Anaplerotic pyruvate carboxylation was examined in hearts perfused with physiological concentrations of glucose, [U-13C]lactate, and [U-13C]pyruvate. Also, a fatty acid, [1-13C]octanoate, or ketone bodies were added at concentrations providing acetyl-CoA at a rate resulting in either low or substantial pyruvate decarboxylation. Relative contributions of pyruvate and fatty acids to citrate synthesis were determined from the 13C labeling pattern of effluent citrate by gas chromatography-mass spectrometry (see companion article, Comte, B., Vincent, G., Bouchard, B., and Des Rosiers, C. (1997) J. Biol. Chem. 272, 26117–26124). Precision on flux measurements of anaplerotic pyruvate carboxylation depended on the mix of substrates supplied to the heart. Anaplerotic fluxes were precisely determined under conditions where acetyl-CoA was predominantly supplied by β-oxidation, as it occurred with 0.2 or 1 mM octanoate. Then, anaplerotic pyruvate carboxylation provided 3–8% of the OAA moiety of citrate and was modulated by concentrations of lactate and pyruvate in the physiological range. Also, the contribution of pyruvate to citrate formation through carboxylation was equal to or greater than through decarboxylation. Furthermore, 13C labeling data on tissue citric acid cycle intermediates and pyruvate suggest that (i) anaplerosis occurs also at succinate and (ii) cataplerotic malate decarboxylation is low. Rather, the presence of citrate in the effluent perfusate of hearts perfused with physiological concentrations of glucose, lactate, and pyruvate and concentrations of octanoate leading to maximal oxidative rates suggests a cataplerotic citrate efflux from mitochondria to cytosol. Taken altogether, our data raise the possibility of a link between pyruvate carboxylation and mitochondrial citrate efflux. In view of the proposed feedback regulation of glycolysis by cytosolic citrate, such a link would support a role of anaplerosis and cataplerosis in metabolic signal transmission between mitochondria and cytosol in the normoxic heart.

In the heart, the existence of anaplerotic and cataplerotic processes is illustrated by the rapid changes in combined pool size of citric acid cycle (CAC) intermediates occurring under various conditions, such as altered fuel substrates or work load and ischemia (1–6). Anaplerotic substrates include pyruvate (supplied as such or derived from glucose (7)), propionate (8), aspartate, glutamate, and the branched chain amino acids (9). Evidence supports the importance of anaplerosis, especially from glutamate and aspartate, for heart function in ischemia-reperfusion (4, 8, 10–11). Less is known about the role and site(s) of anaplerosis and cataplerosis in the heart under normoxia. Regulation of pool sizes by means of anaplerotic and cataplerotic reactions is likely to be involved not only in feedback control of the turnover of the cycle itself (1, 8) but also in the transmission of signals between mitochondria and the cytosol. The latter is illustrated by the possibility of feedback inhibition of phosphofructokinase by cytosolic citrate accumulating during fatty acid oxidation (12–13). However, in the heart, the significance of this regulatory mechanism is questioned because of the low activity of the tricarboxylate transporter (14–15). Anaplerotic pyruvate carboxylation prevented the contractile dysfunction of hearts perfused with ketone bodies (KB; 16–17). In vivo, the normoxic heart could rely on blood lactate and pyruvate for anaplerosis. In the isolated heart, the constant need for anaplerotic substrates is illustrated by the decrease in tissue levels of glutamate, or aspartate, upon perfusion with glucose alone, or glucose and long chain fatty acid, respectively (4, 8). Anaplerosis from pyruvate can occur through either pyruvate carboxylase or NADP-malic enzyme. The relative contribution of these reactions is debated (12, 18–19). Since pyruvate carboxylase and malic enzyme are differently regulated (20–22), they may operate under different circumstances. Aside from oxaloacetate (OAA), pyruvate also supplies acetyl-CoA to the CAC. Both pyruvate carboxylation and decarboxylation were linked to some beneficial effects on heart function (16, 23–27), although the partitioning of pyruvate through these two reactions remains to be clarified. Using 14C- and 13C-labeled substrates, several authors documented the reciprocal regulation of pyruvate decarboxylation and fatty acid oxidation (see for example Refs. 25, and 28–30). However, in these studies, the relative contributions of pyruvate decarboxylation and carboxylation to citrate formation could not be assessed directly because of methodological limitations.

Here we report data on simultaneous measurements of pyruvate carboxylation and decarboxylation, and of fatty acid oxidation, using a strategy described in the companion article (Comte et al. (67)). This was achieved using 13C-substrate(s) and analyzing the 13C labeling pattern of effluent citrate by gas chromatography-mass spectrometry (GCMS). Our 13C data

This paper is available on line at http://www.jbc.org
show how the contributions of anaplerotic OAA and acetyl-CoA from pyruvate for citrate formation are modulated by the nature and the concentration of substrates feeding the CAC. They also suggest another site of anaplerosis as well as probable sites for cataplerosis. Part of this work was presented in abstract form (31–33).

**EXPERIMENTAL PROCEDURES**

**Chemicals—**Chemicals, enzymes, and coenzymes were purchased from Boehringer Mannheim (Laval, Quebec), Fisher (Montreal, Quebec, Sigma, and Anachemia (Derval, Quebec). [U,13C3]Lactate (99%), [U,13C6]pyruvate (99%), and [1,13C]octanoate (99%) were obtained from Isotec (Miamisburg, OH) and Cambridge Isotopes Laboratories (Woburn, MA). The derivatization agent N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide was supplied by Carolina Chemical (Morton Grove, IL). Acetoacetate (AcAc) was prepared as described previously (34). All solutions were made with water purified by a Milli-Q system (Millipore, St. Laurent, Quebec).

**Heart Perfusion—**Procedures for isolation and perfusion of rat hearts were as described in previous publications (35, and Comte et al. (36)). Briefly, hearts from male Sprague-Dawley rats (Charles River, Quebec) weighing 120–220 g (heart wet weight, 1.1–1.3 g) were perfused according to the Langendorff technique at a constant pressure of 80 mmHg with a non-circulating modified Krebs-Ringer bicarbonate buffer (in mM: 119 NaCl, 4.8 KCl, 1.3 CaCl2, 1.2 KH2PO4, 1.2 MgSO4, 25 NaHCO3, 11 glucose, 0.5 or 1 lactate, or 0.05 or 0.2 pyruvate, 0.02, 0.2, or 1 octanoate, 0.15 AcAc, 0.15 β-hydroxybutyrate (BHB) ± 0.001 norepinephrine (NE) or 1 dichloroacetate (DCA)). The perfusion buffer was exchanged with 13C3]pyruvate, and [1-13C]octanoate to citrate formation from the 13C labeling pattern of citrate released from hearts perfused with [U,13C3]lactate, [U,13C6]pyruvate, and [1,13C]octanoate were presented in the accompanying report (67). Note that in the present report, we will refer to these equations using the same numbering.

**Statistical Analysis—**Individual enrichments are averages of three to five GCMS injections. Data are expressed as means ± S.E. when n > 2 or S.D. when n = 2. Two-tailed Student's t test was used to determine the probability of a significant difference between means. Calculations were made by a personal computer (37) developed for feedback control of a syringe pump (Harvard Instruments, Southnatick, MA). Samples of effluent perfusate (20 ml) were collected every 5 min starting 10 min before the labeling period and processed as follows: (i) 7 ml was immediately made with 10 mM hydroxyamine-hydrochloride and sonicated for 1 min to convert α-ketoglutarate (αKG) to its oxime derivative (36), (ii) 10 ml was made 1% sulfosalicylic acid, and (iii) 1 ml was left untreated. Samples were stored at −20 °C until further analyses. At the end of the experiment, hearts were freeze-clamped and stored in liquid nitrogen.

**GCMS Assays—**Procedures for determination of the 13C-mass isotopomer distribution (MID) of tissue and effluent metabolites (citrate, OAA, and acetyl moieties of citrate, αKG, succinate, malate, fumarate, and pyruvate) are described in the accompanying report (67). MID data are expressed as mol fraction (MF) or molar percent enrichment (MPE). The MF in a given mass isotopomer (M1 through M6) is

\[
\text{MF}(M_i) = \frac{A_i}{A_1 + A_2 + \ldots + A_6}
\]

where A represents the peak area of each fragmentogram, determined by computer integration and corrected for naturally occurring heavy isotopes (38–40), and i ranges from 1 to 6, n being the number of carbon atoms. MPE is equivalent to MF

\[
\text{MPE}(M_i) = \frac{A_i}{A_1 + A_2 + \ldots + A_6} 	imes 100
\]

**RESULTS AND DISCUSSION**

Anaplerotic pyruvate carboxylation was examined in hearts perfused with physiological concentrations of glucose (11 mM), lactate (0.5–1 mM), and pyruvate (0.05–0.2 mM). Lactate and pyruvate were present in a fixed ratio to clamp the redox state. Also, a source of acetyl-CoA other than pyruvate, either the medium chain fatty acid, octanoate, or the KB, AcAc, and BHB, was provided. Medium chain fatty acids is not subjected to carnitine palmitoyltransferase I regulation (41–43). Thus, the perfusate octanoate concentration sets the rate of mitochondrial acetyl-CoA generation. In a first series of heart experiments, octanoate was supplied at 0.2 mM or greater, leading to maximal rate of β-oxidation (41–42) and thus of acetyl-CoA formation. This should favor (i) pyruvate carboxylation over its decarboxylation and (ii) citrate synthesis from OAA formed by anaplerotic pyruvate carboxylation. Under these conditions, we attempted to increase substrate flux through pyruvate decarboxylation by adding known activators of pyruvate dehydrogenase, either 1 mM DCA or 1 μM NE. However, results obtained prompted us to favor pyruvate decarboxylation by decreasing the supply of acetyl-CoA from sources other than pyruvate. Thus, in a second series of experiments, hearts were supplied with either 0.02 mM octanoate, leading to submaximal rate of oxidation, or with 0.15 mM AcAc and 0.15 mM BHB. For simplicity, data obtained in these two series of experiments are presented sequentially.

**Conditions Favoring Pyruvate Carboxylation Over Decarboxylation**

Metabolic and Functional Status of Perfused Hearts—Hearts perfused with physiological concentrations of glucose, lactate, and pyruvate and concentrations of octanoate leading to maximal oxidative rates (i.e., >0.2 mM, Ref. 44) maintained spontaneous beating at 305 ± 14 beats/min, a coronary flow rate of 10.0 ± 0.5 ml/min, and a rate pressure product of (23.1 ± 1.9) × 105 mmHg × beats/min (n = 14). The latter parameter was increased significantly by the addition of NE (33.6 ± 2.8) × 105, n = 3). In a previous study, similarly perfused rat hearts consumed oxygen at a rate varying between 3.7 and 5 μmol/min (37) and released minimal quantity of lactate dehydrogenase or glutathione (45). Rates of pyruvate, lactate, or glucose uptake in a single pass could not be calculated from the influent and effluent concentrations since the differences were too small to be measured with precision. Rates of octanoate uptake were similar under all conditions tested, on average 0.57 ± 0.05 μmol/min (n = 17, ranging from 0.24 to 0.88). These rates are in agreement with literature values (44). Finally, KB (AcAc + BHB) were released into the effluent perfusate at rate of 258 ± 13 nmol/min (n = 9, Ref. 32). Although the heart is normally a net consumer of KB, pseudoketogenesis can occur via the reverse of the succinyl-CoA transferase reaction (46). We interpret this KB release as reflecting acetyl-CoA spillover from the CAC.

**13C Labeling of CAC Metabolites—**Table I and Fig. 1 show the 13C labeling of tissue pyruvate and of various CAC metabolites when hearts were perfused for 40 min with physiological concentrations of [U,13C3]lactate, [U,13C6]pyruvate, and [1,13C]octanoate under various conditions. The MPE of influent pyruvate was 96.6 ± 0.4% in M3, 3.0 ± 0.2% in M2, and 0.03 ± 0.02% (n = 3) in M1 isotomers. The effluent pyruvate was slightly diluted (MPE M3 86 ± 1%). Tissue pyruvate showed even more dilution of M3 isotomers (from 61 to 85%; Table I). When corrected for the presence of M2 and M1 isotomers in the infused pyruvate, tissue pyruvate en-
Anaplerosis from Pyruvate in Heart

Other data from heart perfusion experiments described in Figs. 1 and 2. Hearts were freeze-clamped and processed for the analysis of 13C labeling of tissue pyruvate by GCMS. Data, corrected for natural abundance in heavy isotopes and light isotopic impurities of the tracer, are expressed as MPE and are means ± S.E. (n > 2) or S.D. (n = 2).

<table>
<thead>
<tr>
<th>Perfusion conditions (n)</th>
<th>MPE tissue pyruvate</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>M1 (13C enrichment)</td>
</tr>
<tr>
<td>1 mM [U-13C3]lactate + 0.2 mM [U-13C3]pyruvate</td>
<td>0.23 ± 0.11</td>
</tr>
<tr>
<td>+ 0.2 or 0.02 mM [1-13C]octanoate (3)</td>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td>+ 0.2 mM [1-13C]octanoate + 1 mM DCA (5)</td>
<td>0.20 ± 0.07</td>
</tr>
<tr>
<td>0.5 mM [U-13C3]lactate + 0.05 mM [U-13C3]pyruvate</td>
<td>0.10 ± 0.05</td>
</tr>
<tr>
<td>+ 0.2 mM [1-13C]octanoate (4)</td>
<td>1.04 ± 0.50</td>
</tr>
<tr>
<td>+ 1 mM [1-13C]octanoate (2)</td>
<td>0.17 ± 0.05a</td>
</tr>
<tr>
<td>+ 0.15 mM (AcAc + BHB) (4)</td>
<td>0.17 ± 0.05a</td>
</tr>
</tbody>
</table>

a p < 0.05: means tested against the null hypothesis.
b p < 0.05, all conditions versus 1 mM lactate + 0.2 mM pyruvate + 0.2 or 0.02 mM octanoate, using a one-way analysis of variance followed by a Bonferroni multiple comparison test.

richment in M2 or M1 isotopomers was negligible (not significant, means tested against null hypothesis) or very low (MPE M1 = 0.2% with DCA).

Of all tissue CAC metabolites, citrate showed the highest total 13C enrichment (p < 0.001), predominantly M1, M2, and M3 isotopomers (Fig. 1). The enrichments in M4, M5, and M6 citrate were less than 1.5, 0.2, and 0.1%, respectively, under all conditions tested. The total MPE decreased from citrate to αKG and succinate: a greater difference was observed between αKG and succinate than between citrate and αKG (1.5 versus 1.1-fold, respectively). These differences in total MPEs reflect in part the conversion of labeled to unlabeled isotopomers in the CAC due to the loss of 13CO2. When this process is taken into account, the 13C dilution attributed to entry of unlabeled αKG and succinate was estimated (from Eq. 10, Comte et al. (67)) to be 1.13 ± 0.04 (p < 0.05, n = 8) and was similarly increased by NE and DCA (1.29 ± 0.03, n = 9; p < 0.05 unpaired t test).

In all cases, the enrichments in M3, M2, and M1 isotopomers of tissue citrate were not significantly different from that of effluent citrate at 25–40 min of perfusion (not shown; paired t test), a time when isotopic steady state was attained (see Comte et al. (67)). A similar 13C enrichment was also observed for tissue and effluent αKG (not shown). Furthermore, the total 13C enrichment and the 13C MID of the OAA moiety of effluent citrate were similar to that of tissue malate or fumarate (Fig. 1). These data indicate rapid isotope randomization through the reversible fumarase reaction. Note that tissue fumarate and malate, and the OAA moiety of effluent citrate, are more enriched in M3 isotopomers (Fig. 1D) than tissue succinate. This can only be explained by an entry of M3 isotopomers at the level of malate or OAA by anaplerotic carboxylation of [U-13C3]pyruvate.

Relative Contributions of Pyruvate to Carboxylation and Decarboxylation—The 13C enrichment of tissue pyruvate and of the acetyl and OAA moieties of citrate, isolated from the effluent of hearts perfused for 25–40 min with [U-13C3]lactate, [U-13C3]pyruvate, and [1-13C]octanoate, allows calculation of relative substrate fluxes relevant to pyruvate and fatty acid metabolism. Under these conditions, all assumptions on which equations were developed were validated (see Comte et al. (67)).

From the M3 enrichment of tissue pyruvate, we calculate that more than 60% of the pyruvate converted to OAA arose from exogenously supplied pyruvate and/or lactate (FCPYR→OAA; Eq. 3 of Ref. 67; where FC is fractional contribution; PYRi and PYRe indicate intracellular and extracellular pyruvate). The percent contribution of glucose and/or glycogen to pyruvate generation (1 − FCPYR→PYRi) was 33.8 ± 1.6% in all conditions, except with 1 mM DCA where it was decreased significantly to 14.4 ± 2.5% (p < 0.001, unpaired t test). These changes support an inhibitory (47) rather than a stimulatory (25, 48–51) effect of DCA on glycolysis under our perfusion conditions.

From the M1 and M2 enrichment of the acetyl moiety of effluent citrate, we calculate that in all cases, close to 90% of the acetyl moiety of citrate was supplied by octanoate oxidation (octanoate oxidation/citrate synthesis (CS) = 0.89 ± 0.03%, n = 17, Eq. 6 of Ref. 67, not shown). The contribution of pyruvate decarboxylation to acetyl-CoA formation was less than 5% (PDC/CS, Table IIA) and was not significantly modified by 1 mM DCA or 1 mM NE or by increasing the octanoate concentration to 1 mM. Most likely, conversion of pyruvate dehydrogenase to the active form in hearts perfused with 0.2 mM octanoate requires a higher concentration of DCA (i.e. 5 mM) and/or of pyruvate (i.e. 10 mM; Ref. 52).

To calculate the contribution of anaplerotic pyruvate carboxylation to citrate synthesis, the measured M3 enrichment of the OAA moiety of citrate was corrected for the formation of M3 OAA isotopomers through metabolism in the CAC of citrate precursor molecules. The magnitude of this correction was between 16 and 25% of the measured enrichment values (Table IIA). Then, the contribution of anaplerotic pyruvate carboxylation to citrate synthesis varied between 3.4 and 7.4% (PCCS; Table IIA). Among the various interventions studied, only a change in the concentration and/or ratio of lactate and pyruvate significantly modified the PC/octanoate oxidation (PCCS/CS; Table IIA) ratios observed with NE (p < 0.05) and 1 mM octanoate (p < 0.05) were in agreement with the known effects of these interventions on the pyruvate dehydrogenase complex (i.e. activation and inhibition, respectively).

Rates of octanoate uptake, expressed in acetyl-CoA units (Equation 1, below), were used to convert the relative flux ratios (PC/octanoate oxidation; Eq. 4/Eq. 6 of Ref. 67) into absolute fluxes. Rates were corrected for KB release. Indeed, GCMS assay of effluent KB M1 enrichments, which resemble those of the acetyl moiety of citrate, indicated that KB were formed from [1-13C]octanoate oxidation (32).

Rates of acetyl-CoA formation from octanoate oxidation (μmol/min) = (rate of octanoate uptake (μmol/min) × 4) − (2 × rate of KB release (μmol/min)) (Eq. 1)
Alternatively, conversion of relative fluxes to absolute fluxes could be achieved using measured rates of oxygen consumption.

**Conditions Favoring Pyruvate Decarboxylation Over Carboxylation**

In a second series of experiments, pyruvate carboxylation was examined under conditions where substrate flux through pyruvate decarboxylation was increased by perfusing hearts with 0.02 mM octanoate or 0.3 mM KB (AcAc and BHB).

Table I and Fig. 2 show the $^{13}$C labeling of tissue pyruvate and various CAC metabolites when hearts were perfused for 40 min with physiological concentrations of [U-13C]octanoate, [U-13C]pyruvate, and either 0.02 mM [1-13C]octanoate or 0.3 mM KB. Similar to experiments with 0.2 mM octanoate described above (Fig. 1), most of the $^{13}$C dilution occurred between αKG and succinate. The $^{13}$C dilution estimated from $^{13}$C enrichments in citrate and succinate using Equation 10 of Ref. 67 was 1.34 ± 0.02 (n = 2) and 1.38 ± 0.06 (n = 4), respectively. Also, the $^{13}$C MID of tissue citrate was similar to that of effluent citrate after a 25–40-min perfusion period (not shown, using a paired t test). However, precise analysis of the $^{13}$C MID of citrate released by hearts perfused with KB required twice the volume of perfusate (14 instead of 7 ml), suggesting a lower citrate release rate. In addition, the following differences were noted between the $^{13}$C data obtained in the two series of perfusions. (i) Although tissue pyruvate was predominantly enriched in M3 isopomers, a low but significant enrichment in M1 and M2 isopomers was detected in hearts perfused with KB (Table I). (ii) Tissue citrate was enriched in M4, M5, and M6 isopomers (11.4 ± 0.7, 7.5 ± 2.7, and 5.0 ± 4.2%, with 0.02 mM octanoate (n = 2), and 3.8 ± 0.5, 1.5 ± 0.3, 0.2 ± 0.1 with 0.3 mM KB (n = 4)). This indicates that a significant proportion of citrate molecules are labeled in both their acetyl and OAA moieties (45 ± 2, n = 2, compared with 25 ± 1, n = 21, for hearts perfused with 0.2 mM octanoate, p < 0.001). Finally, (iii) the M3 enrichments of the OAA moieties of citrate and of tissue malate and fumarate were not significantly different from that of tissue succinate. This indicates substantial formation of M3 OAA through the metabolism of citrate isopomers in the CAC.

**Relative Contributions of Pyruvate to Carboxylation and Decarboxylation**—As shown in Table II, perfusion of hearts with physiological concentrations of glucose, [U-13C]octanoate, [U-13C]pyruvate, and 0.02 mM [1-13C]octanoate or unlabeled 0.3 mM KB resulted in an increased flux through pyruvate decarboxylation. Under both conditions, as much as 36% of the acetyl moiety of citrate was supplied by pyruvate decarboxylation (PDC/CS, Table II). Also, pyruvate decarboxylation predominated over pyruvate carboxylation (PC/PDC, Table IIB). Tissue pyruvate arose predominantly from external pyruvate and/or lactate (FCpyr→pyr = 0.73 ± 0.01; Equation 3 of Ref. 67) in the presence of 0.02 mM octanoate, whereas more than 50% was formed through glycolysis from glucose and/or glycogen (1 – FCpyr→pyr = 0.52 ± 0.05) in the presence of KB. In hearts perfused with 0.02 mM octanoate, which showed very low KB release (3 ± 2 nmol/min, n = 4, Ref. 32), 20% (mean of 35 and 6; n = 2) of the acetyl moiety of citrate was supplied by octanoate oxidation leaving 44% for the contribution of other sources, most likely long chain fatty acids. In hearts perfused with KB, the contribution of KB to acetyl-CoA formation was not evaluated but is likely to be substantial (58).

The contribution of pyruvate carboxylation to citrate synthesis in hearts perfused with 0.02 mM octanoate or 0.3 mM KB was not significantly different from those perfused with 0.2 mM octanoate (PC/CS; Table II). However, with 0.02 mM octanoate, the contribution of pyruvate carboxylation to citrate synthesis was significantly lower (58).

Also a minimal estimate of the CAC flux was calculated as the sum of acetyl-CoA production from pyruvate decarboxylation and octanoate oxidation (Equations 5 and 6, Comte et al. (67)). Absolute rates of pyruvate carboxylation were similar in all conditions, on average 0.14 ± 0.02 μmol/min (n = 14). These values are in agreement with those reported by others (18–20, 53) assuming a (dry weight)/(wet weight) ratio of 7.7 (18). Minimal estimates of CAC flux were on average 1.98 ± 0.26 μmol/min. These values are also in agreement with those of others (9, 28, 41, 54–57) and with measured rates of oxygen consumption (37) for similarly perfused rat hearts. Note that the conversion of relative to absolute fluxes assumes that all the octanoate molecules taken up by the heart are completely oxidized. Unlike long chain fatty acids, medium chain fatty acids such as octanoate are not esterified in the heart but are completely oxidized, at least under aerobic conditions (42, 44).
or 0.3 mM KB, these flux measurements were imprecise (PC/CS, Table IIB; mean not significantly different from zero for hearts perfused with 0.02 mM octanoate). This imprecision is inherent to the use of highly enriched [U-13C3]lactate + pyruvate under conditions where pyruvate decarboxylation is substantial. Then a high percentage of citrate isotopomers become trapped by decreasing the 13C enrichment of the supplied substrate. However, the lower limit on the enrichment of lactate and pyruvate is imposed by the precision with which one can measure the low enrichments of the OAA moiety of citrate. Therefore, relative flux through pyruvate carboxylation under conditions where the flux ratio PC/PDC is smaller than one remains to be precisely determined.

Physiological Considerations—Our 13C data allow precise quantitation of flux through anaplerotic pyruvate carboxylation in hearts perfused with physiological concentrations of glucose, lactate, and pyruvate and concentrations of octanoate leading to low rates of pyruvate decarboxylation. Anaplerotic pyruvate carboxylation could be catalyzed by mitochondrial pyruvate carboxylase or cytosolic NADP-linked malic enzyme. Our data do not differentiate between these two processes. Since these two enzymes are differentially regulated by acetyl-CoA (7, 19, 22, 59), their relative contributions should vary with the perfusion conditions. In hearts perfused with 0.2 or 1 mM octanoate, the malic enzyme is likely to be inhibited by the high rates of acetyl-CoA generation. The activity of pyruvate carboxylase in rat heart extracts, although lower than that of NADP-malic enzyme, can account for absolute fluxes reported in this and others studies (19–20, 52–53). Also, the modulation of the flux ratio (pyruvate carboxylation)/(citrate synthesis) by lactate and pyruvate concentrations is compatible with the kinetics of heart pyruvate carboxylase: $K_m$ for pyruvate of 0.125 mM (20).

In addition, under all perfusion conditions studied, the 13C dilution between tissue CAC metabolites (Figs. 1 and 2) suggests the presence of another site of anaplerosis aside from pyruvate carboxylation. The 13C enrichments of citrate and αKG (Fig. 1) indicate a low dilution of the αKG pool through exchange or anaplerotic reactions. Such conclusion is supported by the low enrichment of αKG in M5 isotopomers (<4% observed when hearts were perfused with <99% [U-13C5]glutamate (Comte et al. (67)). Most of the dilution occurred at the level of succinate. Unlabeled succinate could be formed from the branched chain amino acids, valine or isoleucine (9, 60–61). In hearts perfused in absence of these amino acids, the maximal rate of succinate formation from valine arising from proteolysis is 5 nmol x min$^{-1}$ x (g wet weight$^{-1}$) (9), representing about 0.2% of the estimated CAC flux. Such flux cannot account for all the 13C dilution in the CAC (estimated at 1.1 with 0.2 or 1 mM octanoate, Equation 10 of Ref. 67). Thus, the anaplerotic flux at succinate (about 10% of the CAC flux in hearts perfused with 0.2 or 1 mM octanoate) would be similar to that attributed to pyruvate carboxylation (5.4 ± 0.5%, n = 29). However, because of evidence pointing to a heterogenous labeling of the myocardial pool of succinate (35), the possibility and extent of anaplerosis at the level of mitochondrial succinate remain to be investigated in experiments with 13C-labeled valine or isoleucine.

Despite uncertainties about the magnitude of the anaplerotic flux at succinate, the demonstration of an active substrate flux through anaplerotic pyruvate carboxylation in hearts perfused with 0.2 or 1 mM octanoate raises the question of a site for cataplerosis. Under steady-state conditions, anaplerotic and cataplerotic fluxes should be of equal magnitude. 13C NMR studies on rat hearts perfused with 13C-substrates pointed out malate decarboxylation as a cataplerotic reaction (62). How-

### Table II

<table>
<thead>
<tr>
<th>Perfusion conditions (n)</th>
<th>MPE OAA, measured M3 (min–max)</th>
<th>Flux ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PC/CS</td>
</tr>
<tr>
<td>A. 1 mM [U-13C3]lactate + 0.2 mM [U-13C3]pyruvate</td>
<td>5.54 ± 0.80 (4.37–4.55)</td>
<td>0.063 ± 0.009</td>
</tr>
<tr>
<td>+ 0.2 mM [1-13C]octanoate + 1 mM DCA (5)</td>
<td>5.73 ± 0.25 (4.46–4.74)</td>
<td>0.056 ± 0.003</td>
</tr>
<tr>
<td>+ 0.2 mM [1-13C]octanoate + 1 mM NE (4)</td>
<td>5.98 ± 0.26 (4.46–4.83)</td>
<td>0.074 ± 0.004</td>
</tr>
<tr>
<td>0.5 mM [U-13C3]lactate + 0.055 mM [U-13C5]pyruvate</td>
<td>2.81 ± 1.19 (2.33–2.37)</td>
<td>0.035 ± 0.003$^a$</td>
</tr>
<tr>
<td>+ 0.2 mM [1-13C5]octanoate (4)</td>
<td>2.51 ± 0.26 (2.11–2.09)</td>
<td>0.034 ± 0.004$^a$</td>
</tr>
<tr>
<td>+ 1 mM [1-13C5]octanoate (2)</td>
<td>2.16 (0.66–2.67)</td>
<td>0.037 ± 0.038$^a$</td>
</tr>
<tr>
<td>B. 1 mM [U-13C3]lactate + 0.022 mM [U-13C5]pyruvate</td>
<td>8.59 ± 2.16 (0.66–2.67)</td>
<td>0.064 ± 0.028</td>
</tr>
<tr>
<td>+ 0.055 mM [U-13C5]pyruvate</td>
<td>5.70 ± 0.96 (1.79–2.84)</td>
<td>0.038 ± 0.004$^a$</td>
</tr>
<tr>
<td>+ 0.15 mM (AcAc + BHB) (4)</td>
<td>2.16 (0.66–2.67)</td>
<td>0.037 ± 0.038$^a$</td>
</tr>
</tbody>
</table>

$^a$ p < 0.05, all conditions versus 1 mM lactate + 0.2 mM pyruvate + 0.2 mM octanoate, using a one-way analysis of variance followed by a Bonferroni multiple comparison test.

$^b$ p < 0.05, 0.5 mM lactate + 0.05 mM pyruvate + 1 mM octanoate versus 0.2 mM octanoate, using an unpaired t test.

$^c$ Nondetectable mean tested against the null hypothesis. NA, not applicable.
Anaplerosis from Pyruvate in Heart

The likelihood of this site in hearts perfused with a medium chain fatty acid is supported by increased tissue levels of citrate (8, 12) and by the presence of citrate in the effluent (this study). Earlier experiments with isolated rat heart mitochondria indicated that citrate accumulation and efflux from the mitochondria occurred at a very high NADH/NAD⁺ ratio or state 4 respiration by exchange with added malate (14). There was no reverse flux of citrate. In rat hearts perfused under normoxic conditions, state 4 respiration (ATP/Pi, limited) prevails with octanoate (see for review Ref. 63). High citrate levels in hearts perfused with octanoate (12) probably explain citrate release in the present study. Tissue citrate levels are also increased by perfusion with long chain fatty acid, pyruvate, and by starvation and diabetes (13). These and other data led to the suggestion that accumulation of cytosolic citrate may feed back on NADH production by the cytosol by inhibiting glycolysis at the level of phosphofructokinase (12–13). In β-cells (64), and more recently in skeletal muscles (65), cytosolic citrate was proposed to be a “signal of mitochondrial fuel abundance.” Also, evidence was presented in these cells (64–65) that cytosolic citrate is a source of malonyl-CoA, an inhibitor of carnitine palmitoyltransferase I, the enzyme governing long chain fatty acid oxidation. However, in the heart, the significance of these regulatory mechanisms is questioned because of the low activity of the tricarboxylate transporter (14–15). Still this low activity is compatible with citrate efflux rates reported in this study (estimated at 50 nmol/min). Thus, on the basis of the above evidence, we speculate that the following sequence of events takes place in hearts perfused with 0.2 or 1 mM octanoate: (i) high productions of mitochondrial acetyl-CoA and NADH from octanoate oxidation inhibit pyruvate decarboxylation and favor anaplerotic pyruvate carboxylation; (ii) this sets up conditions for accumulation of tissue citrate and malate, and for citrate efflux from mitochondria to cytosol. A similar sequence of events could also occur in hearts perfused with high concentrations of long chain fatty acids, although this remains to be shown.

In conclusion, in hearts perfused with a physiological mix of 13C-substrates, the labeling pattern of tissue CAC, OAA, and acetyl-CoA can be probed by that of effluent citrate. Using the 13C protocol that we developed, flux through anaplerotic pyruvate carboxylation was quantitated with precision in hearts perfused with a mix of substrates where acetyl-CoA was predominantly supplied by β-oxidation. Then, anaplerotic carboxylation contributed substantially to citrate synthesis from pyruvate. Other data indicate succinate as another site for anaplerosis and raise the possibility of a cataplerotic citrate efflux from mitochondria to cytosol. In view of the role of cytosolic citrate as a modulator of glycolysis (through inhibition of phosphofructokinase; Refs. 12 and 13) and, possibly, of fatty acid metabolism (through malonyl-CoA; Refs. 43 and 66), further investigations of the relationship between anaplerotic pyruvate carboxylation and citrate efflux from mitochondria to cytosol in heart perfused with long chain fatty acids may clarify the role of anaplerosis and cataplerosis in signal transmission in the normoxic heart.

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A $^{13}$C Mass Isotopomer Study of Anaplerotic Pyruvate Carboxylation in Perfused Rat Hearts

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