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We have used [13C] nuclear magnetic resonance to monitor acetate metabolism in a proline-overproducing strain of *Escherichia coli* growing on [13C]-labeled acetate. The conversion of [13C]-labeled acetate to proline by actively dividing cells was followed in vivo, and the site specificity of the incorporation of the acetate carbons in the proline was determined from spectra of butanol extracts of the growth media. The degree of incorporation of deuterium from partially deuterated water into various sites on the proline was monitored from the β-deuterium-shifted signals in the [13C] spectra. The spectra provide information on the origin of the carbons and the protons during proline biosynthesis.

[13C] nuclear magnetic resonance (NMR) spectroscopy provides a powerful tool by which several metabolic characteristics of [13C]-labeled carbon substrates can be simultaneously determined in vivo or in vitro (3, 6). Substrate utilization, as well as product and byproduct accumulation, is frequently portrayed within the same spectrum, providing information on both fermentation rates and product yields (13, 18). Quantitative distributions of metabolite [13C]-isotopomers, obtained directly from high-resolution spectra, reveal relative carbon fluxes through alternative metabolic pathways (5, 9, 10, 18, 19). When [13C] and [2H] are both incorporated, deuterium changes the [13C] spectrum in a manner that provides insight into the metabolic origins of the incorporated deuterium atoms (1, 7).

Traditionally, metabolism was analyzed by examining the incorporation of radiolabeled compounds after fragmentation of both the biological system under study and its constituent labile molecules. In principle, the types of metabolic information described above can be obtained noninvasively via NMR spectroscopy, since NMR spectra of whole organisms provide snapshots of cellular metabolism. In practice, the utility of this approach for the examination of carbon metabolism is reduced by the limited sensitivity of [13C] NMR spectroscopy. To obtain interpretable spectra within convenient periods, cell extracts or concentrated cell suspensions, in which metabolism is likely altered by anaerobiosis, have often been required (8). The sensitivity problem can be alleviated by using isotopically enriched nutrient supplies (9, 10, 18, 19), by studying metabolites that are normally present at high concentrations (19), and by examining the metabolic characteristics of organisms manipulated to overproduce or excrete particular metabolites (9, 10). Thus, this approach is particularly applicable to industrial microorganisms whose metabolism has been manipulated to cause overproduction of a desired metabolite (9, 10, 18).

In an effort to analyze the impact of membrane permeability changes on fermentative metabolism, we isolated *Escherichia coli* derivatives in which proline biosynthesis was uncontrolled and excess proline was readily excreted (15). Proline production by one of those strains during aerobic growth on [13C]-enriched acetate could be detected by [13C] NMR spectroscopy of the bacterial culture. Bacteria were grown on [1-13C]acetate or [2-13C]acetate in the absence or presence of 20% D2O. Full interpretation of the spectra obtained revealed the efficiency with which acetate was converted to proline, some characteristics of acetate metabolism by this *E. coli* derivative, and the metabolic origins of carbon and hydrogen in the proline excreted by the bacteria.

**MATERIALS AND METHODS**

**Chemicals.** Deuterium oxide (99.8 atom% D), sodium [1-13C]acetate, and sodium [2-13C]acetate (both 99 atom% [13C]) were purchased from MSD Isotopes (Montreal, Quebec, Canada). All other chemicals were obtained from the previously noted sources (15, 20), or the reagent-grade product from BDH Chemicals (Toronto, Ontario, Canada) was obtained. External benzene standards used as intensity reference signals in NMR spectra were prepared by dissolving tris-(acetylacetone)chromium(III) to a concentration of 30 mM in benzene. This solution was sealed in a capillary tube (18 cm by 2 mm [inner diameter]) to give an approximately 8-cm height of liquid.

**Bacterial strains.** The bacterial strains used in this study were derivatives of *E. coli* K-12 (Table 1). Bacteriophage P1-mediated transductions were performed as described by Miller (14). Proline auxotrophic derivatives of strains CSH4 and RM2 were identified after selecting tetracycline-resistant transductants on LB agar plates (14) supplemented with tetracycline (25 μg ml⁻¹). Proline-overproducing derivatives of strains WG187 and WG191 were identified by screening proline prototrophic transductants for constitutive expression of proline dehydrogenase (for derivatives of strain WG187) or for proline excretion (for derivatives of strain WG191) by previously described procedures (20). The isolation of allele pro-203 (strain WG264) was described by Rancourt et al.; the purity and genetic stability of all cultures was verified by the phenotypic testing procedures described in that paper (15).
TABLE 1. E. coli K-12 strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype*</th>
<th>Source, derivation, or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSH4</td>
<td>F’ trp lacZ rpsL thi</td>
<td>Cold Spring Harbor Laboratory (20)</td>
</tr>
<tr>
<td>CSH26</td>
<td>F’ ara Δ(lac pro) thi</td>
<td>Cold Spring Harbor Laboratory</td>
</tr>
<tr>
<td>NK5525</td>
<td>pro-81::Tn10 λ−</td>
<td>B. J. Bachmann</td>
</tr>
<tr>
<td>RM2</td>
<td>CSH4 Δ(pu PA)101</td>
<td>R. Menzel (20)</td>
</tr>
<tr>
<td>WG187</td>
<td>CSH4 pro-81::Tn10</td>
<td>P1 transductant of CSH4 from NK5525</td>
</tr>
<tr>
<td>WG191</td>
<td>RM2 pro-81::Tn10</td>
<td>P1 transductant of RM2 from NK5525</td>
</tr>
<tr>
<td>WG195</td>
<td>CSH4 pro-203</td>
<td>P1 transductant of WG187 from GS3</td>
</tr>
<tr>
<td>WG199</td>
<td>RM2 pro-203</td>
<td>P1 transductant of WG191 from GS3</td>
</tr>
<tr>
<td>WG264</td>
<td>CSH26 F’128 lac pro-203</td>
<td>15</td>
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</table>

* For genetic nomenclature, see reference 2. Δ, Chromosomal deletion; ::, transposon insertion; IN, chromosomal inversion.

Bacterial cultures. A loop of the designated E. coli strain was added to 5 ml of LB medium (14) and incubated at 37°C overnight. This culture was used to inoculate 500 ml of an acetate minimal medium containing potassium phosphate buffer (10 g liter−1, pH 7.0), (NH₄)₂SO₄ as the nitrogen source (1 g liter−1), sodium acetate as the carbon and energy source (3 g liter−1), MgSO₄·7H₂O (10 mg liter−1), L-tryptophan (100 mg liter−1), and thiamine hydrochloride (2 mg liter−1). Aeration and agitation were provided by sparging with air at 1 liter min−1 while the culture was incubated at 37°C. Under these conditions, the culture optical density doubled in 4 h. Typically, cells from 5 ml of culture at an optical density (at 500 nm) of 1.0 were collected by centrifugation and transferred to 10 ml of the same minimal medium in which a 13C-labeled carbon source replaced the naturally abundant sodium acetate.

Cell dry weights were determined from a standard curve relating culture optical density to cell dry weight. Cells were collected in stationary phase, washed with water, and resuspended to 10 ml at optical densities (at 500 nm) of 0.45, 0.60, 0.73, and 1.00. Each of the four 10-ml suspensions was slowly dried to constant weight in a vacuum oven at 70°C with a water aspirator. Cell dry weight was plotted against optical density.

For in vivo NMR measurements, bacteria were provided with [1-13C]acetate (2 g liter−1) as the carbon and energy source in a previously autoclaved sample assembly. A 20-mm-diameter NMR sample tube was fitted with a coaxial 5-mm bubbling tube drawn to a fine capillary tube at the sample end. An external benzene intensity standard was attached to the bubbling tube with an elastic band, and the assembly was closed with a cotton plug. After introduction of the sample, a 4-mm-inner-diameter Tygon hose (Fisher Scientific Co., Pittsburgh, Pa.) was attached to the bubbler and the whole assembly was inserted into a spinner and lowered into the magnet. Air was metered to the culture at 30 ml min−1, and the temperature was maintained at 37°C as described below (see NMR spectroscopy).

n-Butanol extracts containing 13C-labeled metabolites were prepared from cultures provided with [1-13C]acetate or [2-13C]acetate (2 g liter−1) as the carbon and energy source in 20-mm-diameter test tubes. After aeration by bubbling for 6 h at 37°C, the culture supernatants were collected by centrifugation.

Preparation of culture supernatants for NMR analysis. Culture supernatants from bacteria grown on 13C-labeled acetate were filtered through 0.45-μm-pore-size nitrocellulose filters (Nuclepore, Toronto, Ontario, Canada) and evaporated to dryness at 60°C under vacuum. To separate amino acids from the salts of the medium, the dry residue was extracted with 10 ml of n-butanol under reflux for 30 min. The n-butanol was evaporated at 60°C under vacuum, the residue was redissolved in 0.5 ml of D₂O, and the solution was filtered into a 5-mm-diameter NMR tube.

NMR spectroscopy. Carbon NMR spectra of bacterial cultures were obtained at 50.3 MHz with 20-mm NMR tubes (described above) in a Bruker CXP-200 spectrometer. No field/frequency lock was required as a result of the excellent field stability of the magnet. The field homogeneity was adjusted by maximizing the proton-free induction decay from H₂O observed with the decoupler coil. Spectra were acquired with broadband proton irradiation to collapse the proton-coupled multiplets. Dielectric heating by the decoupler, which provided 2 W of power at 200 MHz, was balanced by a flow of cooler air around the sample tube (34°C, 500 liters h−1; maintained with a Bruker VT 1000 temperature control unit). To reduce heating, the decoupler was gated on only during the 1-s spectrum acquisition time and gated off during the subsequent 2-s relaxation decay. The decoupler power setting and air flow rate required to maintain the sample at 37°C were determined by monitoring the temperature with a copper constantan thermocouple inserted into a dummy sample.

Spectra were acquired by using π/4 pulses with a 3-s recycle time and gated decoupling as described above. They were obtained by accumulating 1,024 transients in 8K (1K = 1,024) data points with a spectral width of 10 kHz. Acquisition of each spectrum required 51.2 min, and a set of sequential files spanned 9 to 13 h. An exponential multiplication with 5-Hz line broadening was applied to the carbon-free induction decay before Fourier transformation. Peak heights were measured relative to that of the external benzene standard. Chemical shifts are quoted relative to tetramethylsilane (0.0 δ) but were measured relative to [5,13C]proline (47.0 δ) as the internal standard.

The NMR peak assignments for proline are available from the literature (11) but were checked by standard methods (16). The proton assignments were checked by simple decoupling experiments and two-dimensional proton correlation spectroscopy. The carbon assignments were confirmed by a heteronuclear chemical shift correlation to the previously assigned proton spectra.

In spectra acquired as described above, the signals arising from different carbon sites within the same molecule will not reflect the same apparent concentration. There is a considerable range of spin lattice relaxation rate constants (0.03 to 0.2 s−1) among the 13C sites within the same molecule in these samples so that, with only a 3-s recycle time, there will be differences in intensity due to more or less complete relaxation between pulses. The proton irradiation will generate an intensity gain from the nuclear Overhauser enhancements (NOEs). The differences in relaxation rates also generate differential NOEs from signal to signal. The biological nature of our samples precluded the use of longer delays and longer irradiations to ensure accurate intensity determinations. In general, we have not tried to compare the absolute intensities of the signals from different carbon atoms but instead have calibrated individual signals against a
good signal-to-noise of individual relaxation reagent to ensure that the signal peak heights relaxed fast and had 4,000 transients achieved added back were used with relaxation of extracts were measured relative to the benzene standard, were added back to the sample four times. An NMR spectrum was acquired after each of the four additions. The peak heights were measured relative to the benzene standard, and the quantity of each compound originally present was determined by extrapolating a plot of peak height versus quantity added back to the zero addition point. The correlation coefficients for these plots were greater than 0.999.

The 13C NMR spectra of starting materials or butanol extracts were acquired in 5-mm tubes at 100.6 MHz in a Bruker AM-400 spectrometer. Field stabilization was achieved by locking on the solvent, D2O. To permit significant relaxation of all the signals in the spectrum, π/4 pulses were used with a 5-s relaxation delay. The power level of the decoupler was shifted between 0.5 W during the relaxation delay and 3 W during spectral acquisition. Usually more than 4,000 transients were acquired in overnight runs to obtain good signal-to-noise ratios. The NMR signal was acquired in 32K data points with 20 kHz spectral width. When indicated, an exponential multiplication with 5-Hz line broadening was applied to the free induction decay before Fourier transformation. In some of the butanol extract spectra, resolution enhancement was applied by using Gaussian multiplication. Gaussian multiplication uses two parameters; GB is the fraction of the acquisition time at which the multiplication factor rises to 1, and LB is the resolution enhancement factor defining the Gaussian width. Peak height and chemical shift standards were as noted above.

**RESULTS**

In vivo assessment of acetate metabolism and proline production. E. coli WG195 and WG199 both grew on 13C acetate, but 13C-labeled proline was detected spectroscopically only during the growth of strain WG199. We have previously demonstrated that both strains lack regulation of proline biosynthesis, but only strain WG199, which also lacks the proline catabolic enzymes, is a net producer of proline (15). The proline detected in cultures of strain WG199 was in the culture medium, after removal of the bacteria by centrifugation, the NMR spectrum of the supernatant was essentially the same as the spectrum of the original cell suspension. Furthermore, no significant NMR signals other than those of acetate and proline were observed in any of the experiments reported below.

The NMR signals arising from the low level of 13C (1.1% natural abundance) present at C-2 and C-1 in [1-13C]acetate and [2-13C]acetate, respectively, were employed to examine the level of 13C enrichment at the labeled site in the substrates. Before metabolism, each of the natural-abundance signals was split into a doublet by the 13C in the adjacent, labeled position; no singlet from residual 12C at the labeled site was detectable (Fig. 1A and C). After metabolism, significant singlet resonances were observed representing 8 (from metabolism of [1-13C]acetate [Fig. 1B]) and 12% (from metabolism of [2-13C]acetate [Fig. 1D]), respectively, of 13C at the nominally labeled site. The latter (12%) may be an underestimate, since the methyl 13C may provide a significant relaxation pathway for the slowly relaxing carboxyl 13C (12) generating larger signals in the incompletely relaxed spectra. These observations suggest approximately 10% replacement of the label by naturally abundant carbon in substrate acetate during metabolism. Head-to-head or tail-to-tail coupling of acetate moieties, followed by subsequent metabolism to produce doubly labeled acetate, would increase the intensities of the doublets. Such reactions would be undetectable in these analysis.

Utilization of [1-13C]acetate and [13C]proline production were observed simultaneously by monitoring C-5 of proline

**FIG. 1.** Metabolism of [13C]acetate by E. coli WG199. The resonance originating from the naturally abundant 13C at C-2 (23.8 ppm) (A and B) or C-1 (182.1 ppm) (C and D) of acetate is illustrated. Acetate in the culture medium was examined before inoculation (A and C) and after bacterial cultivation on [1-13C]acetate (B) or [2-13C]acetate (D) as described in Materials and Methods.

**FIG. 2.** Time course of proline production from [1-13C]acetate. Spectra were obtained from a culture of strain WG199 growing in the spectrometer as described in Materials and Methods. Each spectrum is the sum of two sets of 1,024 transients each.
(47.0 ppm) and C-1 of acetate (182.1 ppm), respectively (Fig. 2). Outside the spectrometer, the optical density (at 500 nm) of cultures of strain WG199 doubled in 4 h. The final optical density of those cultures increased linearly as the sodium acetate supply rose from 1 to 4 g liter\(^{-1}\), and 1 g liter\(^{-1}\) of sodium acetate supported an optical density increase of 0.22. During 10 h of incubation within the spectrometer (Fig. 2), 10 mg of sodium [1-\(^{13}\text{C}\)]acetate was consumed in 10 ml of culture within approximately 7.5 h, yielding a culture optical density increase (at 500 nm) of 0.2. Thus, although the spectrometer culture grew slowly, its growth yield on acetate was unaltered. In the spectrometer, 0.37 mg of \([^{13}\text{C}]\)proline and approximately 0.75 mg of cells (dry weight) were produced.

**Metabolic origins of proline carbons.** When strain WG199 was grown on \([^{13}\text{C}]\)-labeled acetate, the proline excreted into the culture medium was highly \([^{13}\text{C}]\) enriched. Different proline-labeling patterns (Fig. 3C) were observed when butanol extracts of the culture media were examined after growth on \([1-^{13}\text{C}]\)acetate and \([2-^{13}\text{C}]\)acetate (Fig. 4; Table 2). As expected in view of the acetate label scrambling noted above, a low background level (approximately 5%) of general labeling of proline was observed in both cases. Growth on \([1-^{13}\text{C}]\)acetate yielded proline containing \([^{13}\text{C}]\), primarily at carbons 1 and 5, as indicated by the singlet resonances at 175.3 and 47.0 ppm, respectively (Fig. 4A). Growth on \([2-^{13}\text{C}]\)acetate yielded proline labeled at carbons 1, 2, 3, and 4, as revealed by doublet or double doublet resonances with coupling constants of 30 to 60 Hz (Fig. 4B). The resonance of proline C-4 (24.6 ppm) was readily resolved from that due to residual \([2-^{13}\text{C}]\)acetate (23.8 ppm). Since essentially no \([^{13}\text{C}]\) from \([2-^{13}\text{C}]\)acetate was detected in C-5 of proline, virtually complete \([^{13}\text{C}]\) enrichment at C-5 must be attained during growth on \([1-^{13}\text{C}]\)acetate. The level of labeling at proline C-1 was underestimated with respect to that at C-5 when peak heights are compared (see Discussion), but the contribution to C-1 of proline from C-1 of acetate was found to be 60% with standard additions.

Resolution enhancement and expansion of Fig. 4B clarified the multiple NMR signals representing the mixture of

![FIG. 3. Key to numbering for isotopic labeling patterns for α-ketoglutarate (A), glutamate (B), and proline (C).](image)

![FIG. 4. Proline isotopomers produced by strain WG199 during growth on [\(^{13}\text{C}\)]acetate. NMR spectra of butanol extracts from the culture media subsequent to growth of strain WG199 on [1-\(^{13}\text{C}\)]acetate (A) or [2-\(^{13}\text{C}\)]acetate (B) were obtained as described in Materials and Methods.](image)

![FIG. 5. High-resolution spectra of the proline isotopomers derived from growth of strain WG199 on [2-\(^{13}\text{C}\)]acetate. The resolution of the NMR spectrum in Fig. 4B was enhanced using a Gaussian apodization window with GB = 0.3 and LB = −2.0. Resonances for C-1, C-2, and C-3 have been enlarged eight-, two-, and twofold, respectively. Residual [2-\(^{13}\text{C}\)]acetate gives the strong resonance at 23.8 ppm. The observed splitting pattern is indicated for each of the proline carbons. The numbering is shown in Fig. 3C.](image)
The levels of deuterium incorporation indicated by the β-D shifts, estimated from the relative peak intensities, were compared with the probabilities for random deuterium incorporation by exchange with the solvent (Table 3; Fig. 3C). The relative amounts of the deuterium-shifted signals were determined from integrated areas as the line widths increase because of deuterium-carbon coupling as the number of deuterons increases. This effect is significant for the signals from C-4. C-2 and C-4 reported approximately random incorporation of deuterium at C-3 and C-5. C-5 reported no deuterium at C-4, so the shift in the signal from C-3 must be due to incorporation of a single deuterium at C-2.

DISCUSSION

Several technical factors contributed to the success of these experiments. Bacteria genetically engineered to excrete proline were used, thereby ensuring that proline levels detectable by NMR would be attained during culture growth. Proline, which was excreted into the culture medium, provided a window through which intracellular metabolism could be viewed (see below).

The NMR signal intensity is, in principle, proportional to the number of resonating nuclei, allowing a quantitative measure of the numbers or concentrations of particular nuclei present within a sample (17). Usually each spectrum obtained reflects a compromise between an adequate signal-to-noise ratio and short time for resolution required to follow metabolism. Rapid signal averaging and NOE can improve signal-to-noise ratios, but the intensity of each signal no longer reflects the absolute concentration of the nucleus from which it originates. The sensitivity with which particular carbon nuclei are detected thus depends on their molecular environments, on the conditions of spectral acquisition such as NOE and proton decoupling, and on postacquisition spectral manipulations. For example, the sensitivity for detection of the carboxyl carbons of proline and acetate in our experiments was poor because of their slow relaxation and small NOE. Introduction of 13C adjacent to those sites would reduce sensitivity by splitting their spectral signals but improve the sensitivity by generating faster spin lattice relaxation (12). For spectra from acetate C-1, our experimental conditions would overestimate the amount of doubly labeled material compared with singly labeled (i.e., 13C at C-2) material.

Metabolic origins of proline hydrogens. 13C NMR signals are shifted upward when deuterium atoms are incorporated at the carbon site(s) adjacent (i.e., 13C-C-deuterium or β-deuterium) to the 13C under observation. The observed shift is 0.05 to 0.1 ppm per deuterium incorporated. Thus, the ratio of the intensities of shifted and unshifted 13C signals reports the extent of deuterium incorporation on adjacent carbon atoms (1, 7). Proline biosynthesis from [2,13C]acetate in the presence of D2O (20%) yielded multiply deuterated proline isotopomers (Fig. 6). β-Deuterium shifts were obvious when the peak multiplets observed after [13C]proline synthesis in the absence (Fig. 5) and presence (Fig. 6) of D2O were compared. No shifts were observed in the signals from C-1, but β-D shifts (i.e., shift generated by one deuteron β to the site in question) were apparent in the multiplets from C-2, C-3, and C-4. Furthermore, β-D2 shifts were visible in the signals from C-3 and C-4, and a β-D3 shift was observed in the multiplet from C-4. In a separate experiment in which [1-13C]acetate and D2O (20%) were provided, less than 1% deuteration at C-4 was reported by the resonance originating from C-5 (data not shown).

TABLE 3. Deuterated proline isotopomers

<table>
<thead>
<tr>
<th>Reporter nucleus</th>
<th>Deuterated carbon site(s)</th>
<th>No. of β-deuterons</th>
<th>% Resonance contribution</th>
<th>Observed</th>
<th>Calculated</th>
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<tbody>
<tr>
<td>C-2</td>
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<td>0</td>
<td>70</td>
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<tr>
<td></td>
<td></td>
<td>1</td>
<td>30</td>
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<td></td>
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<td>2</td>
<td>4.0</td>
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<tr>
<td>C-3</td>
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<td>70</td>
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<tr>
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<td>2</td>
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<tr>
<td></td>
<td></td>
<td>3</td>
<td>5</td>
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<td></td>
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<td>2</td>
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* Estimated to ±5% from Fig. 6.
For the above reasons, we could not base quantitative conclusions about the levels of label incorporation on peak height comparisons among signals originating from different carbon atoms. We employed [1-13C]acetate to monitor the in vivo conversion of acetate to proline since sensitivity did not limit our estimation of substrate acetate utilization. More importantly, the resulting labeling at C-5 (and not C-2, C-3, or C-4) of proline provided a sensitive indicator of proline production because proton decoupling, NOE, and the lack of 13C-13C splitting all contributed to generate a quickly relaxing singlet signal. The result was a sensitivity for proline detection of 5 μg ml−1 (Fig. 2). Quantitative estimates of proline synthesis from acetate were based on the method of standard additions. Most factors influencing sensitivity of the signal were therefore taken into account empirically.

An alternative procedure was used in examining the fraction of proline molecules incorporating 13C or 2H from various metabolic sources. With a knowledge of the expected 13C-13C signal splitting or the β-D shift, we were able to use one 13C signal to report on the fraction of deuterium labeling at a second, adjacent carbon (1, 7, 18, 19). Quantitative data were obtained without comparing relative integrated intensities at different carbon sites (Fig. 3, 4, and 5; Tables 2 and 3).

Before evaluating the site-specific incorporation of acetate 13C into proline, it was necessary to examine the impact of metabolism on labeling of the substrate acetate. Approximately 10% reversal of each singly labeled acetate isopomer was observed during the 10- to 13-h incubation represented in Fig. 1. In addition, the small doublet resonance observed at C-5 when proline was synthesized from [2-13C]acetate (Fig. 4A) provided some evidence for incorporation of doubly labeled acetate. Walsh and Koshol found no evidence for scrambling of the acetate label during synthesis of glutamate from [2-13C]acetate by E. coli (19), whereas Shulman and co-workers interpreted their studies of acetate metabolism by Saccharomyces cerevisiae in terms of a futile cycle in which the acetyl coenzyme A label did become scrambled (4). Our data suggest that acetate label reversal occurs in strain WQ199. The generation of doubly labeled acetate through futile cycling of 13C-labeled oxaloacetate via phosphoenolpyruvate carboxykinase, pyruvate kinase, and pyruvate dehydrogenase cannot be ruled out. Further studies will be required to determine the metabolic basis of the label reversal and to assess whether it is related to the proline-producing capability of this strain. The alteration in acetate labeling during metabolism was insufficient to prevent interpretation of the proline-labeling patterns obtained from [1-13C]acetate and [2-13C]acetate.

Incorporation of [1-13C]acetate yielded proline labeled almost exclusively at C-1 and C-5 (Fig. 4A). C-5 of proline is derived exclusively from C-5 of α-ketoglutarate, which is in turn derived from C-1 of acetate (Fig. 3A, [19]). In contrast, C-2, C-3, and C-4 of proline originated almost entirely in C-2 of acetate (Fig. 5 and text). Our analysis indicates that 60% of proline C-1 originated from acetate C-1 (Fig. 4A and text), whereas 35% of proline C-1 was derived from acetate C-2 (Fig. 4B and Table 2). These values, which accounted quite well for the total proline produced, are consistent with the quantitative carbon flux through the tricarboxylic and glyoxylate cycles reported previously by Walsh and Koshland (19). They examined acetate metabolism by monitoring the labeling of the proline precursor, glutamate, in an E. coli strain with normally regulated biosynthetic pathways.

The incorporation of deuterons observed at C-2 and C-3 of proline was that expected from facile solvent exchange of those protons during transamination and the enolization of α-ketoglutarate, respectively. The lack of deuterium at C-4 indicates that in the steady state, there is virtually no deuterium incorporation in acetyl coenzyme A. Hence, the integrity of the acetate C-H bonds is preserved at C-4 during the synthesis of proline.

Carbon-13 NMR spectroscopy provides a convenient method of examining a range of useful kinetic and structural parameters. NMR is not a sensitive form of spectroscopy, but through the use of specific 13C labels, careful attention to acquisition parameters, and the use of overproducing bacterial strains, one can obtain spectra from cultures of actively dividing cells. Since one sees the entire range of compounds present in the medium, it is not necessary to use assays designed to detect specific and pre-judged metabolites. Hence, the method has considerable potential for the detection of unusual metabolism which may be introduced in the genetic manipulation of the bacteria.

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LITERATURE CITED


