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Pathways of Carbohydrate Metabolism in *Microcyclus* Species

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Radiorespirometric and enzymatic studies were conducted to determine primary and secondary pathways of carbohydrate catabolism in *Microcyclus aquaticus* and *M. flavus*. *M. aquaticus* catabolizes both glucose and gluconate mainly via the Entner-Doudoroff and pentose phosphate pathways with some concurrent participation of the Embden-Meyerhof pathway. *M. flavus*, however, oxidizes glucose mainly via the Embden-Meyerhof pathway and gluconate via the Entner-Doudoroff pathway with some simultaneous operation of the pentose phosphate pathway. Both of the organisms showed evidence of the tricarboxylic acid cycle as a secondary pathway for the oxidation of carbohydrates.

The little-known genus *Microcyclus* includes only two species, *M. flavus* and *M. aquaticus* (1, 21). The organisms in this genus are unique because of their closed ringlike structure and other morphological variations of the basic ring curvature. Very little is known with respect to the metabolism of these aquatic bacteria. In the present work, both radiorespirometric and enzymatic studies were carried out to determine the patterns of primary and secondary pathways of carbohydrate catabolism in these organisms. Part of this work was presented at the 72nd Annual Meeting of the American Society for Microbiology, Philadelphia, Pa., 23-25 April 1972.

MATERIALS AND METHODS

Bacteria used and cultural conditions. Two *Microcyclus* species, *M. aquaticus* (Ørskov, ATCC 25396) and *M. flavus* (Raj, ATCC 23276), described taxonomically elsewhere by Raj (21) were used in this study to determine the pathways of carbohydrate catabolism operative in these organisms. These freshwater bacteria were maintained on slants of tryptone glucose extract agar (Difco) plus 0.1% yeast extract (TGEY) at room temperature.

Radiorespirometric studies. An inoculum was prepared by transferring a 24-hr slant culture of each organism to 100 ml of broth of the same medium. It was then incubated at room temperature on a wrist-action shaker overnight. The resulting culture was transferred to 500 ml of fresh broth and incubated as before for an additional 20 hr. The cells were harvested by centrifugation and washed twice with growth medium from which glucose was omitted. Finally the cell crop was adjusted to 25 mg (dry weight) in 10 ml of the above medium and used as an inoculum for radiorespirometric studies. Practically all of the radioactive substrates used in this study were obtained commercially, except for [2-^{14}C] gluconate and [3,4-^{14}C]gluconate which were prepared from correspondingly labeled glucose by the method of Moore and Link (18). All of the labeled substrates were adjusted to prescribed specific activities prior to administration. The radiorespirometric procedures followed were the same as previously reported from this laboratory (20). Because the radiorespirometric technique essentially involves a time-course experiment, the percent interval recoveries of respiratory $^{14}CO_2$ when plotted against time revealed the kinetic aspects as well as the overall patterns of substrate utilization. Also, by plotting the percent cumulative $^{14}CO_2$ recoveries against time, the nature as well as the extent of the pathway(s) involved in the overall catabolism of the administered substrate can be estimated.

To compensate for differences in the rate of oxidation of different substrates used, a relative time unit (RTU), defined previously (20) as the time required in a given experiment for an organism to consume all of the intact labeled substrate originally added, is shown in the respective graph of each substrate. Because the cumulative respiratory $^{14}CO_2$ recoveries indicate the complete utilization of the substrate, there was no need to assay for any substrate left in the medium at the termination of each experiment.

Because $^{14}C_3$ substrates were not used, the C_3 recovery was calculated separately from that of C_4 by the method of Kitos et al. (13). By this method the C_3 recovery was calculated separately from that of C_4 as if these carbon atoms were channeled solely via the pentose phosphate (PP) pathway as follows: $C_4 = (C_6 \times C_2)/(C_1)$ and $C_3 = 2(C_{3,4}) - (C_4)$.

Also, for the estimation of pathway participation in an organism that utilized Entner-Doudoroff (ED) and PP pathways, the following formulas suggested by

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Stern, Wang and Gilmour (22) were used: $G_{ED} = 1 - (G_{PP})$ and $G_{PP} = (C_1) - (C_4)$, where G_{PP} and G_{ED} stand for glucose (or gluconate) oxidized via the PP and ED pathways, respectively, and where $C_4 = 2(C_{3,4}) - (C_6)$. The subscript numeral shown with each carbon atom (C) indicates the specific position of such an atom in a substrate molecule.

For the estimation of pathway participation in an organism that utilizes both the Embden-Meyerhof (EM) and the PP pathways the formula $G_{EM} = 1 - (G_{PP})$ where $G_{PP} = (C_1) - (C_6)$ was used as suggested by Cheldelin (2).

Enzymatic studies. A slant culture of each organism, maintained on TGEY agar with either glucose or gluconate as the primary carbohydrate source and subcultured twice on the same medium at room temperature for 40 to 48 hr, was used as inoculum. In each case, the slant culture was then transferred to 100 ml of the same broth medium and incubated on a rotary shaker at room temperature for 48 hr. After a purity check by microscopy examination, the broth culture was centrifuged at 8,000 $\times$ *g* for 10 min to harvest the cell crop. The pelleted cells were washed twice in 0.2 M phosphate buffer (*p*H 7.0), resuspended in the above buffer, and adjusted to an OD of 1.0 by using a Klett-Summerson colorimeter fitted with a green filter (no. 54). For the preparation of crude extracts, a 5.0-ml sample of the above cell suspension was placed into a precooled vial maintained in an acetone-ice bath and disrupted by using a Branson sonifier model S-75 with a 0.5-inch (12.7 mm) tip. The sonic treatment was carried out at 20 kc in a succession of 30-sec exposures for a total of 5 min.

The broken-cell suspension, when examined photometrically and microscopically, revealed complete disruption of the cells. It was then transferred to a Nalgene centrifuge tube and centrifuged at 30,000 $\times$ *g* for 30 min at 4 C in a Sorvall RC 2-B superspeed refrigerated centrifuge. The resulting supernatant fraction (1.1 mg of protein per ml) was used immediately as a crude extract for assay of enzymatic activity.

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activity was determined at 340 nm by the method of Kuby and Noltman (15) by using 0.1 ml of crude extract as described by Keele, Hamilton, and Elkan (11). 6-Phosphogluconate dehydrogenase (EC 1.1.1.44) was determined by the method of Pontremoli and Grazi (19) at 340 nm. The reaction mixture contained glycylglycine buffer, *p*H 7.6, 75 μmoles; gluconate-6-phosphate (Sigma), 4.5 μmoles; nicotinamide adenine dinucleotide phosphate (NADP), 0.9 μmoles; $MgCl_2$, 60 μmoles; crude extract, 0.1 ml; and water to a total volume of 3.0 ml. Conversion of 6-phosphogluconate to pyruvate was performed by the method of Katznelson (9) by using his reaction mixture with 0.2 ml of crude extract. Pyruvate produced from this reaction was determined by using the method of Keele, Hamilton, and Elkan (11) at 420 nm. Transketolase (EC 2.2.1.1) activity was determined by coupling formation of 3-phosphoglyceraldehyde from ribose-5-phosphate to triosephosphate isomerase and α-glycero-phosphate dehydrogenase by the method of Gale and Beck (7) at 340 nm. The reaction mixture was identical to that described previously (11), using 0.1 ml of crude extract. Fructose-1,6-diphosphate aldolase (EC 4.1.2.13) was assayed at 340 nm by the method of Taylor (24) by using his reaction mixture with 0.1 ml of crude extract. Isocitrate dehydrogenase (EC 1.1.1.42) was assayed by the method of Cleveland, Thompson, and Barden (3). The reaction mixture described by these workers was modified slightly to include glycylglycine buffer, *p*H 7.6, 50 μmoles; DL-threoisocitrate (Psaltz-Bauer), 4 μmoles; NADP (Sigma), 0.3 μmoles; $MgCl_2$, 20 μmoles; crude extract, 0.1 ml; and water to a total volume of 3.0 ml to prevent precipitation. Malate dehydrogenase (EC 1.1.1.37) activity was determined by the method of Kitto (14) at 340 nm by using the same reaction system. Glutamate-oxalacetate transminase (EC 2.6.1.1) and glutamate-pyruvate transaminase (EC 2.7.1.2) were assayed by the method of Karmen (8) at 520 nm by using a Sigma 505 standard kit. The reaction mixtures were the same as described elsewhere (Sigma technical bulletin no. 505, Sigma Chemical Co., St. Louis, Mo.), by using 0.2 ml of crude extract.

Spectrophotometric readings for all of the above enzymatic assays were performed by using a Beckman DU-2 spectrophotometer at the wavelengths specified. Protein content of the crude extracts was determined by the method of Lowry et al. (16). At least duplicate determinations were made on different days for each enzymatic assay, and values obtained within the experimental margin of reproducibility ($\pm$10%) were averaged and expressed as specific activity as shown in Table 2.

RESULTS AND DISCUSSION

Glucose catabolism by M. aquaticus. Figure 1 shows the preferential oxidation of labeled carbon atoms of glucose and radiochemical recoveries of respiratory CO_2 by *M. aquaticus* yielding a pattern of $C_1 > C_2 > C_{3,4} > C_6$. It is apparent that the EM pathway was not operative in this organism because by glycolysis $^{14}CO_2$ would be expected to arise from C_3 and C_4 in preference to C_1. The greater recovery of C_1 than of any other carbon seems to indicate the operation of the PP pathway in this organism. Glucose, when oxidized primarily via the PP pathway, yields preferential $^{14}CO_2$ recoveries in the order of $C_1 > C_2 > C_3 > C_6 > C_5 > C_4$. This implies that for every hexose that enters that PP pathway C_1 will appear first as CO_2 followed by C_2 and then C_3 , retaining the last three carbons (4-5-6) intact in a triose. Only when two such trioses are condensed to form a hexose will C_6 appear as CO_2, followed by C_5 and then by C_4 as illustrated by Raj (20). This would also imply that respiratory CO_2 from these carbon atoms would be practically insignificant by this pathway. However, if C_3 and C_4 recoveries were calculated separately from the

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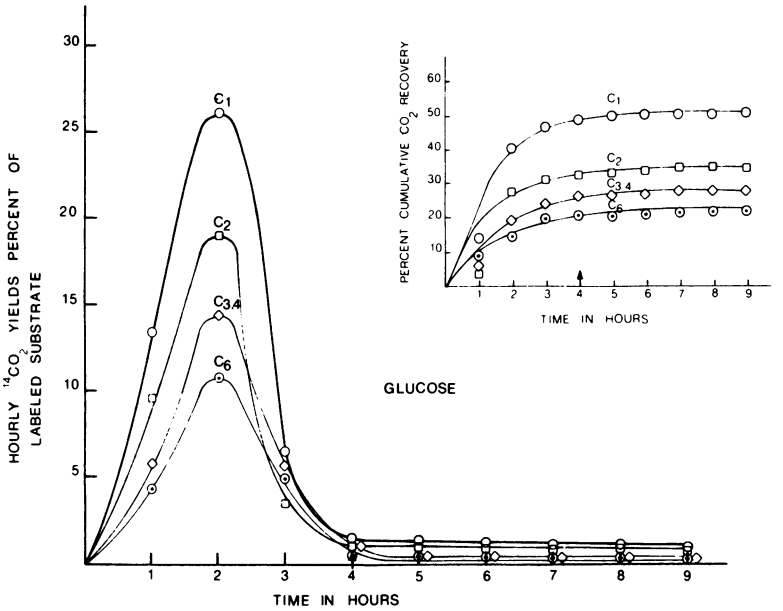

FIG. 1. *Radiorespirometric patterns for the utilization of glucose by M. aquaticus. Arrow indicates a relative time unit of 4 hr.*

cumulative $^{14}CO_2$ recoveries obtained from glucose (Table 1), it was estimated that when these carbon atoms are channeled solely via the PP pathway, the maximum C_4 recovery would be only 16% and the minimum C_3 recovery would be 40% implying, thereby, that the $^{14}CO_2$ recoveries from C_3 (40%) would be higher than that of C_2 (33%). Because it is not possible for C_3 recovery to exceed that of C_2 by the PP pathway, it seems unlikely that the PP pathway is involved as a major primary route of glucose oxidation in this organism.

Because the EM and PP pathways did not seem to be primarily involved in the catabolism of glucose by *M. aquaticus*, the formulas for the estimation of concurrent participation of the ED and PP pathways were applied. The C_3 recovery as $^{14}CO_2$ from glucose was thus calculated to be 25% and C_4 recovery 31%. Considering these values, it is obvious that a $C_{3,4}$ recovery of 28% obtained here was primarily due to the greater recovery of C_4 than of C_3. It is expected on the basis of the ED pathway to have catabolic equivalences between C_1 and C_4 and between C_3 and C_6 with respect to respiratory CO_2. As may be noted from Table 1, the recovery of C_1 or C_4 was greater than that of any other carbon of glucose, and the recovery of C_3 was equal to that of C_6, suggesting operation of the ED pathway. However, the recovery of C_1 (50%) was greater than that of C_4 (31%), indicating concurrent operation of the PP pathway in this organism. It was thus estimated that 81% of the administered glucose was catabolized via the ED pathway as the major primary route and 19% via the PP pathway as the minor primary route. The operation of these metabolic pathways in this organism is indicated by the presence of glucose-6-phosphate dehydrogenase (Table 2). Furthermore, evidence for the operation of the ED pathway is shown by the presence of 6-phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase which convert 6-phosphogluconate to pyruvate. Also, the presence of 6-phosphogluconate dehydrogenase and transketolase is consistent with PP pathway operation in this organism (Table 2).

Gluconate catabolism by M. aquaticus. The aerobic utilization of gluconate by *M. aquaticus* is shown in Fig. 2 and Table 1. There was some adaptation of the cells during the early period of respiration, possibly for the induction of necessary enzymes. The radiorespirometric data showed preferred oxidation of the labeled carbons of gluconate to CO_2 as $C_1 > C_2 > C_{3,4} > C_6$. The greater recovery of C_1 (85%) compared with any other carbon of gluconate indicates the operation of the PP pathway in this organism. By applying the formulas for the sole participation of the PP pathway (13), the C_3 recovery as $^{14}CO_2$ was calculated to be 53% and C_4 recovery 7%. This yields a respirometric pattern with greater recovery of C_3 (53%) than

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TABLE 1. *Utilization of various ^{14}C-labeled substrates*

Substrate	Amt of substrate added (mg)	*M. aquaticus* radioactivity inventory			*M. aquaticus* Total ^{14}C recovery (%)	*M. flavus* radioactivity inventory			*M. flavus* Total ^{14}C recovery (%)
		CO_2 (%)	Cells (%)	Medium (%)		CO_2 (%)	Cells (%)	Medium (%)	
Glucose-*1*-^{14}C	1,5[a]	50	16	30	96	34	46	15	95
Glucose-*2*-^{14}C	1,5	33	35	37	105	31	59	16	106
Glucose-*3,4*-^{14}C[b]	1,5	28	32	38	98	49	38	12	99
Glucose-*6*-^{14}C	1,5	25	40	27	92	30	45	20	95
Gluconate-*1*-^{14}C	1	85	2	14	101	93	2	10	105
Gluconate-*2*-^{14}C	1	36	29	35	100	28	49	17	94
Gluconate-*3,4*-^{14}C[c]	1	30	34	26	90	50	30	19	99
Gluconate-*6*-^{14}C	1	17	30	48	95	32	40	25	97
Pyruvate-*1*-^{14}C	5,2	23	2	85	110	2	99[d]		101
Pyruvate-*2*-^{14}C	5,2	18	10	70	98	4	86		90
Pyruvate-*3*-^{14}C	5,2	11	9	78	108	3	91		94
Acetate-*1*-^{14}C	2,5	25	13	57	95	74	24	6	104
Acetate-*2*-^{14}C	2,5	15	21	54	90	52	37	9	98
L-Glutamate-*1*-^{14}C	2	30	2	67	99	50	6	42	98
L-Glutamate-*2*-^{14}C	2	26	7	70	103	46	9	41	96
L-Glutamate-*3,4*-^{14}C	2	14	11	75	102	31	16	49	96
L-Glutamate-*5*-^{14}C	2	25	6	68	99	46	7	40	93

[a] Second number indicates the substrate level used for *M. flavus*.

[b] Separate C-3 and C-4 recoveries as $^{14}CO_2$ were calculated to be 25 and 31%, respectively, for *M. aquaticus* and 49 and 49%, respectively, for *M. flavus*.

[c] Separate C-3 and C-4 recoveries as $^{14}CO_2$ were calculated to be 17 and 43%, respectively, for *M. aquaticus* and 32 and 68%, respectively, for *M. flavus*.

[d] These values in parenthesis represent combined percent radiochemical recoveries obtained from both cells and medium.

TABLE 2. *Activity of key enzymes in Microcyclus spp. grown in the presence of glucose or gluconate*

Enzyme	Glucose		Gluconate	
	M. flavus	*M. aquaticus*	*M. flavus*	*M. aquaticus*
FDP aldolase[a]	42.0	11.6	24.0	6.6
Glucose-6-phosphate dehydrogenase[b]	41.6	70.6	61.3	78.0
6-Phosphogluconate dehydrogenase[b]	40.0	10.0	37.6	9.6
Transketolase[c]	5.0	16.0	27.3	34.0
6-Phosphogluconate dehydrase and KDPG aldolase[d]	0.0	22.0	4.6	16.6
Isocitrate dehydrogenase[b]	349.3	99.0	305.3	141.3
Malate dehydrogenase[c]	1790.0	3733.6	1813.3	3958.0
Glutamate-oxalacetate transaminase[e]	15.0	32.3	24.0	19.0
Glutamate-pyruvate transaminase[e]	4.0	6.6	5.3	8.0

[a] Expressed in terms of nanomoles of NAD reduced per minute per milligram of protein.

[b] Expressed in terms of nanomoles of NADP reduced per minute per milligram of protein.

[c] Expressed in terms of nanomoles of NADH oxidized per minute per milligram of protein.

[d] Expressed in terms of nanomoles of pyruvate formed per minute per milligram of protein.

[e] Expressed in terms of nanomoles of glutamate formed per minute per milligram of protein.

of C_2 (36%), substantiating the above finding with glucose that the PP pathway is not primarily and solely responsible for carbohydrate catabolism in this organism. Therefore, by applying the formulas for the estimation of concurrent participation of the ED and PP pathways, the values of C_3 and C_4 were calculated to be 17 and 43%, respectively. This yields a pattern of $C_1 > C_4 > C_2 > C_3 = C_6$ where C_1 = 85%, C_4 = 43%, C_2 = 36%, and $C_3 = C_6$ = 17%. This is consistent with the simultaneous operation of the ED and PP pathways. It was thus estimated that *M. aquaticus* metabolizes gluconate 58% via the ED pathway and 42% via the PP

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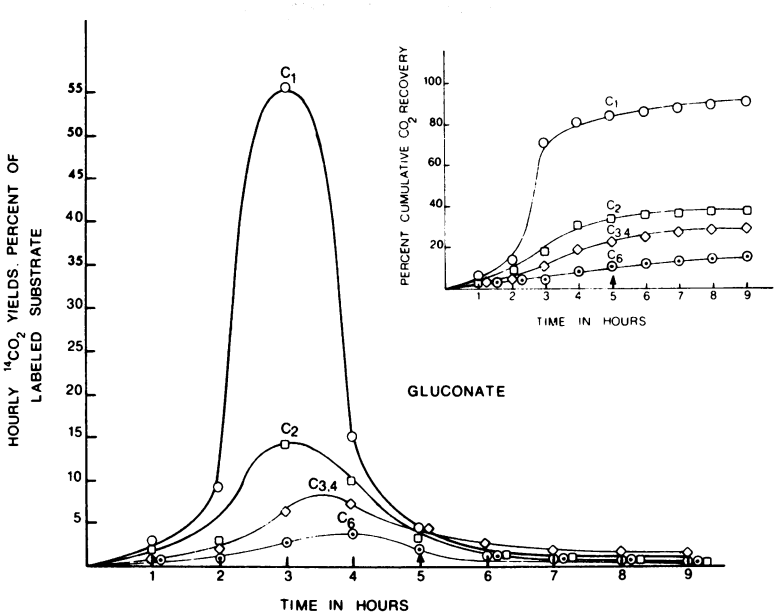

FIG. 2. *Radiorespirometric patterns for the utilization of gluconate by M. aquaticus. Arrow indicates a relative time unit of 5 hr.*

pathway. The operation of these pathways is also indicated by presence of glucose-6-phosphate dehydrogenase (Table 2). Furthermore, the presence of the key enzymes for the ED pathway (6-phosphogluconate dehydrase and KDPG aldolase) and the PP pathway (6-phosphogluconate dehydrogenase and transketolase) substantiated the above findings (Table 2).

It will be noted that the radiorespirometric data (Fig. 1 and 2) for the catabolism of both glucose and gluconate by *M. aquaticus* showed absence of EM pathway in either case. However, enzymatic assays showed the presence of fructose-1,6-diphosphate (FDP) aldolase in this organism when grown with glucose or gluconate as a substrate (Table 2). This might indicate some operation of the EM pathway in this organism for carbohydrate metabolism. However, some strains of *Pseudomonas aeruginosa* which have been reported to contain FDP aldolase do not have a functional EM pathway because of the absence of phosphofructokinase (26). Because radiorespirometric experiments were performed by using glucose or gluconate with a combined label at C_3 and C_4 (instead of using specifically labeled substrate at C_3 or C_4), it was not possible to assay accurately $^{14}CO_2$ recoveries as derived from C_3 and C_4 separately. This handicap is inherent in the mathematical formulas usually applied to estimate quantitative participation of known pathways and is based on the assumption that the EM and ED sequences are mutually exclusive (27).

Glucose catabolism by M. flavus. The differential rate of glucose oxidation by *M. flavus* yielded a higher recovery of $C_{3,4}$ as $^{14}CO_2$ than that of any other carbon, implicating the operation of the EM pathway (Fig. 3). However, because C_2 recovery should exceed that of C_1 via this pathway, the slightly greater recovery of C_1 obtained here as compared with that of C_2 indicated simultaneous participation of the PP or ED pathways, or both. Also, enzymatic studies of *M. flavus* failed to show formation of pyruvate from 6-phosphogluconate, possibly suggesting the absence of both 6-phosphogluconate dehydrase and KDPG aldolase (Table 2). Thus, the ED pathway does not seem to be operative in this organism when grown on glucose. By using the formulas for the concurrent operation of the EM and PP pathways, it was estimated that as much as 96% of the administered glucose was catabolized via the EM pathway and only 4% via the PP pathway by this bacterium. The above radiorespirometric findings were substantiated by the presence of FDP aldolase for the operation of the EM pathway and glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and transketolase for the PP pathway as shown in Table 2.

Gluconate catabolism by M. flavus. The radiorespirometric pattern for the utilization of gluconate by *M. flavus* showed preferred oxidation of C_1 as respiratory $^{14}CO_2$ in comparison with any other carbon (Fig. 4). Although the PP

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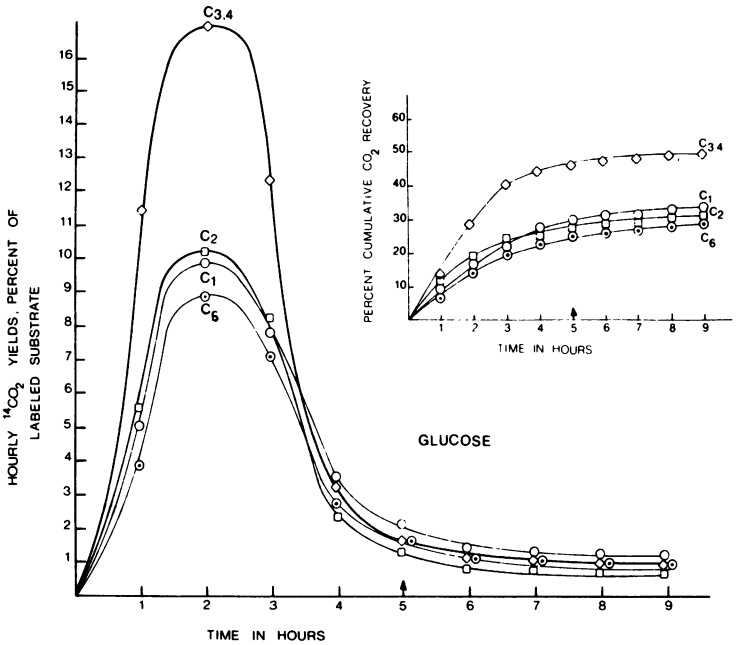

FIG. 3. *Radiorespirometric patterns for the utilization of glucose by M. flavus. Arrow indicates a relative time unit of 5 hr.*

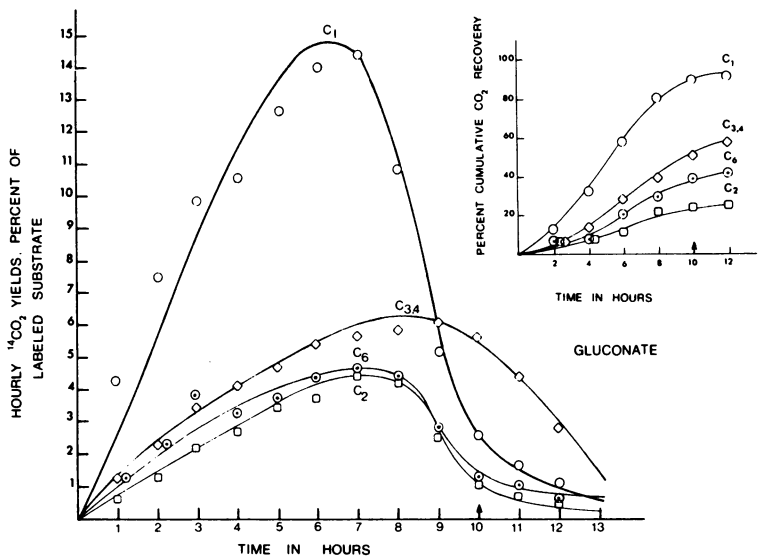

FIG. 4. *Radiorespirometric patterns for the utilization of gluconate by M. flavus. Arrow indicates a relative time unit of 10 hr.*

pathway is obvious due to the highest recovery of C_1 (93%), it seems, on closer examination, that the PP pathway is not solely responsible for gluconate oxidation in this organism. By applying the formulas for the concurrent participation of the PP and EM pathways, it was calculated that 61% of the gluconate administered could be catabolized via the PP pathway yielding percent recoveries of C_1, 57%; C_3, 30%; C_6, 19%; C_2, 17%; and 39% via the EM pathway giving percent recoveries of C_4, 50%; C_1, 36%; C_6, 13%; and C_2, 11%. Because it is not possible for $^{14}CO_2$ recovery from C_3 and C_6 to exceed that from C_2 via the PP pathway, it

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seems unlikely that only these two pathways are responsible for gluconate oxidation. Thus, by using the formulas for simultaneous operation of the ED and PP pathways (22) C_4 recovery as $^{14}CO_2$ was 68% and that of C_3 was 32%. By using these values, it was then calculated that 75% of the gluconate administered could be oxidized via the ED pathway giving percent recoveries of C_1, 70%; C_4, 68%; $C_3 = C_6$, 24%; C_2, 21%; and 25% via the PP pathway yielding percent recoveries of C_1, 23%; $C_3 = C_6$, 8%; C_2, 7%. The slightly higher recovery of C_6 than of C_2, noted above, may be explained by a possibility that a hexosediphosphate with a carbon skeleton of 6-5-4-4-5-6 formed from two such trioses (3-phosphoglyceraldehyde) via the PP pathway could yield additional recovery of C_6. Also, slightly higher recovery of C_3 than of C_2 as noted above cannot be justified on the basis of ED or PP pathway. However, it seems likely that a significant portion of gluconate is being oxidized via these pathways and returned as glucose-6-phosphate through fructose-6-phosphate. The recycled glucose-6-phosphate could then be catabolized via the EM pathway, giving somewhat higher yields of C_3 than that of C_2. The FDP aldolase activity obtained in this organism when grown on gluconate might be due to the involvement of the EM pathway (Table 2). Furthermore, PP pathway operation is substantiated by the presence of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and transketolase, and ED pathway operation is substantiated by the presence of both 6-phosphogluconate dehydrase and KDPG aldolase (Table 2).

Tricarboxylic acid cycle as a secondary pathway. Because it is clear from the above data that the ED or the EM pathway or both are involved in carbohydrate catabolism in these organisms, the operation of the tricarboxylic acid cycle as a secondary pathway was determined by radiorespirometry and enzymatic assays. Figure 5 shows the aerobic utilization of L-glutamate by *M. aquaticus*. *M. flavus* also utilized L-glutamate, yielding similar radiorespirometric patterns for $^{14}CO_2$ release where $C_1 > C_2 = C_5 > C_{3,4}$. Cumulative recoveries of specifically labeled carbons of glutamate are shown in Table 1. These findings are consistent with the expected pattern of glutamate oxidation via the tricarboxylic acid cycle. Also, *M. aquaticus* utilized pyruvate, readily yielding differential rate of $C_1 > C_2 > C_3$ as respiratory CO_2 via the tricarboxylic acid cycle. *M. flavus*, however, failed to utilize pyruvate, possibly due to permeability problems (21). By using acetate as a substrate both organisms showed preferential oxidation of C_1 to C_2. It seems that acetate is primarily derived by oxidative decarboxylation of pyruvate and catabolized further by way of the tricarboxylic acid cycle. At least in the case of *M. aquaticus* this is substantiated by the recovery ratio (1.66) of C_1 to C_2 of acetate which is equal to that of C_2 to C_3 of pyruvate. Similar results

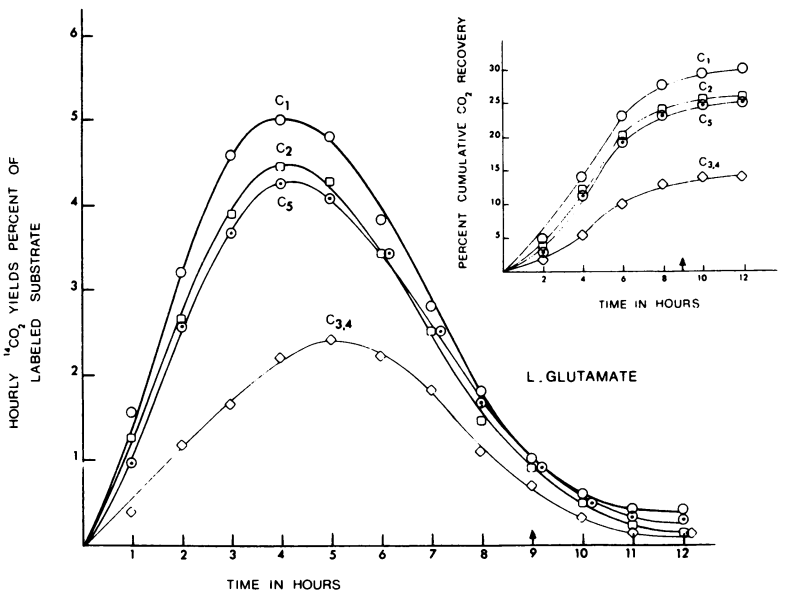

FIG. 5. *Radiorespirometric patterns for the utilization of L-glutamate by M. aquaticus. Arrow indicates a relative time unit of 9 hr.*

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were obtained by Raj (20), Keele et al. (11), and Stern et al. (22). Furthermore, the presence of key enzymes such as isocitrate dehydrogenase, malate dehydrogenase, glutamate-oxalacetate, and glutamate-pyruvate transaminases substantiates the operation of the tricarboxylic acid cycle in these organisms (Table 2). It may be noted that the values of glutamate-oxalacetate transaminase were higher in all cases than those of glutamate-pyruvate transaminase. Similar results have been reported for *Saccharomyces cerevisiae* (25). Specific activities of both dehydrogenases were remarkably high in accordance with the oxidative functions inherent in this pathway. The consistently higher values for malate dehydrogenase than those for isocitrate dehydrogenase may indicate a possibility of the glyoxylate bypass. Similar results for these two enzymes in *Rhizobium* species have been reported by Martinez-De Drets and Arias (17). However, because the glyoxylate bypass is normally induced when cells are grown on acetate or other two-carbon compounds, one would not expect to find appreciable glyoxylate bypass in these cells when grown on glucose or gluconate. Thus, as expected, preliminary assay of malate synthetase (responsible for the condensation of acetyl Co-A and glyoxylate to form malate) failed to show any activity in either organism.

As shown in Table 1, low yields of respiratory CO_2 from certain carbon atoms of various substrates utilized by these bacteria were compensated by their proportionately higher cellular incorporations and vice versa. In the case of glucose or gluconate, *M. aquaticus* showed heavy retention of C_2, $C_{3,4}$, or C_6 as compared with C_1, whereas *M. flavus* incorporated C_1, C_2, or C_6 more than $C_{3,4}$. However, both organisms showed greater incorporation of C_2 than C_1 of acetate and $C_{3,4}$ more than any other carbon of glutamate. As expected, these findings indicate the greater conservation of the methyl carbon atom (C_2 of acetate and $C_{3,4}$ of glutamate) than the carboxyl carbons of these substrates for cellular biosynthesis via the tricarboxylic acid cycle.

Species differences. In a taxometric study of these aquatic bacteria, Raj (21) reported that *M. aquaticus* and *M. flavus* showed a similarity index (on the basis of positive features shared) of only 42%. Because this index is far below that expected of strains within the same species, but is significant enough to justify the same genus status, he concluded that *M. flavus* should be recognized as a separate species within the same genus. This conclusion is further supported by our findings in radiorespirometric and enzymatic studies which indicated certain metabolic differences between these two species. *M. aquaticus* showed evidence for the concurrent operation of the three primary catabolic pathways (ED, PP, EM) whether grown in the presence of glucose or gluconate. *M. flavus* when grown on glucose showed simultaneous participation of the EM and PP pathways, but lacked the enzymes for the ED pathway. However, when grown in the presence of gluconate, it showed evidence for the operation of the ED in addition to the above two pathways. That gluconate seems to induce the ED pathway in this organism is shown by both the radiorespirometric and enzymatic data (Tables 1 and 2). The possibility of such an induction has been shown in *Escherichia coli* (5), *Pseudomonas natriegens* (4) and *Rhizobium japonicum* (12). Similarly, Fraenkel and Horecher (6) had shown earlier that wild and mutant strains of *S. typhimurium* catabolized 20% of the administered glucose via the PP pathway and the remainder via the EM pathway, whereas both strains metabolized gluconate solely via the ED pathway. Evidence obtained here for the operation of the three primary pathways (EM, ED, and PP) in both of the organisms is in agreement with the findings reported in the literature for *Rhizobium* species (9, 10). This is in contrast to the reports of Still and Wang (23) who had suggested, on the basis of radiorespirometric studies, that the ED and EM pathways do not operate simultaneously in microorganisms.

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