ACINETOBACTER PHOSPHADEVORUS: PHOSPHATE UPTAKE AND INITIAL STUDIES OF METABOLISM

by

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My thanks go to Dr. Irving Yall for his interest and help during this study. Also, my thanks go to my mother and to my wife for their encouragement and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

ABSTRACT

Acinetobacter phosphadevorus, a Gram-negative bacterium isolated from activated sludge on the basis of its affinity for phosphate, removed phosphate and formed volutin granules in all media tested in which growth occurred. Removal of phosphate by the organism did not require any pretreatment of the cells, but was inhibited in sewage broth by the presence of iodoacetate, sodium azide, or 2,4-Dinitrophenol. The optimal initial concentrations of medium constituents for phosphate uptake in a chemically-defined medium were 40 mg/liter carbon, 32 mg/liter nitrogen, 30 mg/liter phosphate, 20 mg/liter magnesium, and 100 mg/liter sulfate. The medium used contained sodium acetate as the carbon source.

Differentially-labelled sodium acetate was used to initiate studies of the metabolism of A. phosphadevorus. The organism utilized mainly the methyl carbon of acetate, with a very small amount of the carboxyl carbon being fixed as CO_2. The majority of the carboxyl carbon of acetate was released as CO_2. Sodium ^{14}C-carbonate was not utilized as a carbon source and there was very limited utilization of ^{14}C-formate, indicating a preference for a reduced source of carbon. There was different utilization involved when sodium citrate was present. The pathway of acetate metabolism was blocked by the presence of iodoacetate.

CHAPTER 1

INTRODUCTION

Acinetobacter phosphadevorus is a Gram-negative, rod-shaped bacterium generally occurring in pairs or in large flocs. *A. phosphadevorus* is strictly aerobic, with an optimum temperature between 24 and 30 C and an optimum pH between 7.0 and 8.5. The organism was placed in the genus *Acinetobacter* on the basis of its negative Gram stain reaction, its non-motility, its use of various organic compounds as a source of carbon in a chemically-defined medium, and its negative reaction in the test for oxidase activity (2, p. 436; author's unpublished data).

A. phosphadevorus, first designated plump 7, was originally isolated from activated sludge on the basis of its affinity for phosphates. In sewage, the organism formed dense flocs of cells. These flocs could be readily identified in mixed cultures by intense staining of volutin granules, the organism having high phosphate removal potential. These volutin granules were shown to be composed of inorganic polyphosphate and it was proposed that *A. phosphadevorus* would be useful in the alleviation of the problem of eutrophication (20).

Eutrophication is the process of over-enrichment of a body of water, resulting in the increased proliferation of certain types of algae and aquatic plants. The increase in numbers of these organisms brings about radical changes in the water. Freshwater lakes and streams become choked with algal scums, malodorous, and the recreational value

of the water is lost. Purification of this water for use in cities also becomes more laborious, if not impossible (10, 12). Some investigators maintain that the algae require three nutrients for survival and proliferation; carbon, nitrogen, and phosphorus. Because carbon and nitrogen may be obtained from the atmosphere by these organisms, phosphorus becomes the limiting nutrient. Domestic waste water is the largest source of phosphates in most waterways. It has been shown that the growth rates of algae are inhibited at phosphate concentrations of less than 0.5 mg/liter and almost stop at 0.05 mg/liter (7, 8, 10).

Raw sewage is surprisingly uniform, and it would be desirable to be able to seed A. phosphadevorus into an activated sludge of low phosphate removal capability and increase the potential if the organism were able to compete with other organisms found in the sludge. One purpose of this study was to determine the conditions that would provide an optimal environment for proliferation and phosphate uptake by A. phosphadevorus and ascertain if these are met by the Tucson sewage treatment plant.

Because of the various sources of microbial nutrition found in sewage, it would be desirable to determine which of these sources could be most readily used for the proliferation and phosphate uptake of A. phosphadevorus. For this reason, a second purpose of this study was to initiate studies of the metabolism of this unique organism.

CHAPTER 2

STATEMENT OF PROBLEM

The purpose of this study was to determine the optimal conditions for the accumulation of intracellular polyphosphate by Acinetobacter phosphadevorus, a microorganism isolated from activated sludge. The approach was to vary the amounts of nutrients singly in a chemically-defined medium and determine the effect these varied levels had on the phosphate removal capabilities of the organism. The comparison of nutrient levels found in raw sewage with those providing optimal phosphate removal by A. phosphadevorus should give an idea of the relative effectiveness of the organism.

A second purpose was to begin initial studies of metabolism in A. phosphadevorus through the use of radioactive tracers and metabolic inhibitors and information gained from growth assays on different carbon sources.

CHAPTER 3

MATERIALS AND METHODS

Chemicals

All chemicals used were reagent or analytical grade. The sources of ^{14}C were uniformly-labelled glucose, fructose, and acetate. These and 1-^{14}C-acetate were obtained from the New England Nuclear Corp., Boston, Mass. Also used were 2-^{14}C-acetate and ^{14}C-formate, obtained from the International Chemical and Nuclear Corp., Irvine, Calif. The source of ^{32}P was carrier-free $H_3{}^{32}PO_4$ in 0.02 N HCl, obtained from New England Nuclear. All isotopes had a radiometric purity of 99%.

Equipment

Turbidity of cultures was measured at 540 nm using a Bausch and Lomb Spectronic-20 colorimeter. Cell suspensions were centrifuged at 12,100 x g in a Servall RC-2 refrigerated centrifuge at 0 C for 10 min. Ultraviolet light absorbance of whole RNA was measured using a Beckman model DU spectrophotometer. Color intensity in tests for presence of orthophosphate was measured in a Lumetron colorimeter model no. 401 with 650 nm filter in position. Photographs of smears were taken with a Zeiss microscope and automatic camera.

Organism

Acinetobacter phosphadevorus was obtained from Dr. Irving Yall. The organism was originally isolated from activated sludge from the Rilling Road sewage treatment plant, San Antonio, Texas, and was designated as plump 7 (20). The approximate dimensions of the cells were 0.3-2 μm in diameter with an average of 1.0 μm.

Media

Arginine broth contained 0.2 g arginine, 0.2 g $MgSO_4 \cdot 7H_2O$ and 0.0215 g KH_2PO_4 in 1,000 ml distilled water. The pH was not adjusted. Sewage broth consisted of raw sewage from the Tucson sewage treatment plant which was filter-sterilized using Millipore filters, first with pore size of 0.45 μm, then again with pore size 0.22 μm filter pads. Raw Tucson sewage contained the following approximate concentrations of various nutrients: 30 mg/liter orthophosphate, 83 mg/liter carbon, 35 mg/liter nitrogen, 21 mg/liter magnesium and 178 mg/liter sulfate (6). Acetate I broth contained: 0.284 g sodium acetate, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.15 g $(NH_4)_2SO_4$, 0.0215 g KH_2PO_4, and the pH was adjusted to 7.5 with 1.0 N KOH, all in 1,000 ml distilled water. These concentrations of acetate I broth constituents were chosen to approximate roughly those found in raw Tucson sewage. Acetate I broth, then, was the starting point for the phosphate uptake experiments, and variations were made on the concentrations of nutrients in this medium. In this manner acetate I broth served as the experimental control.

The basal medium used to determine which carbon compounds could support growth consisted of: 0.2 g $MgSO_4 \cdot 7H_2O$, 0.15 g $(NH_4)_2SO_4$, and

0.0215 g KH_2PO_4. Substrates tested for satisfaction of carbon requirement were added in amounts calculated to provide 40 mg/liter of carbon. The volume was made up to 1,000 ml with distilled water and the pH was adjusted to 7.5 with 1.0 N KOH where necessary.

The organism was maintained on nutrient agar slants and on Koser Citrate slants. Koser Citrate (Difco Laboratories, Detroit, Mich.) contained, per liter: 1.5 g sodium ammonium phosphate, 1.0 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 2H_2O$, and 2.5 g sodium citrate. Slants were prepared by adding 2% agar to Koser Citrate broth.

Acetate II broth contained, per liter: 1.5 g sodium ammonium phosphate, 1.0 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 2H_2O$, and 4.0 g sodium acetate. The pH was adjusted to 7.5 with 1.0 N KOH. This medium was used when large numbers of cells were needed for extractions, chromatography, and isotope studies.

Growth Conditions

Broth cultures of A. phosphadevorus consisted of 100 ml of medium in 500 ml Erlenmeyer flasks continuously shaken on a New Brunswick rotary shaker (model C. S.) at 200 rpm. Broth cultures and slant cultures were grown at 24 C.

Determination of Radioactivity

Radioactivity was measured in a Packard Tri-Carb liquid scintillation counting system, Model 3320. The scintillation fluid contained, per liter: 4 g 2,5-bis-[2-(5-tert-Butylbenzoxazolyl)]-

Thiophene (BBOT), 80 g naphthalene, 400 ml ethylene glycol monomethyl ether, and 600 ml toluene.

Stains

Volutin was stained using Neisser's stain (4, p. 10). The procedure was the same as that described by Roinestad (20).

Phosphate Measurement

A modification of the stannous chloride method (1, p. 234) was used to measure orthophosphate. A 10 ml sample was required (the technique was accurate within the limits of 1-6 mg/liter of orthophosphate and dilutions of sample were necessary to get within this range), and to this sample was added 1 drop of 0.05% phenolphthalein; if it turned pink, strong acid, consisting of 28% H_2SO_4 with 0.3% HNO_3 was added until the sample turned colorless. Then 0.4 ml of 2.5% $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ in 28% H_2SO_4 was mixed with the sample. Finally, 0.05 ml of 2% $SnCl_2$ in glycerol was mixed with the sample. Ten min were allowed for formation of molybdenum blue. Percent transmission was read on a Lumetron Colorimeter using a 650 nm filter. A standard curve, using known concentrations of orthophosphate was used for conversion of % T to mg/liter orthophosphate present.

Phosphate Removal

From 100 ml of uninoculated broth, a 1 ml sample was taken and diluted with 9 ml of distilled water. The amount of orthophosphate present was determined by the modified stannous chloride method. The broth was then inoculated with a small inoculum of A. phosphadevorus

cells which were grown 48 hr on a rotary shaker. At the end of the incubation period, the cells were removed by centrifugation and the supernatant fluid was sampled and tested for orthophosphate in the same manner as before. The difference between the orthophosphate present before inoculation and that present after growth of the cells has occurred was taken to be the amount of phosphate removed by the organism. After centrifugation, the cell pellet was saved for subsequent determinations.

Extraction Procedures

RNA, used for measurement of cell mass, was extracted from the cells by a modified version of the Ogur-Rosen extraction procedure (17). The cells were homogenously suspended in 1.0 N perchloric acid (PCA) at 4 C overnight. The debris was removed by centrifugation and washed with another volume of PCA. The combined supernatant fluids contained the extracted RNA.

Extractions used for determining distribution of isotope and for chromatography were accomplished using a slightly-modified Ogur-Rosen procedure (17). Each reagent was used in succession to extract a different fraction of the cellular components. For each of these fractions, the cells were extracted with 5 ml of the reagent, centrifuged at 12,100 x g at 0 C for 10 min, washed with 2 ml of the reagent, and again centrifuged; the combined supernatant fluids from the two centrifugations constituted the fraction.

The extraction procedure was: 70% ethanol for 14 hr at 4 C for the soluble fraction; ethanol-ether (3:1) for 1 hr at 45 C for the lipid

fraction; 0.1 N PCA for 1 hr at 4 C for the cold, acid-soluble nucleotide and nucleoside fraction; 1.0 N PCA for 18 hr at 4 C for the RNA fraction; 1.0 N PCA for 1 hr at 70 C for the DNA fraction; and 0.1 N KOH for 1 hr at 70 C for the alkaline-soluble protein fraction. The residual debris was solubilized with Beckman Bio-Solv BBS-3 for scintillation counting.

Measurement of Total RNA Content

Total RNA was measured by a modified orcinol method (3). The procedure was as follows: to the diluted PCA extract of the sample was added an equal volume of the orcinol reagent. The orcinol reagent consisted of 200 mg of orcinol in concentrated HCl, plus 10 ml of 0.004 M cupric chloride solution in concentrated HCl. After addition of the orcinol reagent to the sample, the vessel was stoppered, the contents mixed well, and held in a boiling water bath for 40 min. The vessel was then cooled under running tap water and the absorbance was measured at 675 nm against a blank containing distilled water and treated in the same manner. The absorbance was measured with either a Bausch and Lomb Spectronic-20 or with Beckman model DU spectophotometer. To obtain a standard curve of absorbance vs μg RNA, yeast RNA was used in various dilutions in PCA and treated in the manner described above. Standards (at least 3) were run with every sample as the orcinol reagent differed slightly with different batches. The orcinol reagent also decomposed with time.

To confirm the orcinol technique of determining RNA, an identical extracted sample was adsorbed on Dowex AG 50w-X8. The RNA was

purified and ultraviolet absorbance was measured using a Beckman model DU spectrophotometer. The measured absorbance at 262 nm was multiplied by the corresponding E-max value and total RNA was calculated. Values obtained were within 10% of that obtained using the orcinol procedure.

Measurement of Protein Nitrogen

Protein nitrogen was measured by the Kjeldahl method, which consisted of three main steps:

(i) Digestion--Into a 10 ml micro-Kjeldahl flask was placed 2-5 ml of the sample. This was heated over a low flame until all the water was evaporated or boiled away. The flask and contents were allowed to cool and then 0.5 g K_2SO_4, 2 ml 36 N H_2SO_4, and 2 Hengar granule (selenized) were added to the contents of the flask. The flask was heated over full flame until the mixture cleared and then heated for an additional 30 min after clearing. The flask and contents were allowed to cool, 1 drop of alcohol was added and then heated again until clearing occurred.

(ii) Reflux distillation--The digestion mixture was decanted into 2 x 20 cm tubes and the Kjeldahl flask washed twice with distilled water, these washings being added to the decanted digestion mixture. The 2 x 20 cm tube was then made into a closed system with a rubber stopper. Air was bubbled through the Kjeldahl digest and exhaust air was passed through 2 N H_2SO_4 by means of glass tubing. When the digestion mixture was made alkaline with 40% NaOH, the NH_4 evolved did not escape as a gas, but was instead trapped in the dilute acid of the collecting tube as $(NH_4)_2SO_4$. The apparatus was kept in an ice bath to reduce the heat of reaction produced when 40% NaOH was added to the digestion mixture.

(iii) Nesslerization--To 1 ml of the dilute acid containing the $(NH_4)_2SO_4$ was added 1 ml of Nessler's reagent and 1.5 ml of 2.5 N NaOH. This was held at room temperature for 10 min and the absorbance at 540 nm was measured in a Spectronic-20 using as a blank distilled water which had been nesslerized in the same manner.

A standard curve of absorbance vs μg NH_4 was constructed by nesslerizing different concentrations of NH_4Cl in the range of 1-20 μg NH_4 per ml. This curve was used to convert absorbance measured to μg NH_4 present.

Isotope Recovery

For determining the distribution of ^{14}C, the cells were grown, harvested in the centrifuge, and resuspended in carbon-free medium. Approximately 600 mg, dry weight, of cells was used per experiment. The cells were then added to a larger vessel containing carbon-free medium plus the isotope being used for that experiment and "cold" carrier where called for in the protocol. Conditions for recovery of CO_2 were met by fitting the growth chamber vessel with a rubber stopper. A glass tube was submerged in the suspended cells and medium and CO_2-free air (washed with 20% KOH) was bubbled through the liquid. Another glass tube, free of the liquid, provided an outlet for the exhaust air and evolved CO_2. This second tube entered a second stoppered vessel containing ethanolamine to trap CO_2. The second vessel also contained another tube for the exhaust of the stream of air. Radioactivity was measured in a liquid scintillation counter. After 30 min, the experiment was

terminated, and the cells were removed by centrifugation and fractionated.

To determine how much CO_2 was soluble as carbonate in the slightly alkaline growth medium, a Warburg apparatus was used. The flask contained 3 ml of supernatant fluid, with 0.1 ml ethanolamine in the center well and 0.5 ml 2 N HCl in the side arm. After the system was closed airtight, the acid was tipped from the side arm into the flask and was shaken for 1 hr to drive off all CO_2, which was then collected in the ethanolamine in the center well. To ascertain that the ^{14}C-activity remaining in the acidified supernatant fluid was due to the presence of ^{14}C-acetate, partition chromatography with silicic acid was used (1, p. 238).

Chromatography

Compounds in the soluble fraction, extracted by ethanol, were separated by means of two-dimensional paper chromatography, using Whatman no. 1 filter paper. The solvent system employed was: first direction; isopropanol:water:urea, 80:20:0.5 (v/v/w), second direction; 1-butanol:pyridine:water, 30:30:30 (v/v/v) (16). After 6 hr of migration at room temperature in each direction, the papers were air-dried, and sprayed with a variety of detection reagents. Other than the location of pyruvic acid, the only success was obtained with 0.5% ninhydrin in acetone, which was sparyed on the paper. The paper was then heated at 50 C for 10 min to develop color. The spots were partially identified using amino acid standards in alcohol and comparing Rf values. Further identification was facilitated by coincidence of

standards and unknown sample using an overlay technique. Pyruvic acid was detected and identified by synthesizing its DNP-hydrazone (13).

Acetate remaining in the supernatant fluid of isotopic-labelling experiments was determined by partition chromatography, using a silicic acid column. The silicic acid was prepared by washing with methanol and with ether for 30 min each, then was dried overnight at 90 C. A 1 g portion of the prepared silicic acid was used to construct the column, to which 1 ml of the acidified sample was added and pulled into the column with a vacuum. The column was then eluted with chloroform: n-butanol:0.5 N H_2SO_4, 30:10:8 (v/v/v), and with methanol:ether, 1:1 (v/v). This elution separated out volatile acids (1, p. 538). Acetate was the volatile acid found in these experiments.

CHAPTER 4

RESULTS

Phosphate removal capabilities of Acinetobacter phosphadevorus were studied at various levels of essential nutrients.

Growth Characteristics

An investigation of the general characteristics of growth by A. phosphadevorus was conducted. In arginine broth and in acetate I broth, the organism was in the exponential phase of growth from 24 hr until 50 hr and in the stationary phase until approximately 150 hr after inoculation (Fig. 1). The doubling time was 12 hr in both arginine and acetate media at 40 mg/liter carbon concentration. Increasing the level of carbon to 700 mg/liter shortened the time required for doubling of cell mass to 3.5 hr. In Fig. 1, the optical density and protein nitrogen determinations remained close as long as the cells were in the exponential phase of growth. As the cells approached stationary phase, the two measurements of mass differed. The reason for this difference was the clumping of cells which began in late exponential phase and continued through the stationary phase of growth. A. phosphadevorus also grew in many natural media, such as nutrient broth, sewage broth, and peptone. Generation times in these natural media were approximately 5 hr. In natural media, there was a great amount of clumping of the cells into macroscopic flocs, presumably due to the presence of some extracellular material which formed a matrix holding the cells together. For later

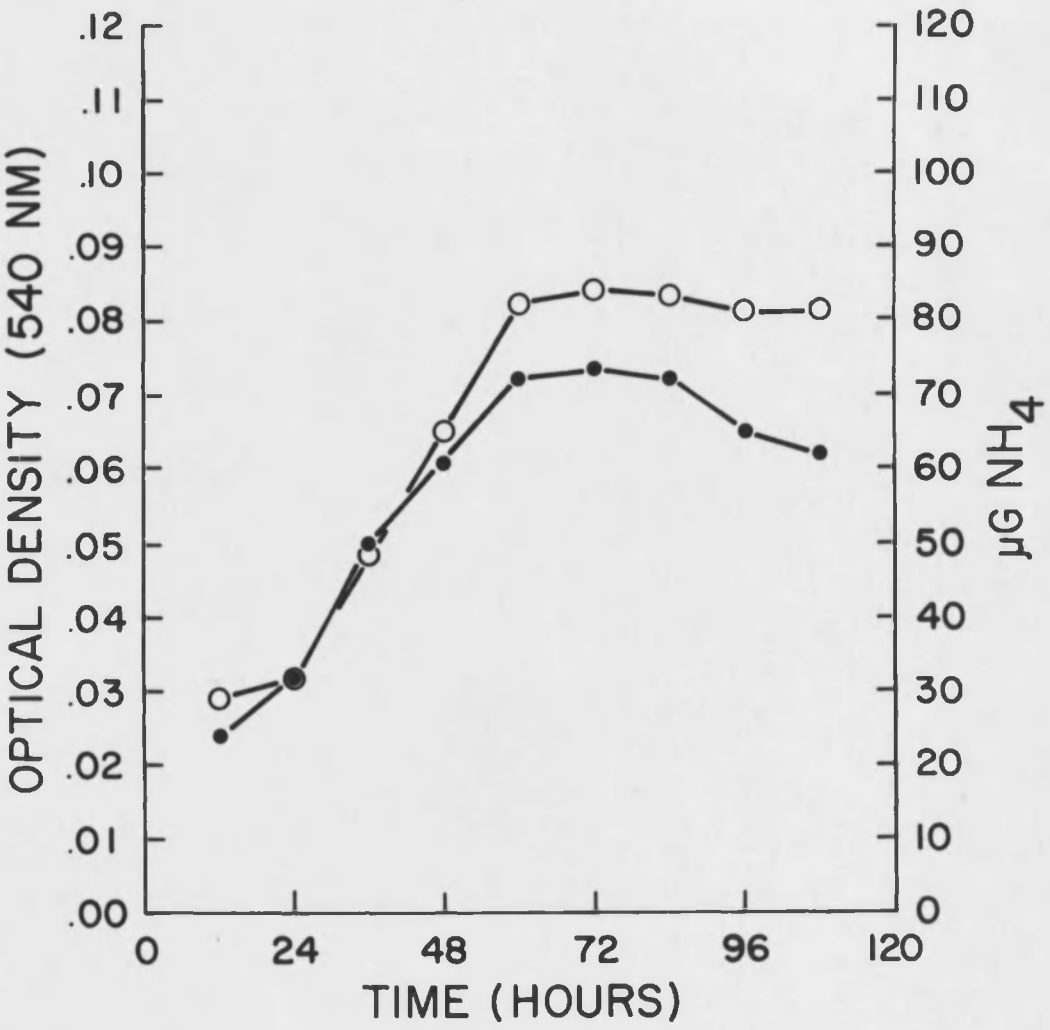

Fig. 1. Growth of
Acinetobacter phosphadevorus in Acetate I broth

Growth was at 24 C on a rotary shaker. The symbols are: ●, optical density (OD) of growth; o, Kjeldahl protein nitrogen (NH_4).

experiments concerning general metabolism, acetate II broth was used to grow the cells. Clumping in this medium, utilizing acetate as sole carbon source, was not found to the same extent as in complex media.

A brief study of the utilization of certain common carbon compounds by the organism at 40 mg/liter for growth was done (Table 1). These media contained the same levels of nutrients as acetate I broth, with the compound in question being substituted for sodium acetate in each case. In all media where growth occurred, the organism removed phosphate.

Phosphate Uptake

Volutin granules were formed by A. phosphadevorus in all media tested. Nutrient broth, sewage broth, peptone, and Koser Citrate broth all showed extensive granulation. When stained using Neisser's method, almost all cells contained a single volutin granule that often filled up the entire cell. A comparison of phosphate accumulation in arginine and acetate (Fig. 2) showed much more extensive granulation when acetate was the carbon source. This was attributed to the fact that acetate was the most abundant carbon source in the organism's natural habitat, sewage, and could be used much more readily by the organism. Except for very striking differences such as that in Fig. 2, it was very difficult to quantitate phosphate uptake by A. phosphadevorus by the appearance of granules. For this reason, uptake of phosphate by the organism was determined by the disappearance of orthophosphate from the medium, and put on a common basis for comparison by dividing this removed phosphate by some unit of cell mass. Kjeldahl protein nitrogen was used to

Table 1. Utilization of Various Compounds as Sole Source of Carbon and Energy, and Uptake of Phosphate

Carbon Source	Growth	Phosphate Uptake
Arginine	+	+
Acetate	+	+
Succinate	+	+
Citrate	+	+
Pyruvate	+	+
Malate	+	+
Glyoxylate	-	-
Glucose	-	-
Sucrose	-	-
Glycerol	+	+
Sodium Bicarbonate	-	-
Urea	-	-
Oxalate	-	-

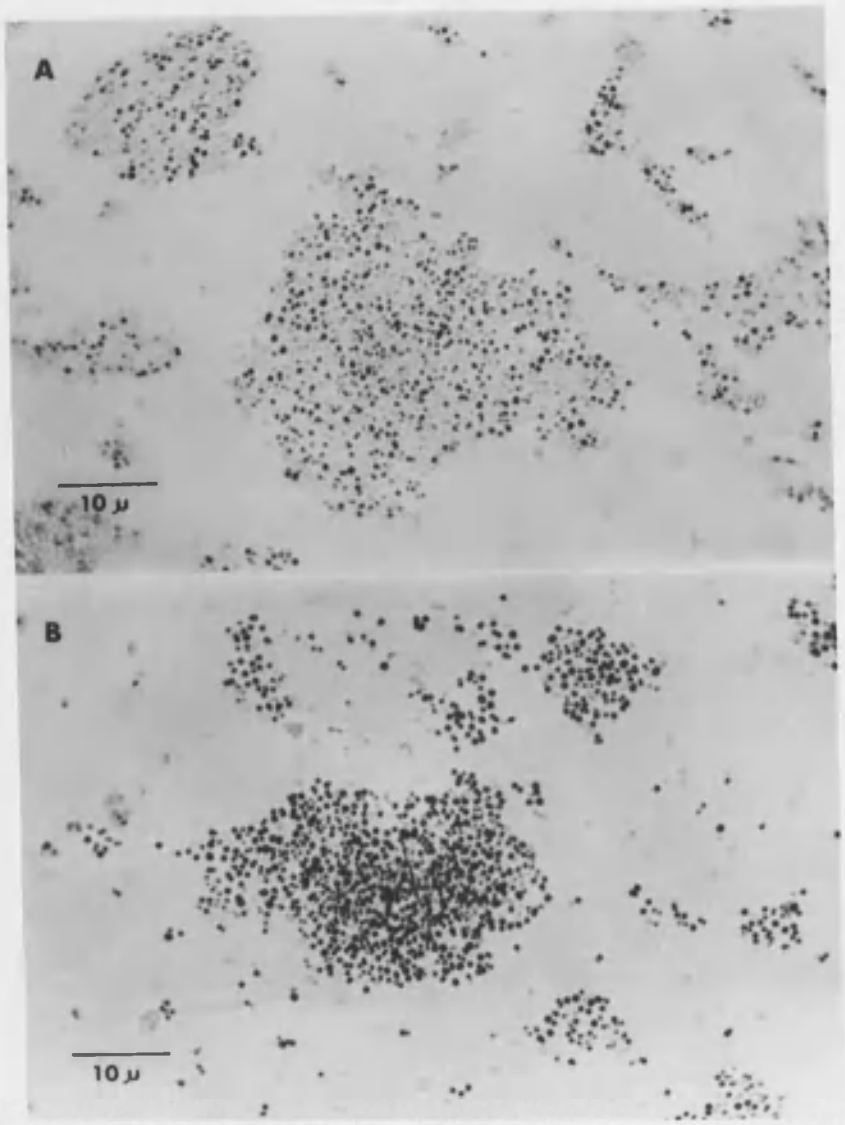

Fig. 2. Photomicrographs of
Volutin Granules in Acinetobacter phosphadevorus

Smears were stained by Neisser's procedure and photographed with 100 X objective. (A) Cells grown in arginine broth; (B) Cells grown in acetate I broth.

measure cell mass and these measurements were correlated by also measuring total RNA and ATP content.

Carbon Variation

Acetate I broth was used as the basal medium in determinations of phosphate uptake. The uptake was measured at different initial concentrations of carbon in the medium (Fig. 3A). As the carbon level was increased, there was a proportional increase in cell growth up to a level of 80 mg/liter of carbon, at which point some other nutrient became limiting. Phosphate uptake by A. phosphadevorus increased to a maximum at 40 mg/liter carbon, and the presence of additional carbon tended to decrease the phosphate uptake per unit of cell mass. In this instance, phosphate uptake seemed to be at the expense of cell growth. These experiments, which used the chemical method of phosphate measurement, were correlated by later experiments in which the phosphate was measured by the more sensitive isotopic method, using ^{32}P. In subsequent phosphate removal experiments, 40 mg/liter of carbon was used in all media.

Nitrogen Variation

The medium contained an inorganic nitrogen source, $(NH_4)_2SO_4$. Cell mass increased as the level of available nitrogen was increased Fig. 3B). But, the phosphate uptake level per unit of cell mass increased to a maximum at 32 mg/liter nitrogen and decreased as more nitrogen was made available. When the cells of A. phosphadevorus began to grow at a fast rate, the uptake of phosphate by the organism became

impaired. Subsequent phosphate removal experiments utilized media containing 32 mg/liter nitrogen.

Phosphate Variation

Phosphate uptake in acetate I broth was measured at different concentrations of orthophosphate. The source of phosphate was KH_2PO_4 and the increase of available phosphate from 7 mg/liter to 45 mg/liter resulted in no significant difference in the level of cell growth (Fig. 3C). So, the level of available phosphate had no effect on the growth of the organism, but there was a maximum amount of phosphate removed per unit of cell mass at 30 mg/liter initial phosphate. Any additional phosphate added to the medium decreased the uptake level. Subsequent phosphate removal experiments utilized media containing 30 mg/liter of phosphate.

Magnesium Variation

Phosphate uptake in acetate I broth was measured at different initial concentrations of magnesium because of the role of magnesium in the phosphorylation process (Fig. 3D). The level of magnesium was varied from 10 mg/liter to 50 mg/liter and no effect on the amount or rate of growth was noticed. A maximum amount of phosphate was removed per unit of cell mass at 20 mg/liter magnesium.

The effect of varying the initial sulfate concentration was approximately the same as that for magnesium, with the maximum amount of phosphate being removed at 100 mg/liter sulfate.

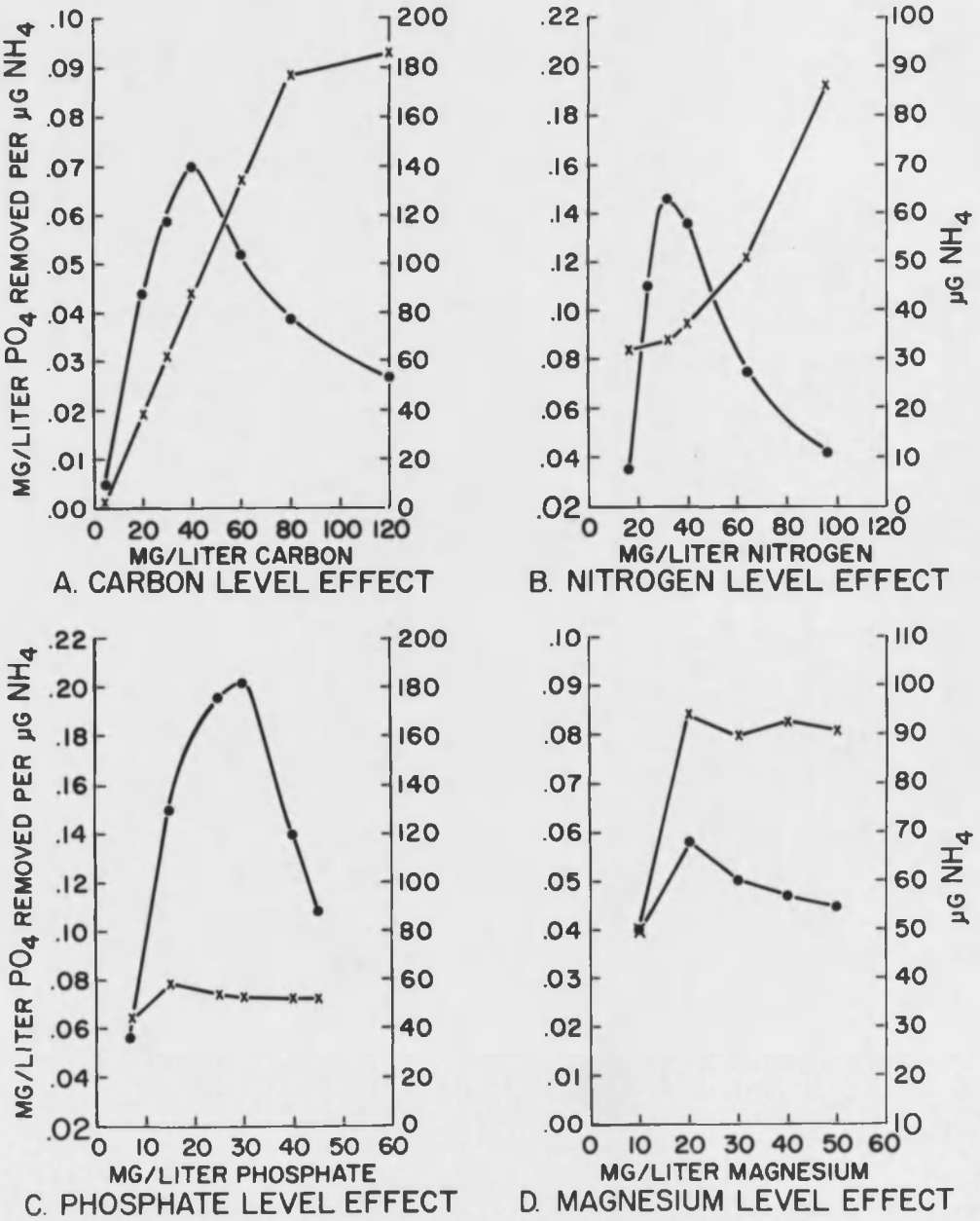

Fig. 3. Effects of Variation of
Medium Constituents on the Uptake of Phosphate

The symbols are: ●, removal ratio; x, cell mass.

Metabolic Inhibitors

The effects of various metabolic inhibitors on phosphate uptake by A. phosphadevorus were investigated. In acetate I broth, all of the inhibitors tested resulted in no growth of the organism when it was inoculated into a flask containing the inhibitor. The inhibitors used were iodoacetate, sodium azide, 2,4-dinitrophenol (2,4-DNP), and ethylenediaminetetraacetic acid (EDTA), present separately in 0.01 M concentrations.

The same group of inhibitors were added to filter-sterilized sewage broth, which was then inoculated with the organism (Table 2). The cells were able to grow in the presence of all but 2,4-DNP as well as in the control, which was sewage broth with no inhibitor. Phosphate uptake was measured and was shown to be affected by all inhibitors to lesser or greater extent. Iodoacetate is an inhibitor of substrate phosphorylation, sodium azide and 2,4-DNP affect oxidative phosphorylation and EDTA damages membrane function.

Radioisotope Studies

A. phosphadevorus seemed to be a unique organism in a physiological sense. For that reason, a study of its metabolic processes was initiated. The approach to the problem was through the use of radioactive labels and by separation and identification of compounds found in cellular extracts using chromatographic procedures.

The three radioisotopes used initially were U-^{14}C-glucose, U-^{14}C-fructose, and U-^{14}C-acetate. There was no removal of the sugars from the medium, even in the presence of "cold" acetate as carrier.

Table 2. Effects of Metabolic Inhibitors on Cell Growth and Phosphate Removal in Sewage Broth

Inhibitor	Kjeldahl protein nitrogen (ug)	Total RNA (ug)	Phosphate removal[a]
Control	350	1600	74
0.01 M Iodoacetate	291	1330	28
0.0001 M Iodoacetate	279	1275	40
0.01 M Sodium azide	252	1150	50
0.01 M 2,4-Dinitrophenol	208	950	0

[a]Expressed as a percentage of the total phosphate initially available in the growth medium.

There was 90% removal of the uniformly-labelled acetate when the cells were exposed to the isotope for 48 hr.

Subsequent experiments, designed to account for all initial radioactivity using 1-^{14}C-acetate (carboxyl-labelled), resulted in only 60% recovery of label when cells were exposed to the label for 30 min, indicating the loss of some volatile compound as the cells utilized the acetate. Presuming the lost activity to be due to loss of CO_2, an ethanolamine trap was devised to catch the gaseous substance. The missing activity was found to be present in the ethanolamine, and to ascertain that this activity was due to CO_2, a similar experiment was set up utilizing 0.1 N NaOH as a trapping agent. The activity trapped in NaOH was determined, then all CO_2 was precipitated out of the NaOH with $BaCl_2 \cdot 2H_2O$. The NaOH was again measured for ^{14}C-activity, and found to have none, indicating that all the activity was due to the presence of CO_2 from the ^{14}C-acetate.

The organism was exposed to 2-^{14}C-acetate (methyl-labelled) for 30 min and removal of ^{14}C-activity by the cells and loss of CO_2 were determined (Table 3). There was much better utilization of the labelled carbon than in the case of carboxyl-labelled acetate, indicating a preference by the organism for the methyl carbon and almost non-utilization of the carboxyl carbon. Approximately the same amount of isotope was removed in both cases, but the difference occurred in the amount found associated with the cells and the amount released as CO_2. The CO_2 released in the case of 2-^{14}C-acetate undoubtedly represented metabolism of the methyl carbon by the organism. To insure that the

Table 3. Radioisotope Removal and Distribution into Cell-Associated Carbon and CO_2

Radioisotope[b,c]	Removal by Cells[a]	Remaining in Medium[a]	Lost as CO_2[a]	Cell-associated Carbon[a]
1-^{14}C-acetate + acetate	65	35	49	8
2-^{14}C-acetate + acetate	67	33	25	37
^{14}C-formate + acetate	40	60	33	5
^{14}C-carbonate + acetate	97	3	95	2
1-^{14}C-acetate + citrate	88	12	57	18
2-^{14}C-acetate + citrate	84	16	24	55
2-^{14}C-acetate	81	19	25	50

[a]Expressed as a percentage of the total radioactivity initially available.

[b]Initial radioactivity was: 1-^{14}C-acetate, 1.04×10^6 CPM; 2-^{14}C-acetate, 919,000 CPM; ^{14}C-formate, 768,000 CPM; ^{14}C- sodium carbonate, 308,000 CPM.

[c]Isotope + acetate or + citrate denotes "cold" acetate or citrate was available as well as isotopic carbon. Where not added on, carbon was available in tracer amounts only.

cells were actually metabolizing and not undergoing carbon starvation, a small amount (40 mg/liter) of carbon was incorporated in the media in the form of "cold" sodium acetate. This carrier carbon overcame the fact that only trace amounts of carbon are provided by the ^{14}C-acetate. These experiments demonstrated that A. phosphadevorus preferred to use only one carbon from the acetate molecule, with relatively small amounts of the carboxyl carbon fixed as CO_2. Experiments with cells grown 48 hr in the presence of the label show this same phenomenon.

When "cold" sodium citrate was used as the carrier carbon instead of acetate, a different utilization of the labelled acetate was seen to occur (Table 3). There was much more extensive removal of ^{14}C-acetate, even more than when the labelled acetate was present with no "cold" acetate. This indicated the interaction of another metabolic pathway with the acetate pathway, resulting in an increased rate of metabolic activity.

With this information, other one-carbon labelled compounds were tested for utilization. These were ^{14}C-formate and sodium ^{14}C-carbonate (Table 3). In the case of formate, there was very little utilization of the labelled carbon, at least in the 30 minute exposure time. Again, "cold" acetate was available as carrier. The utilization of ^{14}C-formate was similar to that of 1-^{14}C-acetate, again indicating a preference for a reduced carbon source. In the case of sodium ^{14}C-carbonate there was substantial removal of ^{14}C from the medium, but the majority of this was found in ethanolamine, indicating volatilization of the $^{14}CO_2$. A minimal amount was found to be associated with the cells.

In an effort to determine the distribution of radioactive carbon once it entered the cell, a fractionation of the cell was performed using a modified Ogur-Rosen procedure (Table 4). With all three labelled compounds, the bulk of cellular ^{14}C-activity was found in the alcohol-soluble fraction. This fraction contained compounds found in metabolic pools. As indicated by the small amount of radioactivity in the alcohol-ether-soluble fraction, very little was lipid in nature. The alcohol-soluble compounds were most likely amino acids or metabolic intermediates. The very small amounts of label in nucleic acids when 1-^{14}C-acetate was available indicated that the organism was not actually using the carboxyl carbon for synthesis. This was also reflected in the low activity of the protein fraction with the carboxyl label. When 2-^{14}C-acetate was the carbon source, nucleic acid and protein fractions approached expected values. When ^{14}C-formate was the carbon source, the distribution of activity followed the pattern expected when an organism is just beginning to utilize a different carbon source than the one it was grown on initially. The formate represented a step-down growth medium. This was evident because of the high activity found in metabolic pools and especially in the RNA fraction, with no activity in the protein, or cell-structure fractions.

In earlier experiments, when iodoacetate was present in the growth medium and sodium acetate was the carbon source, there was no growth of the cells. The following experiment was designed to investigate the effect of iodoacetate on uptake and distribtuion of ^{14}C-acetate. A. phosphadevorus cells were removed from their growth medium, washed,

Table 4. Distribution of Removed Radioactivity[a]

Fraction	1-^{14}C-acetate[b]	2-^{14}C-acetate[b]	^{14}C-formate
CO_2	76	19	84
Alcohol-soluble compounds	8	23	6
Alcohol-ether-soluble compounds	<1	2	0
0.1 N PCA nucleoside & nucleotide pools	2	9	3
RNA	<1	7	4
DNA	<1	4	<1
Protein	3	24	0
Cell residue	<1	5	0
Total recovery	90	97	97

[a]Expressed as a percentage of the radioactive label removed from the medium by the cells.

[b]Initial radioactivity was: 1-^{14}C-acetate, 10^6 CPM; 2-^{14}C-acetate, 1.4×10^6 CPM; ^{14}C-formate, 700,00 CPM.

and resuspended in varying concentrations of iodoacetate. The cells were pre-incubated in iodoacetate for 30 min, then were exposed to 2-^{14}C-acetate plus "cold" carrier acetate. After 30 min more, the cells were centrifuged and fractionated, using a modified Ogur-Rosen technique. The results (Table 5) showed that at lower concentrations, the uptake of label was only affected a little, but the cellular distribution showed that the bulk of the label removed by the cells was localized in the metabolic pools, with lesser amounts being found in the other fractions, as compared to the control. This indicated some blockage of pathways of utilization. When the concentration of iodoacetate was increased to 0.001 M and 0.01 M, the uptake of label and distribution were greatly affected, indicating near complete blocking of pathways of utilization.

In order to gain some insight into the pathways used by A. phosphadevorus for metabolism, a study of compounds inside the cells was initiated. Paper chromatography was used to separate and identify compounds of the metabolic pools of the organism. The alcohol-soluble fraction was spotted on Whatman no. 1 filter paper and developed two-dimensionally using the solvent pair mentioned in Materials and Methods. To date, with the exception of pyruvic acid, only ninhydrin-positive compounds have been present in great enough concentrations to be detected by the methods used. Of these, seven have been identified as members of the pyruvate and aspartic acid families of amino acids plus serine. They are: glutamic acid, valine, leucine, isoleucine,

Table 5. Distribution of Radioactivity in the Presence of Iodoacetate as Inhibitor

Fraction	Control[b]	0.0001 M Iodoacetate[b]	0.001 M Iodoacetate[b]	0.01 M Iodoacetate[b]
Initial[c]	100	100	100	100
Removal of label	80	55	3	<1
CO_2	20	11	≪1	≪1
Alcohol-soluble compounds	14	22	1	≪1
Alcohol-ether-soluble compounds	2	1	0	0
0.1 N PCA Nucleoside & nucleotide pools	6	6	<1	0
RNA	3	2	0	0
DNA	3	2	0	0
Protein	21	8	<1	0
Residue	2	1	≪1	0
Recovery	91	98	99	99

[a]Expressed as a percentage of the total radioactivity initially available in the medium.

[b]The source of radioactivity was 2-^{14}C-acetate, with the cells pretreated with iodoacetate for 30 min in the concentration shown in all cases except the control. There was no inhibitor present in the control.

[c]The labelled acetate was initially available in the amount of 10^6 CPM for each part of the experiment.

threonine, lysine, and serine. Increased sensitivity in detection and identification of compounds may be obtained by the utilization of autoradiographic techniques.

CHAPTER 5

DISCUSSION

A microorganism, isolated from activated sludge on the basis of its affinity for phosphates (20), was found to be capable of growth in various natural media. Some of these natural media were peptone, sewage broth, and nutrient broth. The organism was also found to be capable of utilizing various organic compounds as sole sources of carbon and energy. On the basis of these data plus its non-motility and negative oxidase test, the organism was given the name, Acinetobacter phosphadevorus (2, p. 436; author's unpublished data).

In peptone or nutrient broth A. phosphadevorus was observed to clump into macroscopic flocs of cells. The clumping was less pronounced in sewage broth, because it contained lower levels of nutrients than the former two media. In arginine broth or acetate I broth, clumping never became macroscopic and was not a factor until the cells entered the stationary phase of growth. A comparison of optical density and protein nitrogen in a growth curve in acetate I broth revealed that these measurements were similar until the late exponential or early stationary phase of growth. At this point, the cells produced an extracellular matrix which held cells together in small clumps and rendered optical density measurements inaccurate.

The phosphate removal capacity of A. phosphadevorus fluctuated as various growth medium constituents were varied. In a synthetic

medium, containing approximately the same levels of essential nutrients as raw sewage, optimum conditions for the uptake of phosphate were established. This was accomplished by varying the concentrations of each nutrient while keeping the other constituents at their initial levels and measuring the orthophosphate removed during a prescribed period of growth. The mass of the cells was then measured and a ratio of uptake per unit of cell mass obtained. Stains of the cells using Neisser's stain for volutin confirmed the presence of polyphosphate granules within the cells. These volutin granules were actually polyphosphate, as shown by earlier work in this laboratory (20). The optimum nutritional conditions for removal of phosphate by A. phosphadevorus were: 40 mg/liter carbon, 32 mg/liter nitrogen, 30 mg/liter phosphate, 20 mg/liter magnesium, 100 mg/liter sulfate, a pH of 7.0-8.5, and a temperature of 25-30 C. These were initial concentrations of these nutrients. Increasing the level of carbon brought about an increased rate of cell growth, but a decrease in the amount of phosphate accumulated per cell. This indicated a storage function for the accumulated phosphate, because the faster the cells were growing and dividing, the less phosphate was accumulated. The same situation was found when the initial level of nitrogen was increased and the conclusions were the same. When the level of initial orthophosphate was increased from 30 mg/liter there was no increase in cell mass, but there was a decrease in phosphate accumulated per cell. This could be explained on the basis of end product repression or inhibition. The enzymes involved in the polymerization of polyphosphate were tied up and

the excess phosphate caused repression of a gene responsible for the uptake of phosphate into the cell. The optimal temperatures and pH values reflected those values necessary for biological systems. All of these optimal conditions were approximately the same as those found in raw sewage in the Tucson sewage treatment plant.

The accumulation of polyphosphate in A. phosphadevorus differed from that found in most microorganisms that take up phosphate in that no extreme deprivation of any nutrient was necessary. In other organisms, depletion of sulfate resulted in a cessation of nucleic acid synthesis and diversion of the phosphate required for nucleic acids to an accumulation of polyphosphate. This would most likely occur in late stationary phase of growth. In contrast A. phosphadevorus accumulated polyphosphate more readily in the exponential phase of growth when the cells were actively growing and dividing, if the rate of growth was controlled by availability of nutrients. Also, in certain other organisms, including Aerobacter aerogenes (11), phosphate uptake can be induced by starving the organism for phosphate, then supplying orthophosphate. Starvation of A. phosphadevorus for phosphate brought about depletion of the stored polyphosphate but no increased uptake per cell when phosphate was supplied.

The inhibition of phosphate uptake from sewage by certain metabolic inhibitors sheds some light on the mechanism of phosphate uptake. When either type of phosphorylation, oxidative or substrate level was inhibited, the accumulation of polyphosphate was affected. This indicated involvement of adenosine triphosphate (ATP) as well as some

metabolic intermediate serving as a substrate. Of course, inhibitors of membrane function, such as 2,4-DNP and sodium azide, also inhibited accumulation of phosphate. Elucidation of the exact mechanism involved in the uptake of phosphate will depend on further studies.

Based on the information gained from the present study, it should be possible to seed A. phosphadevorus into activated sludge of low phosphate removal potential and increase this potential. The success of such seeding would depend upon meeting the nutritional requirements set forth in this study, as well as providing adequate aeration since the organism is strictly aerobic.

During the course of this study, it became evident that A. phosphadevorus was a unique organism from a metabolic standpoint. Volatile acids were the primary source of carbon in sewage (6, p. 19), which seemed to be the normal medium for the organism. These volatile acids, of which acetate is the most abundant, were able to function as the sole source of carbon and energy for A. phosphadevorus. Acetate may be utilized by microorganisms in at least two ways. The first is incorporation of the molecule as a whole, two-carbon compound, which enters the tricarboxylic acid (TCA) cycle and also the "glyoxylate bypass". From this series of reactions is gained reduced nicotinamide adenine dinucleotid ($NADH_2$), which later results in the production of energy. Also gained are building blocks such as amino acids to be used for the synthesis of cell constituents. When radioactive acetate is used by a microorganism in this manner, there is a typical pattern obtained in the distribution of differentially-labelled isotope among cell components (9, 15).

However, acetate may be used in a different manner. The molecule can be cleaved and the reduced carbon oxidized as a source of energy and utilized for cell synthetic processes. This would correlate with the proposed utilization of such C-1 carbon sources as methane, methanol, and methylated amines (5, 18, 19). Utilization of acetate as a C-1 compound would result in a different labelling pattern of differentially-labelled acetate than if the molecule were incorporated and utilized as a whole, two-carbon compound.

Experiments with differentially-labelled ^{14}C-acetate showed that this molecule, as the sole source of carbon and energy for the organism, did not enter the metabolic pathways as a whole two-carbon molecule. Rather, the carboxyl carbon was cleaved and released as CO_2, with a small amount of CO_2 fixation. The methyl carbon was then presumably oxidized in part to CO_2 as a source of energy, with the rest of the methyl carbon being used for synthesis of cell constituents. Studies with other C-1 compounds, sodium formate and sodium carbonate, also indicated the preference for a source of reduced carbon. Formate was not utilized as well as the methyl carbon of acetate, but better the sodium carbonate.

Organisms growing on methane, methanol, or other sources of C-1 carbon utilize one or both of the following pathways (14, 18, 19). The allulose pathway, or ribose phosphate cycle, involves the condensation of C-1 with ribose with ribose-5-phosphate to give allulose-6-phosphate, which is epimerized to give fructose-6-phosphate. The fructose-6-phosphate is cleaved to yield two moles of triose-phosphate

and thus synthesis of cell constituents. Some of the triose-phosphate generated undergoes transaldolase and transketolase rearrangements to regenerate ribose-5-phosphate. Sugars are integrally involved in this cycle and, at present, no sugars have been found in cell extracts of A. phosphadevorus in high enough concentrations for detection. The serine pathway involves the transfer of reduced C-1 compounds by tetrahyrofolic acid (THFA) to form serine from glycine. Serine is then converted in a series of metabolic steps to glyceric acid, phosphoglyceric acid and phosphoenolpyruvate. From phosphoenolpyruvate is formed oxalacetate and cell constituents. Glycine is either regenerated from oxalacetate, or formed de novo from reduced C-1 and CO_2. Serine and pyruvate have been detected in the present study using paper chromatography, and it was shown that iodoacetate can block the metabolic pathways. Iodoacetate inhibits substrate-level phosphorylation, in particular, the phosphorylation of glyceric acid, which step is involved in the serine pathway. Thus, it seemed that A. phosphadevorus utilized the reduced carbon from acetate in the same manner as obligate methylotrophs utilize methanol, with the serine pathway. Experiments using ^{14}C-methanol or those which could demonstrate the presence of THFA and certain key enzymes would help establish the existence of this pathway.

There was some evidence to indicate that the serine pathway was not the only one operating in this organism. The cells grew with almost all of the intermediates of the TCA cycle as the sole source of carbon and energy. Growth rates and growth yields were generally faster and larger when such multi-carbon intermediates as succinate and citrate

were used in place of acetate. Also, the presence of "cold" citrate brought about a significant increase in the utilization of 2-^{14}C-acetate over that observed when "cold" acetate was present. Because this increased utilization was even greater than that observed in experiments with tracer amounts of 2-^{14}C-acetate, it seemed possible that there could be an alternate pathway of carbon assimilation which could operate when the proper carbon source was presented to induce it. Further experimentation may help to establish this point.

Further efforts to determine the pathways used by the organism involved the use of paper chromatographic techniques. Due to the relative insensitivity and resolution of the techniques used, only ninhydrin-positive compounds and one keto-acid were identified. The alpha-keto acid was identified as pyruvic acid, and the ninhydrin-positive spots were identified as lysine, threonine, leucine, isoleucine, valine, serine, and glutamic acid. These compounds were found in the fraction of cellular components extracted as metabolic pool compounds. They represented members of the pyruvate and aspartic acid families of amino acids. Because these amino acids and pyruvate were present in sufficient quantities to be detected by the relatively insensitive methods used, they probably played a major role in the cell's metabolism. What that role is will depend on further studies, involving the more sensitive radioautographic techniques.

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