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Bacterial Oxidation of Orthophosphite

GEORGE MALACINSKI AND WALTER A. KONETZKA
Department of Bacteriology, Indiana University, Bloomington, Indiana

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ABSTRACT

MALACINSKI, GEORGE (Indiana University, Bloomington), AND WALTER A. KONETZKA. Bacterial oxidation of orthophosphite. J. Bacteriol. 91:578–582. 1966.—A variety of bacteria grown on a glucose and salts medium were capable of utilizing orthophosphite as a sole source of phosphorus. Two organisms, *Pseudomonas fluorescens* 195 and *Serratia marcescens* 24, were studied in detail. Growth rates and total cell yields of the bacteria grown on phosphite indicated that the bacteria utilized phosphite as efficiently as phosphate. The ability to oxidize the anion was shown to be inducible. A period of adaptation was required prior to growth on phosphite when phosphate-grown cells were transferred to a medium containing a limiting amount of phosphate and excess phosphite. No phosphite-oxidizing activity could be detected in whole cells or cell-free extracts of phosphate-grown cells. Both whole cells and cell-free extracts of phosphite-grown cells possessed phosphite-oxidizing activity.

The biological oxidation of phosphite was first demonstrated by Adams and Conrad (1). They observed that phosphite added to soil samples disappeared with a corresponding increase in phosphate concentration. The oxidation of phosphite proceeded only when microbial activity was not restricted by the presence of a bactericidal agent such as toluene. More recently, Casida (2) investigated the ability of a number of microbial cultures to utilize phosphite during heterotrophic growth. Several organisms were found to utilize phosphite, and one of the bacteria, *Pseudomonas fluorescens* 195, possessed the unique ability of being able to discharge the oxidation product, orthophosphate, into the growth medium.

The purpose of the work described here is to provide more information on the processes by which phosphite is oxidized by microorganisms. The possibility that this process may play a role in a biological phosphorus cycle which involves the change from the $^{+3}$ to the $^{+5}$ oxidation state which accompanies the conversion of phosphite to phosphate was investigated.

MATERIALS AND METHODS

Bacterial strains. A list of cultures employed is given in Table 1. All stocks were maintained on Trypticase Soy Agar (BBL) slants and transferred monthly.

Media and reagents. The following basal medium prepared with glass-redistilled water was employed: 0.5% glucose, 0.2% NH_4Cl, 0.002% Na_2SO_4, 0.05% $MgCl_2 \cdot 6H_2O$, 1.2% tris(hydroxymethyl)aminomethane (Tris), adjusted to *p*H 7.0 with HCl. Components were autoclaved separately and mixed upon cooling Liquid media, unless stated otherwise, contained a single filter-sterilized phosphorus source added to the basal medium at a final concentration of 10 μg of phosphorus per ml. Solid media were prepared by the addition of Ionagar and a phosphorus source to the basal medium, to final concentrations of 0.85% and 20 μg/ml, respectively. All reagents were Fisher "certified reagent" grade except the following: Oxoid Ionagar (Consolidated Laboratories, Inc., Chicago Heights, Ill.), sodium phosphite ("laboratory chemical" grade; Fisher Scientific Co., Pittsburgh, Pa.), and maleic acid (Matheson, Coleman, and Bell, East Rutherford, N.J.).

Phosphorus assay. The phosphorus content of each fraction obtained from anion-exchange chromatography was determined by a modification of the phosphovanadomolybdate method (7). The method of Chen, Toribara, and Warner (3) was employed in all other phosphorus assays.

Growth conditions. All cultures were grown at 30 C with the exception of *Bacillus subtilis*, *B. megaterium*, and *Escherichia coli* which were grown at 37 C, and *Pseudomonas fluorescens* 195 which was grown at 25 C. Shaking during incubation was carried out on a rotary shaker at 270 oscillations per min in DeLong flasks filled to 20% capacity.

Ion-exchange chromatography. A Dowex 1-X8 anion exchange column (3.5 by 60.0 cm) was prepared as described by Pollard et al. (7). The anions were eluted from the column at room temperature by a KCl concentration gradient obtained from the flow of 0.15 M KCl into a mixing vessel containing 750 ml of 0.05 M KCl. The KCl was buffered at *p*H 6.8 with ammonium acetate.

Measurement of growth. Viable counts were obtained by diluting in 0.85% KCl and plating 0.1-ml

samples on Trypticase Soy Agar plates. Protein content was determined by the method of Lowry et al. (6).

Assay of phosphite-oxidizing activity of resting-cell suspensions. Cells were harvested from liquid media containing either phosphate or phosphite as the sole phosphorus source, washed once with 0.85% KCl, and resuspended in Tris-maleic acid buffer (0.1 M, *p*H 7.0) to a protein concentration of 3.8 mg/ml. To initiate the reaction, 0.1 ml of sodium phosphite (32.0 mg/ml) was added to 3.9 ml of the cell suspension, and, at intervals during shaking at 25 C, 0.5-ml samples were diluted into 0.85% KCl and centrifuged for 10 min at 12,000 × *g*. The supernatant liquids were stored at 2 C until assayed for phosphate.

Assay of phosphite-oxidizing activity of cell-free extracts. Cells were harvested from either the phosphite or phosphate medium, washed once with acetate buffer (0.1 M, *p*H 6.0), resuspended in buffer, and crushed in a French press. After centrifugation for 20 min at 17,000 × *g*, the extract was diluted in buffer to a protein concentration of 2 mg/ml. A 0.1-ml amount of acetate-buffered sodium phosphite solution (100 mg/ml) was added to 3.9 ml of the extract to initiate the reaction. For the measurement of the endogenous accumulation of inorganic phosphate, 0.1 ml of acetate buffer was added to 3.9 ml of extract. The reaction mixtures were incubated at 25 C. At intervals, 0.4-ml samples were withdrawn and added to an equal volume of 10% trichloroacetic acid. After standing for 10 min, 1.2 ml of distilled water was added, and the suspensions were centrifuged at 17,000 × *g*. The supernatant liquids were stored at 2 C until assayed for phosphate.

RESULTS

Determination of the phosphate content of sodium phosphite reagent and basal medium. Since small amounts of phosphate are required for the growth of bacteria, it was necessary to determine whether sodium phosphite or reagents used in the basal medium contained significant amounts of phosphate contamination. To determine whether the Dowex 1-X8 anion-exchange column described by Pollard et al. (7) was sufficiently sensitive to detect small quantities of phosphate in a phosphite-phosphate mixture, a solution of 80 mg of $Na_2HPO_3 \cdot H_2O$ and 2 mg of Na_2HPO_4 was passed through the column. Of the phosphate applied to the column, 100% was recovered as the first peak (Fig. 1), revealing that the ion-exchange column was sufficiently sensitive to resolve small quantities of phosphate in a phosphite-phosphate mixture. The sodium phosphite reagent alone was then applied to a fresh column to determine whether a phosphate peak could be detected. Since no phosphate peak was detected, it was concluded that the sodium phosphite reagent used in these experiments did not contain significant amounts of phosphate.

To determine whether the basal medium contained phosphorus contamination, *Serratia marcescens* 24 was grown in liquid phosphate medium, harvested during the logarithmic phase of growth, washed with 0.85% KCl, and resuspended in basal medium lacking an added phosphorus source to a concentration of 5.0 × 10^7 cells per milliliter. Within a 150-hr period, the viable count doubled and the protein concentration increased by only 50%. The results indicated that there was not a significant amount of phosphorus contamination in the basal medium.

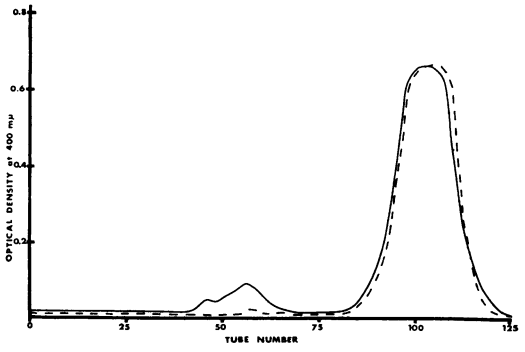

FIG. 1. *Anion-exchange chromatography of phosphite-phosphate mixture (solid line) and phosphite (broken line).*

Ability of bacteria to utilize phosphite as sole source of phosphorus. Several strains of bacteria were found to be capable of growth in the liquid medium containing phosphite as a sole source of phosphorus. However, phosphate-grown cells inoculated in phosphite medium seldom attained an exponential growth rate within 48 hr. Plating techniques were therefore employed which permitted a quantitative estimation of the number of bacteria in a population capable of utilizing phosphite. Bacteria grown in liquid phosphate medium were plated after appropriate dilution on solid

TABLE 1. *Estimation of number of cells in a population of phosphate-grown cells capable of utilizing phosphite*

Organism	Plate count × 10^8/ml	
	Phosphate medium	Phosphite medium
Aerobacter aerogenes 68	17	18
Bacillus megaterium WS	10	$<10^{-7}$
B. subtilis 19	20	$<10^{-7}$
Escherichia coli H	26	25
Pseudomonas aeruginosa Lilly	35	33
P. fluorescens 195	20	22
Serratia marcescens 24	15	15
S. marcescens 235	39	39

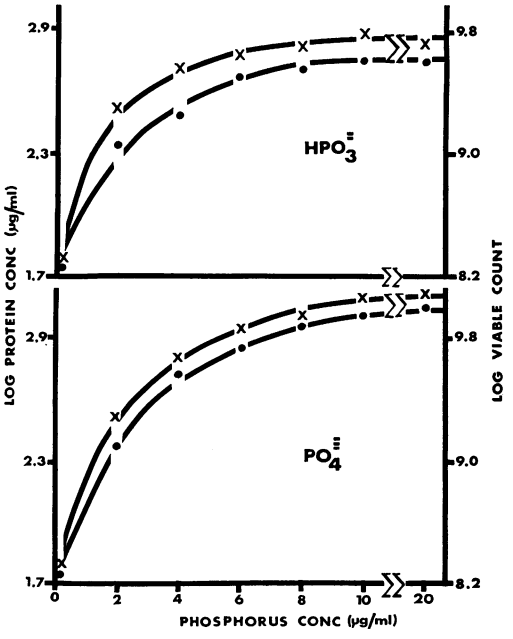

FIG. 2. *Dosage response of Serratia marcescens 24 to phosphite and phosphate. Symbols:* ● = *protein concentration;* × = *viable count.*

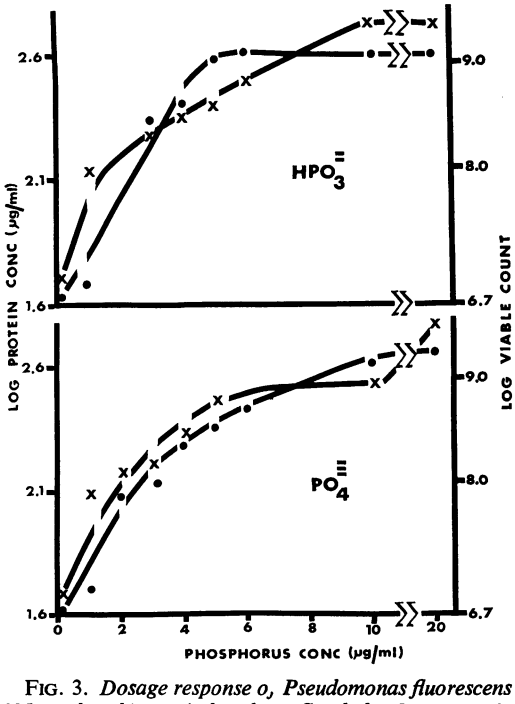

FIG. 3. *Dosage response o, Pseudomonas fluorescens 195 to phosphite and phosphate. Symbols:* ● = *protein concentration;* × = *viable count.*

medium containing phosphate, phosphite, or no added source of phosphorus. The plates were incubated at the appropriate temperatures for 3 to 12 days. Those bacteria capable of growth on phosphite (Table 1) acquired the ability to utilize phosphite by adaptation of the entire population rather than by the growth of small numbers of mutants, since the colony counts on phosphite solid medium were equal to the colony counts on phosphate medium. No growth of any of the bacteria examined was observed on the solid medium lacking an added phosphorus source.

Growth characteristics of S. marcescens 24 and P. fluorescens 195 in media containing phosphate or phosphite as sole source of phosphorus. The total yields of cells grown in the basal medium containing various concentrations of phosphate or phosphite phosphorus after 3 days of incubation are shown in Fig. 2 and 3. *S. marcescens* 24 achieved approximately 85% as much growth on phosphite phosphorus as on phosphate phosphorus, and *P. fluorescens* 195 achieved the same amount of growth on phosphite as on phosphate.

Growth response of P. fluorescens 195 in phosphate-phosphite medium. When phosphate-grown *P. fluorescens* 195 was transferred to medium containing a growth-limiting amount of phosphate (1.0 μg of phosphate P per ml) and excess phosphite (10 μg of phosphite P per ml), a typical diauxie effect was observed (Fig. 4). The initial long lag which preceded growth in fresh liquid medium was characteristic of *P. fluorescens* 195 and was independent of the age or size of the inoculum, the carbon or phosphorus source, and the incubation temperature. After adaptation to growth on phosphite, the reaction of the bacterium when it was transferred to phosphite liquid

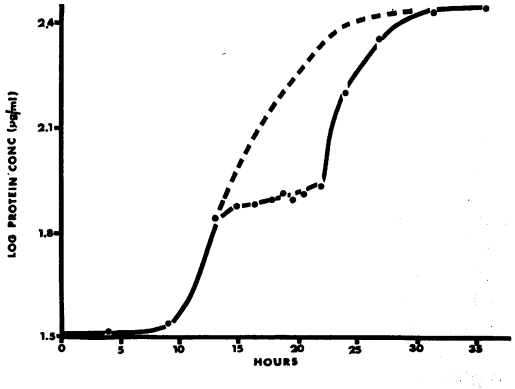

FIG. 4. *Growth of Pseudomonas fluorescens 195 in liquid medium containing 1.0 μg of phosphate phosphorus and 10.0 μg of phosphite phosphorus per ml. Dotted lines represent a control culture grown in medium containing 10.0 μg of phosphate phosphorus per ml.*

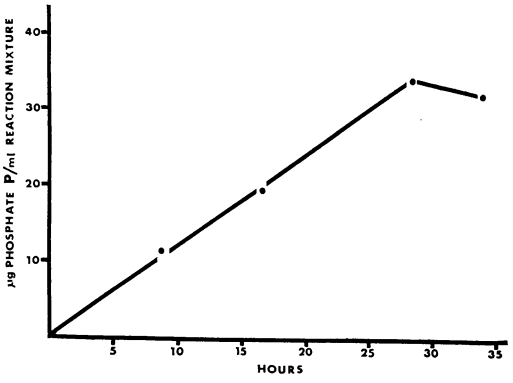

FIG. 5. *Oxidation of phosphite by resting cells of Pseudomonas fluorescens 195. The reaction mixtures contained 3.9 ml of cell suspension in Tris-maleic acid buffer at a protein concentration of 3.8 mg/ml and 0.1 ml of sodium phosphite at 32 mg/ml or 0.1 ml of water. The endogenous phosphate accumulation was subtracted from phosphate accumulation in the reaction mixture containing phosphite.*

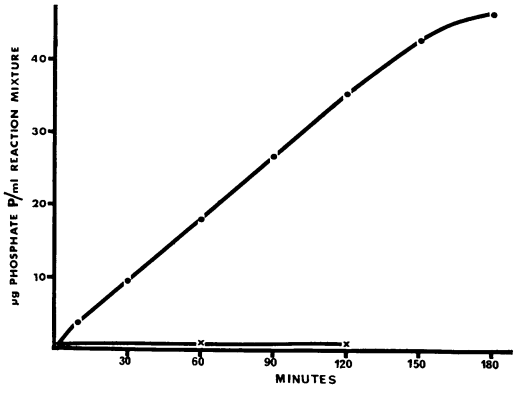

FIG. 6. *Oxidation o, phosphite by cell-free extracts of Pseudomonas fluorescens 195. The reaction mixture contained 3.9 ml of extract in acetate buffer at a protein concentration of 2 mg/ml and 0.1 ml of sodium phosphite (100 mg/ml) in acetate buffer or 0.1 ml of acetate buffer. Endogenous phosphate accumulation was subtrated from phosphate accumulation in reaction mixture containing phosphite. Symbols: ● = extract from phosphite-grown cells; × = extract from phosphate-grown cells.*

medium was identical to that of phosphate-grown cells when they were transferred to phosphate liquid medium. The growth rates were identical in both phosphite or phosphate liquid media.

A diauxie effect was also obtained when *S. marcescens* 24 was employed in a similar experiment.

Oxidation of phosphite by resting cells. Resting-cell suspensions prepared from *P. fluorescens* 195 grown in phosphite medium were capable of oxidizing phosphite to phosphate (Fig. 5), although the activity was quite low. However, no activity was detected in phosphate-grown cells.

Oxidation of phosphite by cell-free extracts. Cell-free extracts of *P. fluorescens* 195 prepared from cells harvested during the exponential phase of growth in phosphite medium possessed considerable phosphite-oxidizing activity (Fig. 6). No activity was detected in cell-free extracts prepared from phosphate-grown cells. Heating the active preparations at 70 C for 2 min completely destroyed the activity. Studies on the purification, cofactor requirements, and kinetics of the phosphite-oxidizing system will be presented elsewhere.

DISCUSSION

A variety of bacteria have been reported by Adams and Conrad (1) and Casida (2) to possess the ability to utilize phosphite. The results of the experiments presented in this report have confirmed their results and, in addition, have established that the ability of certain bacterial populations to utilize phosphite as a sole source of phosphorus is obtained through induction of the entire population rather than by selection of small numbers of mutant organisms. Since only those varieties of bacteria which were able to grow on the synthetic medium were tested, the possibility exists that several other types of bacteria not examined possess the ability to utilize phosphite.

The period of adaptation observed prior to growth on phosphite and the absence of phosphite-oxidizing activity of extracts obtained from cells grown in liquid phosphate medium are similar to the characteristics of the well-documented inducible enzyme systems for the utilization of carbon sources, and suggest that the induction period is required for the synthesis of an enzyme responsible for the oxidation of phosphite.

Assuming that an enzyme is responsible for the oxidizing activity, a specific activity of 6.0 mμ-moles of inorganic phosphite oxidized per min per mg of protein was obtained in cell-free extracts. Since phosphorus comprises only 2 to 3% of the dry weight of most bacteria, this amount of oxidizing activity should be sufficient to obtain the growth rates observed in the experiments reported here. The large difference in activity between intact and cell-free systems is not readily explained. However, it was observed that the presence of an oxidizable carbon source enhanced whole-cell phosphite-oxidizing activity. This suggests that the uptake of the anion may be coupled to an energy-requiring step. Alternatively, since the assay system employed is dependent upon the oxidation product's being present in the

supernatant liquid, the observed low rate of phosphite oxidation may indicate that the discharge of phosphate from the cells is the rate-limiting step in the process.

Natural deposits of phosphite have not been discovered (5), so it may seem improbable that the ability to utilize phosphite would be a property of bacteria. However, Gulick (4) proposed that phosphite deposits existed in nature during the Pre-Cambrian period, and Rudakow (8) and Tsubota (9) reported that phosphate is reduced by microorganisms under anaerobic conditions to phosphite and hypophosphite and that detectable amounts of these compounds appeared in the culture medium.

Since most phosphorus assays involve the oxidation of all phosphorus materials to inorganic orthophosphate, small amounts of phosphite in nature may have escaped detection. Various species of bacteria may, therefore, have retained the ability to oxidize phosphite as a result of continual exposure to the anion. Thus, the possibility exists that phosphorus may be utilized by living forms in a cyclic manner, involving the transition of phosphorus between $+3$ and $+5$ oxidation states.

ACKNOWLEDGMENTS

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