Partitioning of pyruvate between oxidation and anaplerosis in swine hearts

ASHISH R. PANCHAL,1 BLANDINE COMTE,2 HAZEL HUANG,1 TODD KERWIN,1 AHMED DARVISH,1 CHRISTINE DES ROSIERS,3 HENRI BRUNENGRABER,2 AND WILLIAM C. STANLEY1,2

1Department of Physiology and Biophysics and 2Department of Nutrition, Case Western Reserve University, Cleveland, Ohio 44106-4970; and 3Department of Nutrition, University of Montreal, Montreal, Quebec H3C 3Y7, Canada

Received 7 March 2000; accepted in final form 30 May 2000

The heart is dependent on a high rate of citric acid cycle (CAC) flux to support ATP regeneration and contractile function. Although oxaloacetate (OAA) is recycled in the CAC, there is a constant mitochondrial efflux of CAC intermediates (cataplerosis) in normal cardiomyocytes (Fig. 1). Cataplerosis is compensated for by an equivalent entrance of CAC intermediates (anaplerosis). Because the pool of CAC intermediates is small compared with the throughput of the cycle, maintenance of heart function requires exact matching between anaplerosis and cataplerosis. Perfusion of isolated rat hearts with anaplerotic substrates such as pyruvate (4, 29), succinate (5), fumarate (14), or glutamate (2) results in improved postischemic recovery and decreased reperfusion injury. This suggests that anaplerosis plays an important role in the correction of metabolic alterations resulting from ischemia and reperfusion.

The well-perfused heart readily oxidizes pyruvate formed from glycolysis and the oxidation of lactate (25). The primary fate of pyruvate in the normoxic heart is decarboxylation through mitochondrial pyruvate dehydrogenase (25). In isolated, perfused rat hearts, pyruvate is also carboxylated to form OAA or malate, presumably via pyruvate carboxylase or malic enzyme (8, 9, 11, 12, 26, 27) (Fig. 1). Evidence for pyruvate carboxylation in rat hearts is based on substantial activities of pyruvate carboxylase and malic enzyme (13, 27) and the incorporation of label from [1-14C]pyruvate into malate and citrate in perfused hearts (15, 21). Furthermore, inhibition of malic enzyme by hydroxymalonate decreased the incorporation of 13C from [1-13C]pyruvate into citrate and impaired the contractile power of the heart (21). However, although these reports demonstrated the existence and importance of pyruvate carboxylation in the isolated heart, they did not quantify the rate or the relative contribution of pyruvate carboxylation to the CAC flux.

Recent experiments with 13C substrates have expanded the field through the analysis of 13C isotopomers of CAC intermediates. One can now assess either positional or mass isotopomers of the CAC intermediates using NMR or gas chromatography-mass spectrometry (GC-MS), respectively. Studies with [13C]acetate (6, 7), propionate (11), or aspartate (12) and NMR analysis have demonstrated substantial incorporation of carbon into the CAC through anaplerotic pathways. In these experiments, the labeling pattern of glutamate was measured as a surrogate of that of α-ketoglutarate (7). Comte and colleagues (8, 9) recently measured the relative contributions of pyruvate carboxylation and de-
were computed from the MID3 of the CAC intermediates assayed by GC-MS.

MATERIALS AND METHODS

Chemicals

Chemicals, enzymes, and coenzymes were purchased from Boehringer Mannheim (Indianapolis, IN) and Sigma-Aldrich Chemicals (Milwaukee, WI). [3H]oxaloacetic acid, [U-13C3]lactate, and [U-13C3]pyruvate were obtained from Isotec (Miamisburg, OH). The derivatization agent N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide was supplied by Regis Chemical (Morton Grove, IL). Intralipid solution was obtained from Baxter Healthcare (Deerfield, IL).

Experimental Model

An in vivo technique was used to deliver 13C-labeled substrates directly into the left anterior descending (LAD) coronary artery of swine (23, 24). Overnight fasted domestic swine (27–36 kg) of either sex were sedated with Telazol (6 mg/kg im), anesthetized with pentobarbital sodium (25 mg/kg + 5 mg·kg−1·h−1 iv), intubated via a tracheotomy, and ventilated to maintain arterial blood gases in the normal range (PaO2 > 100 mmHg, PaCO2 35–45 mmHg, and pH 7.35–7.45). Rectal temperature was continuously monitored and maintained between 35 and 37°C with heating blankets. The right femoral artery and vein were cannulated for extracorporeal bypass and venous infusions of heparin and Intralipid, respectively. A 7-Fr high-fidelity pressure-transducer catheter (Millar, Houston, TX) was positioned in the left ventricle via the carotid artery. The heart was exposed via a midline sternotomy and suspended in a pericardial sling. The animal was then heparinized (300 U/kg bolus + 150 U·kg−1·h−1 iv) and infused with a 20% triglyceride emulsion (Intralipid 20%, 0.3 ml·kg−1·h−1 iv) to increase plasma free fatty acids (FFA) to 0.6 mM (23). This concentration of FFA is comparable to that measured in overnight-fasted humans (36). Coronary blood flow in the anterior wall was controlled by an extracorporeal circuit as previously described (23, 24). The LAD coronary artery was cannulated and perfused at 30–40 ml/min via roller pumps with blood from the femoral artery (23, 24). The anterior interventricular vein was cannulated to collect venous blood samples from the perfusion territory of the LAD. Previous studies have shown that 91% of the interventricular venous blood is derived from the LAD coronary artery (18). The coronary perfusion pump flow was adjusted to give an interventricular venous Hb saturation of 35–40% (23, 24). Left ventricular pressure was continuously recorded using an online data acquisition system (BioPac Acknowledge).

Experimental Protocols

All three protocols included an equilibration period of 40 min followed by a 60-min intracoronary infusion of labeled substrates. Stock solutions of 99% [U-13C3]lactate and/or 99% [U-13C3]pyruvate and unlabeled octanoate were infused directly into the LAD perfusion circuit from 0 to 60 min at a rate of 6.5 μl/ml of LAD blood flow. In group I (n = 7), the concentrations of [U-13C3]lactate and [U-13C3]pyruvate in the infusate were 154 and 15.4 mM, respectively, so that the lactate and [pyruvate] in LAD blood would be raised by 1.0 and 0.1 mM, respectively. In group II (n = 7), the concentration of [U-13C3]pyruvate in the infusate was 154 mM so that the [pyruvate] in LAD blood would be raised by 1 mM. Group III (n = 7) received [U-13C3]pyruvate as in group II and
unlabeled octanoate from -40 to 60 min at a concentration of 154 mM, to yield an octanoate concentration of 1 mM in the LAD blood.

In all three groups, recordings of left ventricular pressure, end-diastolic pressure, peak first derivative of the left ventricular pressure with time (dP/dt), heart rate, and arterial and venous blood samples were taken at -12, -5, 5, 15, 20, 25, 35, 45, and 55 min. Plasma samples were stored at -80°C until further analysis. At 60 min, a large punch biopsy (3 g) of the LAD bed was quickly taken, freeze-clamped, and stored at -80°C until analysis. The heart was excised and black ink was infused down the right and left main coronary arteries to identify the LAD perfusion bed, which was dissected and weighed (30.8 ± 1.5 g).

Analytical Methods

Arterial and venous pH, PCO₂, and PO₂ were determined on a blood-gas analyzer, and Hb concentration and saturation were measured on a hemoximeter (ABL3 and OSM3, Radiometer America, Cleveland, OH). The concentrations of plasma FFA, glucose, lactate, pyruvate, citrate, and β-hydroxybutyrate as well as tissue lactate, pyruvate, and malate were determined using spectrophotometric enzymatic assays (1, 24, 32). Tissue concentrations were measured immediately following homogenization in neutralized perchloric acid extracts to prevent loss of pyruvate from freeze/thaw (34).

Isotopic enrichments of plasma lactate and pyruvate were determined from the GC-MS analysis of the tert-butyldimethylsilyl (TBDMS) derivatives (8, 9). The MID of tissue lactate, pyruvate, citrate, succinate, fumarate, malate, and the OAA moiety of citrate were assayed as the TBDMS derivatives as described previously (8, 9, 14, 31). Analyses were performed on a Hewlett-Packard 5890 series II gas chromatograph with an HP-5 capillary column (50 m length, 0.2 mm inside diameter, 0.3 μm film thickness) coupled to a model 5970 mass selective detector. Helium gas flow in the capillary column was 0.8–1.0 ml/min. Individual enrichments are averages of two or three GC-MS injections. The tissue concentrations of citrate, succinate, and fumarate were also assayed by GC-MS in samples of 250 mg of tissue spiked with 0.25 μmol of tricarballylic acid and 0.21 μmol of [1,2,3,4-2H₄]succinate as internal standards (10, 14). The TBDMS derivative of tricarballylic acid was eluted at 21.1 min and was monitored at the mass-to-charge ratio (m/z) of 461. Standard curves were used to determine the tissue concentrations.

Calculations. Myocardial oxygen consumption (MV̇O₂) and uptakes of glucose and FFA were calculated as the product of the arteriovenous concentration difference and the myocardial blood flow. The myocardial blood flow was calculated as the LAD perfusion pump flow divided by the mass of the LAD perfusion bed.

Mass isotopomers of metabolites containing 0–n 13C atoms are identified as Mi with i = 0, 1, . . . , n. The molar fraction of a given Mi is

\[ M_i = \frac{A_i}{A_0 - \sum_{j=1}^{n} A_j} \]  

(1)

where \( A_0 \) represents the peak area of the unlabeled ion and \( A_i \) represents the peak area of the ion determined by computer integration and adjusted for the natural abundances obtained from myocardial tissue samples from pigs (n = 3) that were not infused with labeled substrates (3, 10). The MID data are expressed as molar percentage enrichment (MPE), i.e., 100 × M. Total MPE was calculated as

\[ \text{Total MPE} = \sum_{i=0}^{n} M_i \times 100 \]  

(2)

The enrichment in M1 and M2 acetyl-CoA moiety of citrate from the enrichment of M0, M2 citrate and M0, M1, and M2 OAA moiety of citrate (Ref. 8, Eqs. 18 and 19) were determined. The relative rates of pyruvate carboxylation and decarboxylation were calculated from the M3 enrichment of tissue pyruvate, and the M2 and M3 enrichments of the acetyl-CoA and OAA moieties of citrate, respectively (8). In brief, the relative rate of pyruvate decarboxylation (expressed as a fraction of the CAC flux) was calculated as the enrichment ratio (M2 acetyl-CoA moiety of citrate)/M3 pyruvate). The relative rate of pyruvate carboxylation was taken as the enrichment ratio (corrected M3 OAA moiety of citrate)/M3 pyruvate. The measured enrichment of the M3 OAA moiety of citrate was corrected for the fraction of M3 OAA molecules coming from some citrate isotopomers metabolized in the CAC and the dilution of 13C in the CAC, as described in detail by Comte and colleagues (see Ref. 8, Eqs. 8–10).

Fig. 2. Left ventricular peak systolic pressure (LVP), peak first derivative of the LV pressure with time (dP/dt), heart rate, and myocardial O₂ consumption (MV̇O₂).
The absolute rates of pyruvate carboxylation and decarboxylation were calculated from the relative rates of pyruvate carboxylation and decarboxylation and the absolute rate of CAC flux. The latter was calculated from MV\(_{O_2}\) and the stoichiometric relationships between oxygen consumption and citrate formation from fat and carbohydrate. For groups I and II, it was assumed that 1 \(\mu\)mol of consumed O\(_2\) resulted in the formation of 0.6, 0.348, and 0.353 \(\mu\)mol of citrate from glucose, palmitate, and oleate, respectively. It was further assumed that palmitate and oleate supply 40 and 60% of the fatty acids oxidized by the heart, respectively (35). The CAC flux was calculated as
\[
\text{CAC flux} = \frac{\text{MV}_{O_2}}{\text{PDC/CAC}} \times 0.60 + \left(1 - \frac{\text{PDC}}{\text{CAC}}\right) \times 0.351
\]
where PDC/CAC is the relative rate of pyruvate decarboxylation. The same equation was used for group III; however, it was assumed that octanoate was the only fatty acid oxidized, forming 0.364 \(\mu\)mol of citrate per micromol of oxygen consumed.

Statistical Analyses

Data are presented as means ± SE. The hemodynamic variables were compared among the three protocols using repeated measure two-way ANOVA. Statistical significance for the isotopic enrichments and the rates of pyruvate carboxylation and decarboxylation were determined using one-way ANOVA followed by a nonparametric post hoc test (Dunn’s test).

RESULTS

Cardiovascular Parameters

There were no significant changes over the course of the experiment or between groups at any time point in heart rate, peak systolic left ventricular pressure, peak left ventricular dP/dt, MV\(_{O_2}\) (Fig. 2), or in left ventricular end-diastolic pressure or myocardial blood flow (data not shown).

Plasma Metabolites

**Plasma [pyruvate] and [lactate] and enrichments.** Before the infusions of [U-\(^{13}\)C\(_3\)]lactate and/or pyruvate, the arterial plasma [lactate] values were 1.37 ± 0.08, 1.38 ± 0.05, and 1.40 ± 0.10 mM; and [pyruvate] values were 0.11 ± 0.02, 0.17 ± 0.04, and 0.15 ± 0.02 mM, for groups I, II, and III, respectively (Fig. 3). After 15 min of labeled substrate infusion, the plasma [lactate] and [pyruvate] in the blood entering the LAD territory stabilized at 2.16 ± 0.07 and 0.24 ± 0.02 mM in group I, 1.70 ± 0.07 and 1.13 ± 0.07 mM in group II, and 1.51 ± 0.10 and 1.10 ± 0.07 mM in group III, respectively (Fig. 3). The M3 enrichments of lactate and pyruvate stabilized after 15 min at 24.6 ± 4.0 and 44.5 ± 2.6% in group I, 2.3 ± 0.3 and 70.0 ± 2.9% in group II, and 2.3 ± 0.4 and 72.7 ± 3.2% in group III, respectively (Fig. 4).

In all groups, the M3 MPE of plasma pyruvate was lower in the interventricular vein than in the coronary artery (Fig. 4). This fall in MPE likely reflects the lactate/pyruvate equilibration in the myocardial tissue and also in the red blood cells (19). Total equilibration of the M3 MPE of lactate and pyruvate did not occur in any group. Enrichments in M1 and M2 were not detected in plasma lactate and pyruvate.

**FFA and glucose uptakes.** The LAD plasma FFA levels were stable and identical in groups I and II.

![Fig. 3. Concentration of lactate and pyruvate in plasma from the left anterior descending artery (LAD) and interventricular vein for groups I, II, and III.](http://ajpheart.physiology.org/)

![Fig. 4. Molar percentage enrichment (M3) of lactate and pyruvate in plasma from the LAD and interventricular vein.](http://ajpheart.physiology.org/)
(0.62 ± 0.04 and 0.57 ± 0.07 mM, respectively). In group III, the LAD arterial FFA concentration increased significantly from 5 to 60 min (1.76 ± 0.14 mM at 55 min) as a result of the infusion of octanoate (P < 0.05). The net FFA uptakes from 45 to 55 min were similar in groups I and II (0.10 ± 0.05 and 0.07 ± 0.08 μmol·g⁻¹·min⁻¹, respectively) and significantly higher in group III (0.51 ± 0.19 μmol·g⁻¹·min⁻¹) (P < 0.05). The rates of glucose uptake at the end of the study were similar in all groups (0.23 ± 0.06, 0.15 ± 0.04, and 0.12 ± 0.05 μmol·g⁻¹·min⁻¹, respectively).

Citrate and β-hydroxybutyrate balance. The LAD plasma citrate concentrations were unchanged over the time course of the study, and there was no difference among the groups (54 ± 2, 46 ± 3, and 46 ± 2 μM for groups I, II, and III, respectively, from 45 to 55 min). The net citrate releases were stable and similar in all groups (12 ± 7, 13 ± 4, and 9 ± 3 nmol·g⁻¹·min⁻¹ for groups I, II, and III, respectively). The LAD arterial β-hydroxybutyrate concentrations were unchanged over the time course of the study and there were no differences among groups (0.116 ± 0.009, 0.076 ± 0.005, and 0.082 ± 0.009 mM for groups I, II, and III, respectively, at 45 to 55 min). The net balance of β-hydroxybutyrate (arteriovenous difference × flow) was variable and similar among the groups (−1 ± 4, 10 ± 5, and 10 ± 5 nmol·g⁻¹·min⁻¹ for groups I, II, and III, respectively).

**Tissue Metabolites**

Tissue [lactate] and [pyruvate] and enrichments. The tissue [pyruvate] and [lactate] and the [lactate]-to-[pyruvate] ratio were similar in groups I and II despite the high rate of [U-13C3]pyruvate infusion without [U-13C₃]lactate in group II (Table 1). The octanoate-treated myocardium (group III) had more than double the [pyruvate] found in groups I and II without increase in tissue lactate. The lower [lactate]/[pyruvate] ratio in group III probably resulted from a decrease in pyruvate decarboxylation because octanoate provided an alternative source of acetyl-CoA.

The M3 enrichments of tissue lactate and pyruvate were similar in groups I and II (Table 1). Treatment with octanoate (group III) resulted in significantly higher M3 enrichments of lactate and pyruvate in the tissue compared with group I. There was no detectable M1 or M2 lactate and pyruvate present in the tissue in any of the groups.

CAC intermediate tissue concentrations and enrichments. The tissue concentrations of citrate, succinate, fumarate, and malate were similar in groups I and II (Table 2). The infusion of octanoate (group III) resulted in a significant increase in the tissue content of these intermediates.

The total MPE values of the CAC intermediates were similar in groups I and II (Table 3). Inhibition of pyruvate dehydrogenase by octanoate in group III resulted in a large decrease in the total enrichment of CAC intermediates because the entry of M2 acetyl-CoA into the CAC was inhibited (Table 3). The MIDs of the OAA moiety of citrate and malate were identical within each group, reflecting isotopic equilibration via malate dehydrogenase.

The measured enrichment of M3 OAA moiety of citrate was used to calculate the relative flux of pyruvate carboxylation. The enrichment of OAA moiety of citrate has two components: that which results from pyruvate carboxylation, and that which is from the recycling and dilution in the CAC. Only the first component is used in the calculation of pyruvate carboxylation. Therefore, the M3 enrichments of the OAA moiety of citrate were corrected for the proportion of M3 OAA molecules formed through the recycling of some citrate isomers in the CAC and the dilution of 13C in the CAC (8, 9). The calculated dilution factors (8) were 1.22 ± 0.03, 1.26 ± 0.06, and 1.31 ± 0.09 for groups I, II, and III, respectively. The magnitude of the correction of the M3 OAA moiety of citrate for M3 OAA molecules formed through recycling was high in groups I and II (60 and 56%; Table 3). Inhibition of pyruvate decarboxylation with 1 mM octanoate in group III resulted in a large decrease in labeling of CAC intermediates and in the correction for M3 OAA molecules formed through recycling in the CAC (4%; Table 3).

**Table 2. Tissue concentration of CAC intermediates in LAD beds**

<table>
<thead>
<tr>
<th>Group</th>
<th>Citrate</th>
<th>Succinate</th>
<th>Fumarate</th>
<th>Malate</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1.48 ± 0.20</td>
<td>0.10 ± 0.02</td>
<td>0.14 ± 0.01</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>II</td>
<td>1.54 ± 0.38</td>
<td>0.09 ± 0.01</td>
<td>0.12 ± 0.01</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>III</td>
<td>2.91 ± 0.39</td>
<td>0.24 ± 0.05*</td>
<td>0.23 ± 0.01*</td>
<td>0.25 ± 0.01*</td>
</tr>
</tbody>
</table>

Values are means ± SE. CAC, citric acid cycle. *P < 0.05 compared with groups I and II.
Despite more than a 10-fold difference in the degree of 13C labeling of tissue CAC intermediates isolated from LAD bed perfused with [U-13C3]lactate and/or [U-13C5]pyruvate with or without octanoate (Fig. 5). Note the similarity of these fluxes corrected M3 OAA moiety of citrate, and M3 tissue enrichments of the M2 acetyl-CoA moiety of citrate, and shows that it is independent of the rate of pyruvate decarboxylation in the heart in vivo. Using [U-13C5]pyruvate and [U-13C3]lactate, we measured the partitioning of pyruvate between decarboxylation and anaplerotic carboxylation in the swine heart. We found that pyruvate carboxylation accounts for ~3–6% of the CAC flux under normal metabolic and hemodynamic conditions when arterial [pyruvate] is between 0.2 and 1.1 mM. Furthermore, the rate of pyruvate carboxylation was not significantly modulated by inhibition of pyruvate decarboxylation in the presence of octanoate.

The role of pyruvate carboxylation in the maintenance of cardiac function is not well understood. The heart has a high rate of CAC flux (1.6 μmol·g⁻¹·min⁻¹). The rates of pyruvate carboxylation were similar in group I (71 ± 11 nmol·g⁻¹·min⁻¹), group II (68 ± 11 nmol·g⁻¹·min⁻¹), and group III (43 ± 7 nmol·g⁻¹·min⁻¹).

**DISCUSSION**

This is the first quantitative study on anaplerosis via pyruvate carboxylation in the heart in vivo. Using [U-13C5]pyruvate and [U-13C3]lactate, we measured the partitioning of pyruvate between decarboxylation and anaplerotic carboxylation in the swine heart. We found that pyruvate carboxylation accounts for ~3–6% of the CAC flux under normal metabolic and hemodynamic conditions when arterial [pyruvate] is between 0.2 and 1.1 mM. Furthermore, the rate of pyruvate carboxylation was not significantly modulated by inhibition of pyruvate decarboxylation in the presence of octanoate.

The role of pyruvate carboxylation in the maintenance of cardiac function is not well understood. The heart has a high rate of CAC flux (1.6 μmol·g⁻¹·min⁻¹) in the present study with low concentrations of intermediates (Table 2). This results in relatively rapid turnover times of metabolite pools (from 56 s for citrate to 4 s for succinate). Anaplerosis replenishes CAC intermediates lost from the cycle by cataplerosis, thus maintaining the concentrations of the intermediates and optimal CAC flux. In support of this concept, Russell and Taegtmeyer (21) found that inhibition of malic enzyme in perfused rat hearts re-

### Table 3. 13C labeling of tissue CAC intermediates isolated from LAD bed perfused with [U-13C3]lactate and/or [U-13C5]pyruvate with or without octanoate

<table>
<thead>
<tr>
<th>Group</th>
<th>M</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>Total MPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate</td>
<td>63.4 ± 2.1</td>
<td>16.1 ± 0.7</td>
<td>12.5 ± 0.8</td>
<td>6.6 ± 0.8</td>
<td>1.2 ± 0.3</td>
<td>36.6 ± 2.1</td>
</tr>
<tr>
<td>OAA moiety</td>
<td>73.9 ± 1.8</td>
<td>16.3 ± 1.0</td>
<td>7.8 ± 1.1</td>
<td>2.0 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>26.4 ± 1.9</td>
</tr>
<tr>
<td>Corrected OAA moiety</td>
<td>Acetyl-CoA moiety</td>
<td>7.1 ± 0.5</td>
<td>2.1 ± 0.1</td>
<td>1.0 ± 0.2</td>
<td>0.5 ± 0.1</td>
<td>27.8 ± 1.3</td>
</tr>
<tr>
<td>Succinate</td>
<td>69.5 ± 1.1</td>
<td>14.9 ± 0.7</td>
<td>10.7 ± 0.3</td>
<td>3.3 ± 0.4</td>
<td>0.6 ± 0.1</td>
<td>29.5 ± 1.2</td>
</tr>
<tr>
<td>Fumarate</td>
<td>72.2 ± 2.0</td>
<td>15.2 ± 0.6</td>
<td>9.5 ± 0.2</td>
<td>2.6 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>27.8 ± 1.3</td>
</tr>
<tr>
<td>Malate</td>
<td>73.1 ± 0.8</td>
<td>14.9 ± 0.4</td>
<td>8.8 ± 0.3</td>
<td>2.6 ± 0.2</td>
<td>0.5 ± 0.1</td>
<td>26.8 ± 1.2</td>
</tr>
<tr>
<td>Group II</td>
<td>Citrate</td>
<td>61.6 ± 1.2</td>
<td>18.2 ± 0.3</td>
<td>15.1 ± 0.8</td>
<td>5.8 ± 0.4</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>OAA moiety</td>
<td>70.0 ± 0.8</td>
<td>17.2 ± 0.2</td>
<td>10.2 ± 0.4</td>
<td>2.8 ± 0.2</td>
<td>0.5 ± 0.1</td>
<td>30.3 ± 0.7</td>
</tr>
<tr>
<td>Corrected OAA moiety</td>
<td>Acetyl-CoA moiety</td>
<td>7.2 ± 0.6</td>
<td>2.1 ± 0.1</td>
<td>1.0 ± 0.2</td>
<td>0.5 ± 0.1</td>
<td>27.8 ± 1.3</td>
</tr>
<tr>
<td>Succinate</td>
<td>67.7 ± 1.0</td>
<td>16.7 ± 0.4</td>
<td>11.1 ± 0.4</td>
<td>3.6 ± 0.4</td>
<td>0.7 ± 0.2</td>
<td>32.0 ± 0.9</td>
</tr>
<tr>
<td>Fumarate</td>
<td>68.0 ± 0.9</td>
<td>16.5 ± 0.4</td>
<td>10.8 ± 0.6</td>
<td>3.9 ± 0.5</td>
<td>0.6 ± 0.1</td>
<td>32.0 ± 1.9</td>
</tr>
<tr>
<td>Malate</td>
<td>69.0 ± 0.7</td>
<td>15.7 ± 0.4</td>
<td>10.5 ± 0.9</td>
<td>3.9 ± 0.5</td>
<td>0.7 ± 0.1</td>
<td>30.9 ± 2.3</td>
</tr>
<tr>
<td>Group III</td>
<td>Citrate</td>
<td>91.2 ± 0.2</td>
<td>4.9 ± 0.1</td>
<td>3.1 ± 0.2</td>
<td>0.7 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>OAA moiety</td>
<td>93.2 ± 0.5</td>
<td>4.1 ± 0.3</td>
<td>1.8 ± 0.2</td>
<td>0.9 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>6.9 ± 0.5</td>
</tr>
<tr>
<td>Corrected OAA moiety</td>
<td>Acetyl-CoA moiety</td>
<td>0.8 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>6.9 ± 0.6</td>
</tr>
<tr>
<td>Succinate</td>
<td>93.1 ± 0.4</td>
<td>4.1 ± 0.3</td>
<td>2.4 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>6.9 ± 0.6</td>
</tr>
<tr>
<td>Fumarate</td>
<td>93.5 ± 0.7</td>
<td>3.8 ± 0.3</td>
<td>1.4 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>6.5 ± 0.5</td>
</tr>
<tr>
<td>Malate</td>
<td>93.0 ± 0.8</td>
<td>2.6 ± 0.3</td>
<td>2.5 ± 0.3</td>
<td>1.5 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>7.0 ± 1.0</td>
</tr>
</tbody>
</table>

Values are means ± SE. Group I: 1 mM lactate + 0.1 mM pyruvate; group II: 1 mM pyruvate; group III: 1 mM pyruvate + 1 mM octanoate. M₃ acetyl-CoA moiety of citrate was computed from MIDs of citrate and of oxaloacetate (OAA) moiety of citrate. M₄ OAA moiety of citrate was corrected for formation of M₃ OAA from CAC metabolism of citrate isotopomers and dilution of unlabeled carbons in CAC (see MATERIALS AND METHODS). Total molar percentage enrichment (MPE) was calculated as per Eq. 2.

### Flux Parameters

*Relative rates of pyruvate carboxylation and decarboxylation.* The relative rates of pyruvate carboxylation and decarboxylation were calculated from the enrichments of the M2 acetyl-CoA moiety of citrate, corrected M3 OAA moiety of citrate, and M3 tissue pyruvate (see MATERIALS AND METHODS). The relative rates of pyruvate decarboxylation were similar in groups I and II (41.5 ± 2.0 and 34.3 ± 1.3% of citrate formation, respectively) but were reduced by 93% by octanoate infusion in group III (3.1 ± 0.4%; Fig. 5). The relative rates of pyruvate carboxylation were similar in all groups (4.7 ± 0.3, 5.7 ± 0.3, and 3.4 ± 0.3%, respectively; Fig. 5). Note the similarity of these fluxes despite more than a 10-fold difference in the degree of 13C recycling in the CAC, as reflected in the difference between the uncorrected and corrected M3 OAA moiety of citrate (~60% vs. 4%; Table 3). This demonstrates the robustness of our method for calculating pyruvate carboxylation and shows that it is independent of the extent of correction for 13C recycling.

*Absolute substrate fluxes.* Absolute rates of pyruvate decarboxylation and carboxylation were calculated from the rate of CAC flux, which was calculated from the MVO₂ as described in MATERIALS AND METHODS. The rates of CAC flux were not different among the three groups (1.6 ± 0.1, 1.2 ± 0.1, and 1.3 ± 0.2 μmol·g⁻¹·min⁻¹ for groups I, II, and III, respectively). The rate of pyruvate decarboxylation was similar in group I (658 ± 103 nmol·g⁻¹·min⁻¹) and group II (418 ± 80 nmol·g⁻¹·min⁻¹) but was markedly inhib-
results in 1) 75% and 93% decreases in the incorporation of [1-14C]pyruvate into citrate and malate, respectively; 2) depletion of CAC intermediates; and 3) reduction in the contractile power of the heart. The present investigation further supports the concept that pyruvate carboxylation is a constitutive process in the heart, and is necessary for maintenance of normal CAC flux.

Infusion of 1 mM octanoate markedly decreased the contribution of pyruvate to acetyl-CoA production from 34 to 3%, but did not affect the relative (Fig. 5) or absolute rates of pyruvate carboxylation. Despite the dramatic reduction in pyruvate decarboxylation, there were no changes in the net uptakes of pyruvate and lactate (Fig. 3). Although there was a doubling of tissue [pyruvate] (Table 1) that accounted for a small portion of the lactate and pyruvate uptake in this group, it is unclear how the remaining pyruvate was metabolized by the heart. It should also be noted that octanoate increased the tissue content of CAC intermediates (Table 2) without changing the rates of CAC flux or anaplerotic pyruvate carboxylation. There is an increase in the concentration of CAC intermediates when there is an excess availability of acetyl-CoA (7, 28, 33). Our results add further support to the concept that the size of the pool of CAC intermediates is not solely related to the rate of CAC flux, but also to the availability of substrates (28).

Anaplerotic fluxes balance equivalent fluxes out of the CAC through cataplerosis. Because the rate of anaplerosis by pyruvate carboxylation is 3 to 6% of the CAC flux, the minimum rate of cataplerosis must also be ~3–6% of the CAC flux. The exact rate of in vivo cardiac cataplerosis is difficult to derive because the efflux of enriched CAC intermediates is not readily followed. Some potential sites of cataplerosis may be decarboxylation of malate (via malic enzyme) and citrate efflux. In the liver, malic enzyme can operate as either a carboxylase or a decarboxylase; a similar reversibility has been suggested for the heart in 13C NMR studies (10). On the other hand, Sundqvist and colleagues (26, 27) found that the energetics of the malic enzyme reaction in the normal rat heart would not allow for the enzyme to function as a decarboxylase. In the present investigation, cataplerotic decarboxylation of malate to pyruvate would result in substantial M1 and M2 labeling of pyruvate. We observed no M1 or M2 enrichment of pyruvate, demonstrating no detectable loss of CAC carbon from cataplerosis via malic enzyme. A similar finding was reported for the heart perfused ex vivo (9, 31).

Regarding citrate cataplerosis, there was net citrate release by the heart (12, 13, and 9 nmol·g⁻¹·min⁻¹ for groups I, II, and III, respectively). These values are comparable to those obtained in isolated rat hearts (17–22 nmol·g⁻¹·min⁻¹, assuming a heart wet weight of 1 g (31)) and catheterized human hearts (~5 nmol·g⁻¹·min⁻¹, assuming a myocardial blood flow of 1.0 ml·g⁻¹·min⁻¹ (30)). In the present investigation the net citrate efflux amounted to 17, 19, and 21% of the rate of pyruvate carboxylation for the respective groups. This suggests that there are other major cataplerotic processes (e.g., α-ketoglutarate to glutamate). Alternatively, the net release of citrate might underestimate citrate cataplerosis if some citrate is cleaved by cytosolic ATP-citrate lyase, as proposed for skeletal muscle (22). Therefore, the lack of correspondence between citrate release and absolute pyruvate carboxylation cannot be taken as evidence for the contribution of other anaplerotic pathway(s).

We did not observe significant increases in tissue [pyruvate] or MPE, or a decrease in the [lactate] to [pyruvate] ratio when arterial pyruvate was increased fivefold from 0.24 to 1.1 mM (Fig. 3, Table 1). This unexpected finding suggests that pyruvate is either not readily taken up by the myocardium, or it is taken up and rapidly converted to lactate by lactate dehydrogenase. Consequently, there was no difference in the rates of pyruvate decarboxylation or carboxylation between groups I and II. Additional investigations are required to assess whether higher arterial [pyruvate] are needed to increase contribution of pyruvate to citrate formation in vivo. Bunger and Mallet and co-workers (4, 29) demonstrated that adding 1–5 mM pyruvate to the perfusate of isolated guinea pig heart improved contractile function after ischemia. Furthermore, the addition of 5 mM pyruvate resulted in a...
15-fold increase in tissue citrate concentration after ischemia (29), suggesting anaplerosis from pyruvate. It is possible that the heart more readily takes up pyruvate during times of stress, such as increased contractile work, myocardial ischemia, and postischemic reperfusion.

The labeling patterns of CAC intermediates could not shed definite light on whether pyruvate carboxylation in the heart is catalyzed by pyruvate carboxylase or malic enzyme. It is not known which of these two pathways predominates in the heart. The activities of malic enzyme and pyruvate carboxylase have been measured in rat hearts (0.695 and 0.116 μmol·g⁻¹·min⁻¹, respectively) (27). The respective roles of malic enzyme and pyruvate carboxylase have been examined by modulating these activities by pharmacological or nutritional perturbations. In isolated rat hearts, inhibition of malic enzyme with hydroxymalonate results in a decrease in the content of CAC intermediates and a decrease in the incorporation of [1-¹³C]pyruvate into malate (21). On the other hand, biotin deficiency, which results in a 90% reduction in pyruvate carboxylase activity, causes no change in the incorporation of [1-¹⁴C]pyruvate into malate in isolated rat hearts (27). These findings suggest that malic enzyme, and not pyruvate carboxylase, predominates in the isolated rat heart. Individual fluxes through malic enzyme and pyruvate carboxylase cannot be separately measured with current isotopic techniques; thus a thorough understanding of the enzymatic regulation of pyruvate carboxylation awaits new methodological developments.

The fluxes of pyruvate carboxylation measured in our in vivo study are similar to rates measured by Comte and colleagues (8, 9) in hearts perfused with 11 mM glucose, 0.5–1.0 mM lactate, 0.05–0.2 mM pyruvate, and 0.02–0.2 mM octanoate (2.5–8% of citrate synthase flux). More recently, Vincent and coworkers (31) reported a rate of pyruvate carboxylation corresponding to 2.4% of citrate synthase flux in hearts perfused with more physiological substrates (5 mM glucose, 1 mM lactate, 0.2 mM pyruvate, 0.4 mM oleate, and 8 nM insulin). This value is similar to the relative fluxes measured in groups I, II, and III (4.7, 5.7, and 3.4%, respectively).

Methodological limitations encountered in assessing anaplerotic pyruvate carboxylation in vivo were also similar to those reported for perfused rat hearts (8, 9). The first constraint on the use of [U-¹³C₃]lactate and [U-¹⁳C₃]pyruvate to measure the rates of pyruvate carboxylation and decarboxylation of in vivo hearts results from the need to impose a substantial enrichment of substrate in the blood as it enters the organ. The lower limit on the amount of [U-¹³C₃]lactate and [U-¹⁳C₃]pyruvate that needed to be infused was imposed by the precision in the measurement of the enrichment of the OAA moiety of citrate. To achieve the required M3 enrichments of influent lactate and pyruvate, the baseline plasma [pyruvate] was increased from 0.11 ± 0.02 to 0.24 ± 0.02 mM, and the [lactate] was increased from 1.37 ± 0.08 to 2.16 ± 0.07 mM in group I. These high but physiological concentrations of pyruvate and lactate might result in higher rates of pyruvate carboxylation and decarboxylation than would occur in the unperturbed swine heart. Another potential limitation of our method is the use of whole-tissue pyruvate M3 enrichment, rather than intracellular enrichment, in the calculation of the rates of pyruvate carboxylation and decarboxylation. Use of whole tissue pyruvate M3 enrichment would result in a slight underestimation of the actual rates of pyruvate carboxylation and decarboxylation.

In conclusion, under our experimental conditions we found that pyruvate carboxylation in the in vivo heart accounts for at least 3–6% of the CAC flux even when pyruvate dehydrogenase is inhibited by octanoate. Cataplerotic citrate efflux represents at least 17–21% of the rate of pyruvate carboxylation. Our data suggests that pyruvate carboxylation is a constitutive process in the heart that appears to be necessary for maintenance of normal CAC flux.

The authors thank F. David, M. Chandler, and C. Paxson for assistance in conducting this study.

This work was supported by National Institutes of Health Grants HL-58633 (to W. C. Stanley) and HL-59219 (to H. Brunengraber), and Grant-in-Aid 005031N from the American Heart Association National Center (to W. C. Stanley).

REFERENCES


11. Jeffrey FMH, Storey CJ, Sherry AD, and Malloy CR. ¹³C isotopomer model for estimation of anaplerosis substrate oxida-


