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Bacterial Growth on Aminoalkylphosphonic Acids

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ABSTRACT

HARKNESS, DONALD R. (University of Miami School of Medicine, Miami, Fla.). Bacterial growth on aminoalkylphosphonic acids. J. Bacteriol. **92**:623–627. 1966. —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 P_i or 2-aminoethylphosphonic acid (2-AEP) was added to the medium. No compounds containing C–P bonds were detected in *Escherichia coli* B grown on 2-AEP^{32}-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) ha 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-aminoethylphosphonic 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 P^{32}-orthophosphate (P_i^{32}) is incorporated into both free and lipid-bound 2-AEP in growing cultures of *Tetrahymena 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 P_i^{32}, and growth of *E. coli* B on this substrate was compared with that on radioactive P_i. 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. Torriani suppied *E. coli* B_{15}, 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^{-4} M tris(hydroxymethyl)aminomethane (Tris) (*p*H 7.4) for the phosphate buffer. Comparable amounts of phosphorus were added as aminoalkylphosphonic acids, methylphosphonate, 5'-deoxyadenylic acid (5'-dAMP), and P_i prior to autoclaving. Cultures were grown at 37 C in 125-ml Erlenmeyer flasks containing 50 ml of medium shaken aerobically on a New Brunswick gyrotary shaker. Growth was measured as increase in turbidity at 420 mμ.

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

Reagents. The aminoalkylphosphonic acids were purchased 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 phosphatase 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 systems: (i) *n*-butanol-acetic acid-water (160:40:75) and (ii) *t*-butanol-methylethylketone-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 (*p*H 8.6). The aminoalkylphosphonic acids were located on chromatograms with ninhydrin spray reagent and by the phosphate method of Rosenberg (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 absorbance measurements were made in a Hitachi-Perkin Elmer spectrophotometer model 139 (Perkin-Elmer, Norwalk, Conn.) equipped with a photomultiplier tube.

Preparation of 2-AEP^{32}. Six 2-liter flasks, each containing 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, $KH_2P^{32}O_4$ at *p*H 6.0 (5×10^5 counts per min per μmole) was added to a final concentration of 2 mM, and growth was allowed to continue for 54 hr. The cells were then harvested by centrifugation, yielding 84 g (wet weight).

The 2-AEP acid was isolated from the cells as described by Horiguchi and Kandatsu (6). After refluxing 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 dissolved in 500 ml of hot water, decolorized with 10 g of charcoal, and applied to a Dowex 50 (4% 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_4OH. Fractions containing 2-AEP, eluted just prior to the bulk of the amino acids, were identified by radioautograms of chromatograms developed in solvent system ii (R_F for P_i, 0.52; R_F for 2-AEP, 0.25). These fractions were combined, lyophilyzed, and applied 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 treatment was carried out.

A 450-μmole amount of 2-AEP with a specific activity of 200,000 counts per min per μmole of ash phosphate was recovered. By ascending chromatography in solvent ii, two minor contaminating ninhydrin-reactive spots were seen, but neither contained radioactivity or organic phosphate. The purified 2-AEP contained 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 aminoalkylphosphonic acids, methylphosphonate, 5'-dAMP, or P_i. The 5'-dAMP was used to compare growth rates on a phosphate ester. Flasks without added phosphorus 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 organism 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 substituted alkylphosphonic acids was insignificant.

Substrate-dependent growth on P_i and 2-AEP. Not only was the rate of growth on 2-AEP comparable to that on P_i, but the amount of growth was identical. In Fig. 1, growth yield of *E. coli* B on increasing concentrations of P_i is compared with growth on identical quantities of 2-AEP.

TABLE 1. *Comparison of bacterial growth on aminoalkylphosphonic acids and inorganic phosphate*[a]

Substrate	Doubling time (min)							
	Escherichia coli B	*E. coli* Crooke's	*Aerobacter cloacae*	*Serratia marcescens*	*Klebsiella* type 16	*Salmonella typhimurium*	*Proteus mirabilis*	*Shigella sonnei*
Orthophosphate	45	60	50	120	36	90	90	480
2-Aminoethylphosphonic acid	70	60	50	120	40	90	150	480
Methylphosphonate	100	100	240	190	96	—	—	480
Aminomethylphosphonic acid	90	130	300	360	—	—	—	480
3-Aminopropylphosphonic acid	100	110	600	250	—	—	—	480
2-Amino-3-phosphonopropionic acid	500	840	700	280	—	—	—	480
2-Amino-4-phosphonobutyric acid	500	—	720	—	110	—	—	780
1-Aminoethylphosphonic acid	500	—	—	—	—	—	—	—
1-Aminobutylphosphonic acid	—	—	—	600	—	—	—	—
1-Aminopentylphosphonic acid	—	—	—	600	—	—	—	—
5'-Deoxyadenylic acid	45	60	60	130	60	90	72	600

[a] 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 P_i and 5'dAMP.

Total and inorganic phosphate were measured on culture filtrates. No P_i was found in flasks to which 2-AEP had been added, and no organic phosphate was found where P_i had been added. Comparable experiments with *S. marcescens* and *S. typhimurium* gave identical results. Though optimal growth on P_i occurred at 0.2 μmole/ml, no extracellular P_i was recovered until concentrations above 0.3 μmole/ml were added (Fig. 1), indicating the capacity for storage of P_i 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 P_i, 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 (*p*H 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 monosphosphate per hr at 37 C.) Reactions were terminated by adding 0.4 ml of cold 3% perchloric acid. No P_i 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*-nitrophenylphosphate (0.3 μmole/ml) as substrate, the addition of as much as 3 μmoles/ml of methylphosphonate, 1-aminopentylphosphonic acid, 1-aminoethylphosphonic acid, or 2 - AEP caused no inhibition.

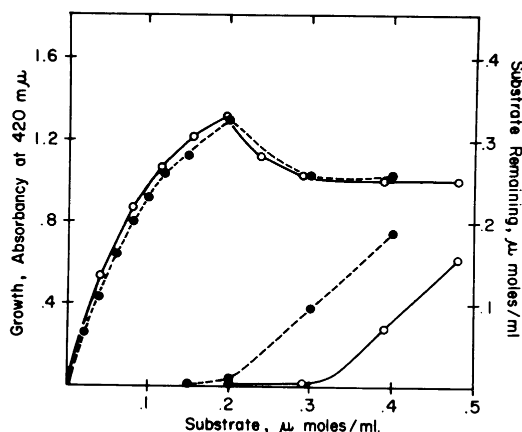

FIG. 1. *Growth of Escherichia coli B on P_i and 2-aminoethylphosphonate. Media containing various quantities of P_i or 2-AEP were inoculated with organisms and shaken at 37 C for 16 hr. Growth and substrate remaining in the culture filtrates were measured. Symbols:* ○, *growth on 2-AEP (upper line) and AEP remaining in medium (lower line);* ●, *growth on phosphate (upper line) and phosphate remaining in medium (lower line).*

As further evidence against involvement of

alkaline phosphatase, we attempted to grow *E. coli* B_{15} on the various phosphonates, expecting to observe growth patterns similar to the wild type. However, *E. coli* B_{15} grew only on P_i. The parent *E. coli* B from which the *E. coli* B_{15} mutant was derived was not tested.

E. coli growth on P_i^{32} *and* $2\text{-}AEP^{32}$. 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_i^{32} (1.2×10^5 counts per min per μmole) or 2-AEP^{32} (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_i 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 (*p*H 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 *p*H 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_i.

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_i and were analyzed by paper chromatography and electrophoresis. No P_i 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.

TABLE 2. *Cell yield and total phosphate incorporation into cells grown on* $2\text{-}AEP^{32}$ *and* P_i^{32}

Addition	Cell yield (mg, dry wt)	Cell phosphate (μmoles)	Ratio of μmoles of P to mg of cells
Control.....	23.4	7.5	0.32
P_i..........	133.4	73.0	0.55
2-AEP......	130.0	64.5	0.50

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_i^{32} or 2-AEP^{32}, all phosphate compounds were acid-labile. In washed cells grown on 2-AEP^{32}, 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_i. 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* B_{15} 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 B_{15} 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 phosphate 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_i 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 phosphoramidate 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.

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 C^{14}-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.

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