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Glucose Catabolism in Two Derivatives of a *Rhizobium japonicum* Strain Differing in Nitrogen-Fixing Efficiency[1]

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Radiorespirometric and enzymatic analyses reveal that glucose-grown cells of *Rhizobium japonicum* isolates I-110 and L1-110, both derivatives of *R. japonicum* strain 3I1b110, possess an active tricarboxylic acid cycle and metabolize glucose by simultaneous operation of the Embden-Meyerhof-Parnas and Entner-Doudoroff pathways. The hexose cycle may play a minor role in the dissimilation of glucose. Failure to detect the nicotinamide adenine dinucleotide phosphate-dependent decarboxylating 6-phosphogluconate dehydrogenase (EC 1.1.1.44) evidences absence of the pentose phosphate pathway. Transketolase and transaldolase reactions, however, enable *R. japonicum* to produce the precursors for purine and pyrimidine biosynthesis from fructose-6-phosphate and glyceraldehyde-3-phosphate. A constitutive nicotinamide adenine dinucleotide-linked 6-phosphogluconate dehydrogenase has been detected. The enzyme is stimulated by either mannitol or fructose and might initiate a new catabolic pathway. *R. japonicum* isolate I-110, characterized by shorter generation times on glucose and greater nitrogen-fixing efficiency, oxidizes glucose more extensively than type L1-110 and utilizes preferentially the Embden-Meyerhof-Parnas pathway, whereas the Entner-Doudoroff pathway apparently predominates in type L1-110.

Recently, four distinct clones were isolated from *Rhizobium japonicum* strain 3I1b110 on the basis of differing colony morphologies formed on yeast extract-mannitol-HEPES-MES (YEM-HM) agar medium [HEPES = *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MES = 2-(*N*-morpholino)-ethanesulfonic acid] (9); isolates L1-110 and L2-110 form large colonies on this medium, whereas isolates I-110 and S-110 form small colonies. These variants are susceptible to the same specific phages and have similar sensitivity to antibiotics and deoxyribonucleic acid synthesis inhibitors (9). However, the rate of free-living and symbiotic nitrogen fixation, as measured by acetylene reduction, shows *R. japonicum* isolates I-110 and S-110 to be 5- to 10-fold more efficient than isolates L1-110 and L2-110 (9). Coincidentally, isolate I-110 nitrogenase and growth rates are more sensitive to Na^+ and K^+ salts than they are in isolate L1-110 (R. G. Upchurch and G. H. Elkan, Can. J. Microbiol., in press).

We set out to elucidate whether the carbohydrate catabolism, as a source of energy and metabolites, differs in *R. japonicum* isolates I-110 and L1-110, as related to their nitrogen-fixing efficiencies.

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In a communication from our laboratory, Keele et al. (7) reported, on the basis of radiorespirometric data and key enzyme assays, that *R. japonicum* ATCC 10324 utilized the Entner-Doudoroff (ED) pathway and the tricarboxylic acid cycle as the sole route for glucose oxidation. The pentose phosphate (PP) pathway was concluded absent by virtue of lack of the nicotinamide adenine dinucleotide (NAD) phosphate (NADP)-linked 6-phosphogluconate (6-PG) dehydrogenase activity, in connection with radiorespirometric data. The possibility of an operative Embden-Meyerhof-Parnas (EMP) pathway was rejected on the assertion (7, 16) that "the Embden-Meyerhof-Parnas (EMP) and ED pathways do not operate simultaneously in a microorganism"; radiorespirometric data, however, did not rule out participation of the EMP pathway in the breakdown of glucose. In addition, Eisenberg and Dobrogosz (3) reported that degradation of gluconate by the ED pathway could occur, in *Escherichia coli*, simultaneously with a glycolytic breakdown of glucose. From our literature review, simultaneous occurrence in an organism of the ED, EMP, and PP pathways is frequent; however, operation of the ED and EMP pathways in organisms lacking the decarboxylating 6-PG dehydrogenase is rather uncommon, having been reported in *Aq-*

uaspirillum gracile (10). As for *Rhizobium*, Katznelson and Zagallo (6) have presented experimental data suggesting the presence of some ED, EMP, and PP pathway enzymes in *R. meliloti*, *R. phaseoli*, and *R. leguminosarum*; in addition, Tuzimura and Meguro (17) have reported that fructose-1,6-diphosphate (FDP) could be oxidized by whole cells of *R. japonicum*.

In this paper, a more thorough investigation of the catabolic enzyme system of *R. japonicum* is presented, and radiorespirometric data for glucose oxidation are considered in the light of this system and compared in variants I-110 and L1-110.

MATERIALS AND METHODS

Chemicals. All radioactive substrates were purchased from New England Nuclear Corp., Boston, Mass. Enzymes, coenzymes, and substrates utilized in the enzyme assays were obtained from Sigma Chemical Co., St. Louis, Mo. 2-Keto-3-deoxy-6-phosphogluconate (KDPG), barium salt, was generously supplied by W. A. Wood, Department of Biochemistry, Michigan State University, East Lansing, Mich. All other compounds were purchased from standard sources.

Bacterial strains. Derivatives, I-110 and L1-110, of *R. japonicum* strain 3I1b110 (originally obtained from the U.S. Department of Agriculture Beltsville Culture Collection by courtesy of D. F. Weber) have been recently isolated in our laboratory by L. D. Kuykendall (U.S. Department of Agriculture, Beltsville, Md.). Isolation of these derivatives and maintenance conditions of the stock cultures have been described elsewhere (9).

Media. The basal growth medium consisted of the YEM-HM of Cole and Elkan (1), from which mannitol and arabinose were omitted. This medium contained 0.1% (wt/vol) yeast extract (Difco). Carbon sources were autoclaved separately as 10% (wt/vol, in distilled water) solutions and added to sterile basal medium to a final concentration of 1% (wt/vol); in the case of mixed carbohydrate media, each carbon source was added to a final concentration of 1% (wt/vol).

Cultural conditions. Primary cultures, in 10-ml quantities, contained the same carbon sources (1%, wt/vol) as the secondary culture. They were started from agar slants and incubated at 28°C, with shaking, until they reached an optical density of approximately 100 Klett units at 660 nm. These cultures provided inocula for the experimental cultures. For radiorespirometric purposes, cultures were grown with shaking at 28°C in 1-liter quantities in 2,800-ml Fernbach flasks, with 0.1% inoculum. Early-stationary-phase cells were harvested by centrifugation at 16,000 × *g*, for 10 min, in a refrigerated centrifuge and washed twice with distilled water and once with the basal growth medium. The resulting cell pellet was resuspended in the basal growth medium to an optical density at 660 nm of 750 Klett units, equivalent to about 35 mg (dry weight) of cells per ml, and equilibrated in a 28°C water bath for 30 min with gentle shaking before use. For enzyme analyses, cultures were grown in 100-ml quantities in 300-ml Delong flasks.

Radiorespirometric analyses. The radiorespirometric apparatus used has been described by Keele et al. (7). Flasks containing 7.5 μmol of specifically labeled glucose, diluted in the basal medium, were placed in a thermostatically controlled water bath set at 28°C and purged with compressed air at a flow rate of 100 ml/min. After 15 min of temperature equilibration of the flasks in the water bath, 7.5 ml of the cell suspension, prepared as described above, was injected with a syringe into each flask. The final volume of the flask content was 20.0 ml. The water bath shaker was adjusted to 150 rpm. Respiratory CO_2 was trapped in 10% KOH. The trapping solution was replaced at 30-min intervals. Samples (0.2 ml) of the trapping solution were diluted in 2.0 ml of absolute ethanol and mixed with 4.0 ml of the scintillation fluid described on the Packard Triton X-100 label. This scintillation fluid was composed of 1 part of Triton X-100 and 4 parts of toluene scintillator solution containing 8.25 g of 2,5-diphenyloxazole (PPO) and 0.25 g of 1,4-bis-[2]-(5-phenyloxazolyl)benzene (POPOP) per liter of toluene. The ^{14}C content was measured with a Packard Tri-Carb liquid scintillation counter. Background activities were subtracted, and corrections for quenching were performed using the automatic external standard method. At the end of the respiration period, the cell suspensions were rapidly chilled in an ice bath, and cells were separated from the respiration medium by a 10,000 × *g* centrifugation for 10 min in a refrigerated centrifuge. Radioactivity of each resulting supernatant fluid was measured in the same way as for the trapping solution. The cell pellets were resuspended in absolute ethanol and assayed for their ^{14}C content.

Preparation of cell extracts. Late-exponential-phase cells were harvested by a 10-min centrifugation at 16,000 × *g*. After being washed twice with distilled water, cells were resuspended in small volumes of ice-cold 0.9% (wt/vol) KCl solution containing 155 μg of cysteine-HCl per ml (KCl-Cys solution); this suspension medium was prepared daily and adjusted to pH 7.5 before use. The suspended cells were broken by two 2.5-min sonic treatments, at 4°C, with 1-min intervals, during which the probe was cooled in an ice-water bath; the Sonifier (Branson) was adjusted to 3-A power output. The crude extracts were obtained after a 15-min centrifugation at 12,000 × *g*, followed by a 120-min centrifugation of the resulting supernatant fluid, at 27,000 × *g*, to minimize the reduced NAD oxidase activity (13). All centrifugations were run at 4°C. The extracts were kept in an ice-water bath and used within 5 h after preparation. The protein concentration in the extracts was determined by the method of Warburg and Christian, as described by Layne (11), and adjusted to 0.75 to 1.25 mg of protein per ml with ice-cold KCl-Cys solution. The final protein concentration in the assay mixtures ranged between 0.15 and 0.25 mg/ml.

Enzyme assays. Enzyme assays were carried out

in a Beckman DUR spectrophotometer equipped with a Gilford Instrument 2000 multiple-sample absorbance recorder, at room temperature.

All dehydrogenases, unless otherwise specified, were assayed according to a modification of the procedure described by Keele et al. for glucose-6-phosphate (G-6-P) dehydrogenase (D-glucose-6-phosphate:NADP oxidoreductase, EC 1.1.1.49) (7). The reaction mixture contained: glycylglycine buffer, pH 8.0, 40 μmol; $MgSO_4$, 12 μmol; the substrate, 3 μmol; NAD or NADP, 1 mg; and cell-free extract, in a total volume of 1.0 ml.

Glyceraldehyde-3-phosphate (GAP) dehydrogenase (D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase [phosphorylating], EC 1.2.1.12) activity was determined according to the procedure supplied by Sigma Chemical Co. with the purchase of DL-GAP diethylacetal, barium salt. Triosephosphate isomerase (D-glyceraldehyde-3-phosphate ketol-isomerase, EC 5.3.1.1) was determined in the same way, except that GAP was replaced by dihydroxyacetone phosphate.

FDP aldolase (fructose-1,6-biphosphate:D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) was measured in the reverse direction, by coupling the formation of FDP from GAP and dihydroxyacetone phosphate with hexose diphosphatase, G-6-P isomerase, and G-6-P dehydrogenase, and by monitoring the reduction of NADP. The assay mixture contained: glycylglycine buffer, pH 8.0, 40 μmol; $MgSO_4$, 12 μmol; GAP, 3 μmol; dihydroxyacetone phosphate, 3 μmol; commercial hexose diphosphatase, 0.5 U; commercial G-6-P isomerase, 0.5 U; commercial G-6-P dehydrogenase, 0.5 U; NADP, 1 mg; and cell-free extract; in a total volume of 1.0 ml. Hexose-diphosphatase (D-fructose-1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) was assayed in the same way, with FDP (3 μmol) as the substrate instead of GAP and dihydroxyacetone phosphate and without the commercial hexose diphosphatase. G-6-P isomerase (D-glucose-6-phosphate keto isomerase, EC 5.3.1.9) was determined in a similar assay, with fructose-6-phosphate as the substrate and in the absence of commercial hexose diphosphatase and G-6-P isomerase.

Transaldolase (sedoheptulose-7-phosphate:D-glyceraldehyde-3-phosphate dihydroxyacetone transferase, EC 2.2.1.2) and transketolase (fructose-6-phosphate:D-glyceraldehyde-3-phosphate glycolaldehyde transferase, EC 2.2.1.1) activities were determined by coupling the formation of fructose-6-phosphate to the oxidation of G-6-P in the presence of excess G-6-P isomerase and G-6-P dehydrogenase. The reaction mixture contained: glycylglycine buffer, pH 8.0, 40 μmol; $MgSO_4$, 12 μmol; commercial G-6-P isomerase, 0.5 U; commercial G-6-P dehydrogenase, 0.5 U; NADP, 1 mg; cell-free extract; and, as substrates, either GAP, 3 μmol, and sedoheptulose-7-phosphate, 3 μmol, for transaldolase measurement, or erythrose-4-phosphate, 3 μmol, and xylulose-5-phosphate, 3 μmol, for transketolase determination; to a final volume of 1.0 ml. These enzyme activities were determined from the initial portion of the curves.

Phosphoglyceromutase (2,3-diphospho-D-glycerate phosphotransferase, EC 2.7.5.3), enolase (2-phospho-D-glycerate hydrolase, EC 4.2.1.11), and pyruvate kinase (adenosine 5'-triphosphate: pyruvate phosphotransferase, EC 2.7.1.40) activities were measured by following reduced NAD oxidation in the presence of excess lactate dehydrogenase. The procedure is supplied by Sigma Chemical Co. to determine the purity of phosphoenolpyruvate, trisodium salt. The reaction mixture for phosphoglyceromutase consisted of: 0.2 mM reduced NAD, 1.6 ml, in 0.3 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.5; $MgSO_4$, 75 μmol; KCl, 75 μmol; 3-phosphoglycerate, 5 μmol; commercial enolase, 0.5 U, in 0.3 M Tris buffer, pH 7.5; commercial pyruvate kinase, 0.5 U, in 0.3 M Tris buffer, pH 7.5; commercial lactate dehydrogenase, 0.5 U, in 0.3 M Tris buffer, pH 7.5; adenosine diphosphate, 5 μmol; and cell-free extract; the final volume was 3.0 ml. The reference cuvette contained no reduced NAD; therefore, reduced NAD oxidase activities were determined by omitting 3-phosphoglycerate in the reaction mixture and were subtracted to obtain the net phosphoglyceromutase activities. In the reaction mixture for enolase determination, 2-phosphoglycerate, 5 μmol, substituted for 3-phosphoglycerate, and commercial enolase was omitted. Five micromoles of phosphoenolpyruvate was used instead of 3-phosphoglycerate in the assay for pyruvate kinase, and both commercial enolase and pyruvate kinase were omitted. In each assay, corrections were carried out for reduced NAD oxidase.

The assay procedure for kinases consisted of coupling the production of adenosine diphosphate to the pyruvate kinase-lactate dehydrogenase system. The reaction mixture was identical to the one described above to measure enolase activities, with the exception that adenosine triphosphate, 5 μmol, substituted for adenosine diphosphate and 5 μmol of the substrate to be phosphorylated was added to a final volume of 3.0 ml. Corrections were made for reduced NAD oxidase.

KDPG aldolase (6-phospho-2-keto-3-deoxy-D-gluconate:D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.14) was assayed following the procedure developed by W. A. Wood, as reported by Keele et al. (8). Determination of 6-PG dehydrase (6-phosphogluconate hydrolase, EC 4.2.1.12) involved coupling its activity with endogenous KDPG aldolase. The reaction mixture was identical to that for KDPG aldolase except that KDPG was replaced by 6-PG.

Transhydrogenase was assayed according to the procedure of Ragland et al. (14) with 3-acetylpyridine adenine dinucleotide.

RESULTS AND DISCUSSION

Enzyme activities in *R. japonicum* isolates I-110 and L1-110. Little effort has been devoted to determine optimal assay conditions for each enzyme, since the purpose of this study was to detect and compare the catabolic enzymes of *R. japonicum* isolates I-110 and L1-110.

To date, glycolytic enzymes have been reported only in the fast-growing species of *Rhi-*

zobium (5, 6). The present study reveals that the enzyme system of *R. japonicum* also includes all the EMP and ED pathway enzymes. Activities of these and other enzymes are listed in Table 1. In both *R. japonicum* isolates I-110 and L1-110, enzyme activities are comparatively in the same range of magnitude, except for the enolase; isolate L1-110 enolase always exhibited activities at least fourfold lower than isolate I-110 enolase; accordingly, this enzyme may determine the rate of carbon flow through the EMP pathway in these strains. Detection of hexose diphosphatase suggests the possibility of gluconeogenesis and the hexose cycle. 6-PG dehydrase was always detected; failure to use, in the assay, excess KDPG aldolase accounts for its low activities.

Among the enzymes of the PP pathway, G-6-P and 6-PG dehydrogenases, transketolase, and transaldolase have been assayed (Table 1). G-6-P dehydrogenase exhibits higher activities with NADP, as the electron acceptor, than with NAD. Because transhydrogenase activities are negligible (Table 1), it can be concluded that *R. japonicum* G-6-P dehydrogenase utilizes either NAD or NADP like the enzyme from other microorganisms (13, 14). No attempt has been made to elucidate whether it consists of two enzymes, different with regard to pyridine nucleotide specificity, like *Pseudomonas multivorans* G-6-P dehydrogenase (18).

The NADP-specific 6-PG dehydrogenase has not been detected in derivatives I-110 and L1-110 of *R. japonicum* strain 3I1b110 (Table 1). Keele et al. (7) and Martinez-de Drets and Arias (12) also mention lack of the enzyme in *R. japonicum*.

However, a NAD-dependent 6-PG dehydrogenase, not reported in *Rhizobium* previously, is active in cell-free extracts of isolates I-110 and L1-110 (Table 1). Table 2 reveals that the NAD-dependent 6-PG dehydrogenase is present in cells of *R. japonicum* grown in various carbohydrates. It is interesting to note that this enzyme is active in extracts from cells grown on arabinose with or without succinate and gluconate; these substrates either fail to induce or repress mannitol dehydrogenase (unpublished data). NAD-linked 6-PG dehydrogenase seems to be constitutive and exhibits high activities in extracts from cells grown in the presence of either mannitol or fructose. We have partially characterized the products of the NAD- and NADP-dependent 6-PG oxidation in *Rhizobium* (K. Mulongoy and G. H. Elkan, submitted for publication). The product of the reaction cata-

TABLE 1. *Enzyme activities in R. japonicum types I-110 and L1-110*

Enzyme	Sp act[a]	
	Type L1-110	Type I-110
Hexokinase	15	20
G-6-P isomerase	38	33
Phosphofructokinase	34	38
FDP aldolase	11	12
Triose phosphate isomerase	5	12
GAP dehydrogenase	77	73
3-Phosphoglycerate kinase	27	38
Phosphoglyceromutase	50	30
Enolase	13	62
Pyruvate kinase	135	81
Hexose diphosphatase	34	39
G-6-P dehydrogenase		
With NAD	8	12
With NADP	23	20
6-PG dehydrogenase		
With NAD	29	24
With NADP (EC 1.1.1.44)	0	0
Transketolase	17	31
Transaldolase	86	41
Gluconokinase	97	97
6-PG dehydrase[b]	4	5
KDPG aldolase[b]	31	46
Isocitrate dehydrogenase		
With NAD	1	0
With NADP	202	170
Malate dehydrogenase		
With NAD	5	7
With NADP	8	16
Transhydrogenase	5	2

[a] Values are expressed as nanomoles of product formed per minute per milligram of protein and are the average of at least two separate measurements.

[b] Assay was performed with extracts from gluconate-grown cells.

TABLE 2. *Activities of NAD-linked 6-PG dehydrogenase in cell-free extracts of R. japonicum type L1-110 grown on various substrates*[a]

Growth substrate(s)[b]	Sp act[c]
Glucose	29
Arabinose	18
Mannitol	59
Fructose	42
Gluconate	19
Sucrose	13
Ribose	13
Glucose and mannitol	45
Arabinose and glucose	22
Arabinose and succinate	27
Arabinose and gluconate	17
Arabinose and mannitol	58
Arabinose and fructose	56
Arabinose and 2-ketogluconate	20
Arabinose and β-hydroxybutyrate	21

[a] Type I-110 6-PG dehydrogenase exhibits similar variations (unpublished data) except that type I-110 does not grow on mannitol as the sole source of carbons.

[b] Cultural conditions are detailed in the text.

[c] Values are expressed as nanomoles of NAD reduced per minute per milligram of protein and are the average of at least two separate measurements.

lyzed by the NAD-dependent 6-PG dehydrogenase is a phosphorylated keto- or diketohexonic compound; this finding suggests that an unknown catabolic mechanism, initiated by the NAD-linked 6-PG dehydrogenase, might be involved in the dissimilation of carbohydrates by rhizobia. On the other hand, we have identified the NADP-specific enzyme as the decarboxylating enzyme of the PP pathway; it catalyzes the production of ribulose-5-phosphate. Its absence in the derivatives of *R. japonicum* strain 3I1b110 indicates a lack of the PP pathway in these organisms. Nevertheless, a partial PP pathway consisting of transketolase and transaldolase reactions functions in *R. japonicum* (Table 1) to provide tetrose and pentose phosphate essential for the synthesis of purines and pyrimidines.

Pyruvate and glucose catabolism in cells of *R. japonicum* types I-110 and L1-110. Figures 1 and 2 present the radiorespirometric kinetics for the catabolism of specifically labeled pyruvate by *R. japonicum* isolates I-110 and L1-110, grown on glucose. The differential rates of $^{14}CO_2$ evolution, for both derivatives, are C1 > C2 > C3. The inventory of ^{14}C utilization in this and other experiments is shown in Table 3. The preferential use of the C1 (first carbon) of pyruvate followed by the C2 and C3 (Fig. 1) is consistent with an operative tricarboxylic acid cycle. In their investigation of pyruvate dissimilation by glucose-grown *R. japonicum* ATCC 10324, Keele et al. (7) obtained similar results that, in connection with the patterns of $^{14}CO_2$ yields from acetate, succinate, and glutamate, demonstrated operation of the tricarboxylic acid cycle. Also, isocitrate and malate dehydrogenases have been detected in cell-free extracts of *R. japonicum* isolates I-110 and L1-110 (Table 1), and earlier investigations (17) have reported the oxidation of α-ketoglutarate, succinate, and malate by whole cells of *R. japonicum*, suggesting operation of the tricarboxylic acid cycle.

Radiorespirometric patterns for use of glucose are basically the same for both derivatives studied, as follows: C3,4 > C1 > C2 > C6 (Fig. 3 and 4). Keele et al. found the same sequence in growing cells of *R. japonicum* ATCC 10324 (7). Our preliminary results show that cumulative yield of $^{14}CO_2$ from glucose C3 equals 69% that from glucose C4; accordingly, the preferential rates of $^{14}CO_2$ evolution become C4 > C3 > C1 > C2 > C6 with C1 = 39%, C2 = 30%, C3 = 57%, C4 = 83%, and C6 = 29% for *R. japonicum* type I-110 and C4 > C1 > C3 > C2 > C6 with C1 = 42%, C2 = 36%, C3 = 40%, C4 = 58%, C6 = 33% for type L1-110. The extensive conversion of the

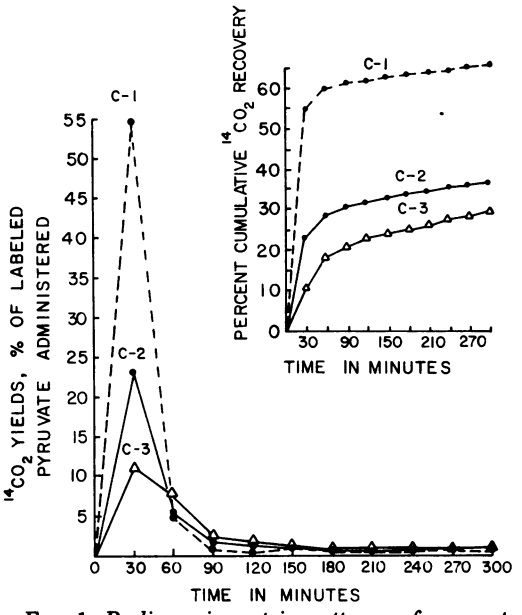

FIG. 1. *Radiorespirometric patterns of pyruvate utilization by glucose-grown cells of R. japonicum type I-110. C-1, C-2, and C-3 (C1, C2, C3 in text) designate specifically labeled carbon atoms.*

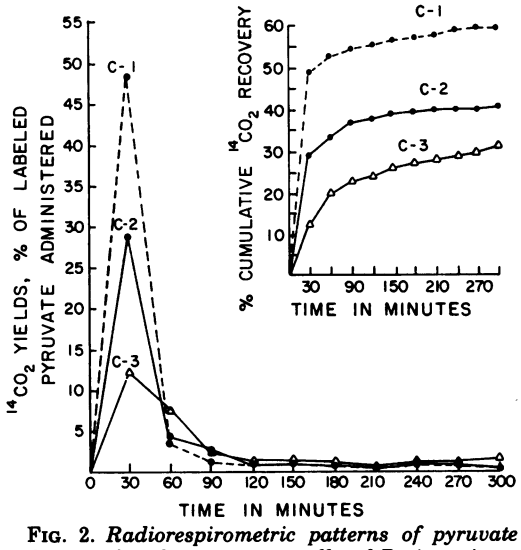

FIG. 2. *Radiorespirometric patterns of pyruvate utilization by glucose-grown cells of R. japonicum type L1-110. C-1, C-2, and C-3 (C1, C2, C3 in text) designate specifically labeled carbon atoms.*

C3 and C4 of glucose to $^{14}CO_2$ indicates the importance of the EMP pathway. Glycolytic reactions convert glucose to two molecules of pyruvate in which the carboxyl groups are derived from C3 and C4 of glucose. In the tricarboxylic acid cycle, the carboxyl carbons of pyruvate, cor-

TABLE 3. *Utilization of ^{14}C-labeled substrates by glucose-grown cells of R. japonicum derivatives I-110 and L1-110*

Derivative	Substrate[a]	Radioactive inventory[b]			Total ^{14}C recovery (%)
		Respiratory CO_2 (%)	Cells (%)	Medium (%)	
L1-110	[1-^{14}C]glucose	42	53	2	97
	[2-^{14}C]glucose	36	60	2	98
	[3,4-^{14}C]glucose	49	49	1	99
	[6-^{14}C]glucose	33	64	2	99
	[1-^{14}C]pyruvate	59	10	33	102
	[2-^{14}C]pyruvate	41	21	38	100
	[3-^{14}C]pyruvate	31	39	31	101
I-110	[1-^{14}C]glucose	39	60	2	101
	[2-^{14}C]glucose	30	70	2	102
	[3,4-^{14}C]glucose	70	26	2	98
	[6-^{14}C]glucose	29	72	2	103
	[1-^{14}C]pyruvate	66	7	29	102
	[2-^{14}C]pyruvate	37	29	31	97
	[3-^{14}C]pyruvate	26	42	29	97

[a] A 7.5-μmol amount of unlabeled substrate was added in each trial.
[b] Determined at end of the experiment.

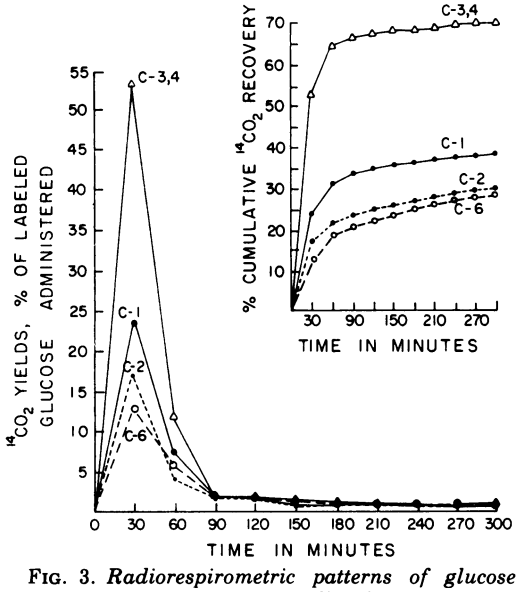

FIG. 3. *Radiorespirometric patterns of glucose utilization by glucose-grown cells of R. japonicum type I-110. C-1, C-2, C-3,4, and C-6 (C1, C2, C3,4, C6 in text) designate specifically labeled carbon atoms.*

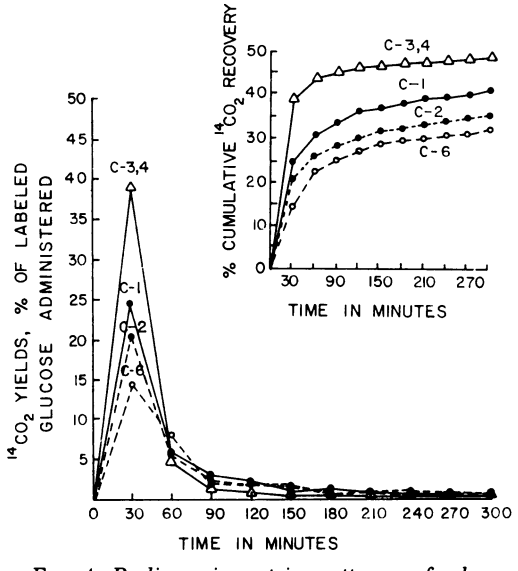

FIG. 4. *Radiorespirometric patterns of glucose utilization by glucose-grown cells of R. japonicum type L1-110. C-1, C-2, C-3,4, and C-6 (C1, C2, C3,4, C6 in text) designate specifically labeled carbon atoms.*

responding to C3 and C4 of glucose, evolve as CO_2 before the other two carbons. Theoretical rates of CO_2 evolution in the EMP pathway are: C3 = C4 > C2 > C1 = C6.

Since glucose C4 is oxidized to CO_2 more extensively than either C3 or C6, and glucose C1 more than either C2 or C6, the ED pathway obviously participates in the breakdown of glucose. In the latter pathway, an aldolase reaction splits the 6-carbon compound between C3 and C4 to yield a molecule of pyruvate in which the carboxyl group is derived from C1 of glucose and a molecule of GAP is subsequently converted, by a sequence of glycolytic enzymes, to pyruvate, whose carboxyl carbon corresponds to C4 of the original glucose. Theoretical rates of CO_2 evolution in this pathway are as follows: C1 = C4 > C2 > C3 = C6.

Thus, concomitant operation of the EMP and ED pathways during glucose catabolism

would yield sequences of CO_2 evolution matching those obtained for *R. japonicum* isolates I-110 and L1-110. Detection of all the enzymes of the EMP and ED pathways in these organisms (Table 1) provides additional evidence for the possibility of simultaneous operation of these pathways.

It is not clear, from the present data, whether the hexose cycle or the "ketogluconate pathway" also participates in the oxidation of glucose. Yet, gluconeogenetic enzymes from FDP aldolase have been detected, and $^{14}CO_2$ yields from glucose C6 are relatively slow as compared to the evolution of $^{14}CO_2$ from the first carbon; this observation might suggest that, after initial breakdown of glucose, the portion containing the sixth carbon of glucose is recycled in pathways such as the hexose cycle. Occurrence of this pathway, however, at the same time as the EMP pathway is justifiable provided that the glycolytic enzymes and the gluconeogenetic system from GAP are located in distinct compartments.

On the other hand, when cells grown in glucose medium, supplemented with mannitol to stimulate the NAD-linked 6-PG dehydrogenase, are used in the radiorespirometric experiments, a 1.2-fold increase, relative to the $^{14}CO_2$ values from glucose C3,4, is noticeable in the formation of CO_2 from glucose C1 and C6. This increase is likely a result of a 1.7- to 2.0-fold induction of 6-PG dehydrogenase and, thus, indicates that the unidentified pathway initiated by the NAD-linked 6-PG dehydrogenase is involved in *R. japonicum* glucose catabolism. At any rate, the latter pathway and the hexose cycle play only minor roles if they function in the dissimilation of glucose; as a matter of fact, they affect very little the overall radiorespirometric patterns resulting from simultaneous operation of the EMP and ED pathways.

The theoretical rates of $^{14}CO_2$ evolution in the PP pathway are: C1 > C2 > C3 > C6 > C4 (15) in the PP cycle in which the hexose phosphate and triose phosphate resulting from transaldolization and transketolization are recycled in the pathway; or C1 > C2 > C3 > C4 > C6 (8) if only the hexose phosphate pool is recycled while triose phosphates are converted to pyruvate and subsequently oxidized in the tricarboxylic acid cycle; or C1 > C4 > C3 > C2 and C4 > C6 in the case that the hexose phosphate and triose phosphate issued from the PP pathway are converted to pyruvate and then oxidized in the tricarboxylic acid cycle. Since radiorespirometric data presented in Fig. 3 and 4 are not consistent with either of these PP pathway theoretical patterns, it can be concluded, in connection with lack of the decarboxylating 6-PG dehydrogenase, that the PP pathway is absent in *R. japonicum*. Keele et al. reached the same conclusion (7, 8).

Comparison of isolates I-110 and L1-110 glucose catabolism. Radiorespirometric and enzymatic analyses reveal an active tricarboxylic acid cycle in both derivatives of *R. japonicum* strain 3I1b110. Whereas the extent of pyruvate decarboxylation is greater in isolate I-110, this derivative produces less $^{14}CO_2$ from either C2 or C3 of pyruvate (Fig. 1 and 2) and incorporates these carbons into cellular materials to a greater extent than does isolate L1-110 (Table 3). These observations suggest that, in isolate I-110, intermediates of the tricarboxylic acid cycle (including acetyl coenzyme A [unpublished data]) are more readily routed into biosynthetic processes than they are in isolate L1-110. Our enzyme study included only two enzymes of the tricarboxylic acid cycle (Table 1) and does not provide information to support our conclusion that deviation of tricarboxylic acid cycle intermediates is more important in isolate I-110.

In Fig. 3 and 4, $^{14}CO_2$ evolution from C3,4 of glucose, in isolate I-110, exceeds that observed in isolate L1-110, whereas the latter derivative releases more $^{14}CO_2$ from either C2 or C3 of glucose; in addition, greater amounts of C2 or C6 of glucose are recovered in the cellular materials of isolate I-110 than in the cell fraction of isolate L1-110 (Table 3). These findings are consistent with concurrent operation of the EMP and ED pathways in connection with the tricarboxylic acid cycle. As a matter of fact, when glucose is catabolized by the EMP and ED pathways, its C2 and C6 always appear as the second and third carbons of pyruvate, respectively, whereas glucose C3 and C4 derived from the EMP pathway and glucose C1 and C4 metabolized by the ED pathway appear in the carboxyl group of pyruvate. In turn, when pyruvate issued from these pathways is oxidized in the tricarboxylic acid cycle, the extent of CO_2 production from the acetyl carbons of pyruvate, corresponding to C2 or C6 of glucose, is expectedly greater in isolate L1-110 than in isolate I-110.

In the same view, the amount of CO_2 evolving from the pyruvate carboxylic group derived from C3,4 or C1 of glucose should be greater in isolate I-110 than in isolate L1-110. This expected difference is true only in the case of C3,4 of glucose. The ratio of respiratory $^{14}CO_2$ evolved from glucose C1 in derivatives L1-110 and I-110 is approximately 1.1 and thus indicates that participation of the ED pathway to the breakdown of glucose is greater in isolate L1-110 than it is in isolate I-110. It can also be

concluded that the EMP pathway is utilized to a lesser degree in isolate L1-110 than in isolate I-110, on the basis of the ratio of CO_2 evolved from C3,4 of glucose in these organisms.

To approximate roughly, in each isolate, the participation of the individual pathways in glucose catabolism when only the EMP and ED pathways are operative, we have used the ratio between cumulative CO_2 yields from C1 and C3 of glucose. The C1/C3 ratio indicates the degree to which the two pathways (ED versus EMP) are operating. The major weakness of this approximation lies in the possible deviation of the glycolytic triose phosphates into anabolic processes, resulting in an underevaluation of the C3 values and the corresponding overestimation of the ED pathway value. C1/C3 < 1, in isolate I-110; therefore, the EMP pathway is predominant in this derivative. In contrast, the ED pathway appears slightly predominant in isolate L1-110 since C1/C3 ≥ 1. As stated above, all the enzymes of the ED and EMP pathways have been detected in both derivatives; enolase activity is relatively low in isolate L1-110. This enzyme might be the rate-limiting step in the flow of carbons through the EMP pathway and account for enhanced importance of the ED pathway in *R. japonicum* isolate L1-110. It should be noted, in addition, that regulation of G-6-P dehydrogenase and G-6-P isomerase becomes of great importance, since these enzymes monitor the channelling of glucose into the ED or the EMP pathways.

Finally, it should be pointed out that derivative I-110 is endowed with a more efficient energy-providing catabolic system than isolate L1-110 since it utilizes preferentially the EMP pathway, a mechanism known to provide more energy in the form of adenosine triphosphate and reduced NAD than the ED pathway (2), and since the sum of the cumulative respiratory $^{14}CO_2$ values shows that this variant oxidizes glucose to a somewhat greater extent than isolate L1-110. Coincidentally, isolate I-110 has a 1.5- to 1.7-fold shorter generation time on glucose (9; unpublished data) and 5- to 10-fold greater nitrogen-fixing efficiencies than isolate L1-110. Comparison of glucose oxidation to CO_2 in variants of other strains of *R. japonicum*, namely, strains 3I1b125 and 3I1b140 (obtained from the U.S. Department of Agriculture, Beltsville Culture Collection), shows that derivatives characterized by greater nitrogen-fixing efficiency also oxidize glucose 1.2- to 1.5-fold more extensively than those with lower nitrogen-fixing ability (unpublished data); the identity and relative participation of the catabolic pathways in these colonial derivatives have not been determined.

Radiorespirometric analyses presented here reveal that *R. japonicum* isolates I-110 and L1-110 dissimilate glucose by concurrent operation of the EMP and ED pathways followed by the tricarboxylic acid cycle. The presence of all the enzymes of the ED and EMP pathways, as well as comparison of the radiorespirometric events in types I-110 and L1-110, supports this conclusion. The $^{14}CO_2$ evolution pattern of glucose catabolism found by Keele et al. (7) in growing cells of *R. japonicum* ATCC 10324 is identical to the sequence obtained with isolate L1-110, indicating the metabolic relatedness between *R. japonicum* strain 3I1b110 and the ATCC strain. It is likely that enolase, G-6-P dehydrogenase, and G-6-P isomerase regulate the flow of glucose through the EMP and the ED pathways.

Operation of the hexose cycle is implied by the finding of an active hexose diphosphatase and by the slow rates of $^{14}CO_2$ yields from glucose C6 as compared to the evolution of $^{14}CO_2$ from glucose C1. Simultaneous occurrence of the hexose cycle and the EMP pathways is justifiable only if they are located in discrete compartments.

Present data do not clearly demonstrate or invalidate the possibility of an unknown pathway initiated by the NAD-linked 6-PG dehydrogenase. This enzyme is constitutive in both derivatives of *R. japonicum* strain 3I1b110 and is stimulated by mannitol and fructose. The absence of the decarboxylating NADP-specific 6-PG dehydrogenase in these organisms indicates that they do not possess an operative PP pathway; they may, however, synthesize the precursors for purines and pyrimidines by means of transketolase and transaldolase reactions.

It has been observed that *R. japonicum* derivative I-110, characterized by greater nitrogen-fixing efficiency and shorter generation times on glucose, oxidizes glucose to a greater extent than isolate L1-110; it also utilizes preferentially the EMP pathway, known as a more efficient source of energy than the ED pathway. The latter mechanism apparently predominates in isolate L1-110.

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