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Biosynthesis of Fosfomycin by *Streptomyces fradiae*

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The antibiotic fosfomycin was produced as a secondary metabolite in a glucose-asparagine medium containing citrate, L-methionine, and L-glutamate. The citrate requirement for antibiotic synthesis was related to its requirement for growth. In contrast, L-methionine and L-glutamate caused a marked stimulation of fosfomycin production and had no effect on growth. L-Methionine had to be added early to effect maximal antibiotic synthesis later in the fermentation. The L-glutamate requirement was not specific, since several tricarboxylic acid cycle intermediates could replace this amino acid. L-Asparagine was the most effective nitrogen source for growth and production of fosfomycin. Glycine, an alternate nitrogen source, supported fosfomycin synthesis only when added in excess of that needed for growth. Cobalt and inorganic phosphate were required also for antibiotic production at concentrations exceeding those supporting maximal growth. Radioactive incorporation studies showed that the methyl carbon of methionine was the precursor of the methyl of fosfomycin. Carbon 1 of fosfomycin was derived from glucose carbons 1 and 6, whereas glucose-*2*-^{14}C labeled fosfomycin carbon 2. Radioactivity from acetate-*2*-^{14}C was distributed equally between fosfomycin carbons 1 and 2. No incorporation of acetate-*1*-^{14}C, asparagine-*U*-^{14}C, citrate-*1,5*-^{14}C, or glutamate-*U*-^{14}C occurred. The labeling pattern of fosfomycin carbons 1 and 2 was similar to that found in 2-aminoethylphosphonate from *Tetrahymena*.

In 1969, Hendlin et al. (9) reported the discovery of a new cell wall-active antibiotic, phosphonomycin. The compound was identified as (—)-(1R, 2S)-1,2-epoxypropylphosphonic acid (3), and has since been renamed fosfomycin. The antibiotic has a unique structure (Fig. 1) containing chemical groups not frequently encountered in antimicrobial substances, namely, epoxide and phosphonate groups. Fosfomycin is produced by several *Streptomyces* species (9, 26). Phosphonates have been detected in a variety of tissues (21); however, before the discovery of fosfomycin, their existence in bacteria and related microorganisms had been doubtful (21).

Jackson and Stapley (14) investigated the nutrition of *Streptomyces fradiae* in complex and semisynthetic media and defined several requirements for fosfomycin production. They found that, in the absence of the critical nutrilites, a low level of antibiotic was produced. This was probably due to the presence, in their media, of low concentrations of compounds essential for fosfomycin synthesis. Therefore, these studies have now been expanded by using a completely synthetic medium and washed-cell inocula. A technique was developed for the isolation of fosfomycin from fermentation broth which enabled us to measure the incorporation of radioactive compounds into the antibiotic. These studies have allowed us to define the physiological role of several compounds in fosfomycin synthesis.

MATERIALS AND METHODS

Organism and media. The culture used was MA-2913 (NRRL B3360) which has been identified as *S. fradiae* (26). The organism was preserved in the lyophilized state and maintained on cornstarch-asparagine agar slants (14). Fosfomycin production medium contained (per liter): glucose (20 g); L-asparagine (5 g; Difco); monosodium glutamate (1 g; Merck); L-methionine (0.15 g); sodium citrate dihydrate (4 g); K_2HPO_4 (1 g); $CaCl_2 \cdot 2H_2O$ (0.5 g); $MgSO_4 \cdot H_2O$ (0.2 g); $CoCl_2 \cdot 6H_2O$ (0.1 g); $FeSO_4 \cdot 7H_2O$ (10 mg); $MnSO_4 \cdot 1H_2O$ (10 mg); $CuCl_2 \cdot 2H_2O$ (2.5 μg); H_3BO_3 (5.6 μg); $ZnSO_4 \cdot 7H_2O$ (2 μg); and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (1.9 μg). The pH was 7.0. Glucose was sterilized separately. Seed medium had the same composition, except that Clinton cornstarch replaced glucose.

Fosfomycin production. Fermentation studies were carried out in 250-ml Erlenmeyer flasks incu-

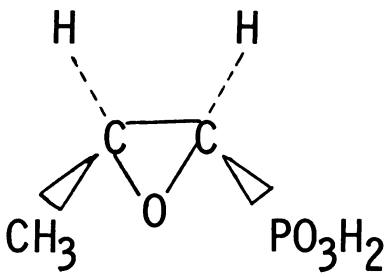


FIG. 1. *Structure of fosfomycin.*

bated at 28 C on a reciprocating shaker (250 rpm). Baffled flasks, containing 40 ml of seed medium, were inoculated from agar slants and incubated for 48 to 72 h. Cells were harvested by centrifugation (12,000 × *g*), washed three times with sterile Davis salts (6), and suspended in Davis salts to the original harvest volume. Unless otherwise indicated, unbaffled flasks containing 30 ml of production medium were inoculated with 1 ml of the washed-cell suspension and incubated at 28 C. At appropriate times, cells were harvested by centrifugation and the supernatant fluid was assayed for fosfomycin.

Preparation of phosphate-starved inoculum. Cells grown for 72 h in seed medium were harvested by centrifugation, washed three times with sterile 0.8% NaCl, and suspended in 40 ml of phosphate-free production medium. These cells were shaken for 16 h at 28 C, harvested, washed three times with 0.8% NaCl, and suspended in 40 ml of 0.8% NaCl. This suspension was used as inoculum in studies of the effect of phosphate on growth and fosfomycin synthesis.

Cell dry weight determinations. Cells (from 1 to 5 ml of broth) were collected on tared membrane filters (0.45 μm pore size; Millipore Corp.), washed with Davis salts, dried overnight at 90 C, and weighed. Growth was expressed as milligrams of dry cells per milliter of broth.

Assays. Fosfomycin was quantitated microbiologically as described by Stapley et al. (26). Glucose was determined by the Glucostat method (Worthington Biochemical Corp., Freehold, N.J.).

Radioactivity measurements. Radioactivity was measured in a Packard Tri-Carb scintillation spectrophotometer. The scintillation fluid contained (per liter): 1,4-bis-2-(5-phenyloxazolyl)-benzene (0.375 g); 2,5-diphenyloxazole (5 g); toluene (688 ml); and Triton X-100 (312 ml). Aqueous solutions of 1.0 ml or less were added to standard counting vials followed by 10 ml of scintillation solution. When less than 1.0 ml of solution was to be counted, sufficient water was added to the vial to give a final aqueous volume of 1.0 ml. In chemical degradation studies, the radioactivity of CO_2 was determined in a scintillation fluid consisting of toluene:methyl cellosolve:ethanol (110:88:13) containing 5.5 g of 2,5-diphenyloxazole per liter of solution (16).

Isolation of radioactive fosfomycin by co-crystallization. Cells grown in the presence of radioactive compounds were harvested from fermentation broth by centrifugation (12,000 × *g*) or by filtration through a Nalgene filter (0.2 μm pore size). The fosfomycin content of the cell-free broth was determined by bioassay. Three milliliters of broth was mixed with 12 ml of ice-cold 2-propanol and placed in ice for 30 min. Insoluble material was removed by filtration through a membrane filter (0.45 μm pore size; Millipore Corp.). Ten milliliters of filtrate was transferred to a Corex centrifuge tube (Corning Glass Corp.), and 600 mg of +-α-phenethylammonium fosfomycin monohydrate (PEA-fosfomycin) was added. The solution was heated at 70 C to dissolve the crystals, cooled to room temperature, and placed in ice for 1 h. The resulting crystals were harvested by centrifugation at 4 C, washed once with ice-cold 90% 2-propanol, and suspended in 80% 2-propanol. Two additional recrystallizations were done similarly. Finally, the crystals were collected on a membrane filter (0.45 μm pore size; Millipore Corp.), washed with ice-cold 80% 2-propanol, and dried for 16 h at room temperature in a vacuum desiccator. The radioactivity of the crystals was used to calculate the specific activity of the fosfomycin synthesized in fermentation medium. After three recrystallizations, the isolated fosfomycin was assumed to be radiochemically pure, since the specific radioactivity of the crystals remained constant upon additional recrystallization. Thin-layer chromatography (23) of the isolated fosfomycin revealed no contamination with structurally related phosphonates or organic phosphates. When glucose-*U*-^{14}C or methyl-labeled [^{14}C]methionine (precursors of fosfomycin) or ^{3}H-labeled 1,2-dihydroxypropylphosphonic acid (diol of fosfomycin) was added to fermentation broth before co-crystallization, no radioactivity was detected in the final crystals, thereby showing the specificity of the co-crystallization procedure.

For the isolation of fosfomycin of low radioactivity, column chromatography was used to concentrate the antibiotic before the co-crystallization step. The pH of cell-free fermentation broth was adjusted to 7 with 85% phosphoric acid. A sample (20 to 40 ml) was applied to a column (0.9 by 25 cm) containing Dowex 1-X2 (chloride cycle) resin. The column was washed with water and eluted with 0.3 M NaCl. Fractions were assayed qualitatively for fosfomycin by using 0.25-inch (about 0.6 cm) paper disks on agar medium seeded with *Proteus vulgaris* (MB-838). Bioactive fractions were combined, concentrated under reduced pressure, and desalted on a Bio-Gel P-2 (200 to 400 mesh) column. The column was eluted with either water or 1% butanol. Bioactive fractions were combined and concentrated. Fosfomycin was isolated from the concentrated fractions by co-crystallization with PEA-fosfomycin as described above.

Chemical degradation of radioactive fosfomycin. Total carbon oxidations were carried out by the Van Slyke-Folch method (30). The combustion fluid was modified in that the CrO_3 concentration was increased to 100 g per liter. For each determination, 0.5 mmol of sample was oxidized and the resulting CO_2 was collected in CO_2-free 2 N NaOH.

To isolate carbons 2 and 3 of fosfomycin, the antibiotic was oxidized initially to acetic acid. Two

millimoles of [^{14}C]PEA-fosfomycin were incubated at room temperature for 15 h in 50 ml of a 1 N CrO_3 solution in a ground glass-stoppered flask. The reaction mixture was steam distilled, and 250 ml of distillate was collected and titrated to pH 8.5 with CO_2-free 0.1 N NaOH. The distillate was concentrated to dryness under reduced pressure, suspended in water, decolorized with charcoal, and evaporated to dryness over an infrared lamp. The resulting white powder was dehydrated at 120 C and cooled to room temperature in a vacuum desiccator. The isolated material was identified as sodium acetate by microanalysis. Acetate obtained in this manner was degraded to its respective carbon atoms. Carbon 1 of acetate (represents carbon 2 of fosfomycin) was obtained by the Schmidt reaction (34) as described by Phares (20). One millimole of sodium acetate was degraded, and the resulting CO_2 was trapped in CO_2-free 2 N NaOH. The methylamine which remained in the reaction mixture (represents fosfomycin carbon 3) was recovered by distillation into 0.4 N H_2SO_4. This solution was evaporated to 0.2 ml by using an infrared lamp, and the methylamine was oxidized to CO_2 by the Van Slyke-Folch procedure (30).

The CO_2 resulting from the various oxidations was isolated from the NaOH samples, and its radioactivity was determined by the procedure of Kornblatt et al. (16). Radioactivity in fosfomycin carbon 1 was determined indirectly by subtracting the sum of the radioactivity in carbons 2 and 3 from the total radioactivity in the antibiotic.

Chemicals. Radiochemicals were purchased from New England Nuclear Corp., Boston, Mass. Phosphonates were purchased from Calbiochem, Los Angeles, Calif. Fosfomycin and PEA-fosfomycin were synthesized in the Process Research Department of Merch & Co., Inc., Rahway, N.J. The 1,2-dihydroxypropylphosphonic acid was prepared by acid hydrolysis of fosfomycin at 37 C, pH 1, for 24 h.

RESULTS

Carbon sources for growth and fosfomycin synthesis. A number of compounds were tested as carbon sources for growth and fosfomycin production in synthetic medium. Of 57 materials tested, cornstarch, glucose, and glycerol were the best for supporting growth and antibiotic synthesis. The optimal concentrations for fosfomycin production were 2% for glucose and 4% for glycerol. Higher levels supported increased growth but had little effect on fosfomycin synthesis. Glucose was chosen as the carbon source for studies in synthetic medium.

Growth and fosfomycin production in synthetic medium. Figure 2 shows the time course of growth and fosfomycin production by *S. fradiae* in synthetic medium. Production flasks were inoculated to give a cell concentration of 0.09 mg of dry cells per ml (Fig. 2A) or 0.2 mg of dry cells per ml (Fig. 2B). With the smaller inoculum, there was an initial lag of 18 to 24 h followed by a phase of rapid growth and sugar utilization. The growth lag could be eliminated by increasing the inoculum size (Fig. 2B). In either case, fosfomycin synthesis was initiated after about 80% of maximal growth was reached, and most antibiotic synthesis occurred after the rapid growth phase. Thus, in analogy to other secondary metabolites, fosfomycin was synthesized during the idiophase of growth. The decrease in cell dry weight after maximal growth indicates that the culture underwent lysis in the later stages of the fermentation. The larger inoculum of 0.2 mg of dry cells per ml (1 ml of a washed-cell suspension as described in Materials and Methods) was adopted as standard in all experiments.

Inorganic nutritional requirements. Jackson and Stapley (14) found that iron was required for fosfomycin synthesis and that cobalt stimulated antibiotic production in semisynthetic medium. The effect of these substances on growth and fosfomycin production in synthetic medium was evaluated (Fig. 3-5). There was a marked difference in the responses to increasing levels of the two cations. Growth was stimulated maximally by the addition of

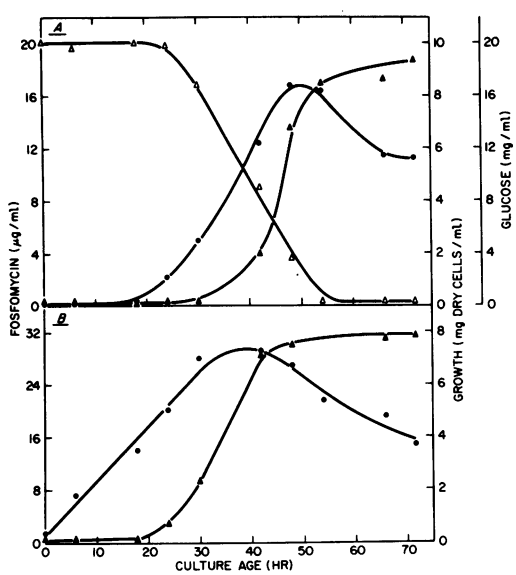


FIG. 2. *Growth, fosfomycin production, and sugar utilization in synthetic medium. (A) Production flasks were inoculated with 0.5 ml of a washed cell suspension to give 0.09 mg of dry cells per ml, and (B) production flasks were inoculated with 1 ml of the cell suspension to give 0.2 mg of dry cells per ml. At the designated intervals, duplicate flasks were analyzed for growth (●) and fosfomycin (▲). In (A), flasks were analyzed also for glucose (△).*

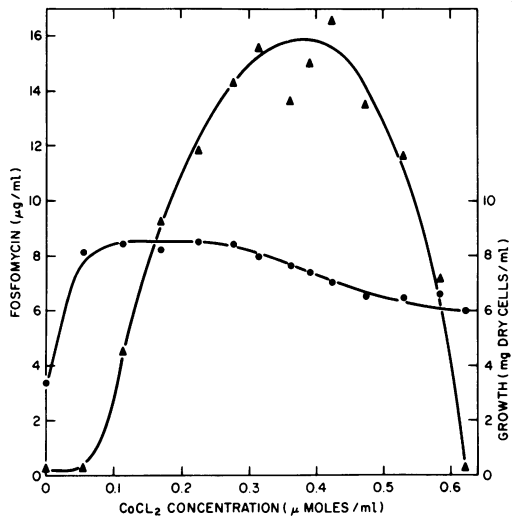


FIG. 3. *Effect of cobalt on growth and fosfomycin synthesis. Growth (●) and fosfomycin (▲) were measured after 72 h of incubation.*

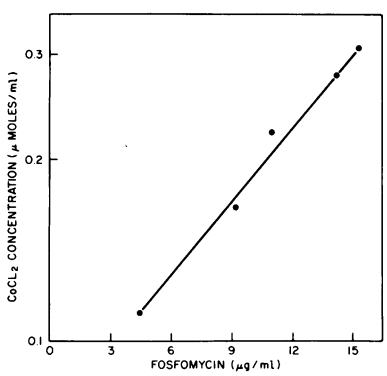


FIG. 4. *Linear relationship between log_{10} of the cobalt concentration and fosfomycin yield. Data were taken from Fig. 3 for $CoCl_2$ concentrations between 0.11 and 0.31 μmol/ml.*

0.06 μmol of cobalt per ml (Fig. 3); however, no fosfomycin was synthesized at this level of cobalt. A much greater cobalt concentration (0.28 to 0.42 μmol/ml) was required for maximal antibiotic synthesis. At cobalt levels above 0.42 μmol/ml, fosfomycin synthesis was inhibited to a greater extent than growth. A linear relationship was observed when fosfomycin yield was plotted as a function of the log of cobalt concentration at metal levels not affecting growth (Fig. 4). This type of response has been observed for the synthesis of several secondary metabolites (33). In contrast to the cobalt response, iron stimulated growth and antibiotic synthesis in a more parallel fashion (Fig. 5). Maximal growth was obtained at 0.01 μmol of iron per ml, whereas slightly more iron was required for maximal fosfomycin synthesis (0.03 μmol/ml). These results suggest that cobalt has a direct role in antibiotic production aside from its function in growth, whereas the role of iron may be indirect and correlated with its stimulation of growth.

The effect of phosphate on fosfomycin synthesis was studied by using normal and phosphate-starved cells (Fig. 6). With unstarved cells (standard inoculum), phosphate was required for fosfomycin synthesis and caused a slight stimulation of growth (Fig. 6A). A clearer picture of the phosphate requirement was obtained by using a phosphate-starved inoculum (Fig. 6B). In this case, phosphate was required for both growth and fosfomycin synthesis. About 3 μmol of phosphate per ml was required for maximal growth, whereas antibiotic synthesis was only 50% of maximum at this level of phosphate. A concentration of 7.2 μmol of phosphate per ml was required for maximal fosfomycin synthesis. This type of dose response was not unexpected since a phosphonate group is part of the antibiotic molecule. At phosphate concentrations above 7.2 μmol/ml, fosfomycin synthesis was inhibited although there was no inhibition of growth, suggesting that phosphate may function also as a regulator of antibiotic synthesis. Phosphate has been shown to inhibit the synthesis of several secondary metabolites (7).

A number of bacteria have been found capa-

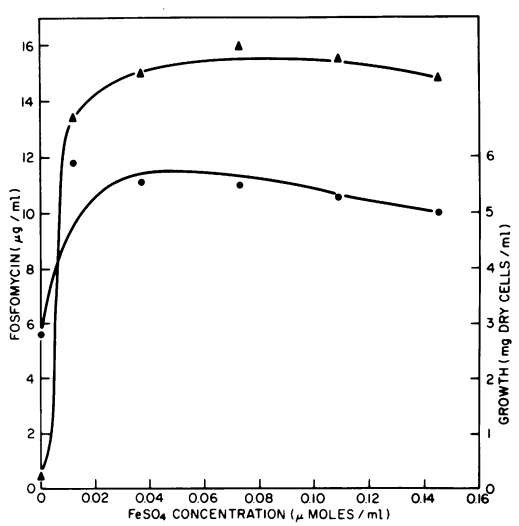


FIG. 5. *Effect of ferrous iron on growth and fosfomycin synthesis. Growth and fosfomycin were measured after 72 h of incubation. Symbols are as for Fig. 3.*

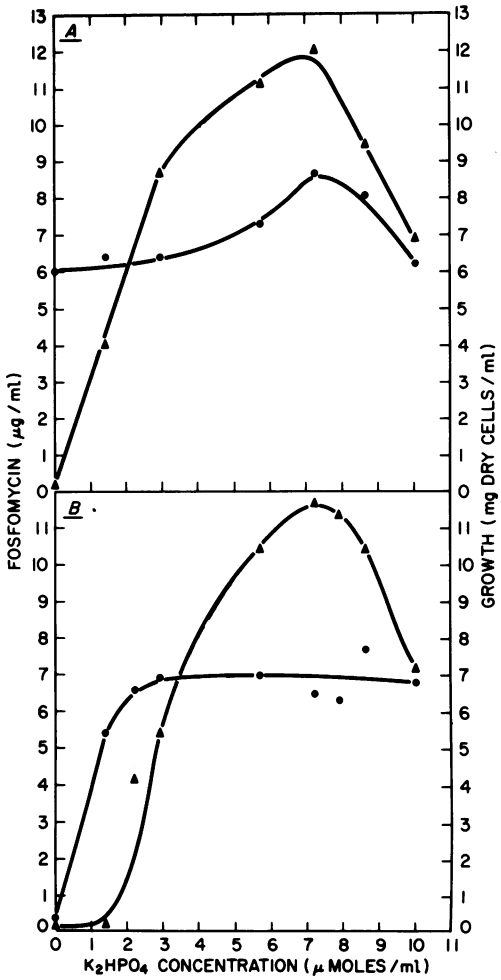


FIG. 6. *Effect of phosphate on growth and fosfomycin synthesis by: (A) phosphate-starved cells and (B) nonstarved cells. Cells were starved for phosphate as described in Materials and Methods. Growth and fosfomycin were measured after 72 h of incubation. Symbols are as for Fig. 3.*

ble of utilizing aminoalkylphosphonic acids as phosphorus sources for growth (8, 22). Three aminoalkylphosphonic acids (2-amino-3-phosphonopropionic acid, 1-aminoethylphosphonic acid, and 2-aminoethylphosphonic acid) were inactive as phosphorus sources for growth of *S. fradiae*. Furthermore, these compounds were without effect on fosfomycin synthesis when added to medium containing 3 μmol of K_2HPO_4 per ml, a suboptimal level of inorganic phosphate (see Fig. 6B). It may be that *S. fradiae* is unable to transport phosphonic acids, although several bacteria possess active transport systems for the uptake of aminoalkylphosphonates (10, 22).

Organic nutritional requirements. An evaluation of the organic nutritional requirements for fosfomycin synthesis was made by deleting various components from the synthetic production medium (Table 1). Removal of L-asparagine prevented both growth and fosfomycin synthesis, suggesting that this compound serves as a nitrogen source for growth. Deletion of citrate caused a reduction in growth and antibiotic production, which may be related to pH since, in the absence of citrate, the pH of the fermentation broth was low. Removal of methionine or glutamate had no effect on growth but caused a marked decrease in fosfomycin production.

Glutamate and methionine requirement. The effect of glutamate and methionine on fosfomycin production was examined further by titrating each compound in synthetic production medium (Fig. 7). Maximal fosfomycin production occurred at a concentration of 5 and 0.7 μmol of glutamate and methionine per ml, respectively. The pronounced stimulation of antibiotic synthesis and the lack of growth effects suggest that these amino acids are directly involved in fosfomycin synthesis as precursors or metabolic regulators.

The importance of time of addition of L-methionine is seen in Fig. 8. Methionine was added at two levels (0.34 and 0.67 μmol/ml) at 0, 25, 31, and 48 h. A control flask received no methionine. All flasks were harvested after 72 h, and growth and fosfomycin were measured. Although not shown, growth was the same in all flasks. The addition of methionine at zero time resulted in good antibiotic yields (only low amounts of fosfomycin were produced in flasks receiving no methionine). When methionine was added at 25 h (approximately the time of initiation of antibiotic synthesis; see Fig. 2B), the synthesis of fosfomycin was reduced greatly. When methionine was added at 31 h, no antibiotic was made in flasks receiving 0.34 μmol of

TABLE 1. *Nutritional requirements for growth and fosfomycin synthesis by S. fradiae in synthetic medium*[a]

Deletions from synthetic medium	Growth (mg of dry cells/ml)	pH	Fosfomycin (μg/ml)
None	4.0	9.0	24.7
L-Asparagine	1.3	8.8	<2.0
Citrate	2.1	4.6	10.1
L-Glutamate	5.4	8.8	13.9
L-Methionine	5.1	8.8	7.8

[a] Incubation was for 72 h. Citrate and glutamate were supplied as the tri- and monosodium salts, respectively.

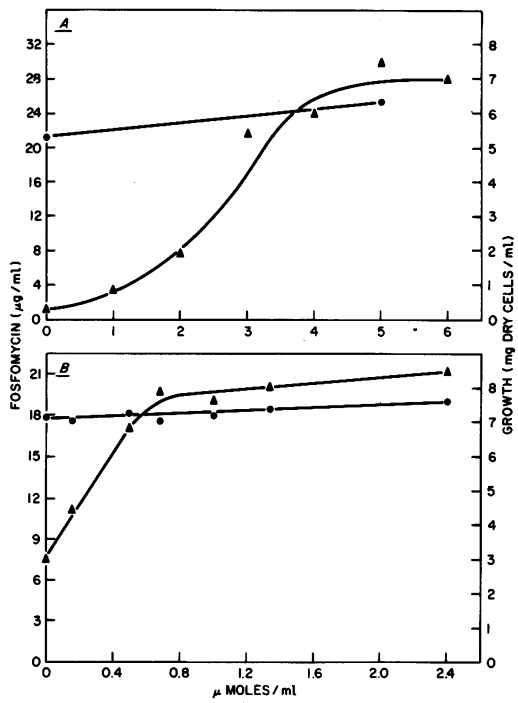


FIG. 7. *Effect of* L-*glutamate and* L-*methionine on growth and fosfomycin synthesis. Growth and fosfomycin were measured after 72 h of incubation. (A)* L-*Glutamate added as the monosodium salt, and (B)* L-*methionine addition. Symbols are as for Fig. 3.*

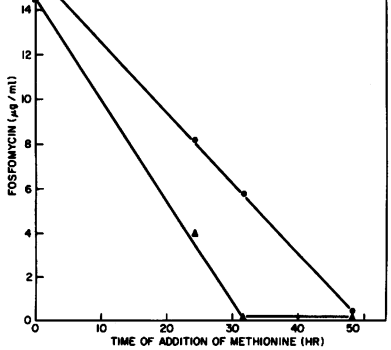


FIG. 8. *Effect of the time of addition of methionine on fosfomycin synthesis. Methionine was added to production flasks to give a final concentration of either 0.34 (▲) or 0.67 μmol/ml (●). Fosfomycin was determined after 72 h of incubation.*

L-methionine per ml, and only 5.8 μg/ml was synthesized in flasks receiving the higher level of the amino acid. No fosfomycin was produced when methionine was added at 48 h. It appears that methionine must be present early in the growth phase of the fermentation to promote the synthesis of fosfomycin during the idiophase.

The specificity of the methionine requirement for high antibiotic synthesis was realized from experiments that revealed that the following compounds could not replace the amino acid: folic acid, cyanocobalamin, betaine, choline, L-serine, L-cysteine, L-threonine, DL-homocysteine, and D-methionine. Further, fosfomycin synthesis was inhibited completely by ethionine at concentrations which caused only a slight inhibition of growth. Ethionine inhibition was overcome by L-methionine. These observations support the notion that L-methionine plays a direct role in fosfomycin synthesis via its function as a methyl donor in transmethylation reactions (24).

Glutamate could be replaced in the medium by several tricarboxylic acid cycle intermediates (Table 2). Pyruvate, acetate, and the early intermediates of the cycle, isocitrate and cis-aconitate (citrate being present in the basal medium), were only partially effective in replacing glutamate. Cycle intermediates, α-ketoglutarate through oxalacetate, were as effective as glutamate in promoting fosfomycin synthesis. Fumarate and malate consistently stimulated antibiotic production to a greater extent than did glutamate. Several amino acids were tested and failed to replace the glutamate function in fosfomycin synthesis. These include L-aspartate, L-proline, L-citrulline, L-ornithine, L-arginine, and L-glutamine, all of which are derived biosynthetically from L-glutamate. The addition of organic acids or amino acids did not affect significantly the growth of *S. fradiae*.

Citrate, asparagine, and glycine requirements. Citrate and L-asparagine were re-

TABLE 2. *Effect of organic acids on fosfomycin synthesis*

Addition[a]	Fosfomycin (μg/ml)[b]
None	9.9
L-Glutamate	17.2
Pyruvate	12.0
Acetate	12.0
Cis-aconitate	12.7
Isocitrate	11.3
Glyoxylate	7.9
α-Ketoglutarate	17.3
Succinate	16.6
Fumarate	19.6
Malate	20.2
Oxalacetate	16.8

[a] At 6 mM.

[b] Assayed at 72 h. Average of five experiments.

quired for growth and fosfomycin synthesis (Fig. 9). Antibiotic yield closely paralleled growth in response to both components. In the absence of citrate, the final pH of production medium was low (pH 6.9) and increased gradually as the citrate concentration was increased to 10.2 μmol/ml. Although the pH remained constant (pH 8.4) within the citrate concentration range of 10.2 to 17 μmol/ml, there was a marked effect on growth and fosfomycin synthesis. Thus, the role of citrate in antibiotic production was not related solely to pH effects. Maximal growth and fosfomycin synthesis occurred at 15 μmol of citrate per ml. Higher levels of citrate inhibited both parameters.

An L-asparagine concentration of 37 μmol/ml resulted in maximal growth and antibiotic synthesis (Fig. 9B). The role of L-asparagine appeared to be that of a nitrogen source for growth. Urea, L-glutamate, L-glutamine, L-methionine, and inorganic nitrogen compounds could not replace L-asparagine in this capacity. Though L-aspartic acid and L-ornithine supported 50% of maximal growth in the absence of L-asparagine, they could not affect fosfomycin synthesis. Glycine was the only organic nitrogen source tested that replaced L-asparagine for both growth and antibiotic synthesis. The effect of glycine in the presence and absence of L-asparagine is shown in Fig. 10. Since fosfomycin synthesis is delayed in cells growing on glycine as a sole nitrogen source, growth and fosfomycin were measured after 96 h of incubation. In the absence of L-asparagine, no antibiotic was synthesized until the glycine concentration exceeded that required for maximal growth (35 μmol/ml); the glycine concentration required for maximal synthesis was about twice this level. In medium containing 20 μmol of L-asparagine per ml, glycine caused a slight stimulation of growth and did potentiate fosfomycin production. In this instance, the level of glycine required for maximal antibiotic synthesis is lower than that required in L-asparagine-free medium. At the higher level of L-asparagine (47 μmol/ml), antibiotic synthesis was at a maximum, and further addition of glycine did not stimulate antibiotic production. Either in the presence or absence of L-asparagine, glycine inhibited fosfomycin synthesis at concentrations which did not inhibit growth. The independent effects of glycine on growth and fosfomycin synthesis suggest that, as with L-glutamate and L-methionine, glycine may have a direct role in antibiotic production.

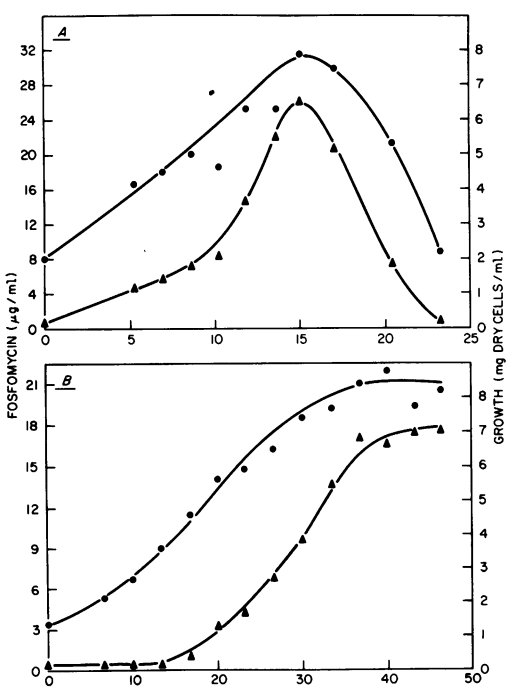


FIG. 9. *Effect of citrate and L-asparagine on growth and fosfomycin synthesis. (A) Effect of citrate (added as sodium salt), and (B) effect of L-asparagine (added as monohydrate). Symbols are as for Fig. 3.*

Radioisotope incorporation studies. The function of citrate, L-asparagine, L-glutamate, L-methionine, glycine, and L-serine in fosfomycin synthesis was evaluated by using ^{14}C-labeled compounds (Table 3). After growth on the indicated radioactive compound, the antibiotic was isolated by the co-crystallization procedure, and its radioactivity was determined. Due to the low incorporation of radioactivity into the antibiotic and the relatively high level of the materials required for fosfomycin synthesis, it was more meaningful to express the incorporation data in terms of dilution of specific activity of the added substrate rather than as percent incorporation. There was a large dilution of label in the antibiotic when L-asparagine-*U*-^{14}C, citrate-*1,5*-^{14}C, and L-glutamate-*U*-^{14}C were used as labeled substrates. The lack of incorporation from L-asparagine and citrate was not unexpected, based on the dose response to these materials; i.e., these compounds promoted fosfomycin synthesis indirectly through their effects on growth (see Fig. 9). It was unexpected, however, that L-glutamate did not contribute label to the antibiotic, since this amino acid promoted fosfomycin synthesis while having no effect on growth (see Fig. 7A). L-Methionine-*methyl*-^{14}C served as the best radioactive precursor. In contrast, L-methionine labeled in either carbon 1 or 2 was a poor precursor. These data support further the role of methionine in

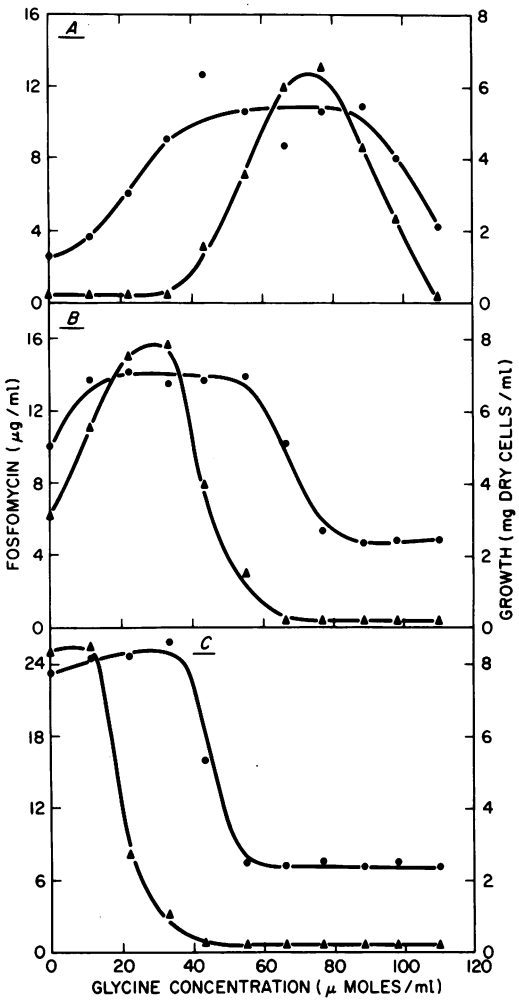


FIG. 10. *Effect of glycine on growth and fosfomycin synthesis in medium containing: (A) no* L-*asparagine, (B) 20 μmol of* L-*asparagine per ml, and (C) 47 μmol of* L-*asparagine per ml. Growth and fosfomycin were measured at 96 h. Symbols are as for Fig. 3.*

fosfomycin synthesis as a methyl donor via an *S*-adenosylmethionine methylation mechanism (24). Because of its function in 1-carbon metabolism, the incorporation of DL-serine-*3*-^{14}C was determined. Carbon 3 of this amino acid was incorporated rather well. Glycine-*U*-^{14}C in contrast to L-asparagine (both amino acids are alternate nitrogen sources for the fermentation) did contribute efficiently radioactivity to fosfomycin. Again, this is in agreement with the dose response data (see Fig. 10).

Radioactive glucose, pyruvate, and acetate were tested also for incorporation into fosfomycin (Table 4). Good incorporation of glucose radioactivity was obtained. Glucose-*1*-^{14}C and glucose-*6*-^{14}C contributed label equally well. Glucose-*2*-^{14}C was only slightly poorer. Glucose-*3,4*-^{14}C was the poorest of all glucose labels, yielding a 36-fold dilution of specific

TABLE 3. *Incorporation of radioactive amino acids and citrate into fosfomycin*

Labeled compound[a]	Sp act (counts/min × 10^3/μmol)	Fosfomycin[b] sp act (counts/min × 10^3/μmol)	Dilution of sp act
L-Asparagine-*U*-^{14}C	900	<0.1	>1,000
L-Glutamic acid-*U*-^{14}C	370	0.4	925
L-Methionine-*1*-^{14}C	1,350	2.6	520
L-Methionine-*2*-^{14}C	1,380	2.5	552
L-Methionine-*methyl*-^{14}C	1,090	130	8.4
Glycine-*U*-^{14}C	80	6.1	13
DL-Serine-*3*-^{14}C	552	15.2	36.4
Citric acid-*1,5*-^{14}C	70	<0.1	>700

[a] Labeled compounds were added at 0 h just before inoculation. L-Asparagine was the major nitrogen source in all flasks except those containing glycine-*U*-^{14}C. In this case, glycine served as the nitrogen source.

[b] Antibiotic was isolated at 72 h postinoculation by the co-crystallization procedure except for the glycine-*U*-^{14}C flasks. In this case, incubation was continued to 96 h, and isolation of the antibiotic was affected by co-crystallization after partial purification by ion exchange and gel filtration chromatography. Details of the isolation are given in Materials and Methods.

TABLE 4. *Incorporation of radioactive glucose, pyruvate, and acetate into fosfomycin*

Labeled compound[a]	Precursor sp act (counts/min × 10^3/μmol)	Fosfomycin[b] sp act (counts/min × 10^3/μmol)	Dilution of sp act
Glucose-*1*-^{14}C	9.5	3.2	3.0
Glucose-*2*-^{14}C	11.0	2.2	5.0
Glucose-*6*-^{14}C	9.1	3.3	2.6
Glucose-*3,4*-^{14}C	10.0	0.28	35.7
Pyruvate-*1*-^{14}C	111	0.13	854
Pyruvate-*2*-^{14}C	154	0.23	670
Pyruvate-*3*-^{14}C	118	0.51	231
Acetate-*1,2*-^{14}C	357	4.5	79
Acetate-*1*-^{14}C	1,000	0.02	>1,000
Acetate-*2*-^{14}C	325	8.9	36.5

[a] Labeled compounds were added at 0 h just before inoculation.

[b] Antibiotic was isolated at 72 h by co-crystallization after partial purification by ion exchange and gel filtration chromatography. Details of the isolation are given in Materials and Methods.

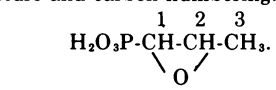
activity. This dilution was 7- and 12-fold greater than that obtained when glucose-2-^{14}C and glucose-*1*-^{14}C (or glucose-*6*-^{14}C), respectively, were precursors. Acetate and pyruvate incorporation was less than that observed for glucose, indicating that the carbon precursor of fosfomycin may be a glycolytic intermediate. Even though the dilution of radioactivity from pyruvate was high, there was a preferential incorporation of carbon 3. Though a poorer precursor than glucose, methionine-*methyl*-^{14}C, and glycine, acetate was a far better progenitor of fosfomycin carbon than were pyruvate, citrate, L-glutamate, or L-asparagine. Furthermore, the pattern of labeling shows that only the methyl carbon of acetate was incorporated into fosfomycin.

The distribution of radioactivity was determined on fosfomycin isolated from broths fed radioactive compounds (Table 5). Since 96% of the label from L-methionine-*methyl*-^{14}C was found in carbon 3 of fosfomycin, it is clear that the precursor of this carbon is the *S*-methyl of the amino acid. Glycine also contributed its label to carbon 3 of fosfomycin and probably did so via L-methionine. Eighty percent of the label from carbons 1 and 6 of glucose was found in carbon 1 of the antibiotic. Glucose-2-^{14}C contributed about 80% of its radioactivity to carbon 2 of fosfomycin. The label from acetate-*1,2*-^{14}C and acetate-2-^{14}C was about equally distributed between carbons 1 and 2 of the antibiotic. It was not possible to obtain sufficient labeled antibiotic from the [^{14}C]pyruvate experiments to do degradation studies.

TABLE 5. *Distribution of radioactivity in fosfomycin*[a]

Labeled compound	Percent distribution of radioactivity in fosfomycin[b]			
	C1	C2	C3	C2 + C3
L-Methionine-*methyl*-^{14}C	4	0	96	
Glycine-*U*-^{14}C	8	5	87	
Glucose-2-^{14}C	14	78	8	
Glucose-*1*-^{14}C	80[c]			20[c]
Glucose-*6*-^{14}C	80[c]			20[c]
Acetate-*1,2*-^{14}C	53	42	5	
Acetate-2-^{14}C	49	46	4	

[a] Structure and carbon numbering:



[b] Distribution of label was determined as described in Materials and Methods.

[c] Labeled fosfomycin formed in the presence of glucose-*1*-^{14}C and glucose-*6*-^{14}C was degraded only to the acetate stage. The radioactivity of the acetate was determined and represents the total label in fosfomycin carbons 2 and 3. The radioactivity of fosfomycin carbon 1 was calculated as described in Materials and Methods.

DISCUSSION

Earlier nutritional studies by Jackson and Stapley (14) had shown that certain compounds were required for or stimulated fosfomycin synthesis in *S. fradiae*. Since fosfomycin was the first example of the synthesis of a phosphonate by a prokaryotic organism, it was of interest to study its biosynthesis in more detail. As is the case with many other antibiotics, fosfomycin synthesis was found to occur mainly during the idiophase of growth, and thus it may be classified as a product of secondary metabolism.

Cations have been found to be important to the production of secondary metabolites. Usually, the synthesis of a secondary product is affected by a single key metal ion, and, as illustrated by Weinberg (33), product yield varies linearly with the log of the metal concentration at metal levels which do not affect growth. This relationship was observed for the cobalt stimulation of fosfomycin production (see Fig. 4). Thus, cobalt can be considered the key metal in fosfomycin synthesis. In the list of key metals affecting secondary metabolism compiled by Weinberg (manganese, iron, and zinc), cobalt was not included (33). However, in addition to affecting fosfomycin production, low levels of cobalt have been shown to cause a marked stimulation of gentamicin production at concentrations having no affect on growth (W. Charney, U.S. Patent 3,136,704, 1964), and furthermore, cobalt has been shown to cause a dramatic shift in product yield in the coumermycin A fermentation (4). In both the gentamicin and coumermycin studies, it was concluded that cobalt functions by stimulating or activating a specific enzyme necessary for antibiotic synthesis. The levels of cobalt required for maximal fosfomycin synthesis are 3.6- and 2,000-fold higher than those required for synthesis of gentamicin and coumermycin A_1, respectively. It is of interest that the levels of cobalt which stimulated fosfomycin synthesis maximally are about threefold greater than those required for vitamin B_{12} overproduction by *Pseudomonas denitrificans* even though cobalt makes up an integral part of the vitamin molecule (L. Kaplan, personal communication). The function of the relatively high levels of cobalt in fosfomycin synthesis is unknown. In bacteria (28) and streptomycetes (19), a cobalamin-dependent transmethylase catalyzing the

final step in methionine biosynthesis has been demonstrated. Although methionine is a precursor of fosfomycin (see below), it is unlikely that cobalt stimulates antibiotic synthesis at the level of the cobalamin-dependent methionine synthesizing system, since high levels of methionine did not spare the cobalt requirement for antibiotic synthesis. Several enzymes have been shown to require cobalt or other divalent cations for activation (5, 31). It may be that an enzyme(s) specific to fosfomycin synthesis requires cobalt for its activity or synthesis.

Methionine caused a dramatic stimulation of fosfomycin synthesis while having little effect on growth, indicating a direct role of this amino acid in antibiotic synthesis. Incorporation studies using methionine labeled in various positions showed that the methyl carbon is incorporated exclusively into the methyl carbon of fosfomycin. The methyl donor function of the amino acid in fosfomycin biosynthesis is supported further by the data showing that ethionine inhibits antibiotic synthesis without affecting growth. The biological role of methionine as a methyl donor (via *S*-adenosylmethionine) in the biosynthesis of several secondary metabolites (1, 13) as well as in the production of primary metabolites (24) is well established.

In addition to its precursor role in fosfomycin production, methionine appears to function also in the regulation of antibiotic synthesis. This was concluded from experiments which showed that methionine must be present during the growth phase (trophophase) in order to promote fosfomycin synthesis during the idiophase. This situation is analogous to the stimulatory effect of tryptophan in ergoline alkaloid synthesis in *Claviceps* (7). Tryptophan, a precursor of alkaloid synthesis, is thought to be required during the trophophase to induce an enzyme(s) necessary for product synthesis during idiophase. It may be that methionine plays a similar inductive role in the biosynthesis of fosfomycin. It has been shown also that the methionine stimulation of cephalosporin C synthesis is greatest when the amino acid is added early in the fermentation (7).

Asparagine was a good nitrogen source for growth of *S. fradiae* but a poor precursor of fosfomycin carbon. Glycine, an alternate nitrogen source for growth, did contribute carbon to fosfomycin synthesis. The majority of the label from glycine was found in carbon 3 of fosfomycin. Since the methyl of methionine was found exclusively in fosfomycin carbon 3, glycine may contribute its carbon to the methyl of methionine which is subsequently incorporated into carbon 3 of fosfomycin. Labeling of the methyl group of methionine could occur by a tetrahydrofolate-mediated cleavage of glycine to yield methylene-tetrahydrofolate (15), which could serve as the methyl donor in the vitamin B_{12}-dependent transmethylation of homocysteine to form methionine (19, 24). Such a reaction sequence would result in methyl-labeled methionine which could then be incorporated into carbon 3 of fosfomycin. DL-Serine-*3*-^{14}C showed rather good incorporation into fosfomycin. Although the labeling pattern of the antibiotic synthesized in the presence of DL-serine-*3*-^{14}C was not determined, this carbon atom could also label fosfomycin via the methyl of methionine by the action of serine hydroxymethyltransferase (15), resulting in the formation of [C^{14}]methylene-tetrahydrofolate. The labeled methyl group could then be transferred to homocysteine to form methyl-labeled methionine. Enzymes which participate in the one-carbon transfer pathway from serine to methionine through the folate derivatives have been demonstrated in cell-free extracts of *Streptomyces olivaceus* (19).

The results from radioisotope incorporation studies with glucose, pyruvate, and acetate and the distribution of label in fosfomycin isolated from broths containing the above compounds suggest a glycolytic intermediate as a precursor of fosfomycin carbons 1 and 2. Glucose carbons 1, 2, and 6 were better precursors of fosfomycin than were pyruvate or acetate carbons. Glucose carbons 1 and 6 preferentially label fosfomycin carbon 1, whereas the majority of radioactivity incorporated from glucose-*2*-^{14}C is found in carbon 2 of the antibiotic. It is of particular interest that the same results were obtained in studies of the biosynthesis of 2-aminoethylphosphonate (AEP) by *Tetrahymena pyriformis* (12, 17, 29) and terrestrial mollusks (18). It was proposed that phosphoenolpyruvate (PEP) was the most likely precursor of AEP, the initial biosynthetic step being an intramolecular rearrangement to yield 2-keto-3-phosphonopropionic acid. Subsequent studies with cell-free extracts of *T. pyriformis* (11, 32) supported a reaction sequence involving, first, the rearrangement of PEP, followed by decarboxylation to yield phosphonoacetaldehyde which is then aminated to give AEP.

In both the case of fosfomycin and AEP, carbon 2 of acetate is preferentially incorporated, and the radioactivity is distributed equally between carbons 1 and 2 of the respective products. Furthermore, only carbon 3 of

pyruvate is incorporated into AEP. Although pyruvate was incorporated poorly into fosfomycin, there was a preferential labeling with carbon 3. In the AEP studies (12, 32), it was proposed that the methyl carbons of pyruvate and acetate are returned to the glycolytic pathway via the tricarboxylic acid or glyoxylate cycle and an inosine triphosphate-dependent PEP carboxykinase. A similar situation may account for the labeling pattern of fosfomycin carbons 1 and 2 by acetate carbon 2.

The similarity between the origin of AEP carbons and carbons 1 and 2 of fosfomycin suggests that the antibiotic carbons may be derived also from PEP. It is clear that carbon 3 of fosfomycin is derived from the methyl of methionine. A methionine-mediated C-methylation of a two-carbon unit resulting from PEP rearrangement and decarboxylation could account for the origin of the three fosfomycin carbons. The biosynthesis of several antibiotics involves methionine-mediated C-methylation (1, 13).

The role of glutamate in fosfomycin biosynthesis is not entirely clear. The amino acid caused a stimulation of antibiotic synthesis while having little effect on growth. Omission of glutamate from the basal medium usually decreased antibiotic yield by about 50%. Maximal yield of fosfomycin could be restored in a glutamate-free medium when one of several tricarboxylic acid cycle intermediates were included. This suggested that glutamate and the dicarboxylic acids can be interconverted. As stated above, the most reasonable interpretation of the acetate and glucose incorporation data is that carbon flows through the tricarboxylic acid or glyoxylate cycles to oxalacetate. The oxalacetate is converted to PEP which, after rearrangement, is decarboxylated to yield a two-carbon intermediate (each carbon with equal distribution of label from acetate-2-^{14}C) for fosfomycin synthesis. It would be expected, therefore, that the addition of labeled glutamate to the fermentation should lead to incorporation of radioactivity into the antibiotic. However, no significant labeling of fosfomycin by L-glutamate-*U*-^{14}C was observed. Thus, exogenous glutamate is not easily converted to tricarboxylic acid or glyoxylate cycle intermediates in *S. fradiae*. The reverse is true in *Tetrahymena*, however, where [^{14}C]glutamate does enter the cycle and does contribute radioactivity to AEP (12). It is possible that exogenous supplied L-glutamate or dicarboxylic acid do not enter the tricarboxylic acid cycle pool but are compartmentalized, thus acting as positive effectors for fosfomycin synthesis without entering the antibiotic precursor pool (27). Further work is necessary in order to elucidate the role of L-glutamate in antibiotic synthesis.

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