Stereoselective Synthesis of Stable Isotope Labeled L-α-Amino Acids: The Enzymatic Preparation of ^{13}C-Labeled L-Glutamic Acids†.

Warren J. Goux*, Linda Rench and Denise S. Weber

Department of Chemistry, University of Texas at Dallas, P. O. Box 830688, Richardson, Texas 75083-0688 USA

SUMMARY

We have developed methods for the preparation of L-glutamic acid, isotopically labeled with ^{13}C, using commercially available purified enzymes. The procedure is sufficiently versatile that L-glutamic acid may be labeled at any carbons or any combination of carbons using ^{13}C-labeled pyruvate, acetate and bicarbonate as isotopically labeled precursors. Using the strategy outlined, we have demonstrated the methodology using as an example the preparation of millimolar quantities of L-[1-^{13}C], L-[4-^{13}C], L-[5-^{13}C] and L-[1,3,4-$^{13}C_3$]glutamic acid in a one-pot incubation. We also outline methods for the preparation of ^{13}C-labeled L-malate and citrate.

Keywords: L-[1-^{13}C]glutamic acid, [4-^{13}C]glutamic acid, L-[5-^{13}C]glutamic acid, L-[1,3,4-$^{13}C_3$]glutamic acid, L-[2,4-$^{13}C_2$]malate, L-[4-^{13}C]malate, [1-^{13}C]citrate, [4-^{13}C]citrate, [5-^{13}C]citrate and [1,3,4-$^{13}C_3$]citrate.

INTRODUCTION

L-Glutamic acid plays a central role in the intermediary metabolism and nitrogen metabolism of living systems. A relatively large glutamate pool provides a buffer between the tightly controlled citric acid cycle intermediates and other L-amino acids directly derived from glutamate, including ornithine, proline, arginine and glutamine[1]. As a nitrogen metabolite, glutamate donates its nitrogen to a wide variety of other amino acids and purine bases. Because of the central role it plays in metabolism, it was felt that L-glutamatic acid, isotopically labeled with isotopes of carbon, at any

† Some of the methods reported are included in an application for patent filed with the US Patent and Trademark Office (SN 07/587,934)

* To whom correspondence should be addressed. Ph. (214)690-2660, FAX (214)690-2925, BITNET WGOUX@UTDALLAS.EDU.

0362–4803/93/030181–13$11.50

Received 24 July, 1992
Revised 30 November, 1992

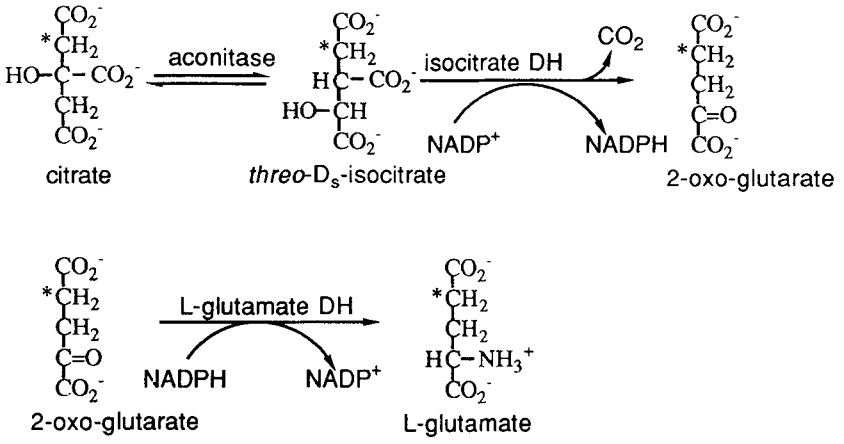
single sites or a variety of single sites, would be of potential value in metabolic studies, particularly those using NMR or mass spectroscopy.

Our general approach for the preparation of L-glutamate has been to use purified enzymes to catalyze the conversion of isotopically labeled citrate to 2-oxo-glutaric acid. The latter product may then be either reductively or nonreductively aminated to L-glutamic acid. As an illustration of the methodology, we describe in this report the enzymatic preparation of [1-^{13}C], [4-^{13}C], [5-^{13}C] and [1,3,4-$^{13}C_3$]citrate from $H^{13}CO_3^-$, [2-^{13}C]sodium pyruvate, and [1-^{13}C] or [2-^{13}C]sodium acetate and the subsequent conversion of these intermediates to the respectively labeled L-glutamic acids. The method which we describe is similar to the chemical/enzymatic method recently described by Cappon et al[2]. with respect to the wide variety of ^{13}C-labeled glutamates which can be synthesized. However, in contrast to the chemical method, all of the reactions which we shall describe take place in aqueous buffered media using commercially available purified enzymes. The enzyme cofactor regeneration systems which we have employed from existing technology[3-6] allow preparations to be carried out using minimal cofactor and enzyme concentrations, making scaleup of the methods capable of producing millimolar quantities of product at costs comparable to other chemical methods.

RESULTS AND DISCUSSION

Isotopically Labeled L-Glutamic Acid from Citrate. Schemes I and II describe two alternative methods which we have used to convert isotopically labeled citrate to L-glutamic acid. Both of the procedures have in common an initial aconitase catalyzed isomerization of citrate to *threo*-D_s-isocitrate followed by a oxidative decarboxylation catalyzed by the $NADP^+$ dependent enzyme isocitrate dehydrogenase. Because of the prochiral nature of the interaction between citrate and aconitase, unique isotopic enrichment at for example, C-4 of L-glutamate is produced from [4-^{13}C]citrate.

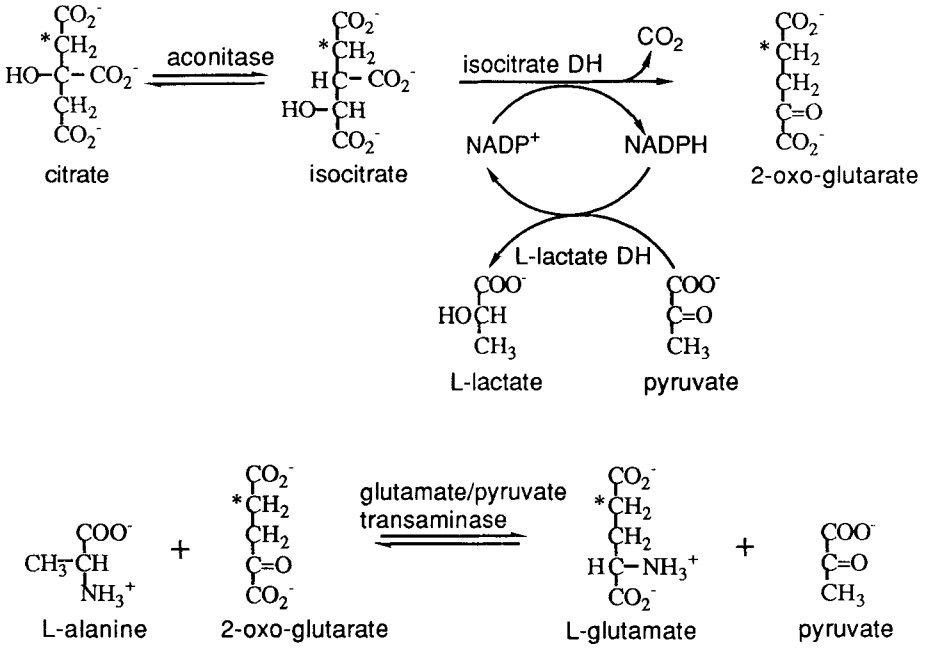
The concentration of the $NADP^+$ cofactor is kept relatively low with respect to substrate concentration (about 2 mM vs 25 mM) in order to minimize the cost of the preparation and to prevent competitive inhibition of isocitrate dehydrogenase by NADPH. Using



Scheme I: Enzymatic preparation of L-[4-^{13}C]glutamate from [4-^{13}C]citrate according to Method I.

Method I (Scheme I), NADP+ is regenerated *in situ* by the coupled reductive amination of 2-oxo-glutarate to the product L-glutamate by the enzyme L-glutamate dehydrogenase[5]. The single L-glutamate dehyrogenase step of Scheme I responsible for NADP+ regeneration and conversion of 2-oxo-glutarate to L-glutamate is replaced in Scheme II by two coupled reactions which together yield the same end result (Method II). In one of the coupled reaction steps, 2-oxo-glutarate is nonreductively aminated to L-glutamate using as a nitrogen donor nonisotopically labeled L-alanine added to the reaction medium. In a subsequent lactate dehydrogenase catalyzed step, NADP+ is regenerated for further isocitrate reduction as pyruvate produced from the deamination of L-alanine is reduced to lactate.

The stoichiometry of both Methods I and II is such that each equivalent of NADP+ regenerated should yield one equivalent of isotopically labeled L-glutamate. In practice, we find that a two day incubation of substrates with enzymes, according to either of the two reaction schemes, yields about a 60/40 molar ratio of L-glutamate to 2-oxo-glutarate, with no ^{13}C-labeled citrate detectable by ^{13}C NMR. The incomplete conversion in all likelihood arises from unlabeled pyruvate present in the reaction medium from a previous step, where it was used in the preparation of citrate (see below). The pyruvate can be reduced either by added (Scheme II) or contaminating lactate dehydrogenase (Scheme I), resulting in the generation of more than one

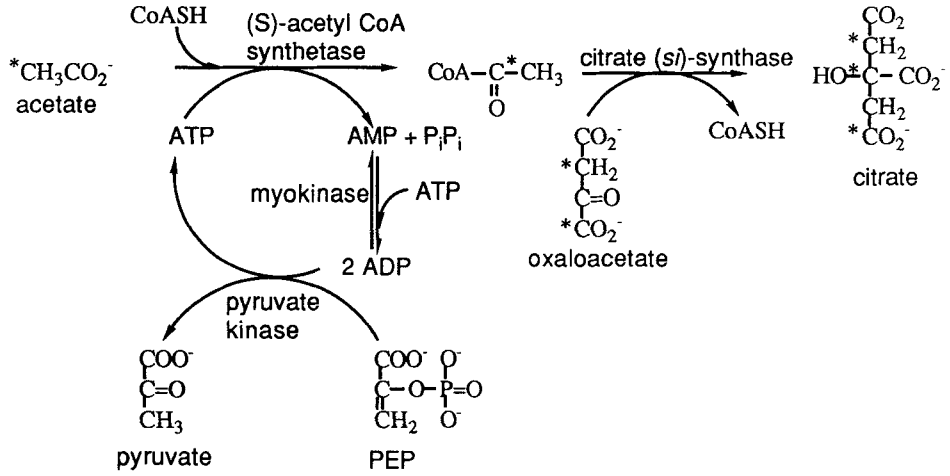


Scheme II: Enzymatic preparation of L-[4-^{13}C]glutamate from [4-^{13}C]citrate according to Method II.

equivalent of NADP+. In the case of Method I, where NADP+ is regenerated by isotopically labeled 2-oxo-glutarate, the final L-glutamate/2-oxo-glutarate ratio reflects the relative rates of the L-glutamate dehydrogenase dependent/NADP+ regeneration system and the contaminating lactate dehydrogenase/NADP+ regeneration system. In the case of Scheme II, the final L-glutamate/2-oxo-glutarate ratio simply reflects the equilibrium concentrations of the pyruvate/L-glutamate transaminase catalyzed reaction ($K_{eq} \approx 1$)[7]. We have been able to carry the reaction to completion following a two day incubation by adding to the reaction medium half an equivalent of D-glucose-6-phosphate along with a few units of glucose-6-phosphate dehydrogenase and L-glutamate dehydrogenase. The D-glucose-6-phosphate/glucose-6-phosphate dehydrogenase is able to act as a NADPH regeneration system[4] in place of a isocitrate/isocitrate dehydrogenase NADPH regeneration system which is unable to function due to the lack of isocitrate.

Isotopically Labeled Citrate. We have prepared L-[4-^{13}C] and L-[5-^{13}C]glutamic acids using as substrates [4-^{13}C] and [5-^{13}C]citrate. Enzymatic preparations of these latter compounds have been carried out in a manner similar to

previously described procedures[3,8], using as substrates [2-^{13}C]acetate and [1-^{13}C]acetate and unlabeled oxaloacetate. In the initial (S)-acetyl-CoA synthetase catalyzed step of the reaction sequence, the free energy of hydrolysis of ATP to AMP and pyrophosphate is used to drive the condensation of the acetate and free CoA to acetyl-CoA (Scheme III). In a subsequent step, citrate (*si*)-synthase catalyzes the condensation between the acetyl moiety and oxaloacetate, forming citrate labeled at C-4 or C-5. Myokinase added to



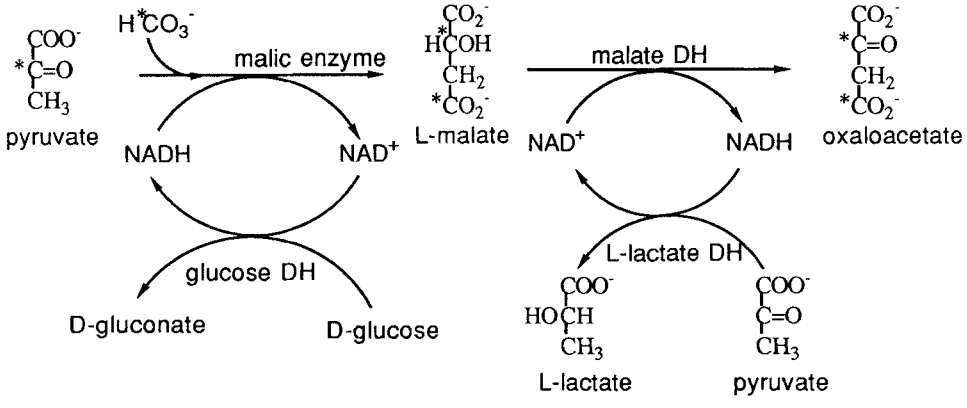
Scheme III: Enzymatic preparation of [1,3,4-$^{13}C_3$]citrate from [2-^{13}C]acetate and [2,4-$^{13}C_2$]oxaloacetate.

the reaction medium catalyzes the transphophorylation of ATP and product AMP to two equivalents of ADP per equivalent of citrate formed. The spent ATP may then be regenerated *in situ* by the pyruvate kinase catalyzed transphosphorylation of ADP using phospho(enol)pyruvate (PEP) as a phosphate donor[6].

Taking into account the large negative free energy changes associated with the citrate (*si*)-synthase and pyruvate kinase catalyzed steps of Scheme III, one predicts quantitative yields from acetate[3,8]. In practice we have found this to be the case only when relatively high concentrations of (S)-acetyl CoA synthetase are used (about ten times those which we have used in our preparations). The probable source of the problem of less than quantitative yields is the precipitation of magnesium from the reaction medium in the form of either magnesium pyrophosphate or magnesium

ammonium pyrophosphate. As magnesium is removed, the (S)-acetyl CoA synthetase catalyzed reaction is slowed substantially. The observation that high initial synthetase concentrations alleviate the problem suggest that there is a competition between the rate of magnesium salt precipitation and the rate of enzyme turnover with the available magnesium in the reaction medium. At lower enzymes concentrations we typically obtain between 65% to 85% conversion to citrate from acetate.

Isotopic labeling of citrate at C-1, C-2 and C-3 can be achieved by replacing unlabeled oxaloacetate by labeled oxaloacetate. Oxaloacetate may be generated *in situ* from pyruvate, ATP and bicarbonate in a reaction catalyzed by pyruvate carboxylase or alternatively, from PEP and bicarbonate in a reaction catalyzed by PEP carboxylase. Problems arise, however, if labeled pyruvate is used as a means of generating labeled oxaloacetate. Unlabeled pyruvate resulting as a product of the ATP regeneration system in the first reaction of Scheme III can be used as a substrate in any concomitant carboxylation to oxaloacetate, resulting in dilution of the label. We have circumvented this problem by first preparing labeled L-malate using the reductive carboxylation of pyruvate as afforded by malic enzyme (Scheme IV). The NADPH cofactor is regenerated *in situ* by coupling the malic enzyme reaction to the oxidation of glucose as catalyzed by glucose dehydrogenase[9]. In practice, a small amount of contaminating L-lactic dehydrogenase in the commercial malic enzyme preparation converts some of the labeled pyruvate to L-lactate, thereby leading to less than quantitative yield of L-malate from pyruvate. Labeling at C-4 of malate may be



Scheme IV: Enzymatic preparation of L-[2,4-$^{13}C_2$]malate and [2,4-^{13}C]oxaloacetate from [2-^{13}C]pyruvate and [^{13}C]bicarbonate.

achieved by carrying out the coupled reaction sequence in a buffered solution containing [^{13}C]bicarbonate while labeling of malate at C-1, C-2 or C-3 may be achieved by using the correspondingly label pyruvate. We have found that boiling of the reaction mixture following the generation of labeled L-malate is an effective way to denature malic enzyme thereby blocking the formation of unlabeled malate from any unlabeled pyruvate added to the reaction mixture in subsequent steps. Labeled L-malate formed in this manner may be oxidized to oxaloacetate by malate dehydrogenase and incorporated into citrate as outlined in Scheme III. NAD^+ needed as a cofactor for this reaction can be regenerated *in situ* by including lactate dehydrogenase as one of the added enzymes. Pyruvate, which is generated from PEP as part of the ATP regeneration system, is reduced to lactate by the enzyme while NADH is oxidized to NAD^+. We have used the series of coupled reactions as outlined in Schemes III and IV to prepare L-[1-^{13}C]glutamic acid, using [^{13}C]bicarbonate as an isotopically labeled substrate, and L-[1,3,4-$^{13}C_3$]glutamic acid, using [^{13}C]bicarbonate, [2-^{13}C]pyruvate and [2-^{13}C]acetate as labeled substrates.

Acknowledgments. This work was supported by The University of Texas at Dallas and Dallas Biomedical Corporation.

METHODS

Materials. Oxaloacetic acid, sodium pyruvate, coenzyme A (sodium salt), ATP, NAD^+, $NADP^+$, L-alanine and D-glucose-6-phosphate, malic enzyme (chicken liver)[EC 1.1.1.40], L-malate dehydrogenase (bovine heart)[EC 1.1.1.37], L-glutamate dehydrogenase (bovine liver)[EC 1.4.1.3], glucose-6-phosphate dehydrogenase (leuconostoc mesenteroides)[EC 1.1.1.49], glucose dehydrogenase (bacillus megaterium)[EC 1.1.1.47], isocitrate dehydrogenase (porcine heart)[EC 1.1.1.42], L-lactic dehydrogenase (rabbit muscle) [EC 1.1.1.27], pyruvate kinase (rabbit muscle)[EC 2.7.1.40], myokinase (rabbit muscle)[EC 2.7.4.3], (S)-acetyl-CoA synthetase (yeast)[EC 6.2.1.1], citrate (*si*)-synthase (porcine heart)[EC 4.1.3.7], aconitase (porcine heart)[EC 4.2.1.3] and glutamic-pyruvic transaminase (porcine heart)[EC 2.6.1.2] were obtained from Sigma Chemical Co.(St. Louis, MO).. Aconitase was activated in ferrous ammonium sulfate/cysteine buffer under an argon purge prior

to use[10]. Those enzymes obtained as crystalline or lyophilized powders were dissolved and stored in glycerol/TRIS buffer pH 7.5 (1:1). Other enzymes obtained as suspensions in saturated ammonium sulfate solutions were used directly. Phospho(enol)pyruvate was prepared as the potassium salt using previously published procedures[6]. [2-^{13}C]sodium pyruvate was obtained from Isotec, Inc. (Miamisburg, OH). Sodium [^{13}C]bicarbonate (99%), [1-^{13}C]sodium acetate (99%) and [2-^{13}C]sodium acetate (99%) were obtained from Cambridge Isotope Laboratories (Woburn, MA).

NMR Methods. Proton-decoupled ^{13}C FT-NMR spectra were obtained at 67.9 MHz using a JOEL FX-270, at 50.3 MHz using a JOEL FX-200 or at 125.75 MHz using a General Electric GN-500 NMR spectrometer. Both JOEL spectrometers were interfaced to pulse programmers obtained from Tecmag, Inc. (Houston, TX) and spectra were processed on MacIntosh IIci personal computers using Tecmag software. For determination of reaction yield, spectra were acquired using a 30° sampling pulse and a 5 s delay time during which the proton decoupler was gated off. Yields and isotopic enrichments were determined by peak integrations using either Techmag or GE software. Isotopically labeled L-glutamic acid samples were dissolved as the free acid in D_2O. Chemical shifts are reported in ppm downfield from TMS using dioxane as an internal standard (67.4 ppm).

Enzymatic Assays. *In situ* concentrations of L-malate and L-glutamate were determined by enzymatic assay. In carrying out the assay, a small test sample of a reaction mixture was boiled for 1 min in order to remove residual enzyme activity. Concentrations were then determined by comparing the initial rates of NAD^+ reduction, as specified in the assay procedure[11,12], to similar measurements determined with known concentrations of substrates.

L-[4-^{13}C] or L-[5-^{13}C]Glutamic Acid (Method I). Into 980 ml of aqueous solution containing 0.1 M 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), 10 mM $MgCl_2$, 2 mM ethylenediaminetetraacetic acid (EDTA), 10 mM β-mercaptoethanol, 0.1 M KCl, 0.05% (w/w) sodium azide, 57 mM oxaloacetate (6.24 g), 53 mM monopotassium PEP (9.36 g), 3.2 mM ATP (1.87 g), 0.13 mM coenzyme A (0.10 g) and 24.8 mM [2-^{13}C]sodium acetate (2.00 g), pH 7.4, was added 50 units of (S)-acetyl CoA synthetase, 1,000 units of citrate (*si*)-synthase, 2,500 units of pyruvate kinase and

1,000 units of myokinase. The reaction mixture was incubated at 30 °C under an argon gas purge. Following a 40 hour incubation it was determined by integration of the ^{13}C NMR that 84% of the [2-^{13}C]sodium acetate in the original reaction mixture had condensed with oxaloacetate to yield [4-^{13}C]citrate. The mixture was then made 1.33 mM in NADP$^+$ (by addition of 1.14 g), 28.8 mM in NH_4Cl (by addition of 1.44 g) and the pH was readjusted to 7.4. To this solution was added 800 units of L-glutamate dehydrogenase, 500 units of isocitrate dehydrogenase and 35 units of aconitase, preactivated in 90 ml of oxygen depleted activating buffer solution[10]. The reaction mixture was then incubated at 30 °C under an argon purge for an additional 70 hours. Following incubation, it was determined by peak integration of the ^{13}C NMR spectrum that the relative percentages ^{13}C-labeled species in solution were L-[4-^{13}C]glutamate, 42%; [4-^{13}C]2-oxo-glutarate, 28%; [4-^{13}C]citrate, 19%; [2-^{13}C]acetate, 12%. The reaction mixture was then made 11 mM in D-glucose-6-phosphate (by addition of 3.00 g) and 200 additional units of L-glutamate dehydrogenase and 50 units of glucose-6-phosphate dehydrogenase were added. The ^{13}C NMR spectrum of the reaction mixture following an overnight incubation at room temperature showed that all of the [4-^{13}C]2-oxo-glutarate had been converted to L-[4-^{13}C]glutamate. L-[5-^{13}C]glutamate was prepared using an identical procedure, but substituting [1-^{13}C]sodium acetate for [2-^{13}C]sodium acetate. Isotopically labeled L-glutamic acids were purified by ion exchange chromatography (see below) and crystallized from ethanol:water (76% yield from [2-^{13}C]sodium acetate). Resonances in the ^{13}C NMR spectrum of L-[4-13C]glutamic acid could be assigned to C-1 (175.2 ppm), C-2 (55.4 ppm, $^2J_{CC}$ = 13.2 Hz), C-3 (26.9 ppm, $^1J_{CC}$ = 37.3 Hz), C-4 (34.2 ppm) and C-5 (178.6 ppm , $^1J_{CC}$ = 54.9 ppm). L-gluamate was chromatographed as its N-trifluoroacetyl diethyl ester and monitored using a flame ionization detector. L-glutamate eluded from the column at 9.8 min. Isotopic purity ,as determined by seconday ion mass spectroscopy (SIMS) following chromatography, was 94.7%. .

L-[1-^{13}C]Glutamic Acid (Method II). To 150 mL of a 0.1 M Tris buffer solution containing 10 mM β-mercaptoethanol, 2 mM EDTA, 0.1 M KCl and 0.05% (w/w) sodium azide are added 7.5 mmol of sodium pyruvate (0.825 g, 50 mM), 7.5 mmol D-glucose (1.35 g, 50 mM), 0.3 mmol of NADP$^+$ (0.23 g, 2 mM) and 6.0 mmol of [^{13}C]sodium bicarbonate (0.50 g, 40 mM). After adjusting the pH of the mixture to 7.5,

8.3 units of malic enzyme and 100 units of glucose dehydrogenase were added. After a 48 hour incubation at 30 °C the ^{13}C NMR spectrum showed only the single labeled carbon resonance of L-[4-^{13}C]malate (180.9 ppm) and a small residual resonance from unreacted [^{13}C]bicarbonate (161.1 ppm). It was determined by enzymatic assay that 62% of the pyruvate had been converted to L-malate (the remainder going to L-lactate as a result of contaminating L-lactic dehydrogenase in the commercial malic enzyme preparation). 15 mmol of sodium acetate (1.23 g, 0.1M), 17 mmol of monopotassium PEP (3.0 g, 0.11 M), 0.41 mmol of ATP (0.25 g, 2.8 mM), 93.4 μmol coenzyme A (71 mg, 0.62 mM) and 0.44 mmol NAD^+ (0.31 g, 2.9 mM) were then added to the reaction mixture. The pH was readjusted to 7.5 and 240 units of citrate (*si*)-synthase, 350 units of myokinase, 4,300 units of pyruvate kinase, 11 units of (S)-acetyl CoA synthetase, 650 units of L-lactic dehydrogenase and 6,000 units of malate dehydrogenase were added. Following a 72 hour incubation at 30 °C, the ^{13}C NMR spectrum showed no detectable labeled carbon resonance from L-[4-^{13}C]malate. This reaction mixture was saturated with nitrogen and to it was added 0.3 mmol more of $NADP^+$ (0.23 g, 4 mM), 22.2 mmol of L-alanine (2.0 g, 0.15 M), 378 units glutamic-pyruvic transaminase, 775 units more L-lactic dehydrogenase, 340 units of isocitrate dehydrogenase and 15.5 units of aconitase in 35 ml of oxygen depleted activating buffer. Following a 2 day incubation under a nitrogen purge at 30 °C, the following integrated intensities and chemical shifts were taken from the labeled carbon resonances in the ^{13}C NMR spectrum: 40% residual [1-^{13}C]citrate (180.0 ppm); 40% L-[1-^{13}C]glutamate (175.5 ppm); 20% [1-^{13}C]2-oxo-glutarate (170.6 ppm). The reaction mixture was then made 11 mM in D-glucose-6-phosphate (by addition of 3.00 g), 200 additional units of L-glutamate dehydrogenase and 50 units of glucose-6-phosphate dehydrogenase were added. The ^{13}C NMR spectrum of the reaction mixture following an overnight incubation at room temperature showed that all of the [1-^{13}C]2-oxo-glutarate had been converted to L-[1-^{13}C]glutamate. L-[1-^{13}C]glutamic acid was purified by ion exchange chromatography and lyophilized to a dry powder (36% yield from [^{13}C]sodium bicarbonate). Resonances in the ^{13}C NMR of the purified product could be assigned to C-1 (175.7 ppm), C-2 (55.4 ppm, ^1Jcc = 52.7 Hz), C-3 (27.3 ppm) and C-4 (34.2 ppm) (C-5 was obscured by the large C-1 resonance).

L-[1,3,4-$^{13}C_3$]Glutamic Acid (Method I). To 3 mL of a 0.1 M Tris buffer solution containing 10 mM β-mercaptoethanol, 2 mM EDTA, 0.1 M KCl and 0.05% (w/w) sodium azide were added 76.3 μmol of [2-^{13}C]sodium pyruvate (8.4 mg, 25 mM), 178 μmol D-glucose-6-phosphate (50 mg, 60 mM), 6.4 μmol of $NADP^+$ (5 mg, 2 mM) and 150 μmol of [^{13}C]sodium bicarbonate (12.6 mg, 50 mM). After adjusting the pH of the mixture to 7.5, 0.2 units of malic enzyme and 10 units of glucose-6-phosphate dehydrogenase were added. Following a 3 day incubation at room temperature, resonances in the ^{13}C NMR spectrum could be assigned to C-4 of L-[2,4-$^{13}C_2$]malate (180.9 ppm), the C-2 of L-malate (71.6 ppm), the residual [^{13}C]bicarbonate (161.1 ppm) and C-2 of L-[2-^{13}C]lactate (69.4 ppm). The C-2 of L-[2-^{13}C]lactate signal was about 21% of the intensity of the C-2 of L-[2,4-$^{13}C_2$]malate signal, indicative of the relative rates at which [2-^{13}C]pyruvate was converted either to L-malate or to L-lactate by contaminating L-lactic dehydrogenase. The reaction mixture was boiled for 1 min to remove the residual malic enzyme activity. To the cooled reaction mixture were added 87 μmol of [2-^{13}C]sodium acetate (7.3 mg, 29 mM), 0.16 mmol of monopotassium PEP (28 mg, 53 mM), 8.3 μmol of ATP (5 mg, 2.8 mM), 0.65 μmol coenzyme A (0.5 mg, 0.21 mM), 30 μmol of sodium pyruvate (3.3 mg, 10 mM) and 8.4 μmol NAD^+ (6 mg, 2.9 mM). The pH was readjusted to 7.5 and 10 units of citrate (*si*)-synthase, 25 units of myokinase, 10 units of pyruvate kinase, 3.75 units of (S)-acetyl CoA synthetase, 50 units of L-lactic dehydrogenase and 200 units of malate dehydrogenase were added. Following a 2 day incubation at room temperature, no L-malate was detectable by ^{13}C NMR. Instead the spectrum showed a singlet arising from the labeled C-1 (180.0 ppm), a doublet from the C-3 (76.3 ppm, $^1J_{CC}$ = 39.1 Hz) and a doublet from the C-4 of [1,3,4-$^{13}C_3$]citrate (46.4 ppm, $^1J_{CC}$ = 39.1 Hz). Resonances were also observed for the C-2 of [2-^{13}C]acetate (24.5 ppm) and C-2 of L-lactate (69.4 ppm). To the reaction mixture was added 6.5 μmol of $NADP^+$ (5 mg, 2.2 mM), 76 μmol of NH_4Cl (4 mg, 25 mM), 2 units of L-glutamate dehydrogenase, 2 units of isocitrate dehydrogenase, and 0.1 unit of aconitase in 1 ml of oxygen depleted activating buffer. The reaction mixture was incubated under an argon purge for three days at room temperature. Following this incubation the ^{13}C NMR spectrum showed resonances for the C-1 (176.0 ppm), the C-3 (28.2 ppm, $^1J_{CC}$ = 35.2 Hz, , $^2J_{CC}$ = 4.4

Hz) and C-4 (34.6 ppm, $^1J_{CC}$ = 33.0 Hz) of [1,3,4-$^{13}C_3$]L-glutamate, the C-2 of [2-^{13}C]L-lactate (69.4 ppm), the C-2 of [2-^{13}C]acetate (24.5 ppm) and the C-1 (180.0 ppm), the C-3 (76.3 ppm, $^1J_{CC}$ = 39.1 Hz) and the C-4 (46.5 ppm, $^1J_{CC}$ = 39.1 Hz) of [1,3,4-$^{13}C_3$]citrate. From integrated intensities we estimate that there was 42% acetate, 36% L-glutamate, 7% citrate and 14% L-lactate contained in the final reaction mixture. L-[1,3,4-$^{13}C_3$]glutamic acid was purified on 0.5 X 10 cm columns of Dowex-50 and Dowex-1 ion exchange resins. In addition to the aforementioned resonances arising from ^{13}C-enriched carbons, the ^{13}C NMR spectrum of the purified product also showed resonances which could be assigned to C-2 (55.4 ppm, broad complex multiplet) and C-5 (178.6 ppm, $^1J_{CC}$ = 54.9 ppm).

Purification of Isotopically Labeled L-Glutamic Acids. Reaction mixtures containing labeled L-glutamic acid were made 2% (v/v) in perchloric acid and the precipitated protein was removed by centrifugation. The supernatant, neutralized with 8 N KOH to pH 7.5, was loaded onto a cation exchange column of Dowex-50 (H^+ form, 200-400 mesh and typically 2.5 X 90 cm) and washed with 1.5 l of deionized water. L-glutamate along with other cationic species were eluted from the column with 2 M NH_4OH and crudely fractionated into 200-500 ml fractions. Samples from the fractions were spotted on paper (Whatman 3 mm), dried with a heat gun and then respotted with a ninhydrin solution (0.5% in methanol:water (1:1)), and developed for 5 min at 100 °C. Those fractions testing positive were evaporated to dryness, taken up in water, adjusted with 1N acetic acid to pH 7.5, and loaded onto a Dowex-1 X 8 anion exchange column (acetate form, 100-200 mesh and typically 2.5 X 90 cm). For those reaction mixture containing L-alanine, the column was washed with deionized water until the eluent tested negative with ninhydrin reagent. For samples which did not contain L-alanine, the column was washed with 1.5 l of deionized water. L-glutamate was then eluted from the column with 0.5 N formic acid and collected in 10 ml fractions. Those fractions testing ninhydrin positive were evaporated to dryness. L-glutamic acid was obtained either as a lyophilized powder or crystallized from ethanol:water (4:1).

REFERENCES

1. Sherry, A. D., Malloy, C. R., Zhao, P. and Thompson, J. R. - Biochemistry 31: 4833-4837 (1992).

2. Cappon, J. J., Baart, J., van de Walle, G. A. M., Raap, J and Lugtenburg, J. - Recl. Trav. Chim. Pays-Bas 110: 158-166 (1991).

3. Billhardt, U.-M., Stein, P. and Whitesides, G. M. - Bioorganic Chemistry 17: 1-12 (1989).

4. Wong, C.-H. and Whitesides, G. M. - J. Am. Chem. Soc. 103:4890-4899 (1981).

5. Lee, L. and Whitesides, G. M.. - J. Am. Chem. Soc. 107: 6999-7008 (1985).

6. Hirschbein, B. L., Mazenod. F. P. and Whitesides, G. M. - J. Org. Chem. 47:3765-3766 (1982).

7. Braunstein, A. E. in *The Enzymes*, Third Edition (ed., P. D. Boyer), pp. 379-481, Academic Press, New York (1973).

8. Wood, T. G., Weisz, O. A. and Kozarich, J. W. - J. Am. Chem. Soc. 106: 2222-2223 (1984).

9. Kuan, K. T., Weber, D. S., Sottile, L. and Goux, W. J. - Carbohydr. Res. 225: 123-136 (1992).

10. Williamson, J. R. and Corkey, B. E. - Methods in Enzymol. 13: 434-509 (1969).

11. Gutmann, I. and Wahllefeld, A. W. in *Methods in Enzymatic Analysis* (ed., H. U. Bergmeyer), v. 3, pp. 15835-1589, Verlag Chemie Weinheim/Academic Press, New York (1971)

12. Bernt, E. and Bergmeyer, H. U.. in *Methods in Enzymatic Analysis* (ed., H. U. Bergmeyer), v. 4, 1705-1708, Verlag Chemie Weinheim/Academic Press, New York (1971)