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$^{14}CO_2$ Fixation, Glutamate Labeling, and the Krebs Cycle in Ribose-grown *Hydrogenomonas facilis*

BRUCE A. McFADDEN,[1] GLENN D. KUEHN,[2] AND H. ROBERT HOMANN[3]
Department of Chemistry, Washington State University, Pullman, Washington

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Exposure of ribose-grown *Hydrogenomonas facilis* to $^{14}CO_2$ for 6 to 12 sec during ribose oxidation resulted in labeling of a number of compounds, three of which were glutamate, phosphoglycerate, and pyruvate. Phosphoglycerate and pyruvate were labeled almost exclusively in C_1, suggesting operation of the reductive pentose phosphate cycle. Glutamate was labeled initially to the extent of 90% in C_1 and 10% in C_5, and this was followed by a concentration of radioisotope in C_5. All of the enzymes of the tricarboxylic acid cycle were detectable in ribose-grown cells, and, in general, specific activities were similar to those found in yeast extract-grown cells. Reduced nicotinamide adenine dinucleotide oxidase, aconitase, and the dehydrogenases for pyruvate, α-ketoglutarate, and succinate appeared to be of particulate origin. In addition to enzymes of the tricarboxylic acid cycle, an acetyl coenzyme A-stimulated phosphoenolpyruvate carboxylase was found, as was isocitrate lyase. Possible participation of these catalysts in glutamate synthesis is discussed.

The mechanism by which carbon dioxide fixation occurs when it is coupled with the oxidation of organic material in facultative autotrophs such as the hydrogen bacteria poses some interesting questions in regulatory biology. Answers may provide a better insight into the molecular basis for heterotrophism and autotrophism.

Our studies to date have focused upon fixation of $^{14}CO_2$ by intact cells of *Hydrogenomonas facilis*. Cells grown, washed, and presented with the growth substrate under growth conditions coupled ribose oxidation with $^{14}CO_2$ fixation very efficiently (15). These studies were extended to investigate the mechanism of $^{14}CO_2$ fixation in very short fixation times (16). Fixation of radiocarbon into glutamate that was dependent upon the oxidation of ribose was observed. In addition, total incorporation was markedly inhibited by uncouplers of oxidative phosphorylation. Thus, characteristics of the observed incorporation of ^{14}C into glutamate suggested that it might be of importance in the biosynthesis of glutamate. Of interest was the fact that intermediates of the tricarboxylic acid cycle were not detectably labeled. Exhaustive efforts to identify the very early products of carbon dioxide fixation were unsuccessful. However, the relatively early labeling of glutamate and of phosphoglycerate and metabolically related C_3 compounds afforded a means by which the mechanism of CO_2 fixation could be examined.

The present communication describes results of the degradation of glutamate, phosphoglycerate, and pyruvate, all of which were labeled in *H. facilis* after short $^{14}CO_2$ fixation times during ribose oxidation. In addition, results of assays for enzymes of the tricarboxylic acid cycle and of ancillary paths are reported. Because it was of interest to determine whether the synthesis of these enzymes was repressed by growth on ribose, the levels in extracts from ribose-grown cells were compared with those after growth on a rich medium. Yeast extract was chosen as the rich medium because it supports two to three times faster aerobic growth than ribose at equivalent concentrations.

MATERIALS AND METHODS

Culture conditions and $^{14}CO_2$ incorporation experiments. Growth of *H. facilis* on D-ribose was as described earlier (16), and involved transfer from an autotrophic culture to the preculture medium described. After growth, the preculture was transferred to 5 volumes of identical medium. The same general procedure was used for culture on 0.3% yeast extract (Difco) in tap water.

All methods employed for $H^{14}CO_3^-$ incorporation

[1] Research Career Development Awardee (1-K3-AI-5,268) of the U.S. Public Health Service.
[2] NASA Trainee.
[3] Predoctoral Fellow (GPM-16,383) of U.S. Public Health Service. Present address: Concordia College, Moorhead, Minn.

experiments with ribose-grown cells, unless otherwise specified, were described in greater detail in an earlier article (16). After fixation experiments, each concentrated extract was divided into several portions. One portion was retained for measurement of label incorporation. The rest were chromatographed in replicate with the solvents appropriate for organic phosphates. Zones containing ^{14}C-glutamate and ^{14}C-phosphoglycerate were then subjected to elution with water. Glutamate and phosphoglycerate were subsequently isolated from these eluants by anion-exchange chromatography (11, 13).

In the case of studies of pyruvate labeling, metabolism was stopped by the addition of 4 volumes of a solution containing 100 ml of 95% ethyl alcohol, 10 ml of saturated solution of 2,4-dinitrophenylhydrazine, and 1 ml of 12 N HCl. After extraction with stirring for 30 min, the suspension was centrifuged, and the sediment was re-extracted with 40 ml of 95% ethyl alcohol. The volume of the combined supernatant fluids was then reduced in vacuo to 15 ml. The 2,4-dinitrophenylhydrazone of pyruvate was subsequently separated and hydrogenolyzed to alanine by the method of Towers and Mortimer (24). The product was then subjected to paper chromatography with butanol-acetic acid-water (12:3:5). The labeled product proved to be alanine, as established by exact coincidence of the spot on a radioautogram with the ninhydrin-positive spot due to standard alanine.

Degradation of labeled products of CO_2 fixation. Carbon five of glutamate was liberated as CO_2 by use of a washed-cell suspension of *Clostridium tetanomorphum* (26), kindly provided by H. A. Barker. Carbon one of glutamate was liberated as CO_2 by use of glutamic decarboxylase (8).

Phosphoglycerate was dephosphorylated (7), and the glycerate was degraded as described by Aronoff (1), except that periodate was substituted for perchloratocerate.

Alanine derived from pyruvate as described earlier was subjected to chloramine T degradation (10), and yielded CO_2 from the carboxyl carbon.

Carbon dioxide was absorbed in sodium hydroxide or Hyamine hydroxide and formaldehyde in dimedone. Data have been corrected for incomplete absorption of CO_2 by Hyamine hydroxide (89%). Radioactivity assay procedures were as described earlier (16).

In all cases, parallel manometric experiments with unlabeled substrates revealed the expected stoichiometry between CO_2 released and substrate provided, confirming the reliability of the methods employed.

Cell-free preparations. After being washed once with 0.05 M tris-(hydroxymethyl)aminomethane (Tris), *p*H 7.7, containing 3 mM MgCl (TM), cells were resuspended in the same buffer. In the case of assays for citrate condensing enzyme, the malate dehydrogenases and reduced nicotinamide adenine dinucleotide ($NADH_2$) oxidase, glutathione was added to the buffer at 1 mM. Cells were disrupted in two ways. In one method, the suspension contained in a beaker immersed in an ice bath was treated for 20 to 40 sec at full power of a 20 kc Biosonik sonic oscillator. In the other technique, the suspension was passed through a French pressure cell at 2 C and a pressure of 7,600 psi.

Except as noted, undisrupted cells were removed by centrifugation for 20 min (2 C) at 1,500 $\times$ *g* to yield supernatant fraction C. A portion of fraction C was then recentrifuged for 1 hr at 144,000 $\times$ *g* (maximal force field) to yield supernatant fraction S.

In all extracts, protein was estimated by the method of Warburg and Christian (27).

Enzyme assays. The general procedures employed in spectrophotometric assays involved, in most cases, slight modifications of standard optical methods. Most of these methods are described in *Methods in Enzymology*, vol. 1, 1955 (Academic Press, Inc., New York). Exceptions will be noted with specific references to the literature. Rate measurements were conducted at ambient temperature, 23 to 26 C. Substrate or enzyme was added at zero-time, and the components were mixed by rapid, repeated immersion of a plastic stirring rod with flattened tip. Absorbancy readings were taken with a Beckman DU spectrophotometer equipped with a Gilford automatic absorbance meter. In general, the rates were calculated in a region of essentially constant initial rate. Concentrations of substrates and cofactors known to be saturating with enzymes from other sources were employed (see *Methods in Enzymology*, vol. 1). All results presented were corrected by data from appropriate controls.

In spectrophotometric assays, unless otherwise noted, conditions were as follows. The reaction volume was 1.0 to 3.0 ml. Concentrations of substrate, pyridine nucleotide (reduced or oxidized), Tris buffer (*p*H 7.7), and $MgCl_2$ were (in millimolar units), respectively: 5 to 10, 0.1 to 0.2, 50, and 3. In the assay of citrate condensing enzyme (21), acetyl coenzyme A (acetyl CoA) and oxaloacetate were used at concentrations of 0.04 and 0.3 mM, respectively. In the assay of succinate dehydrogenase, the succinate-dependent rate of bleaching of 2,6-dichlorophenol-indophenol was determined (22). To assay pyruvate and α-ketoglutarate dehydrogenases, 30 μmoles of substrate was added at zero-time to an incubation mixture (final volume, 3.0 ml) containing 1.5 μmoles of thiamine pyrophosphate, 4.0 μmoles of $K_3Fe(CN)_6$, and extract. The ensuing bleaching of ferricyanide was followed at 418 mμ. It was necessary to correct for appreciable bleaching that occurred in the absence of substrate. Isocitrate lyase was assayed at 30 C by the method of McFadden and Howes (17). Malate synthase activity was determined by adding 2.6 μmoles of glyoxylate to an incubation mixture (2.8 ml) at *p*H 8.5 containing (in micromoles), besides extract: acetyl CoA, 0.33; Tris, 244; and Mg^{+2}, 16. After incubation for 5 min at 30 C, the reaction was quenched by addition of 3 μmoles of 5,5'-dithiobis-(2-nitrobenzoic acid). The coenzyme A (CoA) that had been liberated was then determined (9).

In assays for $^{14}CO_2$ fixation, cells were harvested, washed, and broken in TM supplemented with 4 mM reduced glutathione (TGM) in a French pressure cell as described. Suspensions were then centrifuged (2 C) for 20 min at 20,000 $\times$ *g*. The supernatant fluid was either used directly or 1.0 ml was passed through a

column (1 by 11 cm) of Sephadex G-50 (coarse grade, from Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) equilibrated with TGM. After addition of the sample to the dextran bed, the first 3.2 ml of effluent contained components of a molecular weight > 10,000. The next 5.0 ml of effluent contained components of lower molecular weight. The specific radioactivity of $NaH^{14}CO_3$ employed was determined (16) at the time of each experiment, and incorporated counts were corrected to account for quenching by trichloroacetic acid (2).

Materials. Common organic and inorganic compounds were of high purity. Unusual chemicals were obtained as follows: 5,5'-dithiobis-(2-nitrobenzoic acid) from Aldrich Chemical Co., Inc., Milwaukee, Wis.; *cis*-aconitic acid, CoA, DL-isocitric acid, α-ketoglutaric acid, $NADH_2$, oxaloacetic acid, tricyclohexylammonium phosphoenolpyruvate (PEP), nicotinamide adenine dinucleotide phosphate (NADP) and reduced NADP (NADPH) from Calbiochem, Los Angeles, Calif.; chloramine T, sodium 2,6-dichlorophenol-indophenol (Eastman Grade) from Eastman Chemical Products, Inc., Kingsport, Tenn.; DL-glyceric acid (calcium salt) and thiamine pyrophosphate from Mann Research Laboratories, New York, N.Y.; sodium pyruvate from Nutritional Biochemicals Corp., Cleveland, Ohio; CoA from Pabst Brewing Co., Chicago, Ill.; glutathione and Polidase S from Schwarz Bio Research, Inc., Orangeburg, N.Y.; adenosine diphosphate (ADP), adenosine triphosphate (ATP), NAD, guanosine triphosphate, and inosine diphosphate from Sigma Chemical Co., St. Louis, Mo.; ferredoxin (*C. pasteurianum*) and glutamic decarboxylase (*Escherichia coli*) from Worthington Biochemical Corp., Freehold, N.J. Acetyl CoA was prepared by the method described by Ochoa (21). In all cases, reagents were dissolved in the appropriate buffer and neutralized when necessary.

Guanosine diphosphate was generated in situ by the hydrolysis of guanosine triphosphate catalyzed by heavy meromysin, a gift of R. G. Yount (28).

RESULTS

Labeling pattern of products of $^{14}CO_2$ fixation. Table 1 shows the results of studies of the labeling patterns of glutamate and phosphoglycerate.

In a separate 10-sec fixation experiment conducted on a different batch of cells, glutamate containing 3.8% of the total ^{14}C recovered from paper chromatograms was labeled to the extent of 80% in C_1 and 20% in C_5. In another 10-sec fixation experiment, pyruvate (containing ca. 2% of the label incorporated) was labeled almost exclusively (89%) in C_1.

Enzyme assays. Before conducting assays for NAD- and NAPD-linked dehydrogenases, it was necessary to establish whether $NADH_2$ and $NADPH_2$ oxidases were present in the extracts. $NADPH_2$ oxidase was barely detectable. The data obtained with $NADH_2$ are presented in Table 2, along with data for enzymes of the tricarboxylic acid cycle and related paths. Data for $NADH_2$ oxidase were obtained with molarities of $NADH_2$ considerably higher than those which accumulated in early stages of subsequent studies of NAD-linked oxidations. Because initial rates are reported for the latter reactions, corrections for the competing reaction would have been small and of uncertain magnitude, and were not applied. Only the specific activity of fraction C is shown. As is evident, all of the main enzymes of the tricarboxylic acid cycle are present. In general, there is little difference between enzyme levels in ribose- and yeast extract-cultured cells (Table 2). Only in the case of pyruvate dehydrogenase was there a marked difference. However, large control values (up to 70% of the pyruvate-dependent rate) obtained during the assay of this enzyme rendered this difference of questionable significance. In general, the present data are in accord with those of Truper (25) obtained with *Hydrogenomonas* H16G$^+$, and expand our knowledge of the locus of occurrence of enzymes of the tricarboxylic acid cycle. It is likely that $NADH_2$ oxidase, aconitase, and the dehydrogenases for pyruvate, α-ketoglutarate, and succinate are particulate in *H. facilis*. There seems to be little question of function of the tricarboxylic acid cycle in ribose-grown *H. facilis*. Homann (*unpublished data*) recently found rapid oxidation of succinate, fumarate, malate, pyruvate, oxaloacetate, and α-ketoglutarate by ribose-grown cells.

TABLE 1. *Labeling of glutamate and phosphoglycerate by $H^{14}CO_3^-$ during ribose oxidation by Hydrogenomonas facilis*

Fixation time	Per cent of ^{14}C present in concentrated ethyl alcohol extract as		Per cent distribution by carbon					
			Glutamate			Phosphoglycerate		
	Glutamate	Phosphoglycerate	C_1	C_2–C_4	C_5	C_1	C_2	C_3
sec								
6	3.9 (6.0×10^4)[a]	5.6 (8.5×10^4)	90	—	10	99	0	0
12	3.8 (1.2×10^5)	9.7 (3.0×10^5)	48	—	51	99	0	1

[a] Numbers in parentheses represent ^{14}C found (expressed as disintegrations per minute).

TABLE 2. *Enzyme assays*[a]

Enzyme	Assayed at	Cells ruptured by[a]	Per cent of "C" activity in "S"		Specific activity in "C" (mμmoles per min per mg of protein)	
			Ribose-grown	YE[b]-grown	Ribose-grown	YE[b]-grown
	mμ					
$NADPH_2$ oxidase	340	FPC + SO	—	—	BD	BD
$NADH_2$ oxidase	340	FPC	28	27	21	52
Pyruvate dehydrogenase	418	SO	80	None	19	2.3
Citrate condensing enzyme	232	FPC	86	94	226	323
Aconitase	240	SO	46	83	30	31
Isocitrate dehydrogenase (NADP)	340	SO	93	96	148	71
Isocitrate dehydrogenase (NAD)	340	SO	—	—	UD	UD
α-Ketoglutarate dehydrogenase	418	SO	None	None	2.2	1.5
Succinate dehydrogenase	600	FPC	None	25	323	610
Fumarase	240	SO	95	81	341	243
Malate dehydrogenase (NAD)	340	FPC	106	110	4,360	3,530
Malate dehydrogenase (NADP)	340	FPC	113	105	72	206
Isocitrate lyase	520	SO[b]	—	100[c]	16[d]	23[c]
Malate synthase	412	SO	—	100[c]	102[d]	65[c]
Lactate dehydrogenase (NAD and NADP)	340	FPC + SO	—	—	UD	UD

[a] "C" and "S" represent fractions C and S. YE = yeast extract; FPC = French pressure cell; SO = sonic oscillation; BD = barely detectable; UD = undetectable.

[b] Ribose-cultured cells were disrupted as described for 2 min.

[c] See reference 18. Specific activity is for fraction S.

[d] Specific activity is for fraction S.

Absence of a NAD- or NADP-linked lactate dehydrogenase (Table 2) was of interest because of the rapid proliferation of *H. facilis* on lactate, suggesting that formation of this enzyme may be induced by growth on lactate or that lactate oxidation does not involve pyridine nucleotide. The absence of these enzymes after growth on ribose or yeast extract made it possible to probe spectrophotometrically for carboxylation of pyruvate. Thus, it was anticipated that the product, oxaloacetate, would be rapidly reduced in the presence of reduced pyridine nucleotide by the endogenous pyridine nucleotide-linked malate dehydrogenases which were both known to be present in high levels (Table 2). However, no evidence for carboxylation of pyruvate could be found with this approach. When these studies were extended to an investigation of carboxylation PEP, again no evidence of oxaloacetate synthesis was obtained. However, in the course of probing for an acetyl CoA-dependent PEP carboxylation, a reduction of acetyl CoA by reduced pyridine nucleotides was discovered. In fraction S, after growth on ribose and yeast extract, respectively, the specific activities (millimicromoles per minute per milligram of protein) were 26 and 31 with $NADH_2$ and 14 and 7.7 with $NADPH_2$.

In a continuing search for carboxylation enzymes, a much more sensitive assay procedure was afforded by resorting to the use of $H^{14}CO_3^-$. As is evident in Table 3, the major carboxylation reaction is catalyzed by PEP carboxylase that is apparently dependent upon acetyl CoA. In this experiment, no attempt was made to identify the presumed product, oxaloacetate, because of the likelihood of its partial conversion, in the presence of acetyl CoA, to citrate and isocitrate. Additionally, some of the oxaloacetate may have been decomposed (14). Therefore, another experiment designed to aid in trapping the oxaloacetate by reduction with $NADPH_2$ catalyzed by endogenous malate dehydrogenase was performed. Results of this experiment, also done with varying concentrations of acetyl CoA, are reported in Table 4. The data illustrate the dependence of carboxylation upon acetyl CoA. Whether it acts as a positive allosteric effector of PEP carboxylase (6, 19) remains to be seen. For unknown reasons, omission of $NADPH_2$ in the experiment with the highest concentration of acetyl CoA resulted in increased CO_2 incorporation.

It was of interest to examine the possibility of the presence of pyruvate synthase and α-ketoglutarate synthase (3, 4). In these experiments, extracts were fractionated by gel filtration as described earlier, into two fractions: the early effluent containing compounds of molecular weight > 10,000 and the late effluent with compounds of molecular weight <10,000. No evidence was obtained for an anaerobic exchange of $^{14}CO_2$ with

TABLE 3. *Carboxylation of pyruvate and phosphoenolpyruvate (PEP) by $H^{14}CO_3^-$*

Major components in incubation mixture[a]	$^{14}CO_2$ incorporation, (counts/5 min)	
	After ribose growth	After yeast extract growth
1. PEP, IDP, ADP, GTP, HMM[b]	1,271	3,052
IDP, ADP, GTP, HMM	705	1,613
PEP, IDP, ADP	1,233	2,000
2. Pyruvate, $NADH_2$	630	2,093
$NADH_2$	700	2,021
3. Pyruvate, ATP, biotin	895	1,997
ATP, biotin	1,080	1,526
Pyruvate, ATP	986	2,100
4. Pyruvate, $NADPH_2$	1,876	3,449
$NADPH_2$	1,562	2,623
Pyruvate	703	1,902
5. PEP, acetyl CoA	33,800	—
Acetyl CoA	3,210	—
PEP	8,530	10,650
PEP, inorganic phosphate	8,100	7,838
Inorganic phosphate	2,410	1,964

[a] Cells were disrupted with a French pressure cell in the presence of 4 mM glutathione under the usual conditions. The supernatant fluid from 20,000 × *g* centrifugation (20 min; 2 C) was then used. The following are amounts (in micromoles) used in 1.0 ml of reaction mixture at *p*H 7.7: PEP, 1; pyruvate, 3; biotin, 1; acetyl CoA, 0.2; inorganic phosphate, 1; ATP, ADP, 0.6; IDP, GTP, 0.1; $NADH_2$, $NADPH_2$, 0.06 to 0.07; Tris, 45 to 50; Mg^{+2}, 3; glutathione, 1.6; $NaH^{14}CO_3$, 1.0. After initiation of the reaction with either extract or $H^{14}CO_3^-$ (ca. 1 μc), the mixture was incubated under air for 35 min at 30 C in vials for the liquid scintillation counter. Reactions were stopped with the addition of 0.2 ml of 70% (w/v) trichloroacetic acid. After 4.5 to 6 hr at 30 C, 10.0 ml of Bray solution (2) was added, and the samples were counted (16).

[b] IDP and ADP, inosine and adenosine diphosphate; GTP, guanosine triphosphate; HMM, heavy meromysin.

pyruvate or α-ketoglutarate catalyzed by the early fraction plus clostridial ferredoxin or by the combined early and late fractions. No evidence was obtained for ferredoxin-stimulated production of acetyl phosphate from pyruvate in the presence of early fraction plus thiamine pyrophosphate, CoA, and inorganic phosphate.

DISCUSSION

The present data provide confirmation that glutamate and phosphoglycerate are early intermediates in carbon dioxide fixation by suspensions of *H. facilis* oxidizing ribose under growth conditions (16). More importantly, the labeling patterns observed, coupled with results of enzyme studies, provide some insight into the mechanism of labeling of these compounds by carbon dioxide.

It seems likely that carboxyl-labeled 3-phosphoglycerate arises through function of the reductive pentose phosphate cycle (5) which functions at a suppressed rate during ribose growth. Indeed, the two enzymes of that cycle that might be expected to be repressible during heterotrophic growth, ribulosediphosphate carboxylase and phosphoribulokinase, are detectable after culture on ribose, although at reduced levels compared with those of autotrophic cells (18). The labeling of the pyuvate-carboxyl carbon observed in the present work suggests that pyruvate arises from PEP, derived in turn from phosphoglycerate. Because exchange was not detectable in extracts, the possibility of incorporation by exchange into C_1 of pyruvate or α-ketoglutarate seems unlikely, but cannot be rigorously eliminated.

The mechanism of incorporation of $^{14}CO_2$ into glutamate is also of interest. All major enzymes of the tricarboxylic acid cycle are present, in addition to a PEP carboxylase, which may be dependent upon acetyl CoA, as is the enzyme in enteric bacteria (6, 15). Thus, the, C_1 labeling of glutamate can be explained. The specific enzyme activity of the carboxylase, although lower than that for any other enzymes detected, is nevertheless adequate to account for the observed rate of labeling of C_1 of glutamate. It can be estimated from data in Table 1 and additional information that the rate of CO_2 incorporation into glutamate corresponded to 7.2 mμmoles per min per 66 mg of dry cells at 30 C. This corresponds to a rate of incorporation of 0.4 mμmole of CO_2 per min per mg of protein in the supernatant fluid from centrifugation of extract at 20,000 × *g*—a value somewhat lower than the maximal specific activity observed for the PEP carboxylase (Table 4). These observations, then, raise a question about the failure in earlier experiments (16) to detect labeled tricarboxylic acid cycle intermediates arising from $^{14}CO_2$ fixation during ribose oxidation. Perhaps, in those experiments, this only reflected pool sizes or the loss of these intermediates during volume reduction of the acidified ethyl alcohol extracts.

Of major interest in the present work is the rapid labeling of the γ-carboxyl group of glutamate. One widely overlooked reaction that could partially account for this would involve catalysis

TABLE 4. *Carboxylation of PEP by $H^{14}CO_3^-$ catalyzed by extracts from ribose-grown Hydrogenomonas facilis*

Major components in incubation mixture[a]	CO_2 fixed per min per mg of protein
	mμmoles
PEP, $NADPH_2$, 7×10^{-5} M acetyl CoA	0.44
PEP, $NADPH_2$, 2×10^{-4} M acetyl CoA	0.60
PEP, $NADPH_2$, 3.5×10^{-4} M acetyl CoA	0.38
PEP, $NADPH_2$	0.12
PEP, 3.5×10^{-4} M acetyl CoA	0.61

[a] The reaction mixture of 1.0 ml had the same composition as described in Table 3 except for the final concentration of acetyl CoA, which is specified. $NADPH_2$ was present at 1.4×10^{-4} M. All other experimental conditions are described in the footnote to Table 3.

by isocitrate lyase, which was shown to be present in ribose-grown cells (Table 2). Thus, isocitrate derived from oxaloacetate-*1,4-*^{14}C would be cleaved to succinate labeled in a single carboxyl carbon. Reversal of the reaction would result in incorporation of ^{14}C into the γ-carboxylate group of isocitrate and eventual labeling of C_5 of glutamate. The oxaloacetate-*1,4-*^{14}C would arise from carboxylation of carboxyl-labeled PEP, in turn derived from labeled phosphoglycerate. The amount of label in each of the carboxyls of oxaloacetate would approach equality as a consequence of turnover through catalysis by malate dehydrogenase and fumarase. Under the latter conditions, the specific radioactivity of C_1 of glutamate would be twice that of C_5 in the limit. In the present work, the extent of labeling of C_1 and C_5 was about equal in the longest fixation time. There are other paths that might contribute to synthesis of glutamate-*1,5-*^{14}C. One is outlined in Fig. 1 The relative rates of labeling of C_5 and C_1 of glutamate by path ② would depend critically upon the rate of the aforementioned equilibration of label between the carboxyls of oxaloacetate, and the specific radioactivity of each of these two glutamate carbons would be equal in the limit. Participation of path ② in glutamate biosynthesis by extracts of *Acetobacter suboxydans* can be tentatively inferred from the data of Sekizawa et al. (23), although very long incubations were conducted. If path ② functions in *H. facilis*, this organism might be an excellent source of the enzymes.

A possibility that could account for the observed C_5 labeling of glutamate would first involve conversion of oxaloacetate-*1,4-*^{14}C to pyruvate-*1-*^{14}C. Condensation of the latter compound with acetyl CoA would give rise to citramalate which might then lead to glutamate-*5-*^{14}C (20). Another possibility that would account for C_5-labeling would involve catalysis in path ① by a citrate synthase of abnormal stereospecificity, as found recently by Gottschalk and Barker for *Clostridium kluyveri* (12).

Further work on the biosynthesis of glutamate by *H. facilis* is anticipated in our laboratory.

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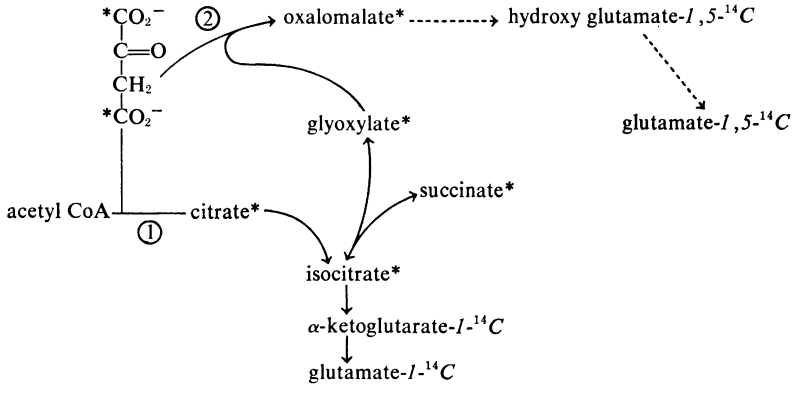

FIG. 1. *Possible path contributing to synthesis of glutamate-1,5-*^{14}C.

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