JOURNAL OF BACTERIOLOGY, Feb. 1969, p. 793-798
Vol. 97, No. 2

Printed in U.S.A.

Conversion of Glucose-1-Phosphate to 3-Keto-glucose-1-phosphate by Cells of *Agrobacterium tumefaciens*

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Received for publication 19 October 1968

Incubation of resting cells of *Agrobacterium tumefaciens* with glucose-1-phosphate resulted in the accumulation of a new sugar phosphate in the suspending medium. Approximately 80% of the glucose-1-phosphate consumed was converted to the new compound, which was identified as α-D-*ribo*-hexopyranosyl-3-ulose-1-phosphate (3-ketoglucose-1-phosphate). Both utilization of glucose-1-phosphate and accumulation of 3-ketoglucose-1-phosphate were inhibited by 2,4-dinitrophenol, polymyxin, and D-glucose, which are inhibitors of the glucoside transport system of this bacterium but are not inhibitors of D-glucoside-3-dehydrogenase, which is the 3-ketoglucose-1-phosphate-forming enzyme. Consequently, it was concluded that glucose-1-phosphate penetrates into intracellular space by means of an active transport system. The glucose-1-phosphate is converted to 3-ketoglucose-1-phosphate by D-glucoside-3-dehydrogenase, and the 3-ketoglucose-1-phosphate formed reaches the extracellular space by passing through the surface layer of the bacterium.

It has been generally assumed that phosphorylated compounds could not pass through cell wall and membrane barriers of microorganisms. Recently, however, the existence of inducible active transport systems for the entrance of glucose-6-phosphate and L-α-glycerophosphate into *Escherichia coli* was demonstrated (5, 10-12).

During studies on sugar phosphate metabolism by *Agrobacterium tumefaciens*, the formation of an unknown sugar phosphate from glucose-1-phosphate was observed when resting cells of the bacterium grown on a sucrose medium were incubated with glucose-1-phosphate. Isolation and purification of the sugar phosphate accumulated in the incubation medium were performed, and the chemical structure of the compound was confirmed to be α-D-*ribo*-hexopyranosyl-3-ulose-1-phosphate (3-ketoglucose-1-phosphate), a novel sugar phosphate. Actions of some effectors on the conversion of glucose-1-phosphate to 3-ketoglucose-1-phosphate by resting cells were also investigated.

In a previous paper, the formation of 3-keto-glycoside-phosphate from glucose-1-phosphate by purified D-glucoside-3-dehydrogenase (9) was reported.

MATERIALS AND METHODS

Microorganism. Strain IAM-1525 of the plant tumor-inducing bacterium *A. tumefaciens* was obtained from the Japanese Federation of Culture Collection of Microorganisms in the Institute of Applied Microbiology, University of Tokyo. Cells grown in the sucrose medium of McIntire et al., as modified by Fukui et al. (4), were harvested at the mid-log phase of growth, washed three times with distilled water, and used as resting cells.

Materials. D-Allose was kindly supplied by N. K. Richtmyer, National Institutes of Health, Bethesda, Md. Glucose-1-phosphate, disodium salt·$2H_2O$, was purchased from Sigma Chemical Co., St. Louis, Mo. Both 3-ketoglucose (7) and α-3-ketoglucosidase (K. Hayano and S. Fukui, *in preparation*) were prepared in our laboratory. α-3-Ketoglucosidase is a new hydrolase having high specificity for α-3-ketoglucoside but not for phosphate.

Determination of 3-ketoglucose-1-phosphate. The amount of 3-ketoglucose-1-phosphate was estimated from the optical density at 340 nm after alkaline treatment (in 0.1 N NaOH; 9); the purified sample gave a molar extinction coefficient of 3.85×10^3 M^{-1} cm^{-1}. When 2,4-dinitrophenol was present in the reaction mixture, the micro-method of Somogyi and Nelson as modified by Fukui et al. (8) was applied.

Determination of 3-ketoglucose. The amount of 3-ketoglucose was estimated from the optical density at 310 nm in 0.2 M phosphate buffer, *p*H 7.0; 3-ketoglucose gave a molar extinction coefficient of 3.80×10^3 M^{-1} cm^{-1} (S. Fukui and K. Hayano, *unpublished data*). This keto-sugar did not show any absorbance at 340 nm in 0.1 N NaOH.

Determination of acid-labile phosphate. Determination of acid-labile phosphate was carried out according to the method of Umbreit et al. (14). The phos-

phate bond in 3-ketoglucose-1-phosphate and in glucose-1-phosphate is acid-labile. In the present paper, an amount of glucose-1-phosphate is calculated as total acid-labile phosphate minus 3-ketoglucose-1-phosphate.

Other analytical methods. Orthophosphate (P_i) and reducing sugar were determined by the methods of Allen (1) and Somogyi and Nelson, respectively.

Paper chromatography. Descending paper chromatography was carried out on sheets of Toyo filter paper (no. 51A) with the following solvent systems: (A) acetone-acetic acid-water (4.0:1.2:1.0), (B) ethylacetate-pyridine-water (4.0:1.0:0.5), and (C) methylethylketone-acetic acid-water saturated with boric acid (9.0:1.0:1.0). Alkaline silver nitrate (13), urea phosphate (15), and molybdate (2) reagents were used for the detection of general sugars, ketosugars, and phosphorylated compounds, respectively. The urea phosphate reagent gave a blue color with 2-ketosugar and its derivatives and gave a clearly distinguishable deep-red color with 3-ketosugar (4, 6).

Paper electrophoresis. Paper electrophoresis was performed in 25 mM sodium tetraborate at *p*H 9.2 in a cooled pressure plate apparatus at 13 v per cm for 2 hr.

Borohydride reduction and periodate oxidation. Both reduction with sodium borohydride and oxidation with potassium periodate were carried out as described in a previous paper (4).

Calculation of molar optical rotation. To establish anomeric configuration of 3-ketoglucose-1-phosphate, the theoretical value of molar optical rotation was calculated by applying Hudson's rule of isorotation (3).

RESULTS AND DISCUSSION

Formation of an unknown sugar phosphate from glucose-1-phosphate by resting cells. Resting cells of *A. tumefaciens* grown on a sucrose medium were incubated with glucose-1-phosphate for 7 hr at 27 C. The reaction mixture of 60 ml contained 1.0 g of glucose-1-phosphate (Na salt), 300 μmoles of tris(hydroxymethyl)aminomethane (Tris)-chloride buffer (*p*H 8.2), and cell suspension (equivalent to 222 mg, dry weight). During the 7-hr incubation period, glucose-1-phosphate was completely consumed, and an unknown sugar phosphate was formed.

After the incubation, the cells were removed by centrifugation and the supernatant liquid was chromatographed on paper. In solvent system A, the ratio of the migration rate of the unknown product to that of glucose-1-phosphate was 1.55 (R_F = 0.25). With spray reagents of urea phosphate and of molybdate, the unknown compound gave reddish and blue colors, respectively. With alkaline silver nitrate reagent, the unknown product readily showed a brown color, whereas glucose-1-phosphate did not react (Table 1). The unknown product was eluted from the paper chromatogram with distilled water and was given an alkaline treatment as described in the method for 3-ketoglucose-1-phosphate determination. By this treatment, the characteristic absorption spectrum having a maximum at 340 nm was revealed. This is specific for 3-ketoglycoside (9). The observations presented here strongly suggest that the product is 3-ketoglycoside phosphate. The compound eluted from the paper was also treated with 1 N H_2SO_4 at 100 C for 7 min. This treatment caused complete release of P_i, suggesting that the product has a phosphate ester at the C-1 position of the sugar moiety.

The properties reported previously (9) for 3-ketoglycoside formed from glucose-1-phosphate by D-glucoside-3-dehydrogenase were the same as those of the product obtained here, so it was concluded that the conversion of glucose-1-phosphate to 3-ketoglycoside by resting cells also occurs as an action of the dehydrogenase.

Isolation and purification of 3-ketoglycoside phosphate from the reaction mixture. To confirm the chemical structure of the new sugar phosphate, isolation and purification were performed. To the clear supernatant liquid (55.0 ml) prepared in the above section was added 160 ml of ethyl alcohol (99.5%) to precipitate the sugar phosphate. The precipitate was collected, washed three times with absolute ethyl alcohol, and dried in vacuo. The preparation obtained (crude sample, 466 mg) was contaminated with P_i. To remove P_i as insoluble phosphate, 12 ml of 1%

TABLE 1. *Paper chromatography of sugar phosphates*[a]

Compound	Migration		Color tests with		
	R_F	R_{G-1-P}	Urea-phosphate	Molybdate	Alkaline silver nitrate
G-1-P	0.16	1.00	No coloration	Yellow	No coloration
Unknown compound (3KG-1-P)	0.25	1.55	Pink	Blue	Brown
Allose-1-P	0.29	1.71	No coloration	Yellow	No coloration
P_i	0.42	2.63	No coloration	Yellow	No coloration

[a] All compounds are sodium salts. Solvent system A was used. Abbreviations: G-1-P, glucose-1-phosphate; 3KG-1-P, 3-ketoglucose-1-phosphate; allose-1-P, allose-1-phosphate.

magnesium acetate solution was added to 30 ml of the solution containing 460 mg of the crude sample, and the *p*H of this mixture was adjusted to 8.2 with 4% NH_4OH. After 5 min, precipitated magnesium phosphate was removed by centrifugation, and 180 ml of ethyl alcohol (99.5%) was immediately added to the clear supernatant liquid. The 3-ketoglycoside phosphate precipitated was collected by centrifugation, washed three times with absolute ethyl alcohol, and dried in vacuo. All steps in the purification were carried out at 4 C. The yield of 3-ketoglycoside phosphate (purified sample, Na salt) was 305 mg.

Identification of α-D-ribo-hexopyranosyl-3-ulose-1-phosphate. The purified sample showed a single spot in both paper chromatography and paper electrophoresis, and gave the following analytical data: 7.7 mg of the sample contained 21.7 μmoles of acid-labile phosphate, 21.7 μmoles of total phosphate (P, 8.73%), 42.0 μmoles of sodium (by flame colorimeter), and 8.8 μmoles of water. Elemental analyses were performed after removal of water and gave: C, 21.21%; H, 2.68% (calculated for $C_6H_9O_9Na_2P$: C, 23.84%; H, 2.98%). Total ash was not determined. After alkaline treatment (0.1 N NaOH), the sugar phosphate showed an absorption spectrum having a maximum at 340 nm (molar extinction coefficient of $3.85 \times 10^3\ M^{-1}\ cm^{-1}$ on the basis of acid-labile phosphate). The infrared-absorption spectrum showed a sharp peak at 1,740 cm^{-1}, indicating a free carbonyl group (Fig. 1). An optical rotation of $[\alpha]D_D^{15} = +68.0$ ($c = 0.72$ in water) was given without mutarotation. In this experiment, the concentration (c) was calculated from an amount of acid-labile phosphate in the sample having a molecular weight of 302 ($C_6H_9O_9Na_2P$). When Hudson's rule of isorotation (3) is applied, the following relationship between molar optical rotations should obtain:

$$M_4 = M_3 + M_2 - M_1$$

where M_4, M_3, M_2, and M_1 designate the molar optical rotations of 3-ketoglycoside phosphate, glucose-1-phosphate, α-D-methyl-3-ketoglucopyranoside, and α-D-methylglucopyranoside, respectively. By using values of $M_3 = 26{,}520$, $M_2 = 24{,}250$ and $M_1 = 30{,}850$ (4), M_4 was calculated to be 19,920, in reasonable agreement with the value of 20,536 obtained from the observed optical rotation of the 3-ketoglycoside phosphate. This result permits the assignment of α configuration to the glycoside moiety.

Hydrolysis with sulfuric acid or with α-3-ketoglucosidase. When the 3-ketoglycoside phosphate was hydrolyzed with 1 N H_2SO_4 at 100 C for 7 min, complete release of P_i was observed, whereas no saccharide was detected by paper chromatography. Thus, the sugar moiety was found to be very unstable under these conditions. On the other hand, 3-ketoglycoside phosphate was hydrolyzed to an equimolar amount of 3-ketoglucose and P_i by treatment with α-3-ketoglucosidase, a new hydrolase specific for α-3-ketoglucosidic linkage.

Reduction with sodium borohydride followed by hydrolysis with sulfuric acid. Sodium borohydride reduction was carried out at 4 C. The latter reagent (50 mg), 1% solution of the hydride in 0.2 M phosphate buffer, *p*H 6.5, was added dropwise with stirring to 50 mg of the 3-ketoglycoside phosphate in 5.0 ml of the same buffer. The mixture was stirred in the cold until the sample was no longer reducing. The *p*H of the mixture was then brought to 4.0 with acetic acid to destroy excess borohydride. The product showed the following properties: (i) it had no reducing power; (ii) the ratio of its migration

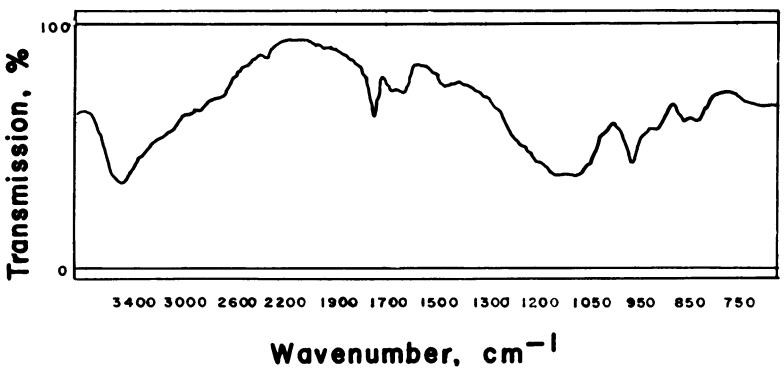

FIG. 1. *Infrared spectrum of unknown product identified as 3-ketoglucose-1-phosphate, 0.5 mg in 100 mg of KBr.*

rate to that of glucose-1-phosphate was 1.71 in paper chromatography with solvent system A, and a yellow color was given by molybdate spray reagent; (iii) complete hydrolysis was observed with 1 N H_2SO_4 at 100 C for 7 min with the formation of an equimolar amount of hexose and P_i (molar ratio, 1.05:1.00). Hexose released was identified as allose by paper chromatography with solvent systems B and C. No sugar other than allose was detected. The results obtained indicate the presence of a free carbonyl at the C-3 position and of a phosphate ester at the C-1 position in the sugar moiety of the 3-ketoglycoside phosphate.

Periodate oxidation. Periodate oxidation was carried out as follows. A solution containing 17.7 μmoles of the 3-ketoglycoside phosphate was treated with 250 μmoles of KIO_4 at *p*H 5.4 in a total volume of 12.0 ml at room temperature in the dark for 18 hr. Acid formed was determined by alkali titration, and periodate utilization was determined by thiosulfate titration. Under these conditions, 2.2 moles of periodate was consumed per mole of the 3-ketoglycoside phosphate, and 2.05 moles of acid was formed. Release of P_i was less than 0.05 moles per mole of 3-ketoglycoside phosphate. These results of periodate oxidation showed good agreement with the results obtained with various 3-ketoglycosides (4, 6, 7), so the compound oxidized could have had hexopyranoside structure with only one free carbonyl group.

Structure of the 3-ketoglycoside phosphate. From the evidence summarized in Fig. 2, the following structure is proposed: α-D-*ribo*-hexopyranosyl-3-ulose-1-phosphate, abbreviated as 3-ketoglucose-1-phosphate.

Accumulation of 3-ketoglucose-1-phosphate by resting cells. The rates of accumulation of 3-ketoglucose-1-phosphate and utilization of glucose-1-phosphate are given in Fig. 3, in which respective rates are calculated to be 12.05 and 15.25 μmoles per min per g of dry cells from the results of 60-min incubation with shaking. Under static conditions, the utilization rate of glucose-1-phosphate decreased to 1.50 μmoles per min per g of dry cells, approximately one-tenth of the rate with shaking (Table 2). When a sonic extract was used as the enzyme preparation in place of resting cells, the utilization rate of glucose-1-phosphate was 5.2 μmoles per min per g (dry weight) under both aerobic and static conditions (Table 2). In these cases, accumulation of 3-ketoglucose-1-phosphate was undetectable, and P_i and acid-labile phosphate were formed. The latter compound has not yet been identified. As shown in Table 3, 2,4-dinitrophenol and polymyxin had an inhibitory effect on the accumulation of 3-ketoglucose-1-phosphate. Both of them were considered to be inhibitors of the transport

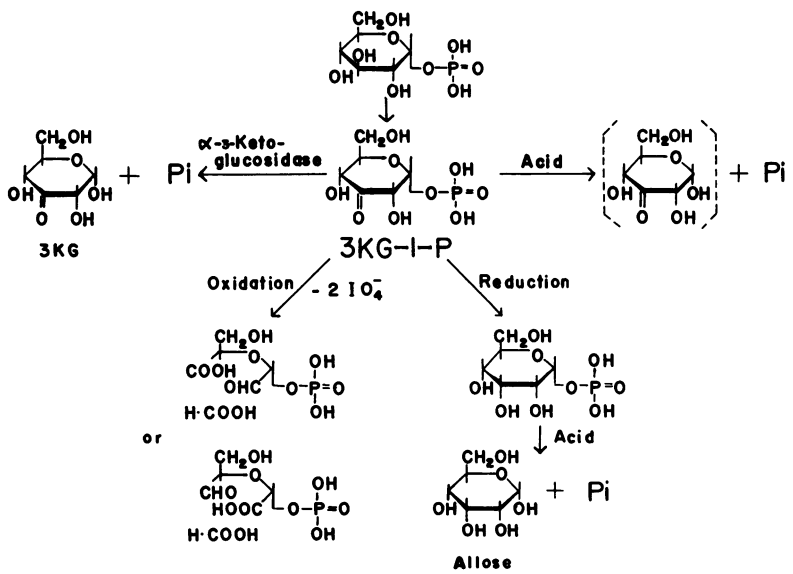

FIG. 2. *Summary of procedure for identification of 3-ketoglucose-1-phosphate. Abbreviations: 3KG-1-P, 3-ketoglucose 1-phosphate; 3KG, 3-ketoglucose.*

system for glucose-1-phosphate, because the 3-ketoglucose-1-phosphate-forming enzyme D-glucoside-3-dehydrogenase was not at all inhibited by them (9). D-Glucose also gave strong

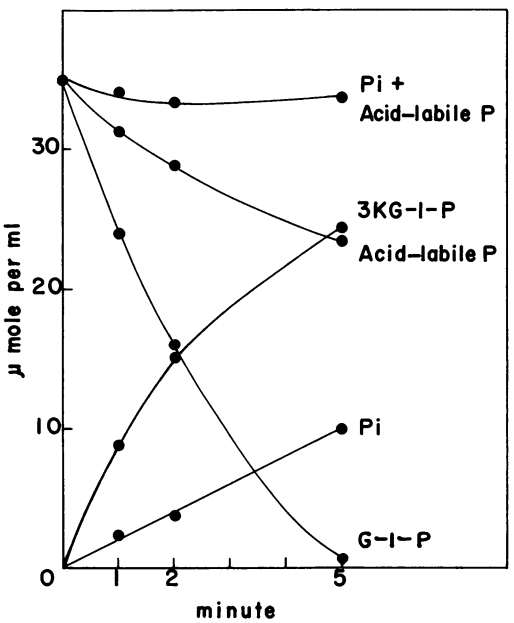

FIG. 3. *Time course of 3-ketoglucose-1-phosphate (3-KG-1-P) formation. Reaction mixture of 10 ml contained 350 μmoles of glucose-1-phosphate (G-1-P), 60 μmoles of Tris-chloride buffer (pH 8.2), and 120 mg of cells (dry weight). Reaction was carried out at 27 C with shaking.*

TABLE 2. *Metabolic rates of glucose-1-phosphate under various conditions*[a]

Conditions	G-1-P consumption	3KG-1-P accumulation	P_i release
Resting cells			
With shaking	15.25	12.05	3.20
Without shaking	1.50	0.2	0.61
Sonic extract			
With shaking	5.22	0.2	1.91
Without shaking	5.20	0.2	—

[a] Numbers represent micromoles per minute per gram of dry matter. Reaction conditions were as follows: reaction mixture of 1.0 ml contained 5 μmoles of G-1-P, 45 μmoles of Tris-chloride buffer, *p*H 8.2, and 6.9 mg of cells or sonic extract (dry weight); reactions were carried out at 27 C. Abbreviations: G-1-P, glucose-1-phosphate; 3KG-1-P, 3-ketoglucose-1-phosphate.

TABLE 3. *Effect of additions on 3-ketoglucose-1-phosphate formation*[a]

Addition	Concn (mM)	Relative activity
None	—	100
P_i	2.5	92
Adenosine triphosphate	3.75	103
Adenosine diphosphate	7.5	100
Pyrophosphate	5.0	100
Phosphoenolpyruvate	1.8	104
Glucose-6-phosphate	12.5	88
Glucose-6-phosphate	5.0	96
D-Glucose	20	22
L-Glucose	20	100
D-Fucose	20	109
D-Xylose	20	72
L-Arabinose	20	107
D-Galactose	20	66
NaF	18	81
2,4-Dinitrophenol	1.0	43
NaN_3	18	41
Polymyxin	100[b]	66

[a] Reaction mixture of 1.0 ml contained 5 μmoles of glucose-1-phosphate, 45 μmoles of Tris-chloride buffer (*p*H 8.2), and 6.9 mg (dry weight) of cells. Reaction was carried out at 27 C with shaking.

[b] Micrograms per milliliter.

inhibition on 3-ketoglucose-1-phosphate-forming activity with a mode of competition (Fig. 4). This phenomenon might be due to the high affinity of D-glucose for both glucose-1-phosphate transport system and D-glucoside-3-dehydrogenase. In the 3-ketoglucose-1-phosphate-forming reaction with resting cells, an apparent K_m value for glucose-1-phosphate was calculated to be 3.8×10^{-3} M from Lineweaver-Burk plots at conditions of 27 C and *p*H 8.2 (Fig. 4), whereas the K_m value of D-glucoside-3-dehydrogenase for glucose-1-phosphate was 1.8×10^{-4} M under these conditions. The difference between K_m values obtained with resting cells and purified enzyme also indicates the presence of a transport system for glucose-1-phosphate. Clear evidence of an active transport system for glucose-1-phosphate entrance will be reported in a separate paper (S. Fukui and S. Miyairi, *in preparation*).

Degradation of 3-ketoglucose-1-phosphate by resting cells. As shown in Fig. 5, degradation of 3-ketoglucose-1-phosphate took place during aerobic incubation with resting cells at a rate of 2.27 μmoles per min per g of dry cells accompanied by a release of P_i at an almost equal rate, 2.18 μmoles per min per g of dry cells. In paper

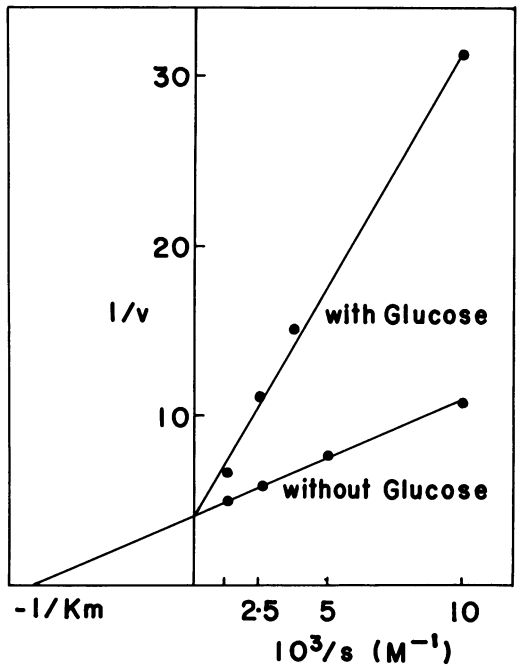

FIG. 4. *Lineweaver-Burk plots for 3-ketoglucose-1-phosphate-forming reaction with resting cells. Reaction conditions were as follows: pH 8.2 in 6 mM Tris buffer, 27 C, and with shaking; v is the rate of formation of 3-ketoglucose-1-phosphate. D-Glucose was used as inhibitor at a concentration of 2 × 10⁻³ M.*

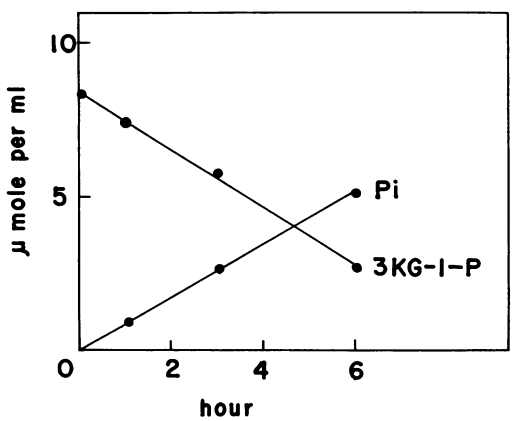

FIG. 5. *Degradation of 3-ketoglucose-1-phosphate by resting cells. Reaction mixture of 10 ml contained 83.8 μmoles of 3-ketoglucose-1-phosphate, 150 μmoles of Tris-chloride buffer (pH 8.2), and 13.8 mg (dry weight) of cells. Reaction was carried out at 27 C with shaking.*

chromatographic and paper electrophoretic analyses, free sugar such as 3-ketoglucose could not be detected. The degradation reaction was inhibited by 2,4-dinitrophenol and D-glucose, as was the glucose-1-phosphate uptake reaction (S. Fukui and S. Miyairi, *unpublished data*). This finding might suggest the presence of an active transport system for 3-ketoglucose-1-phosphate entry into the cells.

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