP, and ATP also inhibited both esterase activities as did α-D-glucose 1-phosphate and α-sodium glycerol phosphate. The isoelectric point of the enzyme was determined to be 8.4.

Many reports have been published on the derepression of alkaline phosphatases in microorganisms (1-7). These reports have focused on the structure and function of the enzyme, its relationship to the physiology of the microorganism, and its location within the cell. In addition to the derepression of alkaline phosphatases, there are a few situations in which the levels of phosphodiesterase activity also increase under phosphate starvation. Taniguchi and Tsugita (8) have reported that in *Aspergillus nidulans* the enzyme possesses phosphomonoesterase activity (12). The enzyme has the ability to hydrolyze a wide variety of monoesters, a thermophilic alkaline phosphatase has the ability to hydrolyze certain phosphodiesterases. Probably the best studied alkaline phosphatase has the ability to hydrolyze certain phosphodiesterases and phosphomonoesterases. The enzyme has been shown to have both activities is a deoxyribonucleic acid phosphatase-exonuclease from *Escherichia coli* (13, 14). The enzyme has the ability to hydrolyze the phosphate residue esterified to the 3'-hydroxyl terminus of a DNA chain, and also has the capacity to cleave 5'-nucleotidyl residues stepwise from the 3'-hydroxyl end of the DNA chain. Unlike the alkaline phosphatase from *E. coli*, the DNA phosphatase is unable to act on 3'-or 5'-deoxynucleotides.

Recently two other enzymes have been reported which have both activities. In the first, a cyclic nucleotide binding phosphodiesterase-phosphomonoesterase has been isolated from *Aspergillus nidulans* (15). The enzyme has acid pH optima and its synthesis appears to be derepressed by nitrogen starvation and not phosphate starvation. The second report described the isolation of an extracellular 5'-nucleotidase with both monoesterase and diesterase activities from *Micrococcus sodononio* (16). This enzyme exhibited a pH optimum of 8.7 and had a molecular weight of approximately 115,000.

Our report demonstrates that in addition to its ability to hydrolyze a wide variety of monoesters, a thermophilic alkaline phosphatase has the ability to hydrolyze certain diesters.

### EXPERIMENTAL PROCEDURES

**Strain—** *Thermus aquaticus* YT-1 used in these experiments was supplied by Dr. Paul Ray, Burroughs Wellcome.

**Preparation and Purification of Enzyme—** The enzyme was purified as previously described (1).

**Enzyme Assays—** Phosphomonoesterase activity was measured by observing p-nitrophenol released from p-nitrophenyl phosphate. The assay was run at 75° and the absorbance was later measured at 410 nm on a Gilford recording spectrometer. The assay mixture contained p-nitrophenyl phosphate (1 mM) and sodium succinate buffer (0.1 M, pH 7.2) in a final volume of 0.5 ml. Phosphodiesterase activity was measured by observing p-nitrophenol released from bis-p-nitrophenyl phosphate. The reaction mixture contained bis-p-nitrophenyl phosphate (1 mM) and sodium succinate buffer (0.1 M, pH 7.2) in a final volume of 0.5 ml. The reactions were stopped 10 min later by placing the reaction tubes in ice and then adding 0.5 ml of 0.4 M NaOH. One unit of enzyme activity is defined as the release of 1 pmol of nitrophenol/min by the enzyme.

**Disc Gel Electrophoresis—** Disc gels were prepared according to the method of Davis (17). The separating gel (8.0 x 0.5 cm) contained 7% acrylamide, whereas the spacer gel (1.5 x 0.5 cm) contained 2% acrylamide. Electrophoresis, in a Canaco model 66 electrophoresis bath, was carried out at 4° in 2.5 mm Tris/1.5 mm glycine buffer, pH 9.5, for 2.5 h at 2 mA. The gels were stained with 0.1% Coomassie brilliant blue in 7% acetic acid and subsequently destained in 7% acetic acid. Instead of being stained, certain gels were sliced into 2-mm discs and placed into phosphomonoesterase and phosphodiester-
phodiesterase Activity

Phosphate in the form of sodium glycerol phosphate (4 x 10^{-5} M) was also added to the reaction mixtures containing 0.5 mM Tris buffer, pH 9.0, and 1 mM p-nitrophophenyl phosphate. The reactions were run at 75°C for 10 min.

Chemicals - The substrates: p-nitrophophenyl phosphate, bis-p-nitrophophenyl phosphate, thymidine 3'-monophospho-p-nitrophophenyl ester, thymidine 5'-monophospho-p-nitrophophenyl ester, and Tris-p-nitrophophenyl phosphate were all purchased from Sigma. *Escherichia coli* alkaline phosphatase was purchased from Worthington. Other chemicals were reagent grade from Fisher and Sigma.

RESULTS

Effect of Phosphate Limitation on Relative Levels of Phosphodiesterase Activity - Since it has been previously shown that the concentration of phosphate in the growth media had a dramatic effect on the relative levels of alkaline phosphatase produced by the cells, it was of interest to see whether the level of phosphodiesterase activity also increased upon phosphate starvation. Some cells were grown with excess inorganic phosphate (8 x 10^{-5} M), whereas others were grown on limiting phosphate in the form of sodium glycerol phosphate (4 x 10^{-3} M). Table I shows that, in addition to the increased levels of phosphomonoesterase activity during phosphate starvation, the phosphodiesterase activity increased over 35-fold when assayed using bis-p-nitrophophenyl phosphate as substrate. This indicates the relative levels of both enzymatic activities are dependent upon the levels of phosphate in the growth media.

Polyacrylamide Disc Gels - Previously, it has been shown that when the purified alkaline phosphatase is subjected to electrophoresis it chromatographs as a single protein band on disc gels and that this band is coincident with the phosphatase activity. Therefore, it was of interest to see whether phosphodiesterase activity also corresponded to the same protein band. Gels were prepared with special care to ensure that they were the same size and were run under similar conditions described under "Experimental Procedures." One gel was stained for protein, while the other two were assayed for phosphomonoesterase and phosphodiesterase activities. Both activities corresponded to the protein band, with a small amount of activity remaining in the stacking gel (Fig. 1).

Substrate Specificity of Phosphodiesterase Activity - Different substrates were assayed in order to test the ability of the purified enzyme to hydrolyze them (Table II). The enzyme was able to hydrolyze bis-p-nitrophophenyl phosphate and thymidine 3'-monophospho-p-nitrophophenyl ester. Only a trace of color could be seen when thymidine 5'-monophospho-p-nitrophophenyl ester was used as substrate, and no color could be seen when Tris-p-nitrophophenyl phosphate was used. *Escherichia coli* alkaline phosphatase was used as a control to monitor against monoesterase activity.

### Table I

<table>
<thead>
<tr>
<th>Substrate (1 mM)</th>
<th>Alkaline phosphatase, A_{650}</th>
<th>Thermus aquaticus</th>
<th>Escherichia coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bis-p-nitrophophenyl phosphate</td>
<td>1.37</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Thymidine 3'-monophospho-p-nitrophophenyl ester</td>
<td>0.58</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Thymidine 5'-monophospho-p-nitrophophenyl ester</td>
<td>0.06</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Tris-p-nitrophophenyl phosphate</td>
<td>0.01</td>
<td>0.01</td>
<td></td>
</tr>
</tbody>
</table>

* The reactions were run in 0.1 M sodium succinate buffer, pH 7.2, for 10 min at 75°C with 0.1 mg of purified enzyme in each assay tube.

### Table II

<table>
<thead>
<tr>
<th>Amendment (3 mM)</th>
<th>Phosphomonoesterase</th>
<th>Phosphodiesterase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inorganic phosphate</td>
<td>0.27</td>
<td>0.05</td>
</tr>
<tr>
<td>α-Sodium glycerol phosphate</td>
<td>86.0</td>
<td>1.78</td>
</tr>
</tbody>
</table>

Effects of phosphate limitation on levels of phosphomonoesterase and phosphodiesterase activities in *Thermus aquaticus*

The cells were grown in 500-ml Erlenmeyer flasks at 75°C in a New Brunswick water bath shaker for 32 h.

### Table III

<table>
<thead>
<tr>
<th>Amendment (3 mM)</th>
<th>Phosphomonoesterase</th>
<th>Inhibition</th>
<th>Phosphodiesterase</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.49</td>
<td>0.40</td>
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<tr>
<td>ATP</td>
<td>0.32</td>
<td>0.35</td>
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<tr>
<td>ADP</td>
<td>0.36</td>
<td>0.26</td>
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<td></td>
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<tr>
<td>AMP</td>
<td>0.34</td>
<td>0.31</td>
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<tr>
<td>Cyclic AMP</td>
<td>0.43</td>
<td>0.18</td>
<td></td>
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</tr>
<tr>
<td>α-D-glucose 1-phosphate</td>
<td>0.41</td>
<td>0.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Sodium glycerol phosphate</td>
<td>0.43</td>
<td>0.23</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Purified enzyme (0.002 μg/assay tube) was added to the reaction mixtures containing 0.5 mM Tris buffer, pH 9.0, and 1 mM p-nitrophophenyl phosphate. The reactions were run at 75°C for 10 min.

* Purified enzyme (0.04 μg/assay tube) was added to the reaction mixtures containing 0.1 mM sodium succinate, pH 7.2, and 1 mM bis-p-nitrophophenyl phosphate. The reactions were also run at 75°C for 10 min.
A thermophilic alkaline phosphatase has been purified and partially characterized, and interestingly, possesses the ability to hydrolyze certain phosphodiesters in addition to a wide variety of phosphomonoesters. The temperature and pH optima for the two catalytic activities are somewhat different. The temperature optimum for the phosphomonoesterase is 75-80°C at a pH of 9.2, whereas that of the phosphodiesterase is slightly higher, 80-85°C, at a pH of 7.2.

There is a large difference in the extent of derepression of the two activities upon phosphate starvation. One possible explanation for this would be the presence of other phosphodiesterases in the crude cellular extracts. This would give an artificially high level of phosphodiesterase activity in the extract which had been grown on excess phosphate.

The phosphodiesterase activity was extremely sensitive to low concentrations of nucleotides such as ATP and ADP, as well as higher concentrations of phosphomonoesters such as sodium glycerol phosphate. Therefore, the question of whether or not the diesterase activity is physiologically important in vivo really cannot be answered at this point.

REFERENCES
Repressible alkaline phosphatase from Thermus aquaticus: associated phosphodiesterase activity.
D H Smile, M Donohue, M F Yeh, T Kenkel and J M Trela


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