Reassessment of $^{14}$CO$_2$ Compartmentation and of [${}^{14}$C]Formate Oxidation in Rat Liver*  

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Our previous report (Marsolais, C., Huot, S., David, F., Garneau, M., and Brunengraber, H. (1987) J. Biol. Chem. 262, 2604–2607) had concluded that a fraction of [${}^{14}$C]formate oxidation in liver occurs in the mitochondrion. This conclusion was based on the labeling patterns of urea and acetoacetate labeled via $^{14}$CO$_2$ generated from [${}^{14}$C]formate and other [${}^{14}$C]substrates. We reassessed our interpretation in experiments conducted in (i) perfused mitochondria and (ii) isolated livers perfused with buffer containing [${}^{14}$C]formate, [${}^{14}$C]gluconolactone, $^{14}$CO$_2$, or NaH$^{14}$CO$_3$, in the absence and presence of acetazolamide, an inhibitor of carboxy anhydrase. Our data show that the cytosolic pools of bicarbonate and CO$_2$ are not in isotopic equilibrium when $^{14}$CO$_2$ is generated in the cytosol or is supplied as NaH$^{14}$CO$_3$. We retract our earlier suggestion of a mitochondrial site of [${}^{14}$C]formate oxidation.

We recently reported (1) a study on the compartmentation of $^{14}$CO$_2$ in the perfused rat liver. We concluded that the specific activity (SA) of the total CO$_2$ system (tCO$_2$ = CO$_2$ + H$_2$CO$_3$ + HCO$_3$) is the same in the extracellular, cytosolic, and mitochondrial compartments of the liver cell. The SA of mitochondrial tCO$_2$ was assessed by the SAs of urea and of carbon-1 of acetocacetate (AcAc) derived from 2-ketoisocaprate (KIC). Mitochondrial bicarbonate is incorporated into urea via carbamyl phosphate synthetase I (Ref. 2). In the course of KIC catabolism, the carbon of bicarbonate, incorporated by methylcrotonyl-CoA carboxylase, becomes C-1 of AcAc (3).

We found that the SA of the mitochondrial CO$_2$ system can be varied by inhibiting carboxy anhydrase with acetazolamide (AZ; Ref. 4). Thus, AZ decreases the SA of urea and AcAc labeled from extracellular NaH$^{14}$CO$_3$. In contrast, AZ increases the SA of urea and AcAc labeled from mitochondrial sources of $^{14}$CO$_2$, such as 2-keto-[1-14C]isocaprate, [1-14C]pyruvate or [1-14C]glutamine.

When experiments were conducted in the presence of [${}^{14}$C]formate, we found that the SAs of urea and AcAc were increased in the presence of AZ. This was unexpected in the light of current concepts that ascribe the generation of $^{14}$CO$_2$ from [${}^{14}$C]formate to cytosolic methylene tetrahydrofolate dehydrogenase and peroxisomal catalase (5, 6). In addition, in the presence of [${}^{14}$C]formate, the SA of C-1 of AcAc was double that of urea. This suggested channeling of $^{14}$CO$_2$ derived from [${}^{14}$C]formate to mitochondrial methylcrotonyl-CoA carboxylase.

In the present study, we first investigated the oxidation of formate in isolated rat liver mitochondria. These were perfused with [${}^{14}$C]formate under conditions that stimulate citrulline synthesis from ornithine + NH$_2$. In isolated mitochondria, the SA of the ureido carbon of citrulline reflects that of matrix bicarbonate.

Upon finding negligible formate oxidation in perfused mitochondria, we reassessed the site of [${}^{14}$C]formate oxidation in liver perfusion experiments. Labeled CO$_2$ was supplied to or generated in the cytosol using tracers of $^{14}$CO$_2$ gas, H$^{14}$CO$_3$, H$^{13}$CO$_3$, [${}^{14}$C]formate, and [1-14C]gluconolactone.

Our data show that the cytosolic pools of CO$_2$ and bicarbonate are not in isotopic equilibrium, when labeled CO$_2$ is generated in the cytosol. We identified an error of interpretation of our previous data on [${}^{14}$C]formate oxidation in liver. Therefore, we retract our earlier suggestion (1) of a mitochondrial site of formate oxidation in rat liver.

**EXPERIMENTAL PROCEDURES**

**Materials**

Enzymes, coenzymes and biochemicals were purchased from Boehringer Mannheim Canada (Montreal, Quebec) and Sigma. 3-Amino-1,2,4-triazole was purchased from Aldrich (Montreal, Quebec), N,O was obtained from Liquid Air Canada Ltd. Acetazolamide was obtained from Lederle (Montreal, Quebec). [${}^{14}$C]Formate (50 Ci/mmol), NaH$^{14}$CO$_3$ (7 Ci/mol) and [1-14C]glucose (65 Ci/mol) were supplied by ICN Radiochemicals (Irvine, CA). NaH$^{14}$CO$_3$ (99% MPE) was purchased from Merck Sharp and Dohme (Pointe Claire, Quebec). N-Methyl-N-(t-butylidemethylsilyl)trifluoroacetamide was supplied by Regis Chemical Company (Morton Grove, IL).

Before use, aliquots of [${}^{14}$C]formate stock were purified from traces of H$^{13}$CO$_3$ as follows. The water ethanol solvent was evaporated with pure CO$_2$. The tracer was dissolved in 0.5 ml of 10 mM Na$_2$CO$_3$, and 0.02 ml of 1.2 M BaCl$_2$ was added to precipitate BaC$_6$. After centrifugation, the supernatant was loaded onto a 0.5 × 3-cm AG50W$^+$ column developed with 1 ml of water. The acidic effluent was neutralized with 1 N NaOH, counted, and used immediately.

D-[1-14C]Glucose was converted to D-[1-14C]gluconolactone by reacting 0.5 mCi of tracer with 10 units of glucose oxidase and 10 units of catalase in 1 ml of 25 mM Tris buffer, pH 7.3. After 1-h incubation at 37°C, pH was raised to 9. Fifteen min later, the reaction mixture was loaded onto a 0.5 × 1-cm column of AG-1-X8-CI resin. The column was first developed with 6 × 0.5 ml of water to elute unreacted...
glucose and then with 6 × 0.5 ml of HCl (0.04 N) to elute [1-\(^{14}\)C] gluconolactone.

**Protocols**

**Perfusion of Mitochondria**—Liver mitochondria from rats fed ad libitum (Sprague-Dawley, Charles River 150-250 g) were prepared by the method of Hogeboom (7). To inhibit catalase activity which contaminates isolated mitochondria (8), 3-amino-1,2,4-triazole was injected into the rats (1.5 g/kg, Ref. 9) 90 min before sacrifice and added to the isolation medium (10 mM). Mitochondria (1 mg of protein) were perfused on a Millipore filter (Millipore) with buffer containing: 80 mM MOPS, 25 mM KCl, 5 mM KH\(_2\)PO\(_4\), 1 mM EGTA, 1 mM MgSO\(_4\), 25 mM KHCO\(_3\), 2 mM ATP, 10 mM ornithine, 10 mM N-acetylglutamate, 10 mM NH\(_4\)Cl, 10 mM succinate, and 0.25 mM \([^{13}\text{C}]\) formate (100-300 dpm/nmol). The perfusion medium was gassed at 37 °C with 95% O\(_2\) + 5% CO\(_2\) and pH was adjusted to 7.40. Effluent perifusate was collected over 5-min periods.

**Liver Perifusions**—Livers from fed or 2-day-starved rats (Sprague-Dawley, Charles River) were perfused (10) with nonrecirculating Krebs-Ringer bicarbonate buffer (30 ml/min) equilibrated with 95% O\(_2\) + 5% CO\(_2\) and containing 1.2 mM N\(_2\), 20 mM ornithine, 20 mM asparagine, 0.5 mM KIC and where indicated, (i) 0.25 mM formate + 1 mM methionine (T1) or (ii) 10 mM glucose + 0.25 mM glutonacol- tone. In the latter case, glutonacol (7.5 mM, pH = 3.0) was infused via a syringe pump just ahead of the liver, to minimize hydrolysis of the lactone. The pH of the perfusate was not affected by the infusion of the acidic stock solution of glutonacolactone. In all perifusions, a 15-min equilibration period was allowed. Tracers were infused for 14 min, and AZ (0.2 mM) was added for the last 7-min period.Radioactive substrate and effluent perifusates were sampled under NaOH perfusions were performed on the same liver.

Analytical Techniques

Citrulline (12) and mitochondrial proteins (12) were assayed by standard colorimetric techniques. All assays of SAs were done as described previously (1). In perfusions with H\(^{14}\)CO\(_3\), MPE of AcAc and urea were determined by gas chromatography-mass spectrometry analysis. Five mill of effluent samples were brought to pH 9-11 with NaOH and made 10 mM NaBH\(_4\) to convert unstable AcAc to stable (R,S)-BHB. After 30 min at room temperature, the samples were saturated with NaCl and extracted four times with 15 ml of ethyl acetate. The pooled extracts containing urea were evaporated. The aqueous phase was acidified with HCl to pH 1-2 and extracted three times with 10 ml of ethyl acetate. The pooled extracts containing BHB were evaporated. Both dry residues were incubated overnight at room temperature with 0.05 ml of N-methyl-N-(\(t\)-butyldimethylsilyl)trifluoroacetamide.

Analyses were performed on a Hewlett Packard 5890 mass spectrometer coupled with a gas chromatograph equipped with a HP-5 capillary column (30 m × 0.20 mm inner diameter, 0.30 μm film thickness; Ref. 14). Carrier gas was helium (0.8 ml/min), and the column head pressure was 20 kilopascals. The injection port was kept at 250 °C and the column temperature, initially set at 150 °C, was programmed to increase by 5 °C/min after a 3-min delay. [\(^{13}\text{C}]\)bicarbonate, the concentration of unlabeled bicarbonate in the stock buffer was reduced to 32.5 mM, and 2.5 mM of pure NaH\(^{14}\)CO\(_3\) was infused into the inflowing perfusate to obtain a total bicarbonate concentration of 25 mM, and a molar percent enrichment (MPE) of 10%.

**RESULTS AND DISCUSSION**

In perifused mitochondria from aminotriazole-treated rats, the rate of \[^{14}\text{C}]\)formate oxidation was 7 pmol/mg protein × min. This rate, equivalent to 1.7 nmol/g dry wt of liver × min, is only one-hundredth of the rate observed in the perfused livers (110-240 nmol/g dry weight × min). We ascribed this very low rate of \[^{14}\text{C}]\)formate oxidation to residual catalase activity in perifused mitochondria which contaminates most mitochondrial preparations (8). Since we could not demonstrate significant rates of \[^{14}\text{C}]\)formate oxidation in isolated mitochondria, we turned back to the perfused liver system. The first protocols were designed to test the hypothesis that part of formate oxidation occurs in mitochondria.

Data from liver perifusions with \[^{14}\text{C}]\)formate ± AZ (Table I, experiment 1) were similar to those (1) reported previously: (i) the SA of urea was equal to that of effluent tCO\(_2\), (ii) the SA of AcAc was 1.5 times that of urea, and (iii) in the presence of AZ, the SAs of AcAc and urea increased 6 to 8 times above that of bicarbonate. We tried to favor putative mitochondrial site of \[^{14}\text{C}]\)formate oxidation and thus to increase the differential labeling of urea and AcAc by inhibiting extramitochondrial formate oxidation with N\(_2\)O (15) + 3-amino-1,2,4-triazole (9). Although the rate of formate oxidation was decreased by 70% as reported by others (16), there was no major change in the labeling patterns of urea, AcAc, and bicarbonate (Table I, experiment 2). Note, however, that the SA of urea was 70% that of effluent tCO\(_2\).

Contrary to expectations, we suspected that we had made an error in the interpretation of some of our original data from perfused liver experiments. We had concluded that a fraction of \[^{14}\text{C}]\)formate oxidation occurs in mitochondria on the basis of: (i) the preferential labeling of AcAc versus urea by \[^{14}\text{C}]\)formate, (ii) the increase by AZ of the SAs of urea and AcAc labeled from either \[^{1-14}\text{C}]\)pyruvate, \[^{1-14}\text{C}]\)glutamine, \[^{1-14}\text{C}]\)KIC, or \[^{14}\text{C}]\)formate, and (iii) the decrease by AZ in the SAs of urea and AcAc labeled from NaH\(^{14}\)CO\(_3\). We had originally equated an infusion of NaH\(^{14}\)CO\(_3\) not only to an extracellular, but also to an extramitochondrial source of \(^{14}\text{CO}_2\). We, therefore, needed an unambiguous metabolic source of extramitochondrial \(^{14}\text{CO}_2\), as a control for experiments using \[^{14}\text{C}]\)formate. We chose to use \[^{14}\text{C}]\)glutonacolactone which generates cytosolic \(^{14}\text{CO}_2\). \[^{14}\text{C}]\)glutonacolactone is activated in the cytosol to \[^{14}\text{C}]\)glutonacolactone (17), a substrate of cytosolic \[^{14}\text{C}]\)glutonacolactone dehydrogenase. Since it was reported (18) that the rate of \(^{14}\text{CO}_2\) production in vivo from \[^{14}\text{C}]\)glutonacolactone was 3 times that from \[^{1-14}\text{C}]\)glutonacolactone, we decided to perfuse livers with \[^{14}\text{C}]\)glutonacolactone as a source of cytosolic \(^{14}\text{CO}_2\). The production of \[^{14}\text{CO}_2\] by livers perfused with 0.25 mM \[^{14}\text{C}]\)glutonacolactone was 0.404 ± 0.045 μmol/g dry weight × min. Production of \[^{14}\text{CO}_2\] from \[^{14}\text{C}]\)glutonacolactone was not significantly affected by AZ or by perfusing the liver in the retrograde mode (not shown).

Contrary to expectations, the SAs of urea and AcAc labeled from \[^{1-14}\text{C}]\)glutonacolactone were increased by AZ (Table I, experiments 3A and 3B), as had been the case with \[^{1-14}\text{C}]\)
bicarbonate does not diffuse through the mitochondrial membranes, let us discuss our data in the framework of Fig. 1. Carbonic anhydrase and a slower spontaneous reaction. On these cytosolic bicarbonate before diffusing into the mitochondrion. There, the SA of bicarbonate is greatly diluted by equilibration with the large pool of bicarbonate shown in Fig. 1. One should keep in mind that at pH = 7.40, the concentration of bicarbonate is 20 times that of CO₂. In addition, the predominant bicarbonate does not diffuse through the mitochondrial membrane, unless it is converted to diffusible CO₂. Finally, the equilibrium between bicarbonate and CO₂ is maintained by two reactions: a rapid enzymatic reaction catalyzed by carbonic anhydrase and a slower spontaneous reaction. On these bases, let us discuss our data in the framework of Fig. 1.

The SA of bicarbonate entering the cell or generated in the cytosol is greatly diluted by equilibration with the large pool of cytosolic bicarbonate before diffusing into the mitochondrion. There, the SA of bicarbonate is further diluted by unlabeled CO₂ generated locally, as it equilibrates with mitochondrial bicarbonate. The latter is incorporated into urea and AcAc via carbamoyl phosphate synthetase and methylcrotonyl-CoA carboxylase. In the presence of AZ, which inactivates cytosolic carbonic anhydrase, dilution of the SA of cytosolic ¹⁴CO₂ is markedly decreased. Thus, the SA of ¹⁴CO₂ that diffuses into the mitochondrion as well as the SAs of urea and AcAc are increased. This occurs in spite of a decrease in the absolute rates of urea (1, 19) and AcAc (1) synthesis caused by a limitation in the supply of mitochondrial bicarbonate.

The SA of ¹⁴CO₂ generated in the mitochondrion is diluted by unlabeled CO₂ generated locally and diffusing from the cytosol, in the presence of AZ, diffusion of unlabeled CO₂ from the cytosol is decreased because of lower production from cytosolic bicarbonate. This leads again to an increase in the SAs of urea and AcAc.

The MPE of H¹³CO₃ entering the liver cell is slightly diluted by equilibration with the small pool of unlabeled CO₂. Cytosolic CO₂ becomes labeled and diffuses into the mitochondrion. There, it is further diluted by unlabeled CO₂ generated locally, as it equilibrates with mitochondrial bicarbonate. In the presence of AZ, labeling of cytosolic CO₂ from extracellular H¹³CO₃ is decreased, leading to a lowering in the MPE of urea and AcAc.

In the presence of AZ, the SAs of urea and AcAc labeled

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**TABLE I**

| Exp. | Labeled substrates or tracers | Conditions | n | SA or MPE area/SA or MPE total CO₂ | SA or MPE AcAc/SA or MPE total CO₂ |
|------|------------------------------|------------|---|-----------------------------------|-----------------------------------|
|      |                              |            |   | Control + Acetazolamide            | Control + Acetazolamide            |
| 1    | 0.25 mM [¹⁴C]formate          | Control    | 6 | 0.98 ± 0.047                      | 5.34 ± 0.01                    |
| 2    | 0.25 mM [¹⁴C]formate          | N₂O + emicrinozole | 5 | 0.70 ± 0.10                      | 4.11 ± 0.27                    |
| 3A   | 0.25 mM [¹⁴C]gluconeolactone  | Anterograde, fed | 7 | 0.69 ± 0.036                      | 4.90 ± 0.19                    |
| 3B   | 0.25 mM [¹⁴C]gluconeolactone  | Retrograde, fed | 7 | 0.73 ± 0.042                      | 4.51 ± 0.34                    |
| 4A   | H¹⁴CO₃ Tracer                 | Anterograde | 7 | 1.24 ± 0.037                      | 3.63 ± 0.45                    |
| 4C   | H¹⁴CO₃ Tracer                 | Retrograde | 7 | 0.89 ± 0.038                      | 2.34 ± 0.10*                   |
| 4D   | H¹⁴CO₃ Tracer                 | Retrograde | 7 | 1.14 ± 0.041                      | 2.20 ± 0.015                   |

* Statistically different from the corresponding figure without acetazolamide (p < 0.01).
* Statistically different from the corresponding SA urea/SA total CO₂ (p < 0.025).
* Statistically different from the corresponding figure in the anterograde mode (p < 0.025).

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**FIG. 1. Compartmentation of the CO₂/HCO₃⁻ system in the liver cell. Bold arrows indicate faster reactions. Other abbreviations used: s, spontaneous; ECF, extracellular fluid; TCAC, tricarboxylic acid cycle. CA, carbonic anhydrase.**
from $^{14}$CO$_2$ increase 3- to 5-fold, whereas the MPE of urea and AcAc labeled from H$^{13}$CO$_2$ decrease by only 20-25%. In this and in the previous report (1), AZ induced large variations in the labeling of urea and AcAc when these are labeled from CO$_2$, the minor species of the CO$_2$/HCO$_3^-$ system. In contrast, when urea and AcAc are labeled from HCO$_3^-$, the predominant species, much smaller variations in urea and AcAc labeling are observed. Thus, in the CO$_2$/HCO$_3^-$ system, the relative size of the pool to which label is added influences the extent to which label is diluted in the other species of the system. Note also that the scales of variation of a parameter (i) from 100 to 0% and (ii) from 100 to + infinity are not symmetrical. Increases are thus much more striking than decreases.

It is puzzling that in the presence of cytosolic sources of $^{14}$CO$_2$ ([1-$^{14}$C]formate and [1-$^{14}$C]gluconolactone; Table I) and in the absence of AZ, the labeling ratio (AcAc/tCO$_2$) is greater than the labeling ratio (urea/tCO$_2$). In contrast, in the presence of mitochondrial sources of $^{14}$CO$_2$, ([1-$^{14}$C]pyruvate, [1-$^{14}$C]KIC, and [1-$^{14}$C]glutamine; Table I of Ref. 1), both labeling ratios were equal to 1. We cannot explain this discrepancy. It might involve some kind of channeling of 14CO$_2$ as has been described for other metabolic precursors such as urea cycle intermediates (20) and oxaloacetate in the tricarboxylic acid cycle (21). We recognize, however, that such an interpretation is speculative.

The above discussion of our data in relation to Fig. 1 requires the presence of an active carbonic anhydrase in the cytosol of liver cells. The liver is known to contain several carbonic anhydrase isoenzymes: carbonic anhydrase V in the mitochondrion (22), and carbonic anhydrase II and carbonic anhydrase III in the cytosol (23). It has been shown immunohistochemically that carbonic anhydrase III, which is insensitive to AZ inhibition (24), occurs throughout the liver but in greater concentration around the perivenous hepatocytes (25). Carbonic anhydrase II, also found throughout the liver, is present at much lower concentrations. Our results suggest the existence of a cytosolic AZ-sensitive carbonic anhydrase. This activity appears distributed throughout the liver lobule, since labeling patterns of urea and AcAc labeled from infused $^{14}$CO$_2$ + NaH$^{13}$CO$_3$ were identical in livers perfused in anterograde and retrograde modes (Table I, experiment 4). A recent study has suggested the presence of carbonic anhydrase activity on the outside surface of the hepatocyte membrane (26). Our data (Table I, experiment 4) show that such extracellular carbonic anhydrase activity, if it indeed occurs, is insufficient to equilibrate the labeling of extracellular bicarbonate and CO$_2$.

The present report illustrates errors of interpretation one can easily make when using labeled bicarbonate to trace the fate of labeled CO$_2$ generated inside the liver cell.

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