GLUCOSE AND MALTOSE TRANSPORT IN BACILLUS POPILLIAE

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ABSTRACT

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By

Daniel Clifford Taylor

Glucose was found to be actively transported by Bacillus popilliae, and the data indicate that the transport is via a phosphoenolypyruvate (PEP) phosphotransferase system. Uptake of [^{14}C] glucose and [^{14}C] methyl α-D-glucoside (αMG) was inhibited by fluoride, and oxidation of glucose was neglibible in the presence of fluoride or azide. Considerable phosphorylation of glucose by toluenized cells occurred with either PEP or ATP as the phosphoryl group donor, but phosphorylation of α MG was largely dependent on PEP. An ATP dependent hexokinase was demonstrated in crude cell extracts, using glucose, fructose, or mannose as substrates. Saturation studies indicated that maltose was diffusing across the bacterial membrane. The uptake of ^{14}C maltose was not inhibited by energy poisons. A constitutive maltose cleaving enzyme was active in crude extracts of B. popilliae.

GLUCOSE AND MALTOSE TRANSPORT IN *BACILLUS POPILLIAE*

By

Daniel Clifford Taylor

A THESIS

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DEDICATION

To my parents, Dr. and Mrs. Clifford B. Taylor, for all their help, love and trust during the past three years.

To Vicki Chessin and Bob and Kay Zahorchak for their warmth and kindness which never allowed me to despair when times were rough.

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I wish to express my deepest appreciation and respect to Dr. R.N. Costilow for his guidance and encouragement during this investigation and his patience throughout the preparation of this thesis.

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INTRODUCTION

The larvae of the Japanese beetle, Popillia japonica Newn., and the European chafer, Amphimallon majalis (Razoum.) are susceptible to milky disease. A massive accumulation of refractile endospores imparts a chalky color to infected larvae. Dutky (11) was the first to demonstrate the causal relationship between the disease and two closely related, gram positive, spore-forming bacilli. Bacillus popilliae, the causitive agent of Type A milky disease, is characterized by the formation of a phase bright parasporal body along with a heat resistant endospore. The parasporal body is a bipyrimidal crystalline structure, and is composed mainly of protein, although some carbohydrate is present (6). Type B milky disease, which differs from the Type A disease in that larvae turn chocolate brown due to extensive formation of blood clots, is caused by Bacillus lentimorbus. Although both organisms are similar in appearance during vegetative stages, B. lentimorbus does not form a parasporal crystal during sporulation.

Many attempts have been made to develop media and other appropriate conditions which would support growth and eventual sporulation of either species, with the hope of mass producing spores and using them as an alternative to chemical insecticides. In vitro sporulation of B. popilliae, albeit in low frequency, was first reported by Steinkraus and Tashiro (41). Later investigators isolated strains of B. popilliae which would sporulate to a low but significant extent in artificial media (31, 36). However, sporulation was inconsistent and in vitro spores were not capable of producing infection via ingestion in test larvae.

In an attempt to compare sporulation of B. popilliae to sporulation in less fastidious Bacillus species, various physiological processes known to be associated with endospore formation were investigated in B. popilliae. Much of this work has been done by Costilow and co-workers (4, 7, 8, 9, 27, 28, 29, 30, 42). Recent studies by Bhumiratama et al. (3) demonstrated that trehalose, which is apparently the natural carbon and energy source for B. popilliae, is transported via a phosphoenolpyruvate dependent phosphotransferase system. Strains of B. popilliae were found to contain phosphotrehalase, and a mutant was isolated that could not grow on trehalose but accumulated trehalose 6-phosphate intracellularly. This report prompted the present investigation into the mode of transport of two other sugars which support the growth of B. popilliae, glucose and maltose.

LITERATURE REVIEW

Bacterial cells transport sugars by several methods. Members of the same genus may transport a specific sugar by facilitated diffusion, active transport, or group translocation, whereas one cell type may transport a variety of sugars by all three processes (34). For example, Escherichia coli and Salmonella typhimurium transport sugars by all three methods, while Staphylococcus aureus appears to transport most sugars by group translocation (34). In addition, E. coli transports galactose by four separate permease systems (34). Many investigations have centered upon group translocation since its discovery in 1964 (22).

Kundig, Ghosh, and Roseman (22) first detected the phosphotransferase system when searching for a kinase in bacterial extracts that catalyzed the phosphorylation of N-acyl-D-mannosamine, which is a step in the biosynthesis of the sialic acids normally occurring in animal tissues. This transport system is unique in that it requires several protein fractions and uses phosphoenolpyruvate (PEP) as the phosphoryl group donor rather than a nucleotide triphosphate. Extensive work with the PEP dependent phosphotransferase system (PEP-PTS) of E. coli and S. aureus has resulted in the characterization of the components in each species (1, 18, 23, 24, 29, 37, 38).

Two proteins, Enzyme I and HPr, appear to be constitutively synthesized members of the PEP-PTS of E. coli, S. aureus, and S. typhimurium (34). Both are soluble proteins and lack sugar specificity. HPr has been purified to homogenity from each of the above organisms. Enzyme I catalyzes the transfer of the phosphoryl group from PEP to HPr. Studies with purified Enzyme I from

S. typhimurium indicate that the phosphoryl group is first attached to a histidinyl residue in the protein; magnesium ion is required for this reaction. In the second step, demonstrated with PEP-PTS components from E. coli, the phosphoryl group is transferred from Enzyme I to the N-1 position of the imidazole ring of a histidinyl residue in HPr. This reaction does not require magnesium ion.

Although most sugar specific proteins are inducible, there are two constitutive systems in E. coli catalyzing the transfer of the phosphoryl group from P-HPr to carbohydrate (34). If E. coli is grown on a salts medium with glucose as the sole carbon source, the membrane fraction is capable of transferring the phosphoryl group from P-HPr to glucose, mannose, and fructose. Extraction of the membrane with a combination of urea and n-butanol gives a soluble protein fraction, designated II-A, and an insoluble fraction. II-A can be further purified into three proteins, each specific for one of the sugars. A protein labeled II-B is obtained from the pellet by fractionation in the presence of deoxycholate, which also removes contaminating lipid. A membrane lipid, phosphatidylglycerol, is an essential component of the Enzyme II system. Using P-HPr, this system is able to phosphorylate a specific sugar (depending upon which II-A is used) if the components of the system are mixed in the following sequence: II-B, Mg^{2+}, phosphatidylglycerol, II-A (24).

The second system found to transfer the phosphoryl group from P-HPr also involves two sugar-specific proteins (34). One is found in the soluble fraction of crude extracts of E. coli, while the other is in the membrane fraction. The soluble protein has been designated Factor III. Factor III is tightly associated with a phosphatase that is inhibited by fluoride.

Fractionation experiments and studies with sulfhydryl reagents indicate that the membrane protein is different from II-B. In contrast to the first system, this one actively phosphorylates the galactoside analog methyl β-thiogalactoside.

S. aureus contains a membrane bound Enzyme II and a soluble Factor III which react with P-HPr to transfer the phosphoryl group to lactose. Factor III is capable of accepting three phosphoryl groups from P-HPr, and chromatographic studies of the phosphohistidine obtained from an alkaline hydrolysate of the phospho-Factor III indicate that the phosphoryl groups are attached to the N-3 position of the imidazole ring of histidine. Enzyme II-B is required for the transfer of the phosphoryl group from P-III to lactose.

Saier et al. (35) demonstrated a PEP-dependent system for the phosphorylation of fructose in two species of purple non-sulfur bacteria. The enzyme system differs from that found in E. coli or S. aureus in that only two protein fractions are necessary for the reaction. In Rhodospirillium rubrum, phosphotransferase activity was found in the particulate fraction of crude extracts. Extracting the pellet with water removed a component which was required in combination with the pellet to transfer the phosphoryl group from PEP to fructose. Only two protein fractions were found essential for the phosphorylation of fructose in crude extracts of Rhodopseudomonas spheroides. In this case, one fraction was associated with the membrane and the other was found in the soluble fraction after centrifugation.

PEP-dependent phosphotransferase activity has been demonstrated in many other bacterial species. Romano et al. (32) assayed a wide variety of bacteria for phosphotransferase activity. After studying representatives of

genera that were either strict aerobes or fermentative species, they concluded that PEP-dependent phosphotransferase activity was limited to those species capable of anaerobic metabolism. Activity was found in species of Bacillus, Cornynebacterium, Escherichia, Salmonella, and Staphylococcus, and was absent in species of Arthobacter, Azotobacter, Micrococcus, Mycobacterium, and Pseudomonas. It has been shown that Pseudomonas aeruginosa actively transports glucose intact, or alternatively, oxidizes it to gluconate by a membrane bound glucose dehydrogenase and transports gluconate inside the cell (17). Eisenberg et al. (13) reported essentially the same mechanism for transport of glucose by Pseudomonas fluorescens.

In addition to the examples mentioned above, PEP-PTS activity has been found in a variety of mesophilic bacilli (21), a strain of a thermophilic Bacillus (21), in all lactic streptococci transporting lactose (25), in Aerobacter aerogenes with respect to mannitol (43), and in Vibrio cholerae growing on galactose or fructose (2). Cirillo and Razin (5) demonstrated that 10 strains of Mycoplasma transported methyl α-D-glucoside by group translocation. This could not be demonstrated in non-fermentative strains.

Romano et al. (32) noted that although phosphotransferase activity is present in B. subtilis and Achromobacter parvulus, most strains are strict aerobes. Both species, he stated, belong to genera that are predominantly facultative anaerobes. However, there have been other reports which directly contradict Romano's hypothesis. Cirillo and Razin (5) could find no phosphotransferase activity for methyl α-D-glucoside in strains of Acholeplasma, a species closely related to Mycoplasma, which metabolizes carbohydrates by fermentation. And Sobel and Krulwich (40) reported that the strict aerobe,

Arthrobacter pyrindinolis, accumulates fructose via an inducible PEP-dependent phosphotransferase system. They were able to separate the transport system into membrane-associated and soluble components. Both fractions were required for the phosphorylation of fructose with PEP. Some mutants of A. pyrindonolis, having a slower growth rate with fructose as substrate than the parent, were shown to have reduced levels of phosphotransferase activity.

It is possible that other strictly aerobic species will be found to possess a phosphotransferase system. However, it is those species which possess the capacity to anaerobically ferment sugars which benefit most from such a system. As Roseman (34) points out, the presence of a phosphotransferase system in facultative organisms enables them to conserve metabolic energy. It takes one molecule of ATP or its equivalent to transport a molecule of sugar against a concentration gradient. In organisms where sugars are oxidized to completion, the expenditure of one ATP to bring the sugar into the cell is not particularly wasteful in view of the large amount of ATP produced. However, under anaerobic conditions, the combined processes of transport and glycolysis result in the production of only one ATP if the result of transport is the non-phosphorylated sugar. If the sugar is phosphorylated during its transport, the subsequent fermentation yields two molecules of ATP. The doubling of ATP production in organisms possessing a phosphotransferase system gives them a tremendous selective advantage. The widespread occurrence of group translocation testifies to such a selective pressure and the logic of microbial economy.

E. coli actively transports the disaccharide maltose, although not by group translocation. Accumulation of maltose was shown to be dependent on

the generation of energy, as sodium azide (0.01 M) inhibited the initial rate of [^{14}C] maltose uptake by 70% (47). Maltose permease activity, as well as the enzymatic activities concerned with the metabolism of maltose, are inducible (47). Receptors for the bacteriophage lambda are also induced by maltose (19).

Once inside cells of E. coli, maltose is cleaved by amylomaltase into glucose and a polymer consisting of α-1,4-glucosidic units (46). In contrast to E. coli, enzyme preparations of Neiserria menengitidis catalyze the direct phosphorolysis of maltose to glucose and β-D-glucose 1-phosphate (14). This is analogous to the phosphorolytic cleavage of sucrose to fructose and glucose-1-phosphate by enzyme preparations from Pseudomonas saccharophila (10). The sucrose phosphorylase of P. saccharophila does not phosphorolyze maltose.

Wiesmeyer and Cohn (46) investigated the mechanism of purified amylomaltase, and demonstrated that glucose from the nonreducing end of maltose is attached to the nonreducing end of the polymer. This reaction is reversible, as shown by Doudoroff et al. (11). They added glucose to bacterial preparations which had synthesized polysaccharide, causing a rapid transformation of the polysaccharide to a shortened form which no longer gave a blue-colored complex with iodine.

If glucose is allowed to accumulate during the breakdown of maltose by amylomaltase, the dextran formed consists of from 4 to 6 glucose units (11). Since amylomaltase activity is reversible, longer polysaccharides can only form in the absence of glucose. Once glucose becomes limiting, the dextran is phosphorolyzed by maltodextrin phosphorylase, producing glucose 1-phosphate. Doudoroff et al. (11) demonstrated the reversibility of this activity by

adding glucose and glucose-1-phosphate to dry bacterial preparations. What was observed was the production of maltose and reducing dextrins.

Doudoroff et al. (11) has proposed that enzymes with catalytic properties similar to amylomaltase be referred to as "transglycosidases". These would include the sucrose phosphorylase of Pseudomonas saccharophila and the dextran- and levan-forming enzymes of certain bacteria. Doudoroff suggested that this type of enzyme plays an important part in the catabolism and synthesis of various carbohydrates.

MATERIALS AND METHODS

Organisms and growth conditions. B. popilliae NRRL B-2309MC was used in all experiments. B. popilliae 2309MC is an asporogenic mutant derived from strain NRRL B-2309M after cultivation in broth for several months (27). It is characterized by a faster growth rate than 2309M. This strain is typical of B. popilliae since (1) it is an aerobic, non-motile rod, (2) vegetative cells are catalase negative, and (3) broth cultures demonstrate a characteristic birefringence. The basal medium used contained 1.5% trypticase, 0.5% yeast extract, and 0.6% K_2HPO_4 (TY). Carbohydrates were filter sterilized separately and added to autoclaved basal media. Media were dispensed in 50 or 200 ml amounts in 125- or 500-ml Erlenmeyer flasks, respectively. Cultures for experiments were started with a 5% inoculum from starter cultures which had reached late exponential or early stationary phase, and incubated at 30 C on a rotary shaker. Stock cultures were maintained on 2% agar slants of TY plus 0.2% glucose or 0.2% trehalose, and transferred every two weeks.

Oxygen uptake experiments. B. popilliae was grown to mid- or late-exponential phase in TY plus 0.2% glucose. Cells were harvested by centrifugation at 12,000 rpm for 15 min., washed twice with 0.05 M phosphate buffer, pH 7.2, and resuspended in the same buffer. Oxygen uptake was measured in a Warburg apparatus, equilibrated at 30 C, by standard manometric techniques (44).

Enzyme assays. All assays were performed at room temperature unless otherwise specified. Hexokinase activity was assayed spectrophotometrically

by coupling the reaction to glucose 6-phosphate dehydrogenase and following the reduction of nicotinamide adenine dinucleotide phosphate ($NADP^+$). Each reaction mixture (0.5 ml) contained 30 µmoles glycylglycine buffer (pH 7.5), 3 µmoles $MgCl_2$, 0.3 µmoles $NADP^+$, 2 µmoles ATP, 15 µmoles carbohydrate, a non-rate limiting amount of glucose 6-phosphate dehydrogenase, crude cell extract, and distilled water. Reduction of $NADP^+$ was monitored by following the increase in absorbancy at 340 nm with a Gilford model 2000 spectrophotometer. One unit of enzyme was defined as the amount that reduced 1 µmole of $NADP^+$ per min. The molar absorbance for reduced $NADP^+$ is $6.2 \times 10^3 M^{-1} cm^{-1}$.

Phosphoenolpyruvate (PEP) dependent phosphotransferase activities were measured by a combination of the methods of Lee et al. (25) and Romano et al. (32). Cells were grown in TY plus 0.2% glucose to late exponential phase, harvested (23,300 x g for 15 min.), washed twice with 0.05 M phosphate buffer, pH 7.2, and either frozen overnight or used the same day. The cells were suspended in 40 ml of the wash buffer; 2 ml of a 1:9 (vol/vol) mixture of toluene-acetone was added, and the suspension was shaken vigorously for 5 min. The cells were centrifuged (27,000 x g for 20 min.) and resuspended in a small volume of buffer. The reaction mixture (5 ml) consisted of 1 ml toluene treated cell suspension, [^{14}C] carbohydrate, 3 mM $MgCl_2$, 0.03 M KF, 0.015 M phosphate buffer (pH 7.2), distilled water, and either 1.5 mM PEP or 1.5 mM ATP. A third reaction contained neither ATP or PEP and served as a control. Reaction mixtures were incubated at 30-31 C in a shaking water bath for 60 min. After centrifugation, (27,000 x g for 20 min.), 0.5 ml of the supernatant liquid was layered on a column (5.0 x 1.1 cm) of Dowex AG 1-x4 (100-200 mesh) in the formate form. The column was rinsed with 5 ml

distilled water, followed by 5 ml 0.5 M ammonium formate in 0.2 M formic acid; 0.5 ml of each wash was counted in handifluor or PCS liquid scintillation counting fluor. Phosphotransferase activity was expressed as percent carbohydrate phosphorylated.

Uptake of [^{14}C] carbohydrates. In this paper, all uptakes refer to the amount of radioactivity accumulated in cells; this includes both the sugar and any products produced by metabolism of the sugar. B. popilliae was grown to mid- or late-exponential phase in TY plus 0.2% glucose, harvested by centrifugation (23,300 x g for 15 min.), washed twice in 0.05 M phosphate buffer, pH 7.2, and resuspended in the same buffer. Reaction mixtures (6.0 ml) contained approximately 3.0 mg (dry weight) cells, [^{14}C] carbohydrate, and buffer. The reaction mixtures were incubated at 30-31 C in a shaking water bath; reactions were started by the addition of carbohydrate. At various intervals, 1 ml samples were filtered (0.45 μm pore size, Millipore Corp.) and washed with 5 ml ice cold buffer. Filters were baked in a hot air oven until completely dry, then counted in 10 ml toluene based scintillation fluid. Where indicated, variations of this procedure were used to determine transport kinetics, competition by analogs, and inhibition by energy poisons.

Purification of maltose and maltase assay. Our lot of maltose (Nutritional Biochemicals Corporation) contained approximately 0.9% glucose contamination as estimated by the spectrophotometric assay for hexokinase activity. A small quantity of this maltose was boiled in 95% ethanol and filtered on Whatmann #1 filter paper. The filter was washed several times with hot 95% ethanol, the crystals were scraped off the filter, and the procedure was repeated three more times. Glucose contamination was reduced to 0.18%. Maltase

activity was assayed using the hexokinase assay. A non-rate limiting amount of commercial hexokinase (yeast) was added to the reaction mixture to enzymatically remove the remaining glucose before cell extract was added.

Other procedures. The dry weight of cell suspensions were estimated by measuring the OD at 620 nm. One OD unit equalled approximately 0.45 mg dry cell weight per ml. Cells used to prepare cell extracts were grown to mid- or late-exponential phase in TY plus 0.2% glucose, harvested by centrifugation (23,300 x g for 15 min.), and washed twice with 0.05 M phosphate buffer, pH 7.2. The pellet was resuspended in an equal volume of 0.01 M tris (hydroxymethyl) aminomethane (Tris)-hydrochloride buffer (pH 7.2), containing 1 mM dithiothreitol. Cells were disrupted by ultrasonic oscillation for four to eight 15-sec. intervals at a peak to peak amplitude to approximately 7 μm; the resulting crude extract was used to enzyme assays. Protein was determined by the method of Lowry et al. (26), using bovine serum albumin, fraction V, as standard.

Reducing sugars were measured using the glucostat reagent (Worthington Biochemical Corp.), Method I-A, according to directions outlined by the manufacturers. The total volume of the assay was reduced to 2 ml. This assay is sensitive to glucose concentrations as low as 5 μg per ml. Glucose was used as a standard, and was measured between 5 and 40 μg per ml. The reaction mixture (2.0 ml) contained 0.015 M phosphate buffer (pH 7.2), approximately 2000 μg maltose, and 0.2 ml crude cell extract. Samples (0.1 ml) taken at different time intervals were diluted in 1.9 ml H_2O and immersed in boiling water to stop the reaction, and 0.2 ml of the dilution was used in the sugar assay.

Radioactivity was measured using a Packard Tri-Carb Liquid Scintillation Spectrophotometer (model 3320). Scintillation fluid consisted of 0.01 g 1,4-bis-2-(5-phenyloxazole)-benzene and 6 g 2,5-diphenyloxazole in 1 liter of toluene. Radioactive liquid samples were counted in handifluor (Mallinckrodt Chemical Works) or PCS solubilizer (Amersham/Searle).

RESULTS

Glucose transport. Enolase, the enzyme which catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate, is inhibited by low concentrations of fluoride (20). When cells of B. popilliae were incubated in the presence of 0.01 M NaF, [^{14}C] glucose uptake was completely inhibited (Fig. 1). Previous data indicated that 0.01 M fluoride inhibited glucose oxidation approximately 50%, and that 0.1 M fluoride inhibited completely (29). The inhibition of glucose oxidation by the cells was rechecked at different glucose concentrations. When the glucose level was 2.5×10^{-3} M or higher, 0.01 M NaF only inhibited by about 50%, but complete inhibition of oxygen uptake was observed when 5×10^{-4} M glucose was used.

Since these findings indicate that glucose is transported by group translocation, assays for phosphotransferase activity were conducted. Trehalose was the first substrate used in the assay, and served as a positive control, since it has been shown that trehalose is transported via a phosphotransferase system (3). In addition to glucose, α-methyl D-glucoside (α MG), which is not readily metabolized by glucose grown B. popilliae cells (4), was also tested. It will be demonstrated later that αMG appears to function as a glucose analog in uptake studies. The results clearly demonstrated the dependence on PEP for the production of phosphorylated sugar from trehalose (Table 1). In contrast, glucose was phosphorylated to about the same extent when either PEP or ATP was present. However, two assays conducted with αMG as substrate indicated that its phosphorylation was largely PEP-dependent.

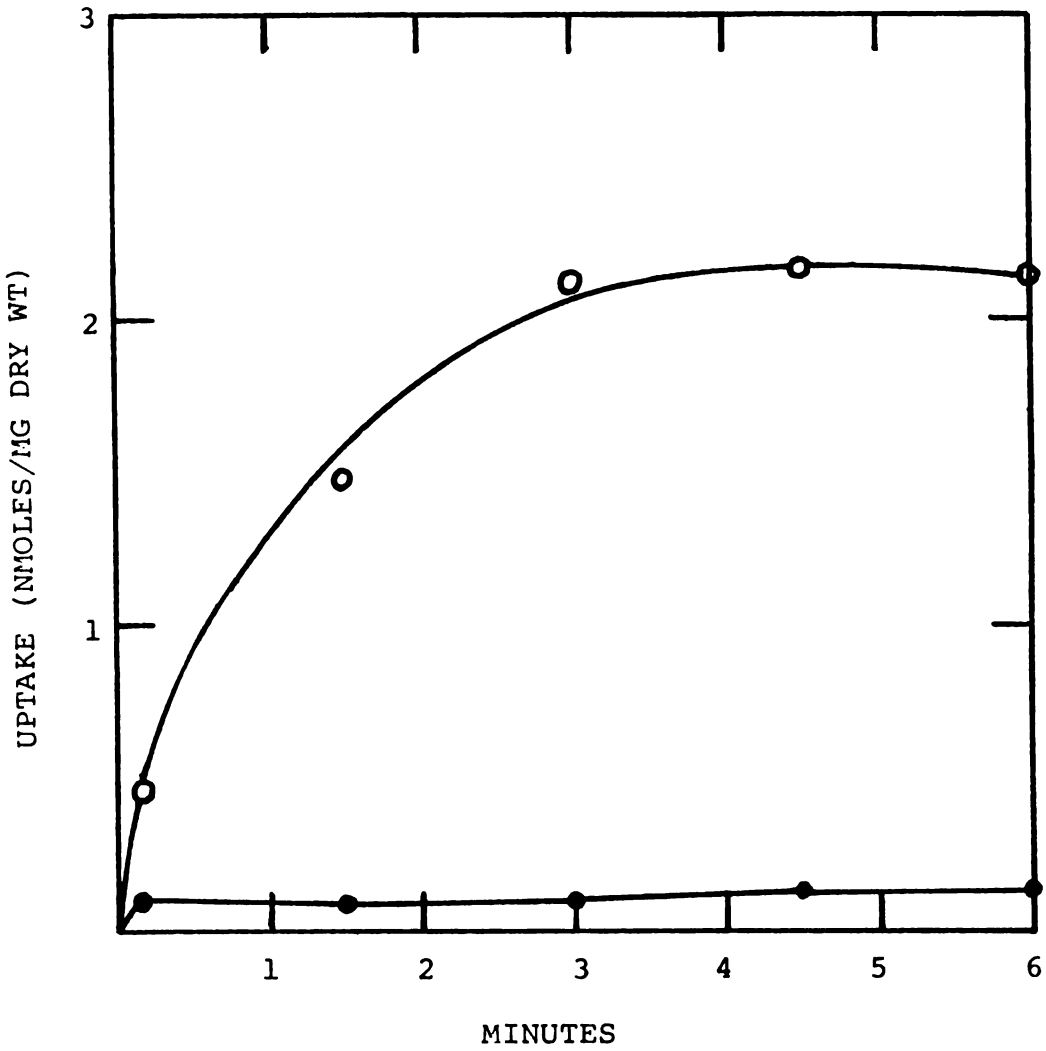


Fig. 1.--Sodium fluoride (0.01 M) inhibition of [^{14}C] glucose (10^{-5}M, 1 μCi/μmole) uptake by B. popilliae. Symbols: O, without NaF; ●, with NaF.

Table 1.—Phosphotransferase activities of *B. popilliae* cells[a].

Dry weight (mg)	Substrate	%Phosphorylation with: ATP	PEP
149	Trehalose[b]	0.2	17.0
109	Glucose[c]	34.3	29.0
81	Methyl α-D-glucoside[d]	3.3	12.5
158	Methyl α-D-glucoside[d]	0.1	4.8

[a]The extent of substrate phosphorylation was determined by the chromatographic procedure of Romano et al (33). Controls were run without ATP or PEP, and the appropriate corrections made.

[b]0.1 M [^{14}C] trehalose (0.02 μCi/μmole)

[c]0.1 M [^{14}C] glucose (0.02 μCi/μmole)

[d]0.1 M [^{14}C] methyl α-D-glucoside (0.1 μCi/μmole)

The generation of a phosphorylated glucose derivative by B. popilliae utilizing ATP as the sole phosphoryl group donor may be due largely to a hexokinase present in the cells since crude cell extracts demonstrated high kinase activities (Table 2). No production of glucose 6-phosphate could be detected when PEP was substituted for ATP in the spectrophotometric assay. Probably the concentration of extract in the reaction was too low for PEP-PTS activity to be detected (16). Fructose and mannose functioned as substrates for the kinase assay, which is indicative of hexokinase, rather than glucokinase, activity. Neither the mannose or fructose were checked for glucose contamination. However, glucose grown B. popilliae oxidizes mannose as rapidly as glucose (4), suggesting that a relatively non-specific hexokinase is involved in the metabolism of both sugars.

To further investigate the transport of glucose, we examined various non-metabolizable analogs of glucose for one which could be accumulated as rapidly as glucose. Romano et al. (32) successfully used 2-deoxy-D-glucose in their survey of phosphotransferase activity in various bacteria, but we discovered that B. popilliae transported this analog very slowly. Uptake of N-acetyl-D-mannosamine could be detected only at very high concentrations. Methyl α-D-glucoside is frequently used as a glucose analog (33), and was found suitable for our studies. This compound was taken up rapidly by cells, and its uptake was inhibited by 0.01 M NaF (Fig. 2). Growth studies with B. popilliae in TY media plus 0.2% αMG indicated that this culture would not metabolize the glucoside, and previous studies (4) demonstrated that cells of this strain could only use it after a fairly long period of induction (4 to 5 hr. lag). The uptake of [^{14}C] glucose (10^{-5} M) was negligible in the presence of 5 x 10^{-4} M

Table 2.—Hexokinase activities in extracts of *B. popilliae* cells[a].

Substrate	TYG grown cells[b]		TYM grown cells[c]	
	ATP	No ATP	ATP	No ATP
Glucose	0.0905	0	0.1037	0
Fructose	0.0851	0	0.0836	0
Mannose	0.0501	0	0.0309	0

[a]Assays were based on the reduction of $NADP^+$ by glucose 6-phosphate dehydrogenase and were conducted as described in Materials and Methods. Enzyme activities are expressed as units of enzyme per mg protein.

[b]TY medium plus 0.2% glucose.

[c]TY medium plus 0.2% maltose plus 0.05% glucose.

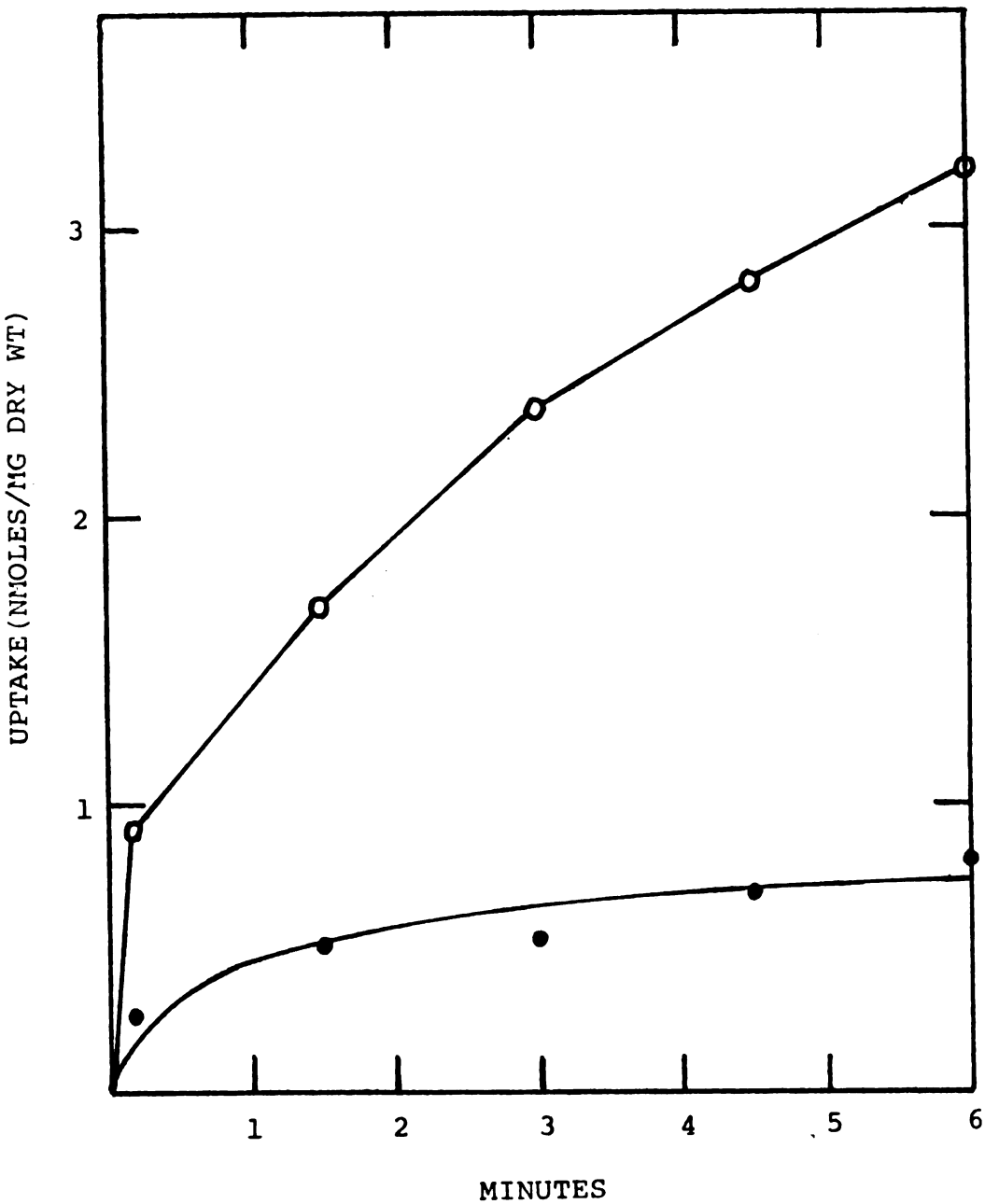


Fig. 2.--Sodium fluoride (0.01 M) inhibition of [^{14}C] methyl α-D-glucoside (10^{-5}M, 1 μCi/μmole) uptake by B. popilliae. Symbols: O, without NaF; ●, with NaF.

glucoside (Fig. 3), and the uptake of [^{14}C] glucoside (10^{-4}M) was lowered in the presence of 10^{-3} M glucose (Fig. 4). Unfortunately, neither the concentrations or ratios were the same in the two experiments and this may account for the difference in the results obtained. However, the data do indicate that the two sugars may share a single uptake system.

Since methyl α-D-glucoside was not immediately metabolized, we were able to study the kinetics of its transport. Initial rates of transport over a range of concentrations were estimated after 20 sec. of incubation with [^{14}C] glucoside (Fig. 5). The transport system appeared to become saturated at 6×10^{-5} M. The apparent K_m value from a Lineweaver-Burk plot (Fig. 6) was 6.7×10^{-6} M. A similar value was obtained with glucose, but a determination of the K_m was not attempted due to the rapid metabolism of glucose once inside the cell.

Studies with maltose. Maltose is taken up by B. popilliae cells at a slower rate than glucose. At 10^{-5} M, an average of 142 picomoles of maltose was taken up per min per mg dry cell weight, while 350 picomoles of glucose was accumulated under identical conditions. Maltose uptake was not affected by 0.01 M NaF or 0.01 M NaN_3 (Fig. 7). However, oxidation of maltose was inhibited by 0.1 M concentrations of fluoride and azide (Fig. 8).

The kinetics of maltose uptake by B. popilliae cells were completely different from those observed with glucose and αMG. Initial rates of transport were calculated after allowing cells to incubate with [^{14}C] maltose for 15 sec. As shown in Fig. 9, intracellular accumulation of maltose increased as the extracellular concentration increased and did not reach a limit even at 10^{-3} M. These data indicate that maltose is diffusing, either mediatively or passively, through the membrane.

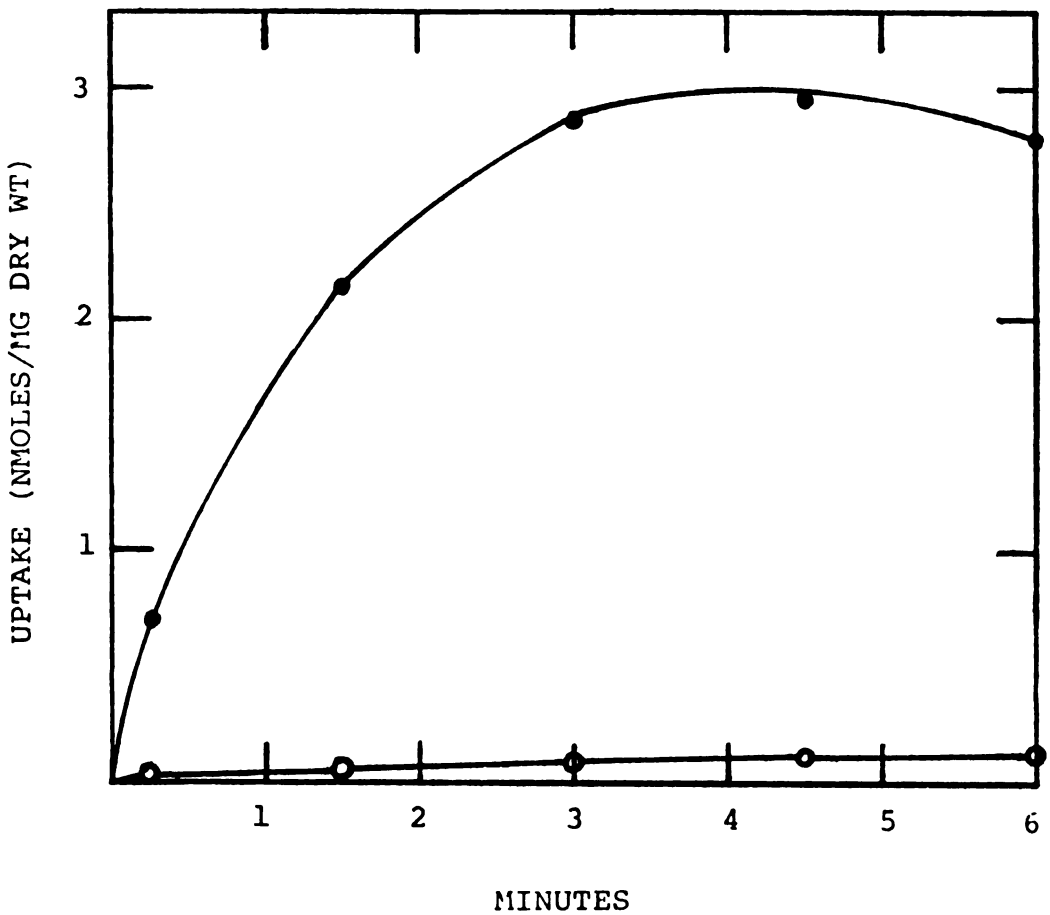


Fig. 3.--Methyl α-D-glucoside (5×10^{-4}M) inhibition of [^{14}C] glucose (10^{-5}M, 1 μCi/μmole) uptake by B. popilliae. Symbols: ●, without αMG; O, with αMG.

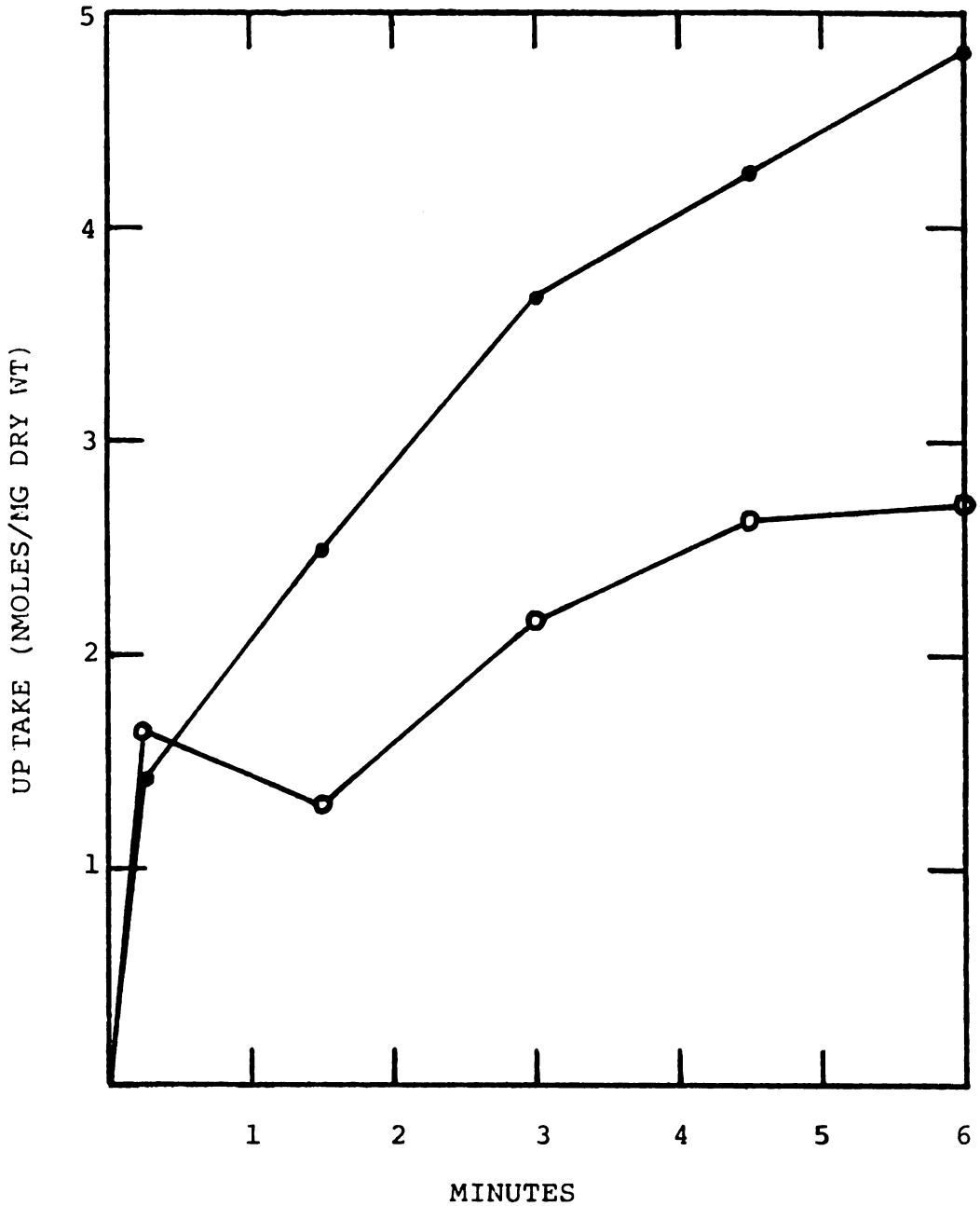


Fig. 4.--Glucose (10^{-3}M) inhibition of [^{14}C] methyl α-D-glucoside (10^{-4}M, 0.5 μCi/μmole) uptake by B. popilliae. Symbols: ●, without glucose; O, with glucose.

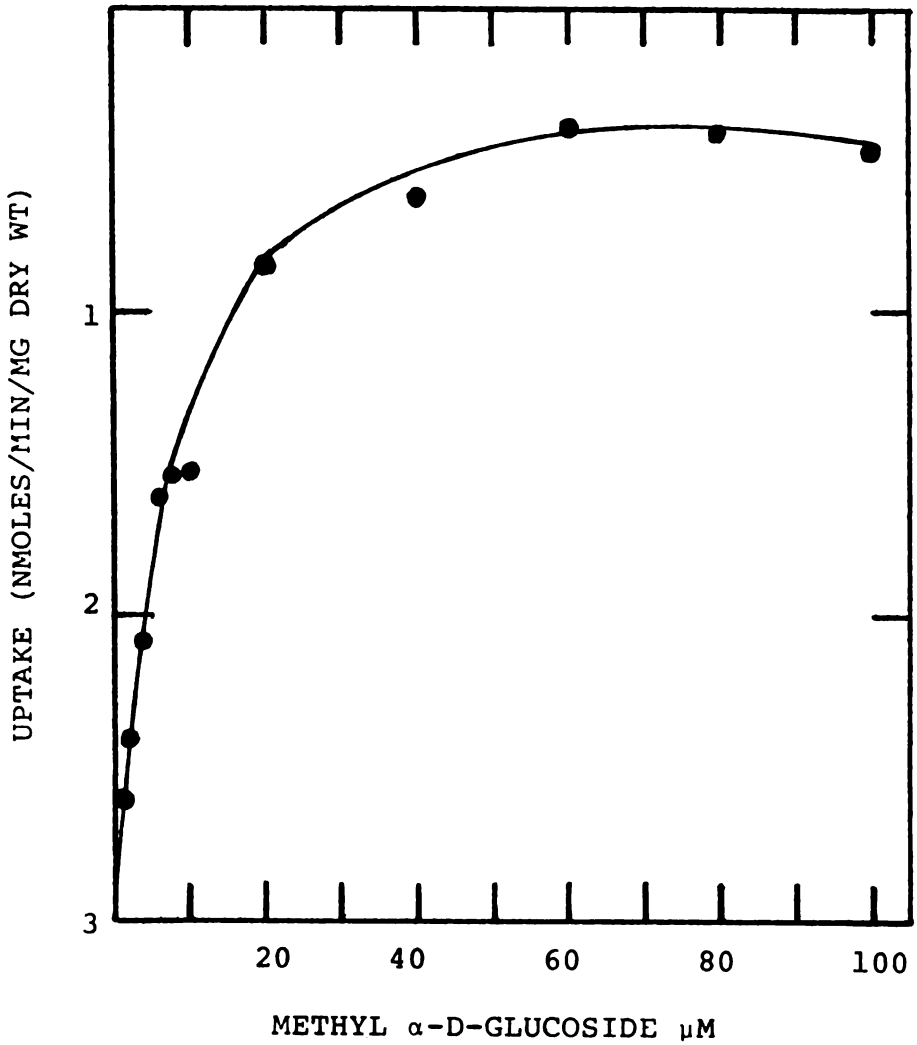


Fig. 5. Kinetics of [^{14}C] methyl α-D-glucoside (0.5 μCi/μmole) transport. Initial rates of transport were estimated after incubation (30 C) for 20 sec. Each reaction mixture contained 0.49 mg (dry weight) cells. Reactions were terminated by the addition of 5 ml ice cold phosphate buffer (0.05 M, pH 7.2).

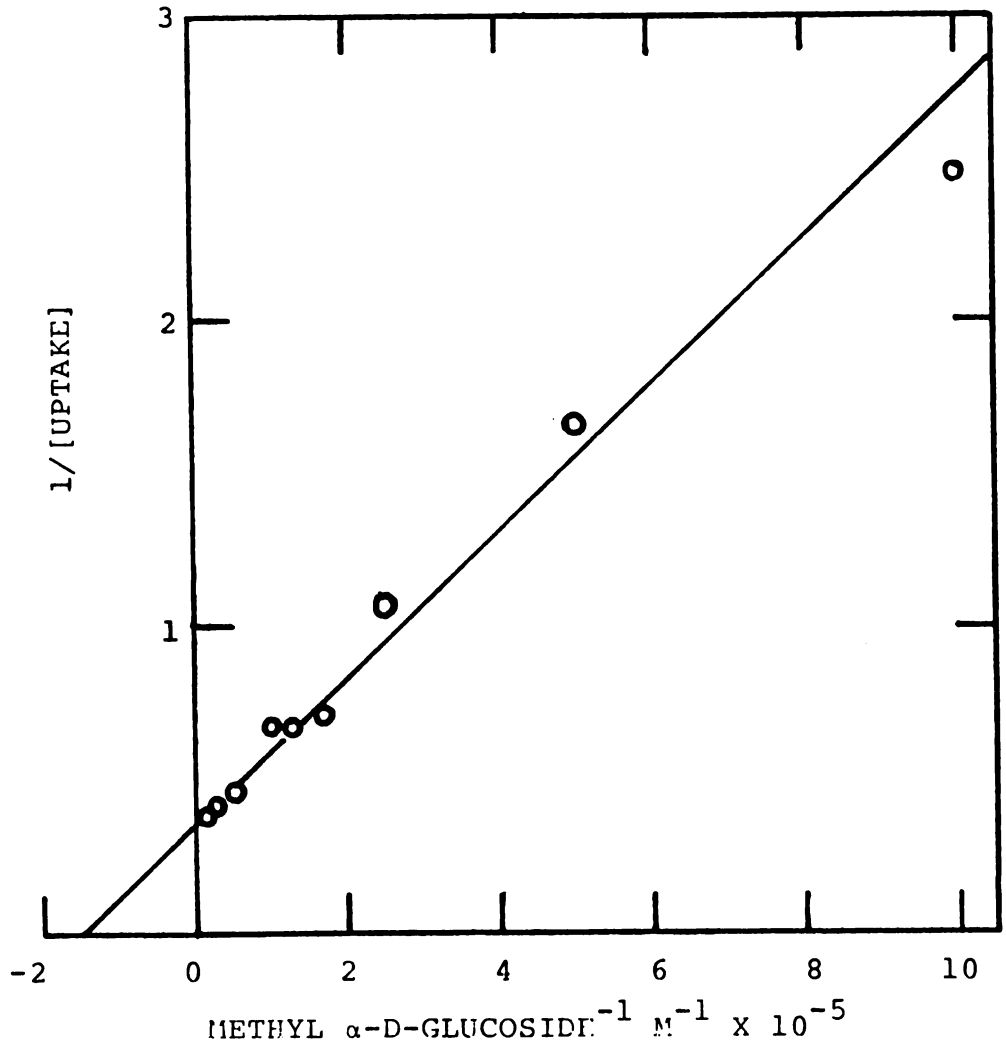


Fig. 6.--Lineweaver-Burk plot of kinetic data from Fig. 5.

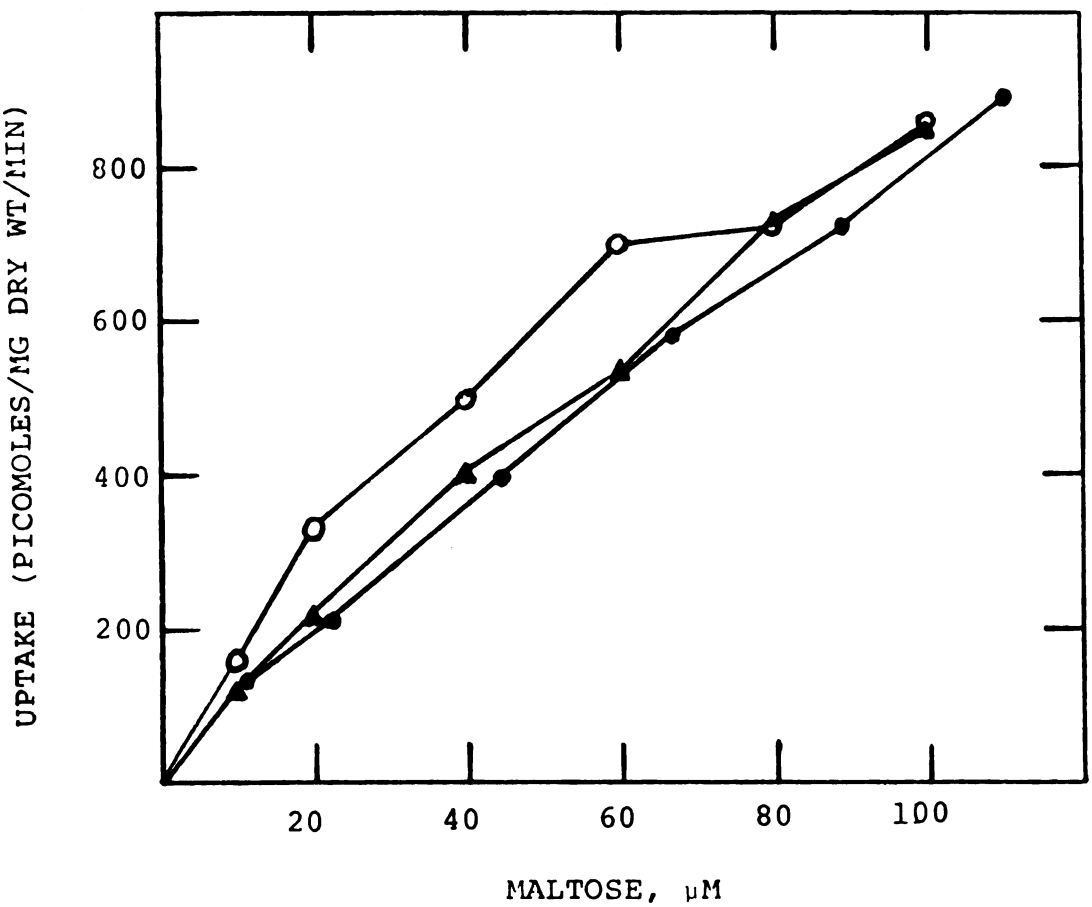


Fig. 7. Effects of inhibitors on [^{14}C] maltose (1 μCi/μmole) uptake. Uptake was determined after 1 min of incubation at 30 C. Each reaction mixture contained 0.5 mg (dry weight) cells. Symbols: ●, without inhibitor; ○, with 0.01 M NaF; ▲, with 0.01 M NaN_3.

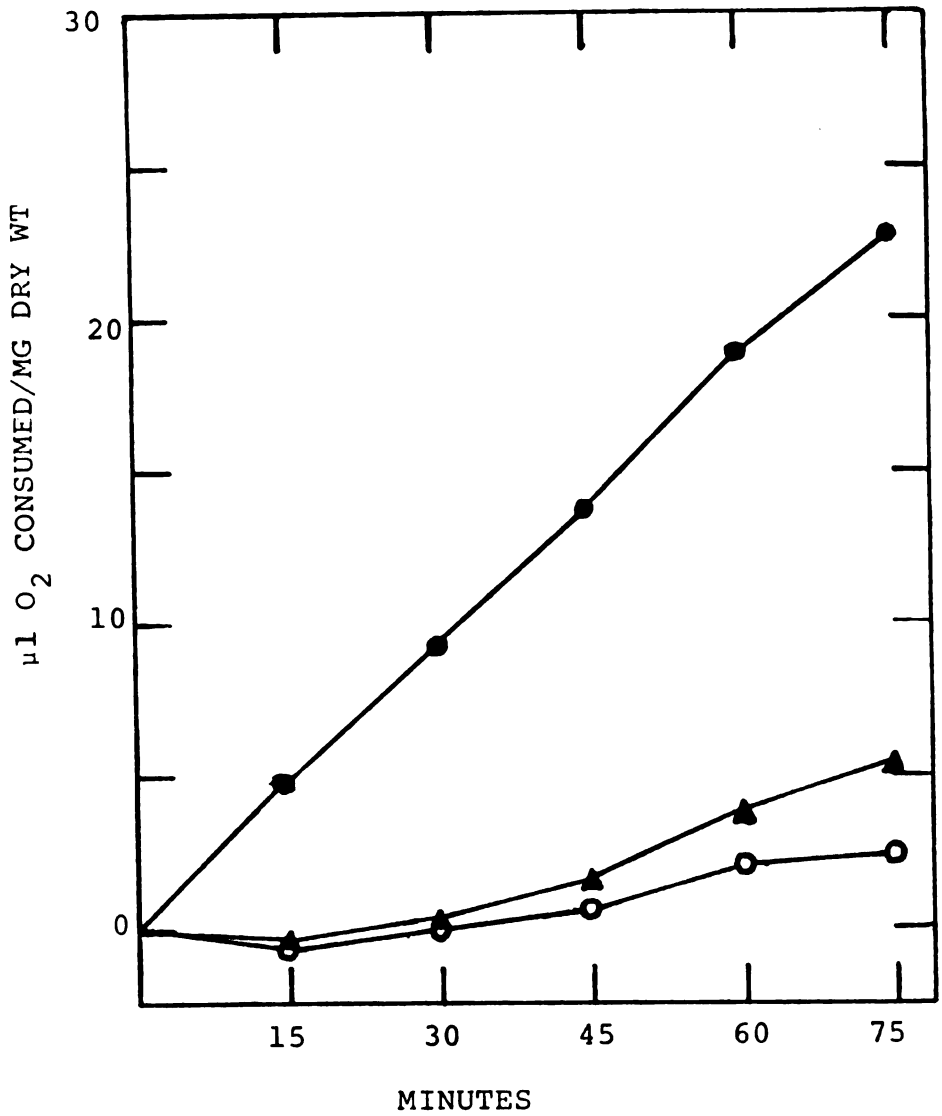
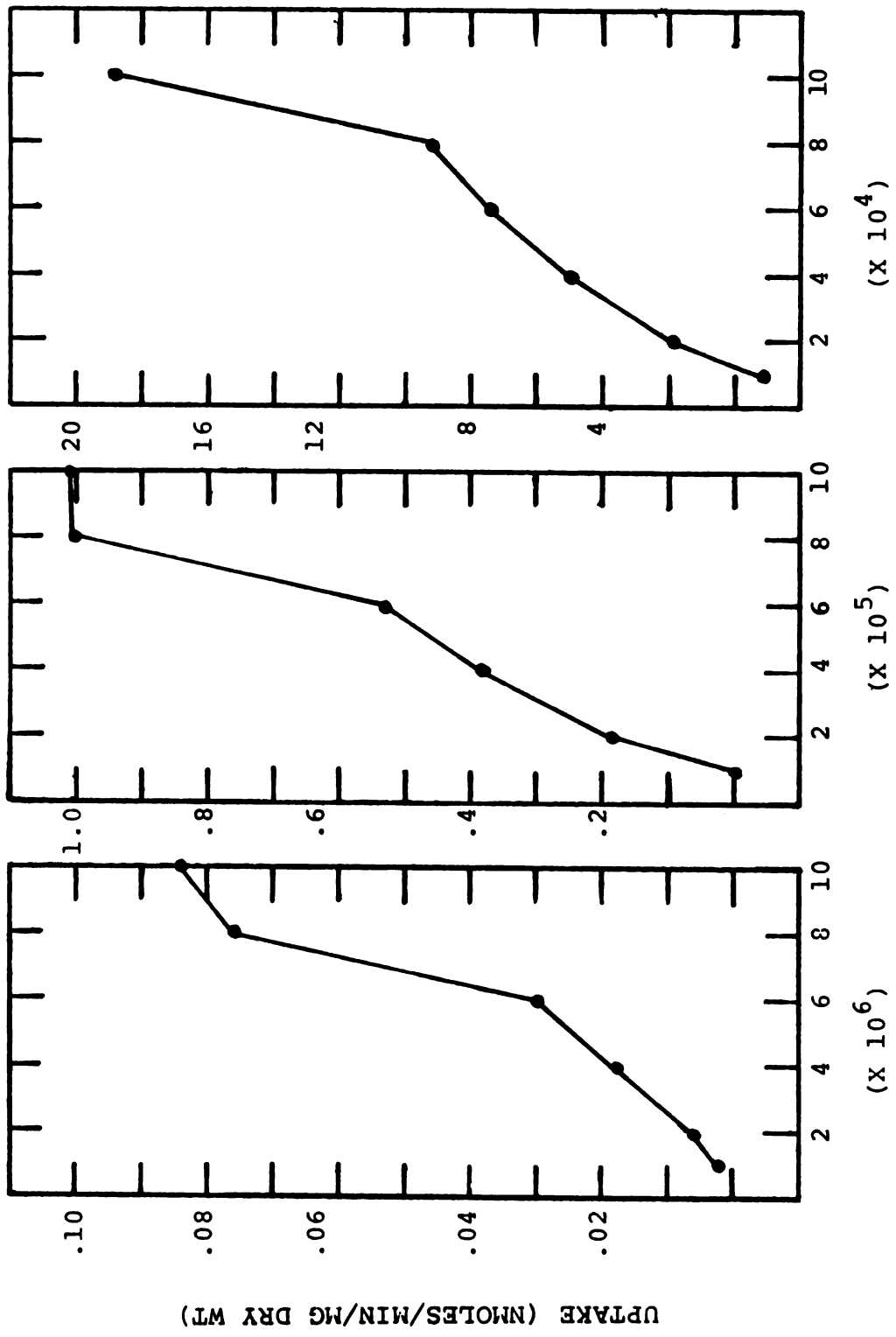


Fig. 8. Inhibition of maltose (0.01 M) oxidation. Oxygen uptake was measured in a Warburg apparatus equilibrated at 30 C. All values were corrected for endogenous activity. Each flask contained 5 mg (dry weight) cells. Symbols: ● , without inhibitors; ▲ , with 0.1 M NaN_3; ○ , with 0.1 M KF.

Fig. 9. Kinetics of [^{14}C] maltose (2 μCi/μmole) uptake by B. popilliae. Initial rates of transport were estimated after incubation (30 C) for 15 sec. Each reaction mixture contained 0.55 mg (dry weight) cells. Reactions were stopped by addition of 5 ml ice cold phosphate buffer (0.05 M, pH 7.2).


UPTAKE (NMOLES/MIN/MG DRY WT)
.10
.08
.06
.04
.02
1.0
.8
.6
.4
.2
20
16
12
8
4
2
4
6
8
10
(X 10^6)
(X 10^5)
(X 10^4)
MALTOSE, M

The cleavage of maltose by crude cell extracts was indicated by the maltose-dependent reduction of $NADP^+$ by glucose 6-phosphate dehydrogenase. The specific activity of "maltase" in the crude extracts was 0.0256 μmole $NADP^+$ reduced per min per mg protein. Cleavage of maltose resulted in the production of either glucose, glucose 6-phosphate, or some derivative of glucose which, in combination with the extract, formed glucose 6-phosphate. No maltase activity was detected by the assay procedure when PEP was substituted for ATP. In addition, no increase in the amount of reducing sugar occurred when maltose was incubated with crude cell extracts. In this experiment, ATP was omitted to prevent the further metabolism of any cleavage produce. Chloramphenicol had no affect on the oxication of maltose by glucose grown cells (Fig. 10), demonstrating that the maltase activity is constitutive. Further support for this conclusion comes from our finding that extracts from glucose grown cells contain spectrophotometrically detectable maltase activity.

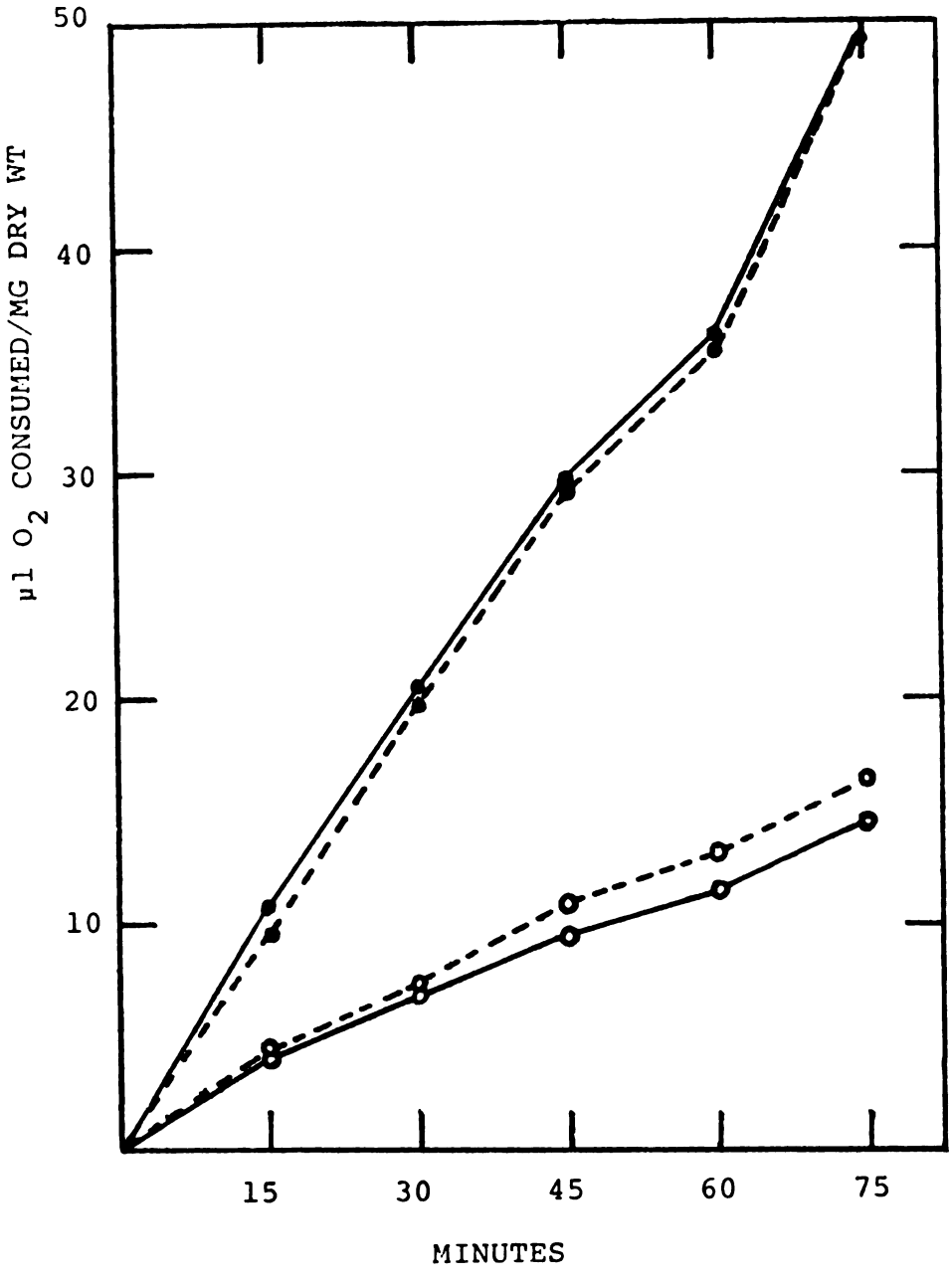


Fig. 10. Constitutive oxidation of maltose. Cells were grown in TY media plus 0.2% glucose. Respiration was measured in a Warburg apparatus equilibrated at 30 C. All values were corrected for endogenous oxygen uptake. Each flask contained 5 mg (dry weight) cells. Symbols: ● , with glucose (0.01 M); ○ , with maltose (0.01 M); dashed lines, with chloramphenicol (0.1 mg/ml).

DISCUSSION

Saturation studies indicate that glucose and αMG are actively transported by B. popilliae. αMG is an appropriate analog of glucose because it appears to be transported as well as glucose. In addition, glucose uptake is inhibited almost completely in the presence of a 50 fold excess of the glucoside. Presumably, αMG competitively saturates the glucose transport system.

Fluoride inhibits the uptake of both [^{14}C] glucose and [^{14}C] αMG by B. popilliae. This inhibition of transport by an enzyme poison which affects the production of phosphoenolpyruvate suggests that these sugars are accumulated by a PEP-dependent phosphotransferase system. Freese et al. (15) reported that that PEP-dependent glucose phosphotransferase system of B. subtilis was able to phosphorylate αMG, whereas the glucokinase was not. Although we did not test whether the hexokinase in 2309MC could phosphorylate αMG, ATP did not appear to serve as a significant phosphoryl donor in the phosphotransferase assays with the glucoside as substrate. Toluenized cell suspensions of B. popilliae, poisoned with fluoride, preferentially used PEP to phosphorylate αMG.

The nearly equal amount of glucose phosphorylation by toluenized cells with either PEP or ATP is presumably a result of both phosphotransferase and hexokinase activity. Since toluenized membranes allow a passive equilibration of an intracellular small molecular weight compound with any given extracellular concentration of the compound (16), glucose would not need to be actively transported to cross the permeability barrier imposed by the cell membrane. Unaltered glucose would be freely accessible for phosphorylation by the ATP-

dependent hexokinase. However, the large amount of phosphorylation obtained with PEP suggests that it too is being utilized directly as a phosphoryl group donor. Lee et al. (25) similarly found that toluenized cells of lactic streptococci would phosphorylate glucose with PEP or ATP and concluded that glucose was transported by a phosphotransferase system.

While the data are not conclusive, it appears probable that B. popilliae possesses a PEP-PTS for glucose transport. This system has been found in many other members of this genus (21), and trehalose, the natural carbon and energy source of B. popilliae, is phosphorylated by a PEP-dependent system during uptake (3). In addition, possession of a phosphotransferase system allows organisms to conserve metabolic energy (34).

Studies of maltose uptake over a range of concentrations indicated that this sugar enters the cell by simple diffusion. There was no evidence of saturation of the transport mechanism at substrate concentrations between 10^{-6} and 10^{-3} M. Also, accumulation of maltose was not affected by the presence of energy poisons at 0.01 M concentrations, although oxidation of maltose was inhibited by 0.1 M fluoride and 0.1 M azide. At the higher concentrations, these poisons inhibit electron transport, which would account for these observations (30).

Once inside the cell, maltose is cleaved into as yet unknown products, at least one of which eventually becomes glucose 6-phosphate. Cleavage of maltose may or may not occur without the simultaneous phosphorylation of one of the products, since no activity could be detected when ATP was omitted from the spectrophotometric assay. In E. coli, maltose is cleaved into glucose and a short dextran by amylomaltase, without the production of a phosphorylated sugar (46). Maltose cleavage in B. popilliae may be dependent upon the presence of ATP, since we failed to find any increase in the amount of reducing

sugar when maltose was incubated with crude extracts without ATP. However, the amount of reducing sugar produced may have been too small to be detected by our assay, since the assay's limit of detection of 5 µg/ml (2.7×10^4 picomoles) would require a relatively large amount of cleavage to cause a change in OD. No ATP was added to the assay for fear of metabolizing, and consequently not detecting, any glucose produced.

Freese et al. (15) found that glucose phorphotransferase activity of B. subtilis declines toward the end of growth. The decrease in the ability to transport glucose correlated with an inability of glucose to suppress sporulation of cells. They noted that a mutant which grew slowly on glucose and could sporulate even when glucose was present throughout growth lacked PEP-PTS activity. It would be interesting to see if group translocation of trehalose by B. popilliae similarly declines as cells enter stationary phase. Since trehalose is cleaved to glucose and glucose 6-phosphate inside the cell, limiting trehalose accumulation would decrease the intracellular glucose concentration. Possibly, this is a method of limiting the accumulation of metabolites which could repress the initiation of sporulation (15). Regulation of transport activity may be one mechanism inherent in the in vivo situation which allows B. popilliae to continue its developmental cycle and sporulate efficiently.

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