13C Nuclear Magnetic Resonance Studies of the Biosynthesis by Microbacterium ammoniaphilum of L-Glutamate Selectively Enriched with Carbon-13*

(Received for publication, July 13, 1981)

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13C NMR of isotopically enriched metabolites has been used to study the metabolism of Microbacterium ammoniaphilum, a bacterium which excretes large quantities of L-glutamic acid into the medium. Biosynthesis from 90% [1-13C]glucose results in relatively high specificity of the label, with [2,4-13C]glutamate as the major product. The predominant biosynthetic pathway for synthesis of glutamate from glucose was determined to be the Embden Meyerhof glycolytic pathway followed by P-enoxyruvate carboxylase and the first third of the Krebs cycle. Different metabolic pathways are associated with different correlations in the enrichment of the carbons, reflected in the spectrum as different 13C,13C scalar multiplet intensities. Hence, intensity and 13C,13C multiplet analysis allows quantitation of the pathways involved. Although blockage of the Krebs cycle at the α-ketoglutarate dehydrogenase step is the basis for the accumulation of glutamate, significant Krebs cycle activity was found in glucose grown cells, and extensive Krebs cycle activity in cells metabolizing [1-13C]acetate. In addition to the observation of the expected metabolites, the disaccharide α,α-trehalose and α,β-glucoylsamine were identified from the 13C NMR spectra.

Several varieties of bacteria have been described which utilize glucose or acetate as a sole carbon source and which excrete large quantities of amino acids (1). One of these is Microbacterium ammoniaphilum, which has an impaired Krebs cycle due to a reduced activity of the enzyme(s) necessary for processing α-ketoglutarate and its metabolites. It can be induced to excrete L-glutamate late in the growth cycle of cells cultured in media of high nitrogen content at sufficiently low concentrations of biotin that the cells develop altered membranes which allow facile transport of L-glutamate formed by transamination of α-ketoglutarate (2-4). We concluded that these characteristics of M. ammoniaphilum made it an excellent potential vehicle for the biosynthesis of L-glutamate selectively labeled with 13C, if it were grown on specifically enriched glucose or acetate as the predominant source of carbon.

Our interest in these systems developed in the continuing program at the Los Alamos National Laboratory on the use of microorganisms and cell-free extracts for the large scale synthesis of natural products, uniformly or specifically labeled with 13C (5). Current work is focused on those L-amino acids for which efficient organic synthesis methods are not now available and for which there is a need either in human metabolic and nutritional studies or in the investigation of the structure and dynamics of proteins enriched with labeled amino acids. In the use of biological systems for the selective enrichment of natural products, there are two prime practical considerations: 1) the optimization of the incorporation of the 13C label from the precursor into the product; and 2) the minimization of the degree to which the label becomes randomized in the product due to the flux of the precursor and its metabolites through various pools. Especially at high levels of enrichment of products for mass spectrometric and nuclear magnetic resonance studies, randomization of the label leads to complications arising from multiple isotope clusters and 13C nuclear spin coupling patterns (6-8).

In this paper we address the latter consideration in a 13C NMR analysis of the flow of 13C label through the Krebs cycle, the phosphogluconate pathway, and the glyoxylate shunt into selectively labeled L-glutamate excreted by M. ammoniaphilum grown in media containing [1-13C]glucose or [1-13C]acetate as the predominant carbon source. The work illustrates the general utility of selective, in contrast to random, 13C labeling of natural products biosynthetically. In addition it is of interest because it demonstrates that it is not generally valid to assume in biosynthetic labeling experiments that the label from 13C-enriched precursors will occupy the sites of a natural product in a statistically independent manner.

EXPERIMENTAL PROCEDURES

Materials—[1-13C]Glucose enriched to 90 atom % (9) and [1-13C]acetate (10) enriched to 70 atom % were gifts of V. Kerr and T. Sanchez, respectively, both of the Los Alamos National Laboratory. Corn steep liquor was a gift of Grain Processing Company of Muscatine, IA. Cultures of M. ammoniaphilum (ATCC 15354) were obtained from the American Type Culture Collection, Rockville, MD.

Nuclear Magnetic Resonance Spectroscopy—Pulsed 13C NMR spectra were obtained with a Varian XL-100-15 spectrometer (25.2 MHz) interfaced to a Data General SuperNova computer. The spectra were recorded at 25 °C with a spectral width of 5000 Hz and 4096 spectral points. The lock signal was obtained from ~5% D2O contained in the sample. Spectra of the production media were obtained on the supernatant after removing the cells by centrifugation at 12,000 x g for 10 min. The supernatant could then be run directly, appropriately diluted, or concentrated by reducing the pH and evaporation in vacuo. Standard samples were dissolved in 0.05 M potassium phosphate buffer and the pH adjusted to 7.5. Proton NMR spectra for...
**RESULTS**

$^{13}$C and $^1$H NMR Spectra—The time dependence of the $^{13}$C NMR spectra of a suspension of *M. ammoniaphilum* containing $[^1-^{13}C]$glucose near the stationary phase of growth is summarized in Fig. 1. Since the production of glutamate in large quantities by this microorganism does not begin until that stage is attained, the initial growth was accomplished with a natural abundance of glucose to minimize loss of the $^{13}$C label. In addition to the expected resonances from $^{13}$C-enriched bicarbonate, glutamate, succinate, and lactate, the spectra in Fig. 1 exhibit prominent resonances from two unusual $^{13}$C-labeled products which are eventually consumed: trehalose and glucosylamine. The accumulation of trehalose, which presumably functions as a storage carbohydrate, has been observed previously by $^{13}$C NMR in growing yeast (11, 12), bacteria (13), and differentiating amoeba cultures (14). To our knowledge, glucosylamine formation in cell culture has not been observed and, at present, we do not know whether its synthesis is under enzyme control and what role it plays in the control of glucose or nitrogen metabolism. The formation of lactate and succinate by *M. ammoniaphilum* depends on the oxygen tension which, for the cultures appropriate to Fig. 1, fell sharply in the later stages of glucose consumption. In fully aerated cultures, the lactate and succinate levels are sharply reduced (Fig. 2). The time dependence of the $^{13}$C NMR spectra of the cell-free production media (see "Experimental Procedures") obtained under fully aerobic and partially anaerobic conditions was qualitatively the same as the spectra of the cell suspensions. This observation, together with the lack of $^{13}$C-enriched resonances in the spectra of medium-free packed cells obtained at the end of the metabolism experiments, suggests that the $^{13}$C spectra monitored (Figs. 1 and 2) were those of extracellular substrates and metabolites. A qualitative appreciation of the flow of labeled carbon in this system can be obtained from the summary of the time dependence of the intensities of the $^{13}$C resonances of the glucose substrate and the $^{13}$C-labeled metabolites tabulated in Fig. 3.

The appearance of glucosylamine in the $^{13}$C NMR spectra of metabolizing cells is not inconsistent with the ease of its formation under a variety of conditions (15–17). In addition, cell-free extracts of *M. ammoniaphilus* will catalyze the formation of $\alpha,\beta[^{1}-^{13}C,N]$ glucosylamine from $[^{13}C]$glucose and $[^{15}N]$urea.

The 360 MHz proton NMR spectrum of a solution of $[^{12},^{13},^{14},^{15},^{16},^{17},^{18}$]glutamate derived from *M. ammoniaphilum* grown on $[^{13}C]$glucose is shown in Fig. 4A. Despite the high level of $^{13}$C incorporation demonstrated by the proton spectrum, the C-2 and C-4 resonances in the $^{13}$C spectrum are predominately singlets (Fig. 4B), suggesting that the $^{13}$C label is introduced into specific sites (C-2, C-4) with a minimum of scrambling to other sites. This site specificity is even greater in the early production phase of glutamate. A plot of the intensity ratio of C-3 to C-4 versus time is presented in Fig. 5, which shows that C-3 is more highly labeled at later times. An even higher degree of site-specific incorporation is illustrated in Fig. 6, which is a reproduction of the $^{13}$C NMR spectrum of a solution of $[^{15},^{13}C]$glutamate obtained from *M. ammoniaphilum* grown on $[^{13}C]$acetate.

**Major Metabolic Pathways in M. ammoniaphilum**—The metabolic pathways of *M. ammoniaphilum* have been established primarily through $^{13}$C labeling studies carried out by Shio and co-workers (Fig. 7). A glucose carbon source is metabolized primarily via the Embden Meyerhof pathway to phosphoenolpyruvate and pyruvate (18, 19). Accumulation of

D$_2$O solutions were obtained on a Nicolet spectrometer in the FT mode operating at 360 MHz. $^{13}$C chemical shifts were measured with respect to external tetramethyl silane and $^1$H shifts with respect to the methyl resonance of internal trimethylsilylpropansulfonic acid.

$^{13}$C NMR Spectroscopy of Cell Suspensions—*M. ammoniaphilum* was grown on a slant of nutrient agar at 30 °C for 3–4 days. One loopfull of the microorganism was precultured in 30 ml of a rich medium containing 5% corn steep liquor, 5% casein, 0.1% MgSO$_4$·7H$_2$O and 0.1% KH$_2$PO$_4$, at pH 7.3 for 24 h at 30 °C in a 500-ml Erlenmeyer flask in a shaker. The fermentation was carried out at 30 °C in a baffled 500-ml Erlenmeyer flask containing 30 or 60 ml in a production medium (pH 7.3) which contained 5% glucose, 1.2% urea, 0.5% KH$_2$PO$_4$, 0.1% MgSO$_4$·7H$_2$O, 0.001% FeSO$_4$·7H$_2$O, 0.001% MnSO$_4$·4H$_2$O, biotin (1.5 μg/liter), and a 2% inoculum of the preculture medium.

*M. ammoniaphilum* cells were grown for 24 h at 300 rpm in an orbital shaker, harvested by centrifugation at 12,000 × g for 10 min, washed twice with 0.2% KCl, and suspended in fresh media containing $[^1-^{13}C]$glucose or $[^1-^{13}C]$acetate. Aeration was provided by continuously bubbling a stream of pure oxygen gas through a very thin capillary tube which reached to within 1–2 mm of the bottom of the spinning 12-mm NMR tube. The total volume in the NMR tube was 4 ml to prevent bubbles breaking the surface from disturbing the magnetic field, and to allow for evaporation of sample due to oxygen bubbling. Other details are given in the figure captions. Spectra were continually obtained for 2–3-h increments over a 20-h period, at which time all of the glucose had been consumed and the glutamate level was reasonably stable.

**Fig. 1.** Time dependence of the proton-decoupled Fourier transform $^{13}$C NMR spectra (25.2 MHz) of a suspension of *M. ammoniaphilum* initially containing $[^{1}-^{13}C]$glucose. T = time interval for accumulation of the spectra after transfer of the cells to fresh medium containing $[^{13}C]$glucose (see "Experimental Procedures"). Enriched $^{13}$C resonances are: HCO$_3^-$ ion; Glc, a and b anomers of $[^{1}-^{13}C]$glucose; Glt, C-2 (56.0 ppm), C-4 (34.6 ppm), and C-3 (28.3 ppm) of glutamate; SA, C-2 of succinate; L, C-3 of lactate; and GA, a and b C-1 resonances of glucosylamine. In the T = 6–8 h and 7–9 h spectra, the glucose C-1 resonances are truncated and the natural abundance glucose resonances are apparent.
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**FIG. 2.** Time dependence of the proton-decoupled $^{13}$C NMR spectra of a fully aerated suspension of *M. ammoniophilum* grown on [1-$^{13}$C (90 atom %)]glucose. See Fig. 1 legend.

**FIG. 3.** Time dependence of the intensities of the $^{13}$C resonances of the enriched glucose substrate and the metabolites identified in Fig. 1. See text also.

**FIG. 4.** Proton NMR and proton-decoupled $^{13}$C NMR spectra of [1,2,3,4-$^{13}$C$_4$]glutamate. a, proton NMR spectrum (360 MHz) of [1,2,3,4-$^{13}$C$_4$]glutamate derived from *M. ammoniophilum* grown on [1-$^{13}$C (90 atom %)]glucose. For each proton, the center of the multiplet arises from $^{13}$C-H moieties with fine structure caused by H-H scalar interactions. The doublets with similar fine structure are due to the $^{13}$C-H splitting from moieties containing the $^{13}$C label. The $^{13}$C populations calculated from the ratio of the doublet to the singlet intensities are: C-2, 34%; C-3, 49%; and C-4, 14%, b, proton-decoupled NMR spectrum (25.2 MHz) of [1,2,3,4-$^{13}$C$_4$]glutamate derived from *M. ammoniophilum* grown on [1-$^{13}$C (90 atom %)]glucose. The spectrum illustrates the nonrandom distribution of the $^{13}$C label among the C-2, C-3, and C-4 sites. If the label were distributed randomly with the $^{13}$C abundances derived from the spectra in B, then, for example, the C-1 and C-5 signals should consist of approximate 1:2:1 multiplets.

Glutamate is due primarily to the reduced activity of α-ketoglutarate dehydrogenase (20) which, in effect, shuts down the Krebs cycle. As a result, α-ketoglutarate levels are elevated and, in the presence of sufficient ammonia, glutamate levels will consequently become elevated. Excretion of glutamate into the medium is found to be dependent on growth on limiting biotin levels (2,3); the effect of the biotin is related to the production of faulty, "leaky" membranes which permit this excretion (21).

As is apparent from Fig. 7, several pathways connecting glucose to glutamate are available to the organism. The shortest pathway for direct conversion follows the glycolytic degradation of glucose to pyruvate and phosphoenolpyruvate, formation of acetyl CoA and oxaloacetate via decarboxylation and carboxylation, respectively, and the first third of the Krebs cycle which combines the oxaloacetate and acetyl CoA to yield citrate and, subsequently, α-ketoglutarate. On the basis of this pathway, the glutamate $^{13}$C-labeling pattern resulting from any initial glucose $^{13}$C-labeling pattern is readily determined: [1-$^{13}$C]glucose leads to the synthesis of [3-$^{13}$C]
Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical shift</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C-1</td>
</tr>
<tr>
<td>α-D-Glucose</td>
<td>93.2</td>
</tr>
<tr>
<td>β-D-Glucose</td>
<td>97.0</td>
</tr>
<tr>
<td>α,α-Trehalose</td>
<td>94.2</td>
</tr>
<tr>
<td>α-D-Glucosylamine</td>
<td>84.2</td>
</tr>
<tr>
<td>β-D-Glucosylamine</td>
<td></td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>175.8</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>175.0</td>
</tr>
<tr>
<td>L-Lactate</td>
<td>183.4</td>
</tr>
<tr>
<td>Succinate</td>
<td>183.3</td>
</tr>
</tbody>
</table>

Assignments are from Ref. 14.
Shifts for α,β-glucosylamine C-2 through C-6 were not observed.

Fig. 5. Time-dependent ratio of C-3 to C-4 intensities in [13C]glutamate. Data were taken from the 13C NMR spectra of the experiment depicted in Fig. 2.

Fig. 6. Proton-decoupled 13C NMR (25.2 MHz) spectrum of [1,5-13C2]glutamate obtained from M. ammoniumphilum grown on [1-13C] (70 atom %)acetate. The cells were grown on natural abundance glucose for 24 h and then transferred to the medium containing [1-13C]acetate and cultured for an additional 96 h. Only C-1 and C-2 are labeled. From the multiplet to singlet intensities of C-4, the 13C enrichment at C-5 is calculated to be 35 atom %.

Fig. 7. Major metabolic pathways in M. ammoniumphilum involving glucose, acetate, and glutamate. Glucose labeled at C-1 produces [3-13C]pyruvate via the Embden-Meyerhof pathway (EMP) and unlabeled pyruvate via the hexose monophosphate shunt (HMS). [3-13C]pyruvate enters the tricarboxylic acid (TCA) and glyoxylate shunt (GS) cycles as [3-13C]oxalacetate and/or [3-13C]acetate, and can result in the formation of [2-13C]glutamate, [4-13C]glutamate, and [2,4-13C]glutamate via α-ketoglutarate formed in a third of a turn of the TCA cycle. Formation of glutamate after one or more turns of the TCA cycle will tend to randomize the label because of the formation of the symmetrical intermediates, succinate and fumarate. Similar scrambling will occur through the flow of citrate through the glyoxylate cycle.

pyruvate and [2-13C]acetate and, consequently, to [2,4-13C2]-labeled glutamate. Reference to Fig. 4 for the glutamate released into the medium indicates a pattern which is substantially in agreement with this prediction, supporting this as the predominant route of glutamate synthesis from glucose.

As is apparent from Fig. 7, the pathway summarized above is not the only route of glutamate synthesis, and the importance of other pathways is suggested by deviations from the above pattern, e.g. labeling of C-1 and C-3 of glutamate, which also occurs to a limited extent. In the following section, we consider the general approach of estimating the contributions of alternative pathways from the available NMR data.

Quantitative NMR Analysis of Metabolic Pathways—Quantitation of the 13C population at specific sites in multiply labeled molecules requires the accurate evaluation of the ratios of singlet-to-multiplet intensities in both 1H and 13C NMR spectra. This is usually a straightforward task, but it must be recognized that the satellite resonances can exhibit different T1 and/or nuclear Overhauser effect values than the center resonance. The differential T1 effect in 1H NMR spectra can be quite significant. For example, a center band (13C-H) to satellite (13C-'2C) T1 ratio greater than 4 has been observed for a 13C-enriched sugar methine proton (22). In such cases, the pulse delay must be sufficiently long that the center band is not overpulsed. A similar, but smaller effect occurs in the 13C NMR spectra of enriched molecules. Although the 13C-'2C dipolar interaction is negligible, relative to the 13C-H dipolar interaction for proton bearing carbon atoms (23), the former interaction can be large for quaternary carbons and lead to significant differences in the satellite (13C-'2C) and center band (13C-H) T1 values. Again, if the pulse delay is not long enough, the intensity of the center band will be...
underestimated due to overpulsing. Another related potential source of error in the analysis of $^{13}$C multiplet intensities of quaternary carbon atoms is a reduction in the nuclear Overhauser effect value due to $^{13}$C-$^{13}$C dipolar interactions and which leads to a reduction in the satellite ($^{13}$C-$^{13}$C)/center band ($^{13}$C-$^{13}$C) intensity ratio ($\gamma$). In the evaluation of the relative intensities of the multiplets discussed in the following, these potential problems have been avoided.

The satellite-to-center band intensities, in the $^1H$ NMR spectrum (Fig. 4a) of the L-[1,2,3,4-$^{13}$C$_4$]glutamate derived from *M. ammonium* grown on [1-$^{13}$C]glucose, directly give the total $^{13}$C enrichment of C-2 (34 atom %), C-3 (39 atom %), and C-4 (14 atom %). Although the relative total integrated intensities of the $^{13}$C resonances of C-2, C-3, and C-4 are consistent with these enrichments, this high level of enrichment is not reflected in the $^{13}$C-$^{13}$C multiplets in the $^{13}$C NMR spectrum (Fig. 4b), as noted previously. The apparent discrepancy arises from the presence of $^{13}$C isotopomers (e.g. $^{13}$C$_4$, $^{13}$C$_3$, $^{13}$C$_2$) in which there is a high degree of negative correlation in the $^{13}$C labeling that does not affect the proton spectrum, but has a major influence on the appearance of $^{13}$C multiplets. For example, the C-2 enrichment obtained from the H-2 satellites is 34 atom %, whereas the apparent enrichment calculated from the C-1 satellites (Fig. 4b) is only 25 atom %. This apparent discrepancy can be explained by a negatively correlated labeling of C-1 and C-2: [3-$^{13}$C]oxaloacetate forms [2-$^{13}$C]glutamate via a $\frac{1}{2}$ turn of the Krebs cycle, whereas in a single turn of the Krebs cycle, [4-$^{13}$C]oxaloacetate is formed which can be the precursor for [1-$^{13}$C]glutamate. Note that these reactions will not introduce label into C-1 and C-2 in the same molecule.

Having the absolute $^{13}$C enrichments from the $^1H$ NMR spectra and the multiplet intensities from the $^{13}$C NMR spectra, one can use several approaches to interpret the data quantitatively, on the basis of the metabolic pathways operative. In the present study, we have used the method of mixtures with the essential elements summarized below.

1) Variables are assigned to each alternative path with the sum normalized to 1.0. 2) A labeling pattern is associated with each path, based on the biochemical pathways expected. 3) A table is formulated in which the various possible labeled species resulting from each pathway are included. 4) From the above table, a second table giving the carbon enrichments and multiplet probabilities can be constructed and then the relative probabilities obtained. As indicated in the table, several compromises have been made in the analysis since in some cases different labeling pathways can result in identical labeling patterns and thus cannot be distinguished. In addition, a small quantity of the labeling patterns indicated under w and y cannot be observed.

<table>
<thead>
<tr>
<th>Pathway designation</th>
<th>Labeling pattern</th>
<th>Source of label</th>
<th>Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>x</td>
<td>C$_1$C$_2$C$_3$C$_4$</td>
<td>Phosphoenolpyruvate carboxylase + $\frac{1}{2}$ Krebs cycle</td>
<td>58</td>
</tr>
<tr>
<td>y</td>
<td>C$_1$C$_2$C$_3$C$_4$</td>
<td>Fumarase activity plus single turn of glyoxylate cycle</td>
<td>26</td>
</tr>
<tr>
<td>z</td>
<td>C$_1$C$_2$C$_3$C$_4$</td>
<td>Multiple turns of Krebs or glyoxylate cycles</td>
<td>10</td>
</tr>
<tr>
<td>w</td>
<td>C$_1$C$_2$C$_3$C$_4$</td>
<td>Single turn</td>
<td>6</td>
</tr>
</tbody>
</table>

*a Asterisk (*) indicates a labeling probability $\alpha$ derived from the enriched pyruvate pool. Zero (0) indicates a labeling probability $\beta$ derived from the enrichment of the bicarbonate pool.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Labeling multiplet probabilities</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1</td>
<td>(x + y + z + w) $\cdot \alpha = \alpha_0$</td>
</tr>
<tr>
<td>C-2</td>
<td>(x + y + $w$) $\cdot \alpha = \alpha_0$</td>
</tr>
<tr>
<td>C-3</td>
<td>(y + z + $w$) $\cdot \alpha = \alpha_0$</td>
</tr>
<tr>
<td>C-4</td>
<td>(x + y + z + w) $\cdot \alpha = \alpha_0$</td>
</tr>
</tbody>
</table>

For the present study, $\alpha_0 = 90\%$, $\alpha_0 = 39\%$, giving $f = 87\%$, or 13%, of the glucose is metabolized via the hexose monophosphate shunt.
additional turns of the Krebs and glyoxylate cycles. Nevertheless, the approach summarized represents a reasonable approximation for quantitative pathway analysis. Since spectral inspection (Fig. 4b) indicates that the number of multiply enriched molecules (z) is small, these approximations are probably fairly good. As can be seen from the table, negative correlations of the labeling of C-2 and C-3 can result from the Krebs or glyoxylate cycle, or from back reactions which reach fumarase. Krebs cycle activity will also result in a negative correlation between C-1 and C-2 labeling. Analytical expressions for carbon labeling and relative multiplet probabilities can be derived in a straightforward manner, and the results are summarized in Table III. Using these expressions and the value of α₀ = 0.39 and the relative multiplet intensities in the $^{13}$C spectrum (Fig. 4b), values of 58, 26, 10 and 6% are obtained for x, y, z, and w, respectively. The relative contribution of these pathways should result in $^{13}$C populations at C-2 and C-3 of 33 and 10%, respectively, in good agreement with those obtained directly from the $^1H$ NMR spectrum (Fig. 4a).

We were unable to grow M. ammoniphilum on acetate as the sole carbon source; however, cells grown on unlabelled glucose were washed and incubated with [l-$^{13}$C]acetate. A $^{13}$C NMR spectrum of the extracted glutamate is shown in Fig. 6. The primary features of this spectrum are the labeling ratio of 2:1 for C-5:C-1 and the absence of label for C-2, C-3, and C-4. The parallel labeling of the C-5 doublet (24) on the glutamate labeling patterns obtained with another glutamate producer, Brevibacterium flavum.

**DISCUSSION**

*Activity of the Hexose Monophosphate Shunt*—The determination that 13% of the glucose metabolized by M. ammoniphilum occurs via the hexose monophosphate shunt agrees well with the 10% value determined by analysis of the pyruvate carboxyl group in metabolically blocked cells (18, 19). The result is significantly lower than the 25% figure determined by recovering $^{14}$CO$_2$ from [1-$^{14}$C]- and [6-$^{14}$C]glucose-grown cells. There is a variety of approximations inherent in both approaches (25). In particular, the hexose monophosphate pathway may be somewhat underestimated since some of the pentose produced via this path will be used for nucleotide synthesis, rather than for production of pyruvate.

*Activities of the Krebs and Glyoxylate Cycles in the Metabolism of [l-$^{13}$C]Glucose*—The major metabolic routes found for the degradation of glucose involve glycolysis to pyruvate and phosphoenolpyruvate, formation of acetyl-CoA and oxaloacetate via decarboxylation and carboxylation, respectively, and the first third of the Krebs cycle to yield α-ketoglutarate and, subsequently, glutamate. Significant activity of the glyoxylate shunt is required to explain the C-3 labeling and reduced C-2 labeling of glutamate. The observed Krebs cycle activity was initially unexpected since this cycle is presumably shut down due to the reduction of α-ketoglutarate dehydrogenase activity. Evidence for the activity of this cycle comes primarily from the observed negative correlation in the labeling of C-1 and C-2 of glutamate.

The time dependence of the $^{13}$C NMR spectra (Fig. 2) and the time-dependent increase in the C-3/C-4 intensity ratio (Fig. 5) is particularly interesting. Since the C-4 enrichment level is constant (α = 0.39), this time-dependent ratio reflects a time-dependent increase in the relative contributions of the y, z, and/or w pathways. Although, in principle, it is possible to quantitate these as a function of time, there are several problems with this approach in practice. 1) The spectra correspond to cumulative pools of glutamate and therefore reflect cumulative average pathways; 2) $^1H$ NMR spectra of purified glutamate in parallel with the $^{13}$C data were not obtained; 3) the signal/noise in the 2-h spectra was insufficient to permit analysis of the C-1 multiplet, which is of value for the demonstration of Krebs cycle activity. In view of these problems, a simplified approach was taken in which the glyoxylate and Krebs cycle activities were both represented by y since it becomes impossible to distinguish between y and w in the absence of C-1 data. Based on this analysis, the cumulative contribution from the y pathway increases from 0 to a steady state value of ~35%, with z increasing to ~12% and x decreasing to a value of ~53%. This increased cycling may reflect the decreasing glucose concentration which might release the inhibition of enzymes in both the glyoxylate and Krebs cycles, i.e. the time-dependent changes may reflect catabolite repression. A similar time-dependent result was recently reported in a study of the glutamate derived from rat hearts perfused with [2-$^{13}$C]acetate (26).

Another interesting result is that the $^{13}$C spectra show that the labeling at C-4 also appears to show a negative correlation with C-5. On the surface, there is no immediately obvious route for C-5 to be labeled, and one would expect C-5 to be at natural abundance regardless of the labeling at C-4. Instead, the analysis of the doublet center band intensities at C-5 leads to an enrichment of 32 atom % $^{13}$C at C-4, compared to the higher value of 39 atom % obtained by the $^1H$ satellites. This apparent discrepancy can be explained using the following pathway in which oxaloacetate breaks down to acetyl-CoA, which leads to an anticorrelated (vis a vis C-4) C-5 enrichment of glutamate (Structure 1).

\[
\text{COO}^+ \rightarrow \text{COO}^- \rightarrow \text{COO}^- \rightarrow \text{CH}_4 \rightarrow \text{CH}_2 \rightarrow \text{HCNH} \rightarrow \text{COO}^- \rightarrow \text{COO}^-
\]

Oxaloacetate  pyruvate  acetyl-CoA  L-glutamate  

**STRUCTURE 1**

The amount by which the acetyl-CoA pool is diluted with material derived from the oxaloacetate pool can be calculated from Equation 2.

\[
D/S = \frac{(1 - f)z\alpha^2 + f\gamma}{(1 - f)\left[\frac{y + w}{2}\alpha + 2z(1 - \alpha)\right] + f(1 - \alpha)}
\]

where D/S is the doublet to singlet ratio for $^{13}$C-5, f is the fraction of acetyl-CoA derived directly from glucose, 1 − f the fraction of acetyl-CoA derived from oxaloacetate, α, y, z, and w are as defined in Table II, and γ is the natural abundance $^{13}$C level for C-5. Solving this equation for f from the data in Table II gives a value of ~96%, suggesting that only ~4% of the acetyl-CoA pool is derived from oxaloacetate. Finally, given f, one can calculate the enrichment of C-5 from Equation 3.

\[
\%^{13}\text{C-5} = f + \alpha(1 - f)(y/2 + w/2 + z)
\]

This value cannot be directly measured from the data, but a value of 1.8% $^{13}$C can be obtained from Equation 2. Using a different approach, one can also calculate the enrichment at C-5 from the apparent enrichment at C-4 using the C-5 multiplets and the actual enrichment at C-4 using the proton data. The discrepancy between these two values and the known distribution of label between the C-5 doublet and singlet from the amount of negatively correlated labeling at C-2 and C-3 of oxaloacetate, allow one to calculate an enrich-
ment of 1.7% at C-5, in excellent agreement with the value determined from Equation 3.

**Metabolism of [1-13C]Acetate**—In principle, it is possible to explain the 1:2 ratio for the C-1/C-5 labeling of glutamate derived from [1-13C]acetate on the basis of a contribution of the unlabeled metabolites initially present in the cell, which would dilute the C-1 label to a greater extent than C-5. However, this explanation proves inadequate since the concentration of initial unlabeled metabolites is far less than would be necessary to produce the observed difference. Thus, the labeling difference must reflect a steady state phenomenon in which the C-1 label is systematically diluted. This conclusion can be made more convincingly in the case of *B. flavum*, in which acetate can be used as the sole carbon source, eliminating the possible complication of an initial pool of unlabeled metabolites.

A steady state analysis of the acetate labeling experiments indicates that the data can be explained on the basis of significant Krebs cycle activity. This reflects the fact that only half of the C-1 carbons processed through the w pathway become labeled (Table II). The possibility of high Krebs cycle activity as an explanation for similar carbon-14 acetate growth results was considered by Shiio and Tsunoda (24), but subsequently rejected on the basis of the demonstrated inhibition of this cycle under glucose growth conditions. The alternative possibility of two acetate pools for a prokaryotic cell seems less likely. We thus conclude that, as in the apparent time-dependent nature of the labeling, differences in carbon source may also have a significant effect on the relative contributions of the different pathways, possibly reflecting catabolite repression.

**Acknowledgments**—We gratefully acknowledge the technical assistance of W. E. Wageman and several helpful discussions with Dr. David LeMaster.

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