Bacterial Growth on Aminoalkylphosphonic Acids

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ABSTRACT

—Of 10 bacterial strains tested, 9 were found to be able to utilize the phosphorus of at least one of eight different aminoalkylphosphonic acids for growth, indicating that the ability to catabolize the carbon–phosphorus (C–P) bond is widespread among bacteria. Several organisms gave comparable growth rates as well as cell yields when an equimolar amount of either P1 or 2-aminoethyolphosphonic acid (2-AEP) was added to the medium. No compounds containing C–P bonds were detected in Escherichia coli B grown on 2-AEP orthophosphate. No degradation of phosphonates by cell-free extracts or suspensions of dried cells was demonstrated. The direct involvement of alkaline phosphatases in cleaving the C–P bond was excluded.

The isolation of aminoalkylphosphonic acids from ciliates (4, 5), the sea anemone (8), a zoanthid (7), and, most recently, from beef brain (14) has stimulated considerable interest. The biological significance of these compounds, unique in containing the very stable carbon-to-phosphorus (C–P) bond, has not been ascertained. The occurrence of 2-aminoethyolphosphonic acid (2-AEP) in complex lipids has been established. Kittredge et al. (8) isolated the glycerol ester of 2-AEP from Anthopleura elegantissima, and Rouser et al. (13) isolated and tentatively identified the intact lipid as a sphingolipid. Quin (10) found 2-AEP in nonsaponifiable lipid in another sea anemone, Metridium dianthus, and also demonstrated its presence in proteins of this organism.

The pathways of biosynthesis and degradation of the aminoalkylphosphonic acids have not been delineated. Rosenberg (12) showed that P32-orthophosphate (P1, P2) is incorporated into both free and lipid-bound 2-AEP in growing cultures of Tetrahymanella pyriformis. Growth of Crooke's strain of Escherichia coli (15) and, more recently, Pseudomonas aeruginosa (E. A. James, T. C. Myers, and E. B. Titchener, Federation Proc. 24: 440, 1965), with methylphosphonic acid as sole phosphorus source, has been described. However, the products formed have not been identified, and nothing is known about the mechanisms involved in breaking the C–P bond.

Efforts in this laboratory have recently been directed toward the study of the bacterial catabolism of the aminoalkylphosphonic acids. The ultimate goal of these studies is to isolate the enzyme or enzymes involved in the cleavage of the C–P bond and to study the mechanism of this reaction. In searching for a suitable organism for these studies, numerous strains of bacteria were tested for the ability to utilize eight different aminoalkylphosphonic acids as a source of phosphorus for growth. In this paper, we report the results of this survey. Most organisms were found able to utilize several of these compounds, and, without exception, growth was most rapid when 2-AEP was added to the growth medium. Radioactive 2-AEP was isolated from T. pyriformis grown on P1, and growth of E. coli B on this substrate was compared with that on radioactive P1. Compounds containing C–P bonds could not be isolated from these organisms. Data are presented which show that nonspecific alkaline phosphatases, known to be present in several of the organisms employed in these studies, are not directly involved in the catabolism of these compounds. We have thus far been unable to demonstrate decomposition of 2-AEP by dried cells or extracts of organisms grown on this compound.

MATERIALS AND METHODS

Organisms. Bacterial strains were obtained from the following sources: E. coli B and Bacillus subtilis from Sheldon Greer (University of Miami School of Medicine), Serratia marcescens and Shigella sonnei from Faith McCoy (Jackson Memorial Hospital Bacteriology Laboratory), and Salmonella typhimurium, Aerobacter aerogenes, A. cloacae, Klebsiella (type 16), and Proteus mirabilis from Mary Jo Carter and Joel Ehrenkranz (University of Miami School of Medicine). A. Torrani supplied E. coli B11, a mutant with a deletion.
in the alkaline phosphatase genome. E. coli Crooke's
strain (ATCC 8739) and T. pyriformis strain W (ATCC
10542) were from American Type Culture Collection,
Rockville, Md.

**Media and growth conditions.** Bacteria were grown
on the medium of Hershey and Chase (3), modified by
substituting 3.4 × 10^{-3} M tris(hydroxymethyl)ami-
nomethane (Tris) (pH 7.4) for the phosphate buffer.
Comparable amounts of phosphorus were added as
aminoalkylyphosphonic acids, methylphosphonate, 5’-
deoxycadenosinic acid (5’-dAMP), and P_{i} prior to auto-
claving. Cultures were grown at 37 °C in 125-ml Elen-
meyer flasks containing 50 ml of medium shaken
aerobically on a New Brunswick gyrotary shaker.
Growth was measured as increase in turbidity at 420
μm.

T. pyriformis was grown aerobically at 27 °C in 2% proteose peptone, 0.5% yeast extract, and 0.85% dext-
rose.

**Reagents.** The aminoalkylyphosphonic acids were
purified from Calbiochem, and methylphosphonate
was a gift from E. A. James. Radioactive inorganic
phosphate was obtained from E. R. Squibb & Sons,
New York, N.Y., and 5’-dAMP was from Sigma
Chemical Co., St. Louis, Mo. Proteose peptone and
yeast extract were products of Difco. Other chemicals,
all of reagent grade, were obtained from Mallinckrodt
Chemical Co., St. Louis, Mo.

Chromatographically purified E. coli alkaline phos-
phatase was purchased from Worthington Biochemical
Corp., Freehold, N.J.

**Methods.** Inorganic and ash phosphate analyses
were done by the method of Ames and Dubin (1).
Ascending chromatography on 3 mm Whatman paper
was performed by use of the following solvent sys-
tems: (i) n-butanol-acetic acid-water (160:40:75) and
(ii) t-butanol-methyleneethylether-formic acid-water
(40:30:15:15). Paper electrophoresis was carried out
in a Durrum chamber on Whatman 3 mm filter paper
in barbital buffer (pH 8.6). The aminoalkylyphosphonic
acids were located on chromatograms with ninhydrin
spray reagent and by the phosphate method of Rosen-
berg (11). The latter method also detected methyl
phosphonate. Radioactivity on paper was located by
autoradiography, with use of standard X-ray film.

Radioactivity was measured quantitatively in a
Packard Tri-carb liquid scintillation spectrometer
(Packard Instrument Co., La Grange, Ill.). All ab-
sorbance measurements were made in a Hitachi-Perkin
Elmer spectrophotometer model 139 (Perkin-Elmer,
Norwalk, Conn.) equipped with a photomultiplier
tube.

**Preparation of 2-AEP.** Six 2-liter flasks, each con-
aining 800 ml of medium, were each inoculated with
5 ml of a 72-hr culture of T. pyriformis and were placed
on a gyrotary shaker at 200 cycle/min. After 18 hr,
KH_{2}PO_{4} at pH 6.0 (5 × 10^{-4} counts per min per
μmole) was added to a final concentration of 2 μmole,
and growth was allowed to continue for 54 hr. The
cells were then harvested by centrifugation, yielding
84 g (wet weight).

The 2-AEP was isolated from the cells as de-
scribed by Horiguchi and Kandatsu (6). After reflux-
ing for 24 hr in constant boiling HCl, the hydrolysate
was extracted four times with 2 volumes of ether,
and the acid was then removed by repeatedly taking to
dryness in a flash evaporator. The residue was dis-
solved in 500 ml of hot water, decolorized with 10 g
of charcoal, and applied to a Dowex 50 (45% cross-
linked, 200 to 400 mesh) column (2 by 28 cm) in the
H^{+} form. The amino acids were eluted with 0.3 N
NH_{4}OH. Fractions containing 2-AEP, eluted just
prior to the bulk of the amino acids, were identified by
radioautograms of chromatograms developed in sol-
vent system ii (R_{f} for P_{i}, 0.52; R_{f} for 2-AEP, 0.25).
These fractions were combined, lyophilized, and ap-
plied to a second Dowex 50-H^{+} column (1 by 40 cm),
which was eluted with 0.6 N HCl. Fractions containing
2-AEP were identified as above, combined, taken to
dryness, and the last traces of HCl were removed by
ion exchange on a Dowex 1-acetate column (1.5 by 2
cm) eluted with 0.3 N acetic acid. The acetic acid was
removed by evaporation, and a final charcoal treat-
ment was carried out.

A 450-μmole amount of 2-AEP with a specific ac-
tivity of 200,000 counts per min per μmole of ash phos-
phate was recovered. By ascending chromatography in
solvent ii, two minor contaminating ninhydrin-re-
active spots were seen, but neither contained radioac-
tivity or organic phosphate. The purified 2-AEP con-
tained no measurable P_{i}, and no P_{i} was released upon
incubation with E. coli alkaline phosphatase.

**RESULTS**

**Survey of organisms for growth on phosphonic
acids.** Organisms were transferred from agar slants to medium containing 0.2 μmole of P_{i} per
ml and were incubated overnight. Samples (1 ml) of
each culture were transferred into 11 flasks containing equivalent amounts (0.2 μmole/ml) of
one of the eight aminoalkylyphosphonic acids, methylphosphonate, 5’-dAMP, or P_{i}. The
5’-
dAMP was used to compare growth rates on a
phosphate ester. Flasks without added phos-
phorus were included as controls. Cultures were
incubated overnight (12 to 16 hr), and growth was
measured. A 1-ml amount of each culture was
then transferred to flasks containing identical
media, and growth was measured each hour. The
doubling times were calculated from semilog
plots of the turbidity readings (Table 1). Of the
organisms tested, only B. subtilis failed to grow on
at least one of the substrates of interest. This organ-
ism grew only on P_{i} and 5’-dAMP. Among the
organisms able to utilize the phosphorus of any of
the phosphonic acids, growth was consistently
most rapid on 2-AEP. Growth on 1-amino substi-
tuted alkylphosphonic acids was insignificant.

**Substrate-dependent growth on P_{i} and 2-AEP.**

Not only was the rate of growth on 2-AEP com-
parable to that on P_{i}, but the amount of growth
was identical. In Fig. 1, growth yield of E. coli
B and a growing concentrations of P_{i} is compared
with growth on identical quantities of 2-AEP.
TABLE 1. Comparison of bacterial growth on aminoaalkylphosphonic acids and inorganic phosphate

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Escherichia coli B</th>
<th>E. coli Crooke's</th>
<th>Aerobacter cloacae</th>
<th>Serratia marcescens</th>
<th>Klebsiella type 16</th>
<th>Salmonella typhi/murium</th>
<th>Proteus mirabilis</th>
<th>Shigella sonnei</th>
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<td>Orthophosphate</td>
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<tr>
<td>1-Aminobutylphosphonic acid</td>
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<tr>
<td>1-Aminopentylphosphonic acid</td>
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<td>5'-Deoxyadenylate acid</td>
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* Numbers represent doubling times; dashes represent no growth. Concentration of phosphorus-containing substrate in each case was 0.2 µmole/ml. A. aerogenes and A. cloacae gave almost identical results, so only one is included in the table. Bacillus subtilis grew only on P1 and 5'dAMP.

Total and inorganic phosphate were measured on culture filtrates. No P1 was found in flasks to which 2-AEP had been added, and no organic phosphate was found where P1 had been added. Comparable experiments with S. marcescens and S. typhi/murium gave identical results. Though optimal growth on P1 occurred at 0.2 µmole/ml, no extracellular P1 was recovered until concentrations above 0.3 µmole/ml were added (Fig. 1), indicating the capacity for storage of P1 in excess of that required for growth. This was not the case with 2-AEP. Here the substrate in excess of that required for maximal growth remained entirely in the culture filtrate.

**Studies with nonspecific phosphomonoesterases.** Since several of the organisms studied here are known to form alkaline phosphatases when grown on medium low in P1, the effect of these enzymes upon 2-AEP was examined. Incubation mixtures (0.5 ml) containing 2 µmoles of 2-AEP, 100 µmoles of Tris-HCl buffer (pH 8.5), and 1, 2.5, 5.0, and 10 units of bacterial alkaline phosphatase were incubated for 1 hr at 37 C. (One unit is defined as that amount of enzyme required to hydrolyze 1 µmole of adenosine monophosphate per hr at 37 C.) Reactions were terminated by adding 0.4 ml of cold 3% perchloric acid. No P1 was liberated. Samples of these incubation mixtures were chromatographed in solvents i and ii, and no compound other than the unaltered substrate was apparent.

In direct spectrophotometric assays of both bacterial alkaline phosphatase and highly purified human placental alkaline phosphatase (crystalline preparations of this enzyme have been prepared in this laboratory, and studies reveal it to be a nonspecific alkaline phosphomonoesterase similar to the enzyme from E. coli) with p-nitrophenolphosphate (0.3 µmole/ml) as substrate, the addition of as much as 3 µmoles/ml of methylphosphonate, 1-aminopentylphosphonic acid, 1-aminooethylphosphonic acid, or 2 - AEP caused no inhibition.

As further evidence against involvement of
alkaline phosphatase, we attempted to grow *E. coli* B15 on the various phosphonates, expecting to observe growth patterns similar to the wild type. However, *E. coli* B15 grew only on P$_1$. The parent *E. coli* B from which the *E. coli* B15 mutant was derived was not tested.

*E. coli* growth on P$_m$ and 2-AEP$_3$. A 5-ml amount of an overnight culture of *E. coli* grown on 2-AEP was inoculated into each of three 2-liter flasks containing 450 ml of minimal medium. Sufficient P$_m$ (1.2 × 10$^4$ counts per min per μmole) or 2-AEP$_3$ (4.3 × 10$^4$ counts per min per μmole) was added to two flasks to give final concentrations of 0.2 μmole/ml. The third flask was kept as a control. The flasks were shaken at 37 C, and samples were removed every 2 hr to measure cell growth and substrate remaining in the medium after centrifugation. Samples chromatographed in solvent systems i and ii showed a gradual disappearance of radioactivity with no evidence of new radioactive materials appearing. During 8 hr of growth, about 90% of the P$_1$ was utilized, whereas only 75% of the 2-AEP disappeared.

The cells were harvested by centrifugation, washed twice with 0.005 M Tris-HCl buffer (pH 7.5), and lyophilized. Cell yield (dry weight) and ash phosphate were determined (Table 2). The cells grown on the two substrates were then refluxed in constant boiling HCl for 24 hr. After ether extraction and charcoal treatment, acid was removed by repeatedly taking the samples to dryness in a flash evaporator. Samples were then chromatographed in both solvent systems and electrophoresed at pH 8.6 for 1.5 hr at 300 V, 40 ma. The sheets were stained with ninhydrin, and autoradiograms were prepared. In neither hydrolysate was any 2-AEP present; all radioactivity migrated as P$_1$.

Experiments with cell-free extracts. Extracts of *E. coli* B and *S. marcescens* grown on 2-AEP were prepared with a French pressure cell. These were incubated at 37 C for 30 min with either unlabeled or radioactive 2-AEP at several hydrogen ion concentrations, with and without reduced nicotinamide adenine dinucleotide and reduced nicotinamide adenine dinucleotide phosphate generating systems, and with glucose added as a possible phosphoryl group acceptor. After deproteinizing with 5% perchloric acid, neutralized samples were assayed for P$_1$ and were analyzed by paper chromatography and electrophoresis. No P$_1$ appeared, and there was no evidence of either substrate disappearance or the appearance of possible products. Similar negative results were obtained in experiments with dried cell suspensions.

**DISCUSSION**

Though only a small number of organisms have been tested, and these of necessity were limited to bacteria able to grow on synthetic medium, it appears from these studies that the ability to catabolize the C—P bond is widespread among bacteria. It will be of interest to extend this survey to organisms other than bacteria. In terms of mechanism of the reaction, it will be of interest to test anaerobic bacteria, as well.

In *E. coli* grown on P$_m$ or 2-AEP$_3$, all phosphate compounds were acid-labile. In washed cells grown on 2-AEP$_3$, no substrate remained, and in neither experiment were new phosphonates detected. Whereas the cell yields in these experiments were almost identical, the phosphate-to-dry weight ratio was somewhat higher in the cells grown on P$_1$. In both cases, this ratio was considerably higher than in cells grown without added phosphorus-containing compounds. Similar information can be inferred from the data appearing in Fig. 1.

The failure of the alkaline phosphataseless mutant *E. coli* B15 to grow on any of the phosphonic acids tested was unexpected, and the explanation is not readily apparent. Our experiments show that nonspecific alkaline phosphatases are not directly involved. These compounds are neither substrates nor inhibitors of the phosphatases tested. The deletion in B15 might affect a portion of the genome responsible for phosphonate metabolism, as well as that for phosphatase. Perhaps a more likely explanation is that a phosphatase ester intermediate is formed either by a direct insertion of an oxygen atom between the carbon and phosphorus atoms or by a phosphoryl group transfer. The phosphatase is then required to liberate P$_1$ for growth. Korman et al. (9) demonstrated a somewhat analogous situation in the metabolism of monothiophosphate by *E. coli* in which various hexoses serve as acceptors, forming hexose phosphates. Similarly, phosphoryl transfer to glucose from phosphoromidade by an enzyme from *E. coli* was described by Fujimoto and Smith (2). Phosphoryl transfer by both specific and nonspecific phosphatases is a well-studied phenomenon.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Cell yield (mg, dry wt)</th>
<th>Cell phosphate (μmole)</th>
<th>Ratio of μmole of P to mg of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23.4</td>
<td>7.5</td>
<td>0.32</td>
</tr>
<tr>
<td>P$_m$</td>
<td>133.4</td>
<td>73.0</td>
<td>0.55</td>
</tr>
<tr>
<td>2-AEP</td>
<td>130.0</td>
<td>64.5</td>
<td>0.50</td>
</tr>
</tbody>
</table>

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**TABLE 2. Cell yield and total phosphate incorporation into cells grown on 2-AEP$_3$ and P$_m$**

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*HARKNESS J. BACTERIOL.*
Having been thus far unable to detect any catabolism of 2-AEP by cell suspensions or extracts of E. coli B or S. marcescens under many different experimental conditions, we have been forced to alter our approach somewhat. We have turned our attention toward a study of C₁⁴-2-AEP utilization by growing cultures of E. coli B. It is hoped that intermediates or products of the catabolism of this compound can be isolated and identified. In this way, perhaps a more sensitive assay can be devised to facilitate attempts at purification of the enzyme or enzymes involved.

ACKNOWLEDGMENTS

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LITERATURE CITED