The branch point of the tricarboxylic acid and glyoxylate shunt has been characterized in the intact organism by a multidimensional approach. Theory and methodology have been developed to determine velocities for the net flow of carbon through the major steps in acetate metabolism in Escherichia coli. Rates were assigned based on the $^{13}$C NMR spectrum of intracellular glutamate, measured rates of substrate incorporation into end products, the constituent composition of E. coli, and a series of conservation equations which described the system at steady state. The in vivo fluxes through the branch point of the tricarboxylic acid and glyoxylate cycles were compared to rates calculated from the kinetic constants of the branch point enzymes and the intracellular concentrations of their substrates.

As the study of cellular metabolism progresses, it is becoming increasingly apparent that regulatory processes must be understood in the context of their cellular environment. Historically, our understanding of metabolic regulation has been developed through in vitro studies with purified enzymes. The results of these studies can be misleading since enzyme assays are generally performed under nonphysiological conditions such as low enzyme levels or high concentrations of substrates and putative regulators. Thus, a combination of in vivo and in vitro data is needed and ideally these should be applied to a critical regulatory step.

In bacteria, a key metabolic branch point exists between the tricarboxylic acid and glyoxylate cycles when cells are grown on acetate as the sole carbon source. The substrate at this branch point, isocitrate, can react with isocitric dehydrogenase leading into the energy-producing steps of the tricarboxylic acid cycle, or isocitrate lyase which leads into the glyoxylate cycle (1) (Fig. 1). The latter cycle is essential to the bacteria during growth on acetate since without it both carbons would be burned to CO$_2$ with no net formation of intermediates which are required for the synthesis of cellular materials. It was discovered some years ago by Holms and Bennett (2) that isocitrate dehydrogenase is regulated. Recently it has been established by Garnak and Reeves (3) and in our laboratory (4, 5) that this regulation is achieved by reversible phosphorylation. The kinase/phosphatase which acts on isocitric dehydrogenase has been purified and some kinetic aspects of this regulation have been clarified (5, 6).

In this study, we have performed in vivo measurements to characterize the branch point between the tricarboxylic acid and glyoxylate cycles in Escherichia coli. Methodology was developed to measure flux, from the nutrient acetate through the major enzymatic steps which provide the system with cell constituents and energy. In the first part of the article, the theoretical approaches are described, and this is followed by the experiments which allowed us to determine values for each reaction. The measurements of velocities through the cycles allowed us to calculate the total rate of ATP production and the efficiency of ATP utilization by the cell.

**Experimental Procedures**

**Materials**—Sodium $[^{1-13}$C]acetate and sodium $[^{2-13}$C]acetate (approximately 60 mCi/mmol) were obtained from Amersham and purified by HPLC (see below) prior to use. Sodium $[^{2-13}$C]acetate (enriched to 99 atom %) was obtained from Merck, Sharp and Dohme and was used without further purification. L-[$^{3-4}$H]Glutamic acid (44 Ci/mmol) was from New England Nuclear. Dowex AG 50W-X8 (200–400 mesh) and Dowex AG 2-X10 (200–400 mesh) were obtained from Bio-Rad and precoated sheets of microcrystalline cellulose for thin layer chromatography were from Brinkmann Instruments, Inc.

**Preparation of Cells**—E. coli 23559 (Met-) cells were grown in MOPS minimal medium (7) containing 100 mM acetate and 0.2 mM methionine. Cells were grown to exponential phase on a rotary shaker at 37°C. Prior to labeling, enzyme extraction, or small molecule extraction, the cells were harvested by centrifugation, washed, and resuspended in fresh growth medium containing 100 or 50 mM acetate.

All growth experiments were performed with seeded cells growing at 37°C.

**Nuclear Magnetic Resonance Spectroscopy**—$^1$H-decoupled $^{13}$C NMR spectra were obtained at 45.3 MHz using a Bruker 180 superconducting magnet interfaced to a Nicolet 1180 computer. Spectra were obtained using a 96° pulse width and a spectral width of 6024 Hz. Extracted metabolites, purified intracellular glutamate, or standards were dissolved in 98% D$_2$O containing 10 mM sodium azide and 250 mM KCl at pH 5.1. Chemical shifts are expressed relative to [99%-$^{13}$C]acetate as the internal standard (6 = 25 ppm).

$^{13}$C-enriched glutamate was purified from cellular extracts by Dowex 50 chromatography. In this purification, the sample was labeled with carrier-free L-[$^{3-4}$H]glutamate prior to chromatography. The $^1$H-containing fractions were pooled and desalted on a Dowex 2 column. Glutamate was eluted from this column with 1 N acetic acid which was later removed by rotary evaporation.

**Liquid Chromatography**—High performance liquid chromatography was performed with a Waters Associates model 6000A solvent delivery system with a U6K injector. The liquid chromatograph was equipped with a Bio-Rad HPLC-87H organic acid analysis column. Compounds were eluted with 0.01 M H$_2$SO$_4$ at a flow rate of 0.5 ml/min. Effluents were monitored by collecting fractions and assaying for radioactivity by scintillation counting and with a refractometer or a UV spectrophotometer.

**Acetate Incorporation into Metabolic End Products**—Table II sum-

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1. The abbreviations used are: HPLC, high performance liquid chromatography; MOPS, 3-(N-morpholino)propanesulfonic acid.
Fig. 1. Scheme of known metabolic steps in acetate utilization. Major pathways in E. coli growing on acetate as the sole carbon source. The abbreviations used as subscripts in the velocity expressions are FA, fatty acids; Ac, acetate (net) acetate influx minus efflux; CS, citrate synthase; CE, citrate efflux; Ac, aconitase; IDH, isocitrate dehydrogenase; GluDH, glutamate dehydrogenase; KGDP, α-ketoglutarate dehydrogenase; SuccDH, succinate dehydrogenase; Fum, fumarase; ME, malic enzyme; MDH, malate dehydrogenase; PEPC, phosphoenolpyruvate carboxykinase; OAANH₂, glutamate-oxaloacetate transaminase; L, isocitrate lyase; MalS, malate synthase.

Figures show the fluxes through competing cycles, with the major pathways highlighted. The scheme illustrates the metabolic intermediates and enzymes involved in the metabolism of acetate in E. coli. The figure is designed to provide an overview of the metabolic pathways and their interconnections, emphasizing the key enzymes and intermediates involved in acetate catabolism.

When NADP⁺ and NADPH levels were measured, cells were collected on 0.45-μm Nylon-66 filters (Ranin Instruments) and alkaline extracts were prepared to prevent the decomposition of NADPH (12).

Metabolite Level Measurements—Metabolite levels in extracts were determined fluorometrically (12, 13). All measurements were made with a Spex Fluorolog which was equipped with a refrigerated RCA C31034 photomultiplier tube. Excitation and emission wavelengths were 392 and 474 nm, respectively, and the band pass was 10 nm.

Isocitrate Dehydrogenase Extraction and Assay—Isocitrate dehydrogenase activity was determined in cell extracts which were prepared by a modification of the method of Holms and Bennett (2). In this technique, 1 ml of 16 mM EDTA (pH 7.6) and 24 mM β-mercaptoethanol were added to 6 ml of culture in an aluminum sonication rosette. Essentially complete cell lysis was achieved by four 35-s bursts of ultrasonic vibration with a 20-s pause between each burst. A Heat Systems model W-220 cell disruptor was used at a setting of 5 with a 1/2-inch probe. Diluted cell suspensions were cooled by immersing the rotor in an ice-water slurry. After cell lysis, extracts were assayed for isocitrate dehydrogenase activity at 37 °C by following NADP⁺ reduction spectrophotometrically at 340 nm. One-mi reaction mixtures contained 15 mM MOPS (pH 7.5), 5 mM MgCl₂, 200 μM NADP⁺, 250 μM threo-D- (+)-isocitrate and 200 μl of the sonicated extract. The maximal velocity was 22% greater than the measured velocity as calculated with the Michaelis constants for NAD⁺ and isocitrate.

Isocitrate Lyase—Isocitrate lyase activity was assayed at 37 °C using the sensitive stop-time assay described by Roache et al. (14). Reaction mixtures typically contained 25 mM MOPS (pH 7.5), 200 mM KCl, 2.5 mM MgCl₂, and 60 mM β-mercaptoethanol. Reactions were initiated by the addition of threo-D- (+)-isocitrate and terminated by the addition of oxalic acid to a final concentration of 100 mM. The intracellular concentration of isocitrate lyase was determined with the purification data from E. coli strain 23559. The kinetic constants of isocitrate lyase were measured with the pure enzyme.

RATIONAL FOR EVALUATION OF FLUX MEASUREMENTS

The pathways involved in acetate metabolism in E. coli are shown in Fig. 1. The extensive literature on this organism indicates that these are the major pathways through which carbon flows during growth on acetate (15-18). The figure shows the velocities for individual enzymes and defines the
symbols used in this article (e.g. $V_{CS}$ for velocity of citrate synthase). The units for all velocities are in terms of moles of an individual substrate consumed (or product formed) per liter of cytoplasmic volume per min. It was possible to develop a series of equations for the pathways of Fig. 1 by expressing conservation relationships. The relationships deduced in this manner are summarized in Table I.

The fluxes through the steps in Fig. 1 could be determined after making the measurements which are shown in Table II. The experimental observations in Table II were related to the theoretical equations in Table I by solving simultaneous equations and by considering the origin of various cellular materials. These relationships are shown in Table III. For example,

\[
V_{CS} = V_{AOX} + V_{CE}
\]

\[
V_{AOX} = V_{L} + V_{IDH}
\]

\[
V_{L} = V_{MSA}
\]

\[
V_{IDH} = V_{GDH} + V_{KGDH}
\]

\[
V_{KOXDH} = V_{KGDH} + V_{L}
\]

\[
V_{KOX} = V_{KH} + V_{L}
\]

\[
V_{SDH} = V_{KDH} + V_{OA} + V_{FA} + V_{ME}
\]

\[
V_{L} = V_{GDH} + V_{PEPCK} + V_{OA} + V_{ME}
\]

\[
V_{CC} = V_{AOX} + V_{CC} + V_{SH}
\]

\[
V_{PA} - V_{SH}
\]

\[
V_{CC} - V_{SH} = 0.25V_{PEPCK} + 0.25V_{ME} - 0.25V_{GDH}
\]

\[
0.8 \left( \frac{V_{CC} - V_{SH}}{2} \right) < V_{L} < 1.33 \left( \frac{V_{CC} - V_{SH}}{2} \right)
\]

The following were measured utilizing the techniques outlined in Column 2. These measurements are described in detail under "Experimental Procedures." The observed rates are given in Column 3.

| Parameter       | Outline of experimental procedure | Observed rates mmol/min/liter cell water |
|-----------------|----------------------------------|----------------------------------------|
| $V_{AOX}$       | Rate of radioactive acetate incorporation into saponified, chlorormsoluble constituents (expressed in acetate units) | 3.1                                     |
| $V_{CE}$        | Rate of appearance of radioactive citrate in the medium in cells ingesting radioactive acetate. | 0.3                                     |
| $V_{CC}$        | Total rate of radioactive acetate incorporation into cellular constituents determined by counting of filtered washed cells (expressed in acetate units). | 68                                     |
| $V_{CO_{2}}$    | Rate of $^{14}$CO$_{2}$ production from radioactive acetate. | 150                                     |
| $V_{FB}$        | Rate of radioactive metabolite efflux (other than acetate) into the medium (expressed in acetate units). | 2.5                                     |
| $V_{IR/KL}$     | Ratio of rates through isocitrate dehydrogenase vs. isocitrate lyase determined from NMR and the $^{13}$CO$_{2}$ results. | 2.6 (ratio)                             |

The following equations were derived from combining one or more of the theoretical relationships in Table I and the experimental measurements in Table II. ($V_{CC}$, $V_{AOX}$, $V_{FA}$, $V_{FB}$ and $V_{IR/KL}$ are expressed in acetate units. Other values are in molar units as described in Table I)

\[
V_{AOX} = V_{CO_{2}} + V_{CC} + V_{SH}
\]

\[
V_{PA} - V_{SH}
\]

\[
V_{CC} - V_{SH} = 0.25V_{PEPCK} + 0.25V_{ME} - 0.25V_{GDH}
\]

\[
0.8 \left( \frac{V_{CC} - V_{SH}}{2} \right) < V_{L} < 1.33 \left( \frac{V_{CC} - V_{SH}}{2} \right)
\]

The net acetate influx ($V_{AOX}$) equals the sum of the rates at which $^{13}$C-carbons are released as CO$_{2}$ ($V_{CO_{2}}$), converted into cell constituents ($V_{CC}$), and excreted as compounds other than acetate ($V_{FA}$) (Equation 12). In addition to these equations, the ratio of the velocity through isocitrate dehydrogenase relative to the flux through the glyoxylate bypass was determined by examining the distribution of $^{14}$C atoms in the glutamate produced or by comparing the relative rates of $^{13}$CO$_{2}$ release from $[1^{13}]$C and $[2^{14}]$C acetate. The rationale for this analysis is shown in Fig. 2 and will be discussed in detail in the text.

The relationships used to calculate the fluxes to a common standard were: (a) suspensions of bacteria at 0.1 $A_{600}$ contain 41 $\mu$g of cells dry weight/ml, (b) the total volume of cells were 2.5 ml/g of dry weight, and (c) since the periplasmic volume is 25% of the total cell volume, the rates and concentrations were calculated using 0.75 of the total cell volume because we are dealing with cytoplasmic processes. Values close to these have been used by others for calculating various properties of a cell (20). Since the same values were used for all calculations, the relative values of flux rates can be compared directly.

### RESULTS

#### Isotopic Steady State—An isotopic steady state exists when the specific activity of cycle intermediates does not change with time. In this system, an isotopic steady state was indicated by three lines of evidence. First, intermediates were extracted at intervals following the addition of the carrier-free (1,2-$^{13}$C)acetate. These intermediates were separated on an HP$^{14}$C column giving a typical profile as shown in Fig. 3. The peak that eluted at the same position as a citrate standard was pooled. Greater than 95% of the radioactivity in this peak was present as citrate as determined by thin layer chromatography in a chloriform:methanol:formic acid (80:20:1) solvent system. The radioactivity eluting at this peak at 1, 3, 6, and 12 min after addition of the radiolabeled acetate to the medium were 1173, 1367, 1051, and 1316 cpm, indicating that isotopic equilibrium had been reached in the cycle within 1 min.

A second indicator of isotopic steady state was the accumulation of radioactivity in CO$_{2}$ as shown in Figs. 4 and 7. The lack of a lag period indicates linear accumulation of CO$_{2}$ and rapid isotope equilibrium. Linear rates were also obtained for the fatty acid fraction and the amount of label in total cell constituents (Fig. 4). After 30 min, the plots became parabolic because cell growth became a significant factor.

Another line of evidence consistent with the presence of an isotopic steady state is the $^{14}$C-$^{13}$C splitting pattern of the
Fluxes through Competing Cycles

FIG. 2. Isotopomer distribution in the Krebs and glyoxylate cycle. The patterns of acetate carbon distribution are shown after the first turn of each cycle and at steady state. The specific activities of the carbons in glutamate and CO₂ are indicative of the relative contributions of the Krebs and glyoxylate cycles in the formation of these compounds. This was the basis for determining the distribution of the flux through the branch point. The specific activity of the glutamate at a steady state was determined assuming equilibration of isotope between malate and fumarate.

enriched intracellular glutamate (Fig. 6). As will be discussed below, the splitting pattern of certain positions indicates that they are enriched with ¹³C to the same degree as the [99%-2-¹³C]acetate in the medium.

Measured Rates—The experimentally observed rates listed in Table II were obtained after isotopic steady state had been reached in a 100 mM acetate medium. Typical time courses of these measurements are shown in Fig. 4. The incorporation of acetate units into fatty acids (VSapo) was 3.1 ± 0.3 mM/min (S.E.). The rate of CO₂ evolution was 150 ± 7 mM/min and the rate of incorporation of acetate units into stable cellular constituents (VCC) was 68 ± 4 mM/min. Separation of the media by HPLC established that the amount of citrate efflux (VCE) was 0.3 mM/min and the total efflux of radiolabeled cellular material in acetate units (VEff) was 2.5 mM/min, indicating that it is a minor end product of acetate metabolism.

Derived Relationships—From the direct measurements of radioactive label and the theoretical relationships of Tables II and III, some of the intracellular rates can be obtained.

The determination of VSapo establishes the rate VFA, which is the rate of acetyl coenzyme A incorporation into saponified fatty acids via the acetyl-CoA carboxylase step (Equation 13). The observed rates of VCO₂, VCC and VEff given in Table II allow the calculation of VAm,E, the net rate of acetate influx (Equation 12). From the values given above, this rate becomes 150/2 + 68 + 2.5 = 145 mM/min.

The net rate (influx minus efflux) of acetate flux to Ac-CoA equals the rate of acetate utilization to make fatty acids (VFA), plus the component going through the Krebs cycle via citrate synthase (VCS) and the amount utilized by the glyoxylate cycle via the malate synthase step (VMalS) (Equation 9). Given the value of VFA (3.1 mM/min), the sum of the flux through the other two enzymes is equal to 142 mM/min. Since at steady state VMalS = VL (Equation 3), and since VCE is negligible (0.3 mM/min), the value of 142 mM/min also equals VAm,E + VL. The flux through VDH and VL was distinguished by ¹³C NMR and radioactive carbon experiments.

¹³C NMR—The enrichment pattern of glutamate and other metabolites has been useful in determining the relative contributions of pathways which lead to their synthesis from glucose (21, 22) and pyruvate or acetate (23-25). We have used this technique to study acetate metabolism in E. coli. Fig. 5 is a ¹H-decoupled ¹³C NMR spectrum of extracted

FIG. 3. Separation of intracellular metabolites by HPLC. Metabolites were extracted 6 min after the addition of carrier-free (1,2-¹³C)acetate to a culture growing on 100 mM acetate. Compounds were eluted as described under "Experimental Procedures." Peak B, eluting at 5.9 min, was isotopically pure citrate.

FIG. 4. Time courses of acetate incorporation into metabolic end products. Labeling reactions were initiated by the addition of carrier-free (1,2-¹³C)acetate to cultures growing on 100 mM acetate. The rates of ¹³CO₂ evolution (VCO₂), and the rates of acetate incorporation into cellular constituents (VCC) and fatty acids (VSapo) were determined as described under "Experimental Procedures."
metabolites from a culture which was grown aerobically in the presence of [99\%\,2\,\textsuperscript{13}C]acetate for 10 min. The most intense peaks in this spectrum had chemical shifts which correspond to the C-2, C-3, and C-4 of glutamate. The strong signal from glutamate is due to the high intracellular concentration of this metabolite (50 mM). Since glutamate had a strong signal and its labeling pattern reflects the isotopic distribution of α-ketoglutarate, we purified this compound from the extract (see "Experimental Procedures"). The spectrum of \textsuperscript{13}C-enriched glutamate is shown in Fig. 6.

The \textsuperscript{13}C-\textsuperscript{13}C splitting of the C-3 and C-4 peaks indicates that intracellular glutamate was highly enriched by the [99\%\,\textsuperscript{13}C\]acetate. This splitting pattern and the absence of a C-5 peak at 187 ppm (Inset A of Fig. 6) indicates that there were two predominant glutamate isotopomers, [\textsuperscript{2,3,4}\,\textsuperscript{13}C\]glutamate and [\textsuperscript{1,2,3,4}\,\textsuperscript{13}C\]glutamate. The complex splitting pattern of the C-2 peak arises from the superposition of the spectra from the two species.

The relative peak intensities of the purified intracellular glutamate were 0.110:0.1:1.1:1:0 for C-1 through C-5, respectively. The variations in the resonance intensity at the different carbon positions is due to the nonstatistical distribution of the \textsuperscript{13}C label as differences in relaxation time and nuclear Overhauser enhancement. To determine the actual isotopomer ratio, natural abundance glutamate was run under identical conditions of pulse width, cycle time, pH, etc., to account for the differences in relaxation times and nuclear Overhauser enhancement at the different positions (Inset B of Fig. 6). Correcting for these differences, we found that the relative enrichment of the intracellular glutamate was 0.4:1.0:0.9:1.00 for C-1 through C-5, respectively. This indicates that approximately 40\% of the glutamate was in the 1,2,3,4-\textsuperscript{13}C isotopomer form.

\textbf{Determination of Flux at the Branch Point}—The determination of the flux distribution at the branch point was complex because both cycles lead to common products. The logic of the determination was deduced from Fig. 2 as follows. The atoms of acetyl coenzyme A and oxalacetate are labeled a, b, r, s, t, u, respectively. Next to each of the key compounds the predicted relative labeling is given for one turn of the cycle. For example, the formation of succinate, which is symmetric, scrambles the label between the two carboxyls and also between methylene carbons and therefore the specific activities become \((b + s)/2\) and \((a + t)/2\), respectively, for the succinate derived from α-ketoglutarate. The malate formed from the glyoxylate shunt is asymmetric initially but, if there is equilibration with fumarate, the C-1 and C-4 as well as the C-2 and C-3 of malate are scrambled. The oxalacetate formed at the end of the first cycle will be derived from two four-carbon compounds produced by the glyoxylate cycle and the one four-carbon compound coming from the Krebs cycle. At steady state, the specific activity of every carbon atom will approach a fixed function of \(a\) and \(b\) depending on the relative contributions of each pathway. In Fig. 2, the predicted steady state specific activities are given for glutamate and CO\textsubscript{2} based on the "Krebs cycle only" and the "glyoxylate cycle only." Note that only C-1 of glutamate and CO\textsubscript{2} have specific activities which reflect the relative fluxes between pathways.

The proportion of the flux through isocitrato dehydrogenase, \(f_a\), can be related to the specific activity of the C-1 position of glutamate, \(C_{\text{C02}}\), by Equation 18.

\[
\begin{equation}
\[2C_{\text{C02}}\] = \frac{2\left(C_{\text{C02}}\right)}{a + b}
\end{equation}
\]

Using the NMR data, the calculated value of \(f_a\) was 0.8 (Equation 18) since 40\% of the glutamate had \textsuperscript{13}C-nuclei at the C-1 position when cells were incubated with [\textsuperscript{2,\textsuperscript{13}C\]acetate (99 atom %)). The value of \(f_a\) was also calculated from the relative rates of \textsuperscript{14}CO\textsubscript{2} evolution from [\textsuperscript{1-\textsuperscript{14}C\]acetate and [\textsuperscript{2-\textsuperscript{13}C\]acetate. As shown in Fig. 2, the specific activity of the evolved \textsuperscript{14}CO\textsubscript{2} should equal that of the [\textsuperscript{1-\textsuperscript{14}C\]acetate or the [\textsuperscript{2-\textsuperscript{13}C\]acetate if the Krebs cycle was the only operative pathway. Likewise, if all of the carbon was diverted through the glyoxylate shunt, \textsuperscript{14}CO\textsubscript{2} would only be evolved from [\textsuperscript{1-\textsuperscript{14}C\]acetate. The amount of \textsuperscript{14}CO\textsubscript{2} evolved from [\textsuperscript{2-\textsuperscript{13}C\]acetate was 36\% of the total rate (Fig. 7) which gives a value of 0.72 for \(f_a\). If the flux through the branch point is calculated from the \textsuperscript{14}CO\textsubscript{2} data, no assumptions regarding the extent of isotope equilibration by fumarase are required. This is true because the \textsuperscript{14}CO\textsubscript{2} released by the Krebs cycle will represent a mixture of isotopes from the C-1 and C-4 of oxalacetate.

Previously, we obtained the relationship, \(V_{\text{Vox}} + V_{\text{C02}} = 142\text{ mm/min}\). This can be related to \(f_a\) by Equation 19.

\[
\begin{equation}
142\text{ mm/min} = \left(\frac{f_a + 2}{1 - f_a}\right) V_{\text{Vox}}
\end{equation}
\]

Using the \(f_a\) value determined from the CO\textsubscript{2} data, the calculated flux through the glyoxylate shunt (\(V_{\text{C02}}\)) was 31 mm/min (Equation 19). Combining Equations 1 and 2 of Table 1, one calculates that \(V_{\text{DH}} = 80\) and \(V_{\text{CS}} = 111\text{ mm/min}\). Using the

\[3\text{ Since the steady state specific activities of the carbons C-2 through C-5 of glutamate are independent of the pathway, Equation 17 can be used to express the specific activity of the C-1 of α-ketoglutarate (as detected by the C-1 glutamate) in terms of the fraction of the flux going through the Krebs cycle (\(f_a\)) relative to the fraction going through the glyoxylate bypass (\(1 - f_a\)). In this equation \(C_a\) represents the specific activity of the C-1 atom in the nth cycle and \(C_{\text{f}}\); the specific activity of the same atom in the n + 1 cycle.

\[4\text{ Although no assumption is made about fumarate equilibration with the calculation from the } \textsuperscript{14}C\text{ data, it is assumed that the flux through phosphoenolpyruvate carboxykinase, the malic enzyme and glutamate dehydrogenase, is small relative to the rate of CO\textsubscript{2} release by α-ketoglutarate dehydrogenase and isocitrate dehydrogenase.} \]
FIG. 6. Proton-decoupled $^{13}$C NMR spectrum of purified intracellular glutamate. Glutamate was purified, as described under "Experimental Procedures," from 600 ml of cells which were grown as described in Fig. 5. The spectrum consists of 6,600 scans with a 1.5-s repetition rate. Inset A, peaks which occur downfield in the spectrum. Inset B, spectrum of natural abundance $[^{13}$C]$[^{13}$C$]_2$ glutamate which was determined with the same conditions as the intracellular glutamate. The relative peak intensities of the natural abundance $[^{13}$C]$[^{13}$C$]_2$ glutamate were 0.25:1.0:1.1:1.2:0.23 for C-1 through C-5, respectively.

Using this relationship, the flux through the bypass was calculated to be between 26 and 43 mM/min. These limits agree well with the values determined independently for $V_L$ by the methods described previously.

Other Steps in the Two Cycles—Having obtained the velocities through the branch point, it was possible to determine the flux through other steps in the two cycles using the conservation equations of Table I and by performing the appropriate additions and subtractions (Fig. 8). Velocities were assigned to the cycle-depleting reactions based upon composition data for $E. coli$ and Equation 11. This equation demonstrates that at steady state the flux through the glyoxylate bypass (31 mM/min from the CO$_2$ data) must equal the sum of the fluxes which deplete cycle intermediates, or $V_{GIDH} + V_{ME} + V_{PEPCK} + V_{GAANN} = 31$ mM/min.

The ratio of the biosynthetic flux through these enzymes was calculated from the known weight percentages of cellular constituents which are derived from the different cycle-depleting reactions. For example, intermediates depleted through glutamate dehydrogenase will give rise to glutamate, glutamine, arginine, proline, and polyamines which comprise a total of 10.8% of the dry weight of $E. coli$ (15, 26). The flux through glutamate-oxalacetate transaminase gives rise to 15.6% of the dry weight and the gluconeogenic flux through the malic enzyme and phosphoenolpyruvate carboxykinase leads to the biosynthesis of 22.6% of the dry weight. Using these weight percentages, the ratio $V_{GIDH}:V_{ME}:V_{PEPCK} + V_{GAANN} = 1:1.4:3.6$. The values for lipid, RNA, and DNA were not included in these calculations. Assuming the gluconeogenic flux is shared equally by the malic enzyme and phosphoenolpyruvate carboxykinase, the following velocities were assigned to the intermediate depleting reactions: $V_{GIDH} = 5$ mM/min, $V_{GAANN} = 7$ mM/min, $V_{ME} = 9.5$ mM/min, and $V_{PEPCK} = 9.5$ mM/min. Using these values, velocities could be assigned to the reaction categories of Table I.

In this calculation, it was assumed that the biosynthesis of RNA and DNA do not significantly affect the ratio of the fluxes through the cycle-depleting steps. The weight percentage value for fatty acids was not included in the calculation because they are synthesized from carbon before it enters the cycle.
assigned to all of the major pathways associated with the early stages of acetate metabolism and this is summarized in Fig. 8. Upon closer examination, it can be seen that these assignments predict that the value of $V_{CO_2}$ is 174 mM/min. Experimentally, we have determined that this value is 150 ± 7 mM/min. The 16% discrepancy most likely arises from a slight error in our determination of the distribution of the flux at the branch point since this will have a significant influence on the velocities calculated for the other steps in the cycle. For example, using the larger $f_a$ value, which was calculated from the NMR experiment, the discrepancy was 32% between the measured and the calculated values.

In Vitro Rates and the Concentrations of Intermediates and Enzymes—To further characterize the system, we have determined the intracellular levels of intermediates and enzymes at the branch point of the two cycles (Table IV). Some of these values have been determined before with different strains of E. coli and, in general, the values agree well (13). The intracellular concentrations of isocitrate lyase and isocitrate dehydrogenase were calculated from the purification data. Homogeneous preparations of lyase were obtained after a 22.6-fold purification indicating that the concentration of 45-kDa subunits is 200 pM inside the cell. Similar calculations with purified isocitrate dehydrogenase indicated that the intracellular concentration of this enzyme was 40 pM. The total concentration of isocitrate was 160 pM and the concentration of free isocitrate was calculated assuming hyperbolic binding to the lyase and the dehydrogenase and that the Michaelis constants were equal to the dissociation constants. These calculations indicated that approximately half of the total isocitrate was bound to enzyme. A similar situation probably exists for NADP$^+$ but we could not calculate the levels of the free dinucleotide due to the prevalence of NADP$^+$-linked dehydrogenases in E. coli.

Using the kinetic constants of the enzymes (Table IV), we calculated the fluxes through the branch point as an independent test for the rates measured in vivo. Using the free isocitrate value, the calculated rate through the lyase was 39 mM/min. This agrees well with the flux of 31 mM/min observed in vivo. Similar calculations were made for the dehydrogenase using the parameters in Table IV. When bacteria grow on acetate, much of the isocitrate dehydrogenase is in the phosphorylated form which is inactive. Under these conditions, the dehydrogenase has a maximal velocity of 138 mM/min in the cell. The intracellular velocity through the dehydrogenase was calculated using a random bireactant rate equation with $\alpha = 1$. The calculated rate of 80 mM/min agrees exactly with the observed rate. This agreement is fortuitous since the calculations did not account for the amount of NADP$^+$ sequestered by enzymes in the cell. Studies

| Parameters of the branch point enzymes |
|----------------------------------------|
| Kinetic constants and intracellular concentrations of enzymes and substrates at the branch point of the Krebs cycle and the glyoxylate shunt. These parameters were used to calculate the flux through the branch point. Assay conditions are described under "Experimental Procedures." |
| Isocitrate lyase constants |
| $K_m$ for isocitrate = 604 μM |
| $V_{max}$ = 289 mM/min |
| Isocitrate dehydrogenase constants |
| $K_m$ for isocitrate = 8 μM |
| $K_m$ for NADP$^+$ = 22 μM |
| $V_{max}$ = 126 mM/min |
| Intracellular concentrations |
| Total NADP$^+$ = 50 μM |
| Total isocitrate = 160 μM |
| Free isocitrate = 200 μM |
| Isocitrate dehydrogenase = 40 μM |
| *Free* isocitrate = 95 μM |

* D. C. LaPorte and D. E. Koshland, Jr., unpublished results.
are currently under way to refine the calculations from in vitro data, but the data reported here are consistent with the in vivo data.

**DISCUSSION**

The above experiments and theoretical approaches have allowed us to assign a value for the major enzymatic steps in both the glyoxylate cycle and the tricarboxylic acid cycle (Fig. 8). Since bacteria do not have compartments and numerous parameters were measured experimentally, it was possible to assign velocities for all steps by assuming only that (a) the major pathways of acetate metabolism have been correctly identified in Fig. 1 and (b) that the ratio of weight percentages of various cellular materials correctly estimates the ratio of fluxes which deplete cycle intermediates. The accuracy of these values can be evaluated in several cases since they were determined by more than one method. For example the rate of CO₂ evolution was measured directly (Table II) and could be calculated by summing the fluxes of the major CO₂-evolving steps (Equation 10). The discrepancy between the two values was 16% and this is probably a good indicator of the error in the values. The velocities assigned to the steps which deplete cycle intermediates probably have larger errors.

Isocitrate lyase and isocitrate dehydrogenase compete for isocitrate at a major metabolic branch point. The conservation equations reveal that a change in the rate of intermediate flux through the lyase would necessitate an equal change in the rate at which intermediates are depleted for biosynthesis (Equation 11). Thus, at this branch point, the cell coordinates biosynthetic fluxes with the energy-producing flux through the oxidative steps of the Krebs cycle. The ratio of the flux calculated for the branch point is a critical parameter in the assignment of velocities to the other steps in the two cycles. The distribution of the flux at the branch point was determined by several methods: ¹³C NMR, the relative ratio of ¹°CO₂ evolution from [1-¹°C]acetate and [2-¹°C]acetate, and calculations from kinetic constants. For example, limits were set for the flux through the bypass from the rate of carbon incorporation into chloroform-insoluble cell constituents (V_ex-V_in). The values obtained from these different measurements were similar, indicating that we have correctly determined the ratio of the flux through the branch point.

The measurement of metabolite and enzyme levels at the branch point revealed that the concentration of isocitrate-binding sites exceeded the total concentration of this intermediate. This suggests that a large proportion of isocitrate was bound to enzymes in vivo. A similar situation is believed to exist with fructose 1,6-diphosphate and oxalacetate in rat liver (28). The fluxes through the branch point were calculated from the kinetic constants of the enzymes, the total concentration of NAD⁺, and the calculated concentration of free isocitrate. These values were in agreement with the in vivo measurements, indicating that our in vitro assays are performed under conditions which reasonably approximate intracellular conditions.

Besides the quantitative data, ¹³C NMR measurements provided additional information about the system. The proton-decoupled spectra of purified intracellular glutamate showed that there were two predominant isotopomer species present, [2,3,4,¹³C]glutamate and [1,2,3,4,¹³C]glutamate, when cells were grown on [99%-2,¹³C]acetate (Fig. 6). These are the same two species which are predicted from the flow of carbon through the pathways outlined in Fig. 1 at steady state. The lack of other isotopomer species is consistent with the supposition that the pathways in Fig. 1 are the quantitatively important reactions in acetate metabolism. For example, the absence of ¹³C enrichment in position 5 of glutamate suggests that there was no significant futile cycling through phosphoenolpyruvate carboxykinase, pyruvate kinase, and pyruvate dehydrogenase. The cycling of carbon through this pathway would scramble the label in Ac-CoA and be reflected by the appearance of ¹³C-nuclei in the C-5 position of glutamate. The lack of ¹³C-nuclei in this position is indicative of regulation at the level of pyruvate kinase or pyruvate dehydrogenase which promotes a gluconeogenic flux. This is in contrast to experiments in yeast where ¹³C measurements indicated significant futile cycling through these pathways (23). The presence of two predominant isotopomers of glutamate was also indicative of a minimal dilution of cycle intermediates by nonlabeled endogenous compounds or by the incorporation of nonlabeled CO₂. Dilution by nonlabeled carbon would be expected to give rise to multiple isotopomers and this would result in a more complex splitting pattern than was observed in Fig. 6.

In the past, studies of microbial energetics have primarily been performed under anaerobic conditions since ATP yields could be precisely calculated from substrate catabolism (27, 29). Under aerobic conditions, the molar production of ATP has been calculated from oxygen consumption data but this relationship is complex and can only be used as an approximation (30–32). Using the fluxes listed in Fig. 8, we were able to exactly calculate the intracellular rate of ATP production during aerobic growth on acetate. This value was 855 mmoles/min. Since the intracellular concentration of ATP is approximately 2 mM (13), the half-life of this metabolite is less than 0.1 s under these conditions. From the calculated ATP-requiring pathways deduced by Stouthamer (27), 301 mM ATP/min is needed for biosynthesis and transport during growth on acetate. This gives an overall efficiency of 35%, for the ATP synthesis-hydrolysis cycle. The uncoupling of energy production from growth may be due to inefficiencies which result from the high metabolic rate of bacteria relative to eukaryotic organisms (32). Extensive studies in eukaryotes indicate that the rates of carbon flow through the major metabolic pathways are substantially less than those found in E. coli (33–36).

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