[14]C DISTRIBUTION IN THE CARBOXYL GROUPS OF GLUTAMIC ACID IN *CLOSTRIDIUM ACIDI-URICI* FED WITH [2-^{14}C]FORMIMINOGLYCINE[1]

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Abstract

[^{14}C]Glutamic acid was isolated in the pure state from *Clostridium acidi-urici* fed with [2-^{14}C]formiminogloycine. Of the total radioactivity in the labeled glutamic acid, 34.1% was found in α-carboxyl group whereas 1.2% in the γ-carboxyl group. The results indicated that in *C. acidi-urici* cells glutamic acid was predominantly synthesized via an atypical pathway.

Introduction

It has become clear that biosynthesis of glutamic acid in animal tissues, yeast, and facultative aerobic bacteria is via the upper portion of the citric acid cycle by the sequential action of citrate synthase, aconitase, isocitrate dehydrogenase, and glutamate dehydrogenase (Stern and Bambers, 1966). Gottschalk and Barker (1966, 1967) reported that the citrate synthase of the strictly anaerobic bacterium *Clostridium kluyveri* is atypical in that it forms the isotopic antipode of the usual citrate. However, at about the same time Stern, Hegre and Bambers (1966) showed that the citrate synthase in cell-free extracts of *C. kluyvrei* had the usual (or typical) stereospecificity described for this citric acid cycle enzyme in animal heart. The opposite results obtained in the two laboratories have remained unexplored. The present study was undertaken to evaluate the physiological significance of the typical and atypical citrate synthases in a chosen strictly anaerobic bacterium, *Clostridium acidi-urici.*

Previous results suggest that when *C. acidi-urici* cells were labeled with [2-^{14}C]formiminoglycine (FIG), the ^{14}C distribution in oxalacetate and acetyl

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CoA would be as those shown in Fig. 1, namely, two-thirds and one-third of
the total radioactivity in the oxalacetate molecule would be located in C-2
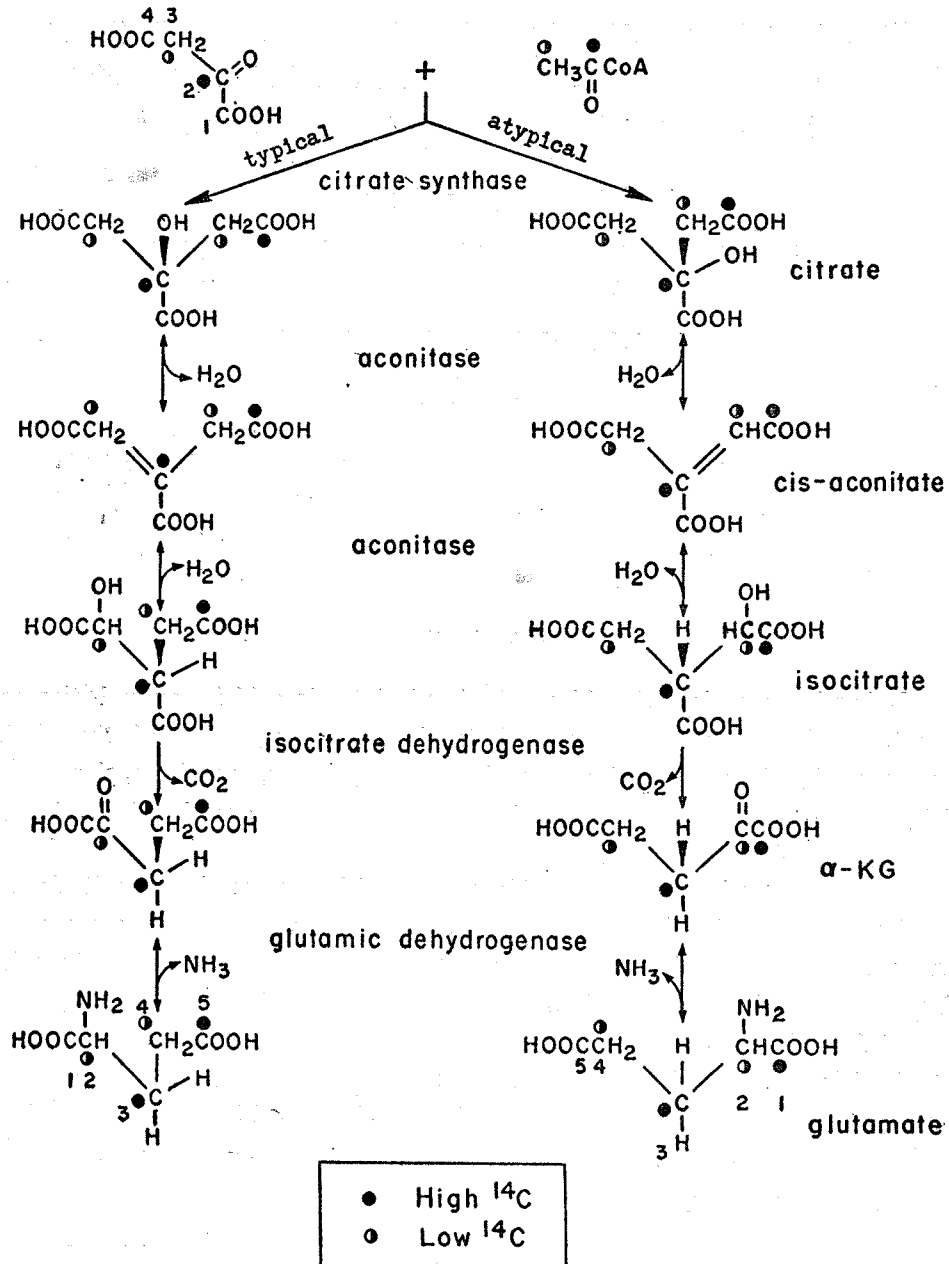and C-3 the oxalacetate, respectively; in the acetyl CoA molecule, two-thirds

Fig. 1. The typical and atypical pathways of L-glutamate synthesis. The presumed
    radiolabeled substrate is [2-14C]FIG. In the case of *C. acidi-urici*, the carbons
    marked with (●) has radioactivity twice that of the carbons marked with (◗).
    Unlabeled carbons are not marked.

and one-third of the total radioactivity would be in carbonyl and methyl carbons of the acetyl group, respectively (Chen and Rabinowitz, 1973).

The theoretical labeling patterns of glutamate by the typical and atypical citrate synthases are also shown in Fig. 1. When [2-^{14}C]FIG is employed as a tracer for *C. acidi-urici*, according to the atypical citrate synthase, C-1 of glutamate will be labeled and the percentage of ^{14}C in C-1 will be about one-third of the total radioactivity, there will not be any ^{14}C in C-5. On the other hand, if glutamate synthesis is via the typical pathway, glutamate C-5 becomes labeled, but C-1 will not be labeled. Therefore, determinations of radioactivity in C-1 and C-5 of glutamate would distinguish these two pathways. The results of such determinations are presented in this paper.

Materials and Methods

[2-^{14}C]FIG was chemically synthesized by the method of Tabor and Rabinowitz (1957).

Isolation of [^{14}C]amino acids from proteins of C. acidi-urici incorporating [2-^{14}C]FIG.

(1) Isolation of *C. acidi-urici* proteins. 250 ml of rapidly growing cells was transfered to 250 ml of urate minimal medium which contained in a final volume of one liter: uric acid, 2 g; 10 N KOH, 1.2 ml; 70% $K_2HPO_4 \cdot 3H_2O$, 1.3 ml; 5% $MgSO_4 \cdot 7H_2O$, 0.7 ml; 0.25% $FeSO_4 \cdot 7H_2O$, 1.3 ml; 0.6% $CaCl_2 \cdot 2H_2O$, 0.7 ml; and mercaptoacetic acid (80%), 2 ml. The medium was neutralized to pH 7.5 with 10 N KOH. After incubation at 37° for two hours, 1.33 ml of 8 mM [^{14}C]FIG and 3 ml of 1 M NaCl were added to start the incorporation of [^{14}C]FIG. The incorporation was allowed to proceed for 100 min. The ^{14}C-labeled cells were harvested by centrifugation at room temperature at 10,000 × g for 10 min and washed three times with cold 0.15 M Tris–HCl buffer, pH 7 containing 0.4 M NaCl. The washed cells were suspended in 10 ml of 5% trichloroacetic acid (TCA) and heated at 90° for 10 min. After cooling to 4°, the 5% TCA insoluble fraction was washed three times with 5% cold TCA and twice with absolute ethanol. The TCA insoluble fraction thus obtained was dried over KOH pellets and phosphorus pentaoxide under reduced pressure in a desiccator. 45.7 mg of the dried TCA insoluble fraction was obtained. This fraction contained 80% protein as estimated by the amino acid content in its acid hydrolysate. Since some amino acids might be destroyed during acid hydrolysis, 80% could represent the lower limit.

(2) Isolation and purification of [^{14}C]amino acids from the acid hydrolysate of *C. acidi-urici* proteins. 20 mg of the clostridial TCA insoluble fraction were hydrolyzed with 6 N HCl at 120° for 24 hours under reduced pressure

in a sealed ignition tube. The hydrolysate equivalent to 6–7 mg clostridial protein was applied to an automatic amino acid analyzer (column size, 1.2× 57 cm). Amino acids were eluted with 0.2 N sodium citrate buffer, pH 3.22, containing 0.2% dimethyl sulfoxide. Column temperature started at 50° and shifted to 60° as soon as serine was emerged from the column. Elution was stopped after cystine and valine came off the column. The column was connected to a fraction collector in such a manner that the eluate could be collected and assayed for radioactivity as previously described (Chen and Rabinowitz, 1973). Fractions fell under each peak were pooled and further purified by paper chromatography. Perier to paper chromatography, the amino acid samples were desalted according to the method of Dreze, Moore and Bigwood (1954) and concentrated by means of a rotary evaporator.

Decarboxylation of the α-carboxyl group of glutamic acid.

Ninhydrin and chloramine–T were employed for the decarboxylation of the α-carboxyl group of glutamic acid by the method of Van Slyke *et al.* (1941) and Kemble and McPherson (1954), respectively. For ninhydrin reaction, 66.88 μmoles of the twice recrystallized L–glutamic acid and 3.49 μmoles of labeled glutamic acid were dissolved in 2.5 ml of 0.2 M sodium citrate buffer, pH 2.5. 1.25 ml water was also added. The mixture was bubbled with nitrogen gas for 20 min to remove dissolved carbon dioxide, then 1.25 ml of 6% CO_2 free ninhydrin was introduced. The mixture was refluxed for 20 min. Carbon dioxide formed was trapped in 0.2 N NaOH. The trapped carbon dioxide was precipitated as barium carbonate by adding 0.3 ml of 0.7 M barium chloride to the trap. The isolated barium carbonate was weighed and assayed for radioactivity by the Diffusion–Scintillation Method described by Baker (1970). For chloramine–T reaction, the procedure was essentially the same as that for ninhydrin reaction except that 0.8 ml of 12% chloramine–T was used instead of ninhydrin and the reaction was performed at room temperature for 60 min.

Microbiological assay for the determination of ^{14}C in the γ-carboxyl group of glutamic acid.

The C–5 of glutamic acid was specifically released as CO_2 by fermenting the glutamic acid with a cell suspension of *Clostridium tetanomorphum* as described by Wachsman and Barker (1955). The experimental tube contained in a final volume of 2 ml: 40 μmoles of potassium phosphate buffer, pH 7.6; 1 μmole of sodium sulfide; 10.8 μmoles of glutamate (24850 dpm) isolated from [^{14}C]proteins of *C. acidi-urici* and approximately 30 mg of dry weight equivalent of cell suspensions of *C. tetanomorphum*. The fermentation was carried out under an oxygen free N_2 atmosphere at 37° for 3 hours. At the

conclusion of the fermentation, the tube was chilled and 0.5 ml of the fer-mentation mixture was removed for determination of $^{14}CO_2$ by the Diffusion-Scintillation Method. The activity of the cell suspension was assured by fermenting a commercial source of [U–^{14}C]glutamate. In this case, 20.8% of total ^{14}C was found to be in C–5 of the [U–^{14}C]glutamate.

Paper and thin layer chromatography.

The following solvent systems were employed for either paper chromato-graphy or thin layer chromatography. Solvent 1: *t*-butanol/formic acid/H_2O 70/15/15 (V/V); Solvent 2: [methanol/chloroform/90% formate 3/3/1 (V/V); Solvent 3: *n*-butanol/acetic acid/H_2O 4/1/1 (V/V); Solvent 4: *n*-butanol/acetic acid/H_2O 12/3/5 (V/V). Solvents 1, 2 and 3 were preferentially used for descending paper chromatography employing Whatman No. 1 paper. Solvent 4 was mainly used for thin layer chromatography, pre-coated plastic sheets of MN–POLYGRAM cel 300 PEI were employed for this purpose.

Results and Discussion

Isolation and purification of ^{14}C-labeled acidic and neutral amino acids with short-side-chains from C. acidi-urici.

The profile of chromatography of the labeled amino acids with an automatic amino acid analyzer is shown in Fig. 2. Aspartic acid was com-
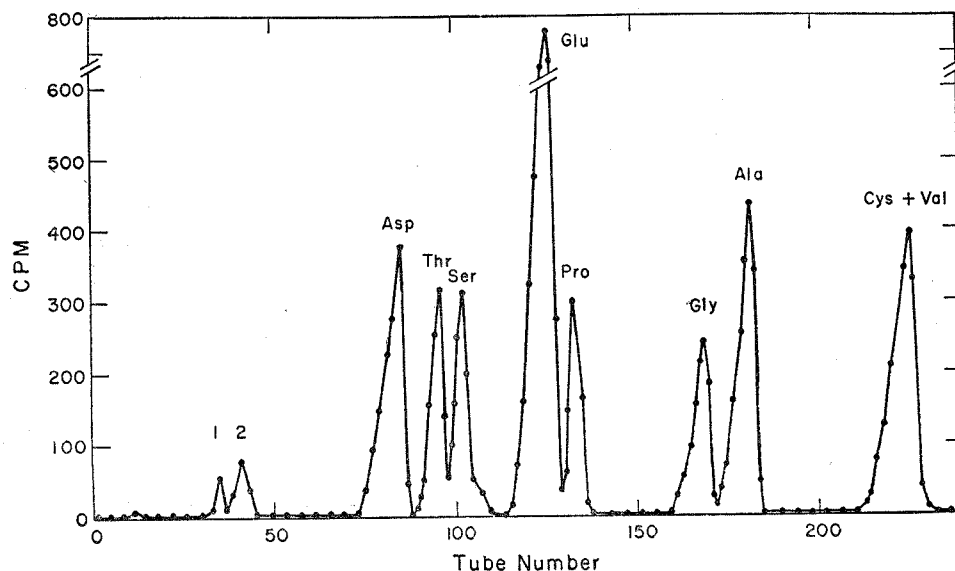

Fig. 2. Separation of labeled acidic amino acids and neutral amino acids with short-side-chains. The experimental conditions are given under the "Materials and Methods".

pletely separated from other amino acids. Threonine-serine, glutamic acid-proline, and glycine-alaine came off the colum as three pairs. Further separation and purification of the amino acids in each pair were performed as follows: the fractions which possibly consisted of both amino acids of each pair were combined, desalted, and concentrated. The concentrated samples were separated by paper chromatography as described under the "Materials and Methods". Each pair could be separated completely into two amino acids without cross contamination as examined by either paper chromatography or thin layer chromatography. The other fractions of glutamate, proline, glycine and alanine were pooled separatly and their purity was confirmed by either paper or thin layer chromatography. The purified [^{14}C]amino acids obtained by this procedure were found to be 99% radiochemically pure as determined by radioautography. The purified [^{14}C]glutamate was employed for determinations of ^{14}C in the carboxyl groups of glutamic acid. Peaks 1 and 2 in the elution profile have not been identified.

^{14}C distribution in the carboxyl groups of [^{14}C]glutamate from proteins of C. acidi-urici labeled with [2-^{14}C]FIG.

^{14}C distributions in the α– and γ–carboxyl groups of C. acidi-urici [^{14}C]-glutamate were found to be 34.1% and 1.2% of the total radioactivity in glutamate, respectively, as shown in Tables 1 and 2. In other words, two-thirds of the total ^{14}C were in C–2, C–3, and C–4. Although ^{14}C distribution in these three carbons has not been determined, the probable distribution is that C–3 has the same radioactivity as C–1 and either C–2 or C–4 has one-half of the radioactivity of either C–1 or C–3. Such ^{14}C distribution indicate that in C. acidi-urici the formation of glutamate is via the atypical pathway in which citrate synthase has opposite stereospecificity of the typical citrate synthase. As a consequence of the atypical stereospecificity, an isotopic antipode of the usual citrate was formed. Therefore, C–5 of glutamate was practically not labeled instead glutamate C–1 was labeled to 34.1% of the total radioactivity in the glutamate carbons. These results are in agreement with that obtained by Gottschalk and Barker (1966, 1967) who employed cell-free systems. The origin of the trace amount of radioactivity found in C–5 of glutamate is unclear, It could possibly reflect that 3.5% (1.2/34.1) of total glutamate in C. acidi-urici was synthesized via the typical pathway or it could have resulted from the incomplete stereospecificity of the atypical citrate synthase.

Since an *in vivo* system was employed in this study, possible artifact could be reduced to minimum. Therefore, it is concluded that in C. acidi-urici most, if not all, of glutamic acid is synthesized via the atypical pathway.

Table 1. ^{14}C *Distribution in the α-Carboxyl Carbon of* [^{14}C]*Glutamate Isolated from C. acidi-urici Fed with* [2-^{14}C]*Formiminoglycine.*

100 μmoles of [^{14}C]glutamate were used for each analysis.
Experimental details are given under "Materials and Methods".

Glutamate degraded (dpm/μmole)	CO$_2$ formed	
	dpm/μmole	%
526	179	33.94[a]
590	203	34.32[b]

[a] By ninhydrin.
[b] By chloramine-T.

Table 2. ^{14}C *Distribution in the γ-Carboxyl Carbon of* [^{14}C]*Glutamate Isolated from C. acidi-urici Fed with* [2-^{14}C]*Formiminoglycine.*

10.8 μmoles of [^{14}C]glutamate were used for each analysis.
Experimental details are given under "Materials and Methods".

Glutamate degraded (dpm)	CO$_2$ formed	
	dpm	%
24850	297	1.20±0.35 (SD)*

* SD stands for standard deviation. It was obtained from data of two experiments.

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以 [2-^{14}C]Formiminoglycine 標示 *Clostridium acidi-urici* 麩酸的二羧基的 ^{14}C 分佈

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　　以 [2-^{14}C]formiminoglycine 供給梭狀芽胞桿菌 (*Clostridium acidi-urici*) 後，自其蛋白質水解物分離純放射性麩酸。^{14}C 在 [^{14}C]麩酸的 α-羧基及 γ-羧基的分佈各佔總放射性的 34.1% 和 1.2%。這種結果表示在梭狀芽胞桿菌細胞中麩酸主要是經由一非典型的路徑合成的。