Control of Citric Acid Cycle Activity in Rat Heart Mitochondria*

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KATHRYN LA NOUE, WILLIAM J. NICKLAS,† and JOHN R. WILLIAMSON§

From the Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania 19104

SUMMARY

Rat heart mitochondria were incubated in state 3 (with ADP) or state 4 (without ADP) in the presence of [3-14C]pyruvate, [3-14C]pyruvate plus unlabeled malate, or unlabeled pyruvate plus uniformly labeled [14C]malate. The net accumulation or loss of each intermediate in the citric acid cycle was determined by specific enzyme assays. In addition, the incorporation of radioactivity into cycle intermediates was measured after resolution of mitochondrial extracts by means of ion exchange column chromatography. In some experiments, mitochondrial suspensions were filtered through Millipore filters to determine the extramitochondrial content of metabolites.

In the absence of malate, pool sizes of the citric acid cycle intermediates were very low in comparison to flux through the cycle. Recycling of 14C caused the specific activity of cycle intermediates to exceed that of the substrate, [3-14C]pyruvate.

In the presence of a large pool of unlabeled malate, relatively large accumulations of 14C intermediates occurred during the oxidation of 14C-pyruvate in both state 3 and state 4. With malate added, 14C does not recycle but is diluted into the large malate pool. Thus, calculations of flux through the various steps in the cycle are made possible by comparing pyruvate disappearance with accumulation of radioactivity into the various intermediates and the final accumulation of radioactivity into malate. Rates of pyruvate utilization and oxygen consumption were linear under all conditions. Accumulations of citrate, α-ketoglutarate, and succinate were linear over the 8 min of incubation in state 4 and were largely extramitochondrial. In state 3, the accumulations of citrate, α-ketoglutarate, and succinate were higher in state 3 than in state 4. On the other hand, the accumulations of α-ketoglutarate and succinate were higher in state 3 than in state 4 and were mainly extramitochondrial. The accumulation of fumarate was not affected by the respiratory state of the mitochondria and was formed directly from the added malate. Addition of pyruvate alone or pyruvate plus malate to the mitochondria in both state 3 and state 4 resulted in a fall of aspartate and a stoichiometric rise of glutamate.

It is suggested that the increased flux through each of the steps of the citric acid cycle observed in state 3 is mediated by coordinated interactions at several sites and that this effect is exerted mainly by changes in the ratio of NADH to NAD. The evidence indicates that the accumulations of citrate, α-ketoglutarate, and succinate in the extramitochondrial space occur by anion exchange reactions with the added malate.

Current investigations in this laboratory are concerned with elucidating metabolic control of the citric acid cycle. Because the pathway involves a series of oxidative reactions linked via NADH and reduced flavoproteins to the cytochrome chain, overriding control may be imposed by the coupling of phosphorylation to electron transport. In heart and liver, but not in brown fat (1-3), there is good evidence that respiration is controlled by ADP availability (4, 5). In tissues subject to respiratory control, the activity of individual steps of the citric acid cycle are adjusted to the electron transport rate, i.e., to the ATP requirements of the tissue. Three major mechanisms mediating control of citric acid cycle reactions may be considered: (a) allosteric control of the initial reactions of the citric acid by ATP or ADP (6-8), (b) control by the oxidation-reduction state of the flavin and pyridine nucleotides (5, 9-12); and (c) control of the transport of intermediates across the mitochondrial membrane (13, 14).

Rat heart mitochondria were chosen for this study because of their low pyruvate carboxylase activity (15). Pyruvate disappearance, after correction for the small ketone body formation, can thus be equated with the rate of citrate formation. Attempts have been made by other workers to evaluate control at different sites in the citric acid cycle by following the accumulation of radioactivity into cycle intermediates after addition of 14C-pyruvate (16-18) or 14C-succinate (19, 20) to mitochondrial suspensions. However, interpretation of the results is difficult because of insufficient analyses, lack of specific activity measurements, or lack of knowledge of the relative amounts of intermediates inside and outside the mitochondrial matrix space.

It is known that externally added malate relieves the inhibition of respiration which occurs when rat heart mitochondria...
oxidize pyruvate for more than a few minutes (21). Malate has also been shown to alter the permeability of the mitochondrial membrane to citrate and α-ketoglutarate (14, 22). Most of the experiments reported here were performed in the presence of malate and pyruvate, with either 14C-pyruvate or 14C-malate. The accumulation and incorporation of isotope into each of the intermediates has been followed kinetically with mitochondria respiring under state 4 or state 3 conditions. A complete knowledge of the metabolic balance over each time interval has allowed a calculation of the flux through individual steps of the cycle. Additionally, the effect of malate on the efflux of the cycle intermediates from the mitochondria to the incubation medium has been studied. The data to be presented indicate that without added ADP there is concerted control of flux at all the NAD-linked reactions, whereas flux through succinate dehydrogenase appears to be controlled mainly by substrate availability. A preliminary account of part of this work has been published (23).

**MATERIALS AND METHODS**

**Preparation of Mitochondria**—Rat heart mitochondria were prepared according to the method of Chance and Hagihara (24) with slight modifications. The isolation medium consisted of 75 mM sucrose, 225 mM mannitol, and 0.1 mM EDTA, pH 7.0. The yield was approximately 10 mg of mitochondrial protein per rat heart. The respiratory control of these mitochondria during pyruvate or pyruvate plus malate oxidation was consistently between 7 and 10. Oxygen consumption was measured polarographically with a Clark electrode.

**Incubation Conditions**—The basic incubation medium consisted of 130 mM KCl, 20 mM Tris-chloride, 20 mM KH₂PO₄, 5 mM MgCl₂, and 30 mM glucose, pH 7.2. The incubation temperature was 28°C. State 3 respiration was initiated by addition of yeast hexokinase (40 units per ml of medium) and 0.5 mM ADP. In state 4 experiments, hexokinase and ADP were omitted. Mitochondria (2 to 4 mg of protein) were preincubated in 1 ml of oxygenated medium for 30 sec before starting the reaction by addition of substrate. Perchloric acid, 0.3 ml, 18% (w/v), was added to stop the reaction after the required interval of time. Deproteinized extracts were neutralized to pH 6 with 6 N NaOH and then added to stop the reaction after the required interval of time.

**Preparation of Mitochondria**—Rat heart mitochondria were prepared according to the method of Chance and Hagihara (24) with slight modifications. The isolation medium consisted of 75 mM sucrose, 225 mM mannitol, and 0.1 mM EDTA, pH 7.0. The yield was approximately 10 mg of mitochondrial protein per rat heart. The respiratory control of these mitochondria during pyruvate or pyruvate plus malate oxidation was consistently between 7 and 10. Oxygen consumption was measured polarographically with a Clark electrode.

**Incubation Conditions**—The basic incubation medium consisted of 130 mM KCl, 20 mM Tris-chloride, 20 mM KH₂PO₄, 5 mM MgCl₂, and 30 mM glucose, pH 7.2. The incubation temperature was 28°C. State 3 respiration was initiated by addition of yeast hexokinase (40 units per ml of medium) and 0.5 mM ADP. In state 4 experiments, hexokinase and ADP were omitted. Mitochondria (2 to 4 mg of protein) were preincubated in 1 ml of oxygenated medium for 30 sec before starting the reaction by addition of substrate. Perchloric acid, 0.3 ml, 18% (w/v), was added to stop the reaction after the required interval of time. Deproteinized extracts were neutralized to pH 6 with 6 N NaOH and then added to stop the reaction after the required interval of time. Deproteinized extracts were neutralized to pH 6 with 6 N NaOH and then added to stop the reaction after the required interval of time. Deproteinized extracts were neutralized to pH 6 with 6 N NaOH and then added to stop the reaction after the required interval of time. Deproteinized extracts were neutralized to pH 6 with 6 N NaOH and then added to stop the reaction after the required interval of time. Deproteinized extracts were neutralized to pH 6 with 6 N NaOH and then added to stop the reaction after the required interval of time. Deproteinized extracts were neutralized to pH 6 with 6 N NaOH and then added to stop the reaction after the required interval of time. Deproteinized extracts were neutralized to pH 6 with 6 N NaOH and then added to stop the reaction after the required interval of time. Deproteinized extracts were neutralized to pH 6 with 6 N NaOH and then added to stop the reaction after the required interval of time. Deproteinized extracts were neutralized to pH 6 with 6 N NaOH and then added to stop the reaction after the required interval of time. Deproteinized extracts were neutralized to pH 6 with 6 N NaOH and then added to stop the reaction after the required interval of time. Deproteinized extracts were neutralized to pH 6 with 6 N NaOH and then added to stop the reaction after the required interval of time.

**Assay Procedures**—Citrato acid cycle intermediates, pyruvate, ketone bodies, glutamate, aspartate, and reduced pyridine nucleotides were measured by methods published elsewhere (25). Protein was determined by the biuret method (26).

**Separation of Intermediates by Ion Exchange Chromatography**—The citric acid cycle intermediates and related metabolites were separated on a Dowex 1-formate column (Bio-Rad AG 1-X10) as indicated in Fig. 1. The mitochondrial extract was carefully adjusted to pH 6.0, and an 0.5-ml aliquot was added to the resin. The procedure was similar to that of Busch (27) except that the formic acid elution gradient was more gradual and was interrupted when 150 ml of solution had passed through the column. The gradient was produced by allowing 3 N formic acid to enter a 250-ml mixing chamber filled with distilled H₂O (16). Subsequently, 2 N ammonium formate, pH 2.7, was used as the eluent. The column dimensions were 0.9 x 14 cm and the elution rate was 2 ml per min. Either 2- or 4-ml fractions were collected with an automated fraction collector. The peaks were shown by pipetting 0.5 ml of each fraction into 10 ml of counting solution (7.5 ml of toluene, 2.5 ml of Triton X-100, 50 mg of 2,5-dihydroxybenzoic acid) and 1 mg of dimethyl 1,4-bis(5-phenyloxazolyl)benzene. The scintillations were counted in a Packard Tri-Carb scintillation counter. The output of the counter was then analyzed either manually or by feeding into a PDP-6 computer via punched tape. The computer was programmed to yield data in terms of total radioactivity under each peak. Specific activity is defined as the ratio of the total radioactivity in each intermediate to the total amount of that intermediate in the sample, as measured by enzymatic analysis.

A satisfactory separation of most intermediates was achieved with this modified system. Citrate and isocitrate were not resolved and were treated as a unit (Fig. 1). The complex peak between tubes 13 and 18 containing glutamate, aspartate, acetoacetate, and β-hydroxybutyrate was evaporated to dryness under vacuum and reconstituted to 2 ml; the pH was adjusted to 7.0. β-Hydroxybutyrate and acetate were volatile and were lost on evaporation. An aliquot of this sample was then put on a Dowex 1 acetate (Bio-Rad AG 1-X4) column (0.7 x 20 cm), which separated glutamate from aspartate (28). When the column was run without previous evaporation, β-hydroxybutyrate and acetate were eluted with 0.1 N acetic acid before the aspartate, but after the glutamate emerged from the column. The identification of peaks was made by comparison with the elution position of known 14C-labeled compounds. The acetoacetate peak was identified by enzymatic assay.

**Chemicals**—[5-14C]Sodium pyruvate (3 mCi per mmole), uniformly labeled L-[14C]aspartic acid (180 mCi per mmole), and [1,5,14C]citric acid (10 mCi per mmole) were obtained from...
New England Nuclear. Uniformly labeled l-[14C] malic acid (23 mCi per mmole), DL-[3-14C] sodium 3-hydroxybutyrate (12 mCi per mmole), [5-14C] sodium 2-ketoglu tarate (17 mCi per mmole), and [1,4-14C] fumaric acid (12 mCi per mmole) were purchased from Nuclear-Chicago. L-[1,4-14C] Glutamic acid (20 mCi per mmole) was obtained from Calbiochem. The scintillators used in the radioactivity measurements and the Triton X-100 were purchased from the Packard Instrument Company. All unlabeled organic substrates were commercial preparations of the highest purity available. Pyruvic acid was distilled twice under vacuum and stored at 2°C.

Enzymes—Hexokinase, Type V, was obtained from Sigma. Some batches of the commercial enzyme were found to be contaminated with glutamate and aspartate. All enzymes used in the fluorometric analyses of metabolites were purchased from Boehringer-Mannheim except for succinate thiokinase, which was the generous gift of Dr. P. D. Boyer.

RESULTS

Oxidation of Pyruvate with No Added Malate—[3-14C]Pyruvate (1 mM) was oxidized rapidly by rat heart mitochondria in the absence of added malate. The oxygen consumption was constant at 350 nanatoms per min per mg of protein for at least 4 min in the presence of ADP. This rate decreased after 6 to 8 min but could be restored by addition of malate. The rate of glucose-6-P formation was also linear and equal to 3 times the rate of oxygen uptake. These findings are in basic agreement with those of Davis (21, 29). Measurements of pyruvate showed that pyruvate utilization was linear in both state 3 and state 4 (Fig. 2). Significant quantities of β-hydroxybutyrate and acetoacetate were formed, particularly in state 3, during the course of the experiment (Table I). The ratio of β-hydroxybutyrate to acetoacetate was considerably greater in state 4 than in state 3, indicating an oxidation of pyridine nucleotides during a state 4 to 3 transition (30). Acetate, estimated by measuring the amount of volatile radioactivity in the glutamate-acetate-aspartate peak, was produced in relatively large amounts (10 nmol per mg of protein) in some experiments. Zero time samples also contained radioactive acetate in variable amounts. This was due presumably to nonenzymatic decarboxylation of pyruvate.

Table II shows the accumulation of radioactivity from [3-14C]pyruvate into citrate, α-ketoglutarate, succinate, and malate. No radioactivity could be detected in fumarate. There were no very significant changes in any of the intermediates after 1 min. In both states 3 and 4, the pool sizes of citrate, α-ketoglutarate, and malate suggested by the 14C accumulation data were very small in comparison with flux through the cycle estimated from the rate of pyruvate disappearance (Fig. 2). Measurement of

### Table I

Acetoacetate and β-hydroxybutyrate production by rat heart mitochondria

<table>
<thead>
<tr>
<th>Intermediate</th>
<th>State 3</th>
<th>State 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate</td>
<td>2.3 ± 0.4</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>1.2 ± 0.1</td>
<td>2.2 ± 0.7</td>
</tr>
<tr>
<td>Succinate</td>
<td>8.5 ± 1.0</td>
<td>15.8 ± 0.6</td>
</tr>
<tr>
<td>Malate</td>
<td>1.5 ± 0.3</td>
<td>2.3 ± 0.9</td>
</tr>
</tbody>
</table>

* Results are expressed as nanomoles of 14C-pyruvate incorporated into each of the intermediates per mg of mitochondrial protein.

### Table II

Incorporation of radioactivity from [3-14C]pyruvate into citric acid cycle intermediates

Rat heart mitochondria (3 to 4 mg per ml) were incubated with 1 mM [3-14C]pyruvate in media containing 130 mM KCl, 20 mM Tris-Cl, 20 mM KH2PO4, 5 mM MgCl2, and 30 mM glucose, pH 7.2. Values shown are means ± standard error of the mean of 4 experiments. State 3 respiration was initiated by addition of hexokinase and 0.5 mM ADP.

<table>
<thead>
<tr>
<th>Intermediate</th>
<th>1 min</th>
<th>2 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate</td>
<td>2.3 ± 0.4</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>1.2 ± 0.1</td>
<td>2.2 ± 0.7</td>
</tr>
<tr>
<td>Succinate</td>
<td>8.5 ± 1.0</td>
<td>15.8 ± 0.6</td>
</tr>
<tr>
<td>Malate</td>
<td>1.5 ± 0.3</td>
<td>2.3 ± 0.9</td>
</tr>
</tbody>
</table>

* Results are expressed as nanomoles of 14C-pyruvate incorporated into each of the intermediates per mg of mitochondrial protein.

Fig. 2 (left). Pyruvate utilization by rat heart mitochondria respiring in state 3 or state 4 in the absence or presence of 5 mM malate. Mitochondria (3 to 4 mg per ml) were incubated with pyruvate in media containing 130 mM KCl, 20 mM Tris-Cl, 20 mM KH2PO4, 5 mM MgCl2, and 30 mM glucose, pH 7.2, in a total volume of 1 ml. The initial concentration of pyruvate was 1 mM in the top section and 2 mM in the lower one. State 3 respiration was initiated by addition of hexokinase and 0.5 mM ADP.

Fig. 3 (right). Contents of aspartate and glutamate in rat heart mitochondria incubated with 1 mM pyruvate. See legend to Fig. 2 for experimental details.
the intermediates by enzyme assays confirmed that pool sizes are of this order of magnitude or somewhat smaller. The amount of radioactivity incorporated into succinate was 4 to 6 times greater in state 3 than in state 4. This accumulation of succinate in state 3 may reflect an increased rate of exchange of intramitochondrial succinate with phosphate (14). Because there was only a small accumulation of radioactivity in cycle intermediates but a very sizable loss of radioactivity from the pyruvate pool, we conclude that most of the pyruvate metabolized was completely oxidized to CO₂, as observed by von Korff (17), who used rabbit heart mitochondria.

Fig. 3 shows the changes of glutamate and aspartate in mitochondria incubated with pyruvate. Aspartate fell rapidly during the first minute of incubation, and glutamate increased. Endogenous aspartate was presumably the immediate source of the oxalacetate required for the synthesis of citrate.

The specific radioactivity of glutamate after 1 min was the same or slightly higher than that of [3-¹⁴C]pyruvate (Table III). The specific radioactivity of glutamate relative to that of pyruvate was always greater in state 3 than in state 4 at the same time point. After 2 min in state 3, there were 2 to 3 equivalents of ¹⁴C from pyruvate incorporated into each mole of glutamate. This follows from randomization of isotope in oxalacetate after several turns of the cycle, so that several carbon atoms of α-ketoglutarate became highly labeled (31). It suggests that the specific activities of all cycle intermediates change with time and that radioactivity data indicate maximum pool sizes only.

**Oxidation of Pyruvate in Presence of Malate**—The rate of pyruvate uptake was about 25% greater in both state 3 and state 4 when 5 mM malate was included in the reaction medium (Fig. 2). The presence of malate decreased ketone body formation by about 50% in state 3 (Table I). No effect of malate on ketone body formation was apparent in state 4. However, the ratio of β-hydroxybutyrate to acetoacetate was much greater in the presence of malate in both state 3 and state 4 (Table I). Thus, the mitochondrial pyridine nucleotides appeared to be more reduced in state 3 with malate than they were in state 4 without malate. This was confirmed in separate experiments in which the pyridine nucleotide fluorescence changes of rat heart mitochondria were monitored in conjunction with analytical measurements of NADH and NADPH (Figs. 4 and 5). The levels of NADPH remained approximately constant after substrate addition, so that the changes of the total fluorescence were associated only with NADH changes. An oxidation of NADH was observed in the state 4 to 3 transition with pyruvate alone (Fig. 4) and with pyruvate plus malate as substrates (Fig. 5).

With [3-¹⁴C]pyruvate (2 mM) and 5 mM unlabeled malate, a quantitative recovery of all the radioactivity added to the reaction medium was obtained in the intermediates eluted from the ion exchange columns. In order to produce ¹⁴CO₂ from [3-¹⁴C]pyruvate, more than one turn of the citric acid cycle is needed. Evidently, the large unlabeled pool of malate diluted the ¹⁴C-malate formed in the citric acid cycle sufficiently that the oxalacetate pool remained virtually unlabeled. The specific activity of oxalacetate could not be measured directly, but very little radioactivity was incorporated into aspartate, which may be presumed to be in equilibrium with oxalacetate.

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**Table III**

Specific activity of glutamate isolated from rat heart mitochondria incubated with [3-¹⁴C]pyruvate

<table>
<thead>
<tr>
<th>Respiratory state</th>
<th>Incubation time (min)</th>
<th>Specific activity of glutamate (cpm/mole)</th>
<th>Ketoacid specific activity of glutamate</th>
</tr>
</thead>
<tbody>
<tr>
<td>State 3</td>
<td>1</td>
<td>2917</td>
<td>1.48</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6892</td>
<td>3.52</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7558</td>
<td>3.86</td>
</tr>
<tr>
<td>State 4</td>
<td>1</td>
<td>1414</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2694</td>
<td>1.55</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3785</td>
<td>1.93</td>
</tr>
</tbody>
</table>

* Specific activity of glutamate divided by the specific activity of pyruvate.

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**Fig. 4.** Changes in pyridine nucleotide reduction associated with state 4 → state 3 transition in rat heart mitochondria incubated with 2.5 mM pyruvate. The initial volume in the cuvette was 2.5 ml and 0.5 ml samples were taken for analysis at the indicated places (A, B, C, D). The incubation medium was the same as that of Table I.

**Fig. 5.** Changes in pyridine nucleotide reduction associated with state 4 → state 3 transition in rat heart mitochondria incubated with 2.5 mM pyruvate (Pyr) and 5 mM malate (Mal). See legend to Fig. 4 for further experimental details.
FIG. 6. Accumulation of citrate in mitochondrial suspension after incubation of rat heart mitochondria with 2 mM pyruvate and 5 mM malate during state 3 or state 4 respiration. See legend to Fig. 2 for other experimental details.

FIG. 7. Intramitochondrial and extramitochondrial concentration of citrate in rat heart mitochondria respiring in state 4 and incubated with 2 mM pyruvate and 5 mM malate. The extramitochondrial content was obtained by rapid filtration of the mitochondrial suspension through Millipore filters (0.65 μ) and the intramitochondrial content by difference. See legend to Fig. 2 for other experimental details.

FIG. 8. Accumulation of α-ketoglutarate in mitochondrial suspensions after incubation of rat heart mitochondria respiring in state 3 or state 4 with 2 mM pyruvate and 5 mM malate. See legend to Fig. 2 for other experimental details.

Support of this hypothesis is derived from the fact that after 1 min of incubation, the specific activities of citrate, α-ketoglutarate, succinate, and glutamate were the same as that of the added \(^{14}C\)-pyruvate, indicating that the labeled carbon essentially passed around the cycle only once. Thus, it is evident that pool sizes can be determined equally well from total radioactivity or from direct enzyme assays. A comparison of the two methods is presented later in this paper (Table IV). The specific activity of malate increased gradually to a value 20% that of the pyruvate after 8 min of incubation in state 3. The specific activity of fumarate was always much lower than that of malate, indicating that a significant portion of it was formed predominantly from extramitochondrial malate, and did not mix during the reaction with the labeled intramitochondrial pool. When samples were rapidly filtered through Millipore filters before addition of perchloric acid, virtually all the accumulated fumarate was found to be extramitochondrial.

Kinetics of Changes of Intermediates in States 3 and 4 during Oxidation of [3-\(^{14}C\)]Pyruvate in Presence of 5 mM Malate—The content of aspartate in mitochondrial suspensions was initially about 10 nmoles per mg of protein, whereas that of glutamate was less than 1 n mole per mg of protein. The endogenous aspartate content fell and the glutamate content rose rapidly in both state 3 and state 4 upon addition of pyruvate plus malate. This again indicates that endogenous aspartate is the immediate source of oxalacetate for citrate formation (cf. Fig. 3).

Fig. 6 shows the formation of citrate by respiring mitochondria. In state 3, a steady state was rapidly attained, and thereafter the size of the citrate pool remained approximately constant at 5 to 7 nmoles per mg of protein. In state 4, however, citrate accumulated at the rate of 3 to 4 nmoles per min of mg of protein. Rapid filtration of the mitochondria showed that in state 3, over 90% of the citrate was intramitochondrial. On the other hand, in state 4, citrate accumulated in the medium after the first minute (Fig. 7). If a matrix water space of 1 μl per mg of protein is assumed (32), it is apparent that significant amounts of citrate do not leave the mitochondria until the intramitochondrial citrate concentration exceeds about 4 mM. By subtracting the extramitochondrial citrate, it can be calculated that the intramitochondrial citrate in state 4 reached a maximum of 8 to 10 nmoles per mg of protein after 4 min, i.e., a value similar to the total citrate accumulation in state 3.

When [1,5-\(^{14}C\)]citrate (0.2 mM) was added to mitochondria respiring in state 4 in the presence of unlabeled pyruvate and malate, no radioactivity was detected in any metabolite other than citrate after 8 min of incubation. Thus, although intramitochondrial citrate is able to leave the mitochondria when its concentration becomes high enough, simultaneous exchange with extramitochondrial citrate does not occur when the external citrate concentration is low.

Unlike citrate, more α-ketoglutarate accumulated in state 3 than in state 4 (Fig. 8). After 4 min in state 3, the amount of
α-ketoglutarate present in the mitochondrial suspension reached a maximum. Filtration studies showed that more than 95% of the α-ketoglutarate was present in the incubation medium with both state 3 and state 4 respiration conditions. The rate of α-ketoglutarate accumulation was proportional to the amount of mitochondria used, but the final concentration of extramitochondrial α-ketoglutarate was not. The maximum concentration of α-ketoglutarate reached in the incubation medium in state 3 was instead a function of the malate concentration (Fig. 9). For example, the accumulation of α-ketoglutarate was about 50 times greater in the presence of 5 mM malate than in its absence.

As with α-ketoglutarate, much more succinate accumulated in state 3 than in state 4 (Fig. 10), and virtually all the succinate accumulation was extramitochondrial. The rate of succinate formation in state 3 was approximately linear (40 nmoles per min per mg of protein). The final amount of succinate accumulated, but not its rate of formation, was much greater than that of α-ketoglutarate. Even up to 8 min of incubation in state 3, succinate formation showed no signs of reaching a maximum. In state 4, the rate of succinate accumulation was low (2 nmoles per min per mg of protein).

The rate of fumarate appearance was linear (20 nmoles per min per mg of protein) and was the same in the presence and absence of ADP. The fact that the specific activity of the fumarate was lower than that of malate (Tables IV and V) and that it was extramitochondrial suggests that most of the fumarate was formed directly from malate, and was compart-

**Table IV**

Comparison of enzymic and isotopic methods of determining accumulations of intermediates of citric acid cycle: state 3 conditions

Rat heart mitochondria (3 to 4 mg per ml) were incubated in 1 ml of media containing 120 mM KCl, 20 mM Tris-Cl, 20 mM KH2PO4, 5 mM MgCl2, and 30 mM glucose, pH 7.2, followed by addition of 40 units of hexokinase and 0.5 mM ADP. State 3 respiration was initiated by addition of 2 mM [3-14C]pyruvate and 5 mM malate. Incorporation of 14C isotope into intermediates [14C] was determined by dividing the total counts in each intermediate after separation by ion exchange chromatography by the specific activity of pyruvate. Values shown are the means of up to 4 experiments for each intermediate.

<table>
<thead>
<tr>
<th>Time</th>
<th>Pyruvate uptake</th>
<th>Citrate</th>
<th>α-Ketoglutarate</th>
<th>Succinate</th>
<th>Malate</th>
<th>Fumarate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[14C]</td>
<td>[14C]</td>
<td>[14C]</td>
<td>[14C]</td>
<td>[14C]</td>
<td></td>
</tr>
<tr>
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<td></td>
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<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
<td>0</td>
<td>0.9</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>45</td>
<td>6</td>
<td>4.4</td>
<td>4.0</td>
<td>45.9</td>
<td>44.5</td>
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<tr>
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<td>107</td>
<td>98</td>
<td>5.0</td>
<td>5.4</td>
<td>61.2</td>
<td>64.0</td>
</tr>
<tr>
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<td>189</td>
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<td>72.1</td>
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<td>413</td>
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<td>6.7</td>
<td>71.8</td>
<td>70.3</td>
</tr>
<tr>
<td>6.0</td>
<td>668</td>
<td>634</td>
<td>6.7</td>
<td>7.7</td>
<td>71.8</td>
<td>70.3</td>
</tr>
<tr>
<td>8.0</td>
<td>848</td>
<td>880</td>
<td>7.4</td>
<td>7.4</td>
<td>71.8</td>
<td>70.3</td>
</tr>
</tbody>
</table>
into the malate pool (Fig. 11).

The initial reaction, pyruvate to acetyl-CoA, was obtained from the pyruvate disappearance rate (Fig. 2). The flux, acetyl-CoA to citrate, was obtained by subtracting the net accumulation of acetyl-CoA and ketone bodies (Table I) from the rate of pyruvate disappearance. Subsequent fluxes were similarly calculated from the fumarate which was the precursor of the 14C incorporation from [3-14C]pyruvate into malate is shown in Fig. 11. After an initial lag, the appearance of isotope in malate was linear. In the steady state, the rate of formation of 14C-malate was about 20% lower than that calculated from isotope incorporation, but this could be caused by different mitochondrial preparations because in this case the same samples were not used for the two determinations. Because fumarate was formed directly from malate, which always had a much lower specific activity than that of pyruvate, the incorporation of counts into fumarate was only about 5% of the total fumarate formation. The amount of malate in the mitochondrial suspension remained approximately constant, so that formation of malate by the citric acid cycle could be determined only isotopically. On the other hand, changes of glutamate and aspartate had to be determined by enzyme assays. The results clearly establish the accuracy and reliability of the isotope method for determining the accumulations of citrate, a-ketoglutarate, succinate, and malate, and removal of pyruvate from mitochondrial suspensions in both state 3 and state 4 conditions.

<table>
<thead>
<tr>
<th>Time</th>
<th>Pyruvate uptake</th>
<th>Citrate</th>
<th>a-Ketoglutarate</th>
<th>Succinate</th>
<th>Malate</th>
<th>Fumarate</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>0.5</td>
<td>2.2</td>
<td>8.2</td>
<td>7.5</td>
<td>2.6</td>
<td>1430</td>
</tr>
<tr>
<td>6.0</td>
<td>7.7</td>
<td>15.8</td>
<td>30.6</td>
<td>16.0</td>
<td>59.9</td>
<td>98.6</td>
</tr>
</tbody>
</table>

**Table VI**

Net flux through individual steps of the citric acid cycle during oxidation of [3-14C]pyruvate in presence of 5 mM malate

The initial reaction, pyruvate to acetyl-CoA, was obtained from the pyruvate disappearance rate (Fig. 2). The flux, acetyl-CoA to citrate, was obtained by subtracting the rate of accumulation of acetyl-CoA and ketone bodies (Table I) from the rate of pyruvate disappearance. Subsequent fluxes were similarly calculated by subtracting the rate of accumulation of substrate (Figs. 6, 8, and 10) from the flux of the previous reaction. Because no accumulation of counts in fumarate is seen, the flux, succinate to fumarate, calculated from pyruvate disappearance and accumulation of precursors should equal the flux, fumarate to malate, obtained from direct measurement of the incorporation of 14C into the malate pool (Fig. 11).

Control of Citric Acid Cycle Intermediates

Measured by Enzymatic Assay with Values Calculated from Isotope Data—In many of the above experiments, the accumulation of citric acid cycle intermediates was measured both by specific enzyme assays and by the incorporation of isotope from [3-14C]pyruvate into intermediates after separation by ion exchange chromatography. A comparison of the results obtained by the two procedures in mitochondria respiring in state 3 or state 4 in the presence of 2 mM [3-14C]pyruvate and 5 mM malate is shown in Tables IV and V. Very similar results were obtained for estimates of pyruvate uptake and productions of citrate and a-ketoglutarate. In state 3, the formation of succinate as measured enzymically was about 20% lower than that calculated from isotope incorporation, but this could be caused by different mitochondrial preparations because in this case the same samples were not used for the two determinations. Because fumarate was formed directly from malate, which always had a much lower specific activity than that of pyruvate, the incorporation of counts into fumarate was only about 5% of the total fumarate formation. The amount of malate in the mitochondrial suspension remained approximately constant, so that formation of malate by the citric acid cycle could be determined only isotopically. On the other hand, changes of glutamate and aspartate had to be determined by enzyme assays. The results clearly establish the accuracy and reliability of the isotope method for determining the accumulations of citrate, a-ketoglutarate, succinate, and malate, and removal of pyruvate from mitochondrial suspensions in both state 3 and state 4 conditions.

Unlabeled Pyruvate with 14C-Malate—A few experiments were carried out in which uniformly labeled L-[14C]malate was the source of labeled carbon during the oxidation of unlabeled pyruvate. In these experiments, no radioactivity was detected in acetacetate, β-hydroxybutyrate, or acetate. Less than 1 nmole of 14C-malate was incorporated into pyruvate during the course of a 4-min experiment. Radioactivity was lost from the mitochondrial suspension during the reaction, indicating loss of radioactivity to CO2. The specific activities of aspartate, citrate, and fumarate were the same as that of the added malate. The specific activities of α-ketoglutarate and succinate relative to that of malate were 0.77 and 0.62, respectively. Loss of radioactive carbon atoms is expected during conversion of citrate to α-ketoglutarate and α-ketoglutarate to succinate. The fact that the specific activity of fumarate was the same as that of the malate again suggests that the accumulated fumarate was formed directly from the malate. Other experiments showed that the rate of fumarate...
production from 14C-malate was independent of the presence of pyruvate, and was not affected by the addition of rotenone which inhibited respiration.

**Flux through Individual Steps in Citric Acid Cycle**—A complete knowledge of the major metabolic transformations in mitochondrial suspensions permits the average flow rate between two time intervals to be calculated for each of the steps in the citric acid cycle. Values for state 3 and state 4 respiration are shown in Table VI. During the first minute of incubation with substrate in state 3, 100 of the 105 nmoles per min per mg of carbon flux through pyruvate dehydrogenase appeared as \( \alpha \)-ketoglutarate. However, because of \( \alpha \)-ketoglutarate accumulation, and loss of carbon into glutamate, flux through the \( \alpha \)-ketoglutarate step was decreased to 49 nmoles per min per mg. Most of the flux was lost from the mitochondria, so that finally only 50 nmoles per min per mg of malate were formed. Thus, the reactions of the citric acid cycle operated essentially not as a cycle, because of carbon loss as \( \alpha \)-ketoglutarate and succinate. After 6 min of incubation in state 3, loss of carbon as \( \alpha \)-ketoglutarate ceased, but loss of succinate continued. In state 4, the losses of carbon as citrate, \( \alpha \)-ketoglutarate, and succinate were about equal, and amounted to 50 to 70% of the flow rate through pyruvate dehydrogenase.

**DISCUSSION**

It is clear from recent reviews (33, 34) on the control of the citric acid cycle that there is a poor understanding of the nature of the many factors contributing to its integrated function. Factors which complicate extrapolation of findings with isolated mitochondria to the situation found in vivo are the artificial nature of incubation medium, the possibility that normal physiological substances which affect the permeability of the mitochondria to anions or cations may be missing (35), and establishment of unphysiological concentration gradients between the incubation medium and the mitochondrial matrix space. Thus, in studies with isolated mitochondria, substrates have to be added in high enough amounts not to be limiting as metabolism proceeds, whereas intermediary products equilibrate with the external medium, and so are lost from the matrix space. However, with these limitations in mind, a general understanding of the feedback regulations between the phosphorylating electron transport chain and the individual reactions of the citric acid cycle may be gained from the present data. Because many of the reactions are irreversible under normal conditions, there must be a coordination of control among multisite interactions.

This was most evident in experiments performed in the absence of malate, which showed only a small accumulation of intermediates despite a 5-fold increase of pyruvate utilization in state 3 compared with state 4. In the presence of malate, the control properties of the system are temporarily upset. Malate addition had two obvious effects. The first was to increase the levels of NADH in both state 3 and state 4. The second effect was to alter the equilibrium between the intra- and extramitochondrial pools of citrate, \( \alpha \)-ketoglutarate, and succinate.

**Malate-driven Anion Exchange**—Chappell (14) and Chappell and Haarhoff (36) have shown that in mitochondria depleted of phosphate and endogenous substrates, the mitochondrial membrane is relatively impermeable to succinate unless malate or phosphate are also present. Furthermore, \( \alpha \)-ketoglutarate penetration is stimulated by malate or malonate (22). Harris and Manger (13) have suggested that these activation phenomena essentially represent exchange reactions, so that those metabolites which can exchange for each other across the membrane compete for entry into the mitochondria. In heart mitochondria, entry of malate requires the influx of either phosphate, succinate, or \( \alpha \)-ketoglutarate. Citrate permease activity is very low in rat heart mitochondria, although a recent report (37) is in agreement with the present work, showing that a slow efflux of citrate occurs. In state 4 respiration, anion exchange with phosphate is presumably very low, and efflux of citrate, \( \alpha \)-ketoglutarate, and succinate may be accounted for by exchange with malate. The lack of appreciable accumulations of intermediates in mitochondria respiring on pyruvate in the absence of malate indicates that exchange with phosphate is also low in state 3. The rate of efflux of a particular intermediate from the mitochondria must be determined by its relative affinities for the enzyme which it serves as substrate, and its exchange carrier. The relatively high affinities of the malate-activated carriers for \( \alpha \)-ketoglutarate and succinate result in a net loss of these intermediates from the mitochondria until the external concentration reaches a limiting value when net movement across the membrane becomes zero. The steady state value was about 250 \( \mu \)M with \( \alpha \)-ketoglutarate but was greater than 1 mM with succinate, suggesting that the affinity of the respective permeases for \( \alpha \)-ketoglutarate is higher than that of the succinate permease for its substrate. Measurement of the \( K_m \) values for \( \alpha \)-ketoglutarate and succinate oxidations in intact mitochondria respiring in state 3 showed, in fact, that the \( K_m \) for \( \alpha \)-ketoglutarate was 30-fold less than that of succinate (Table VII). Malate was a competitive inhibitor of both \( \alpha \)-ketoglutarate and succinate oxidations.

The physiological significance of the anion exchange reactions may well be related to the transport of reducing equivalents across the mitochondrial membrane. Thus, in the scheme proposed by Chappell (14), an exchange of malate with \( \alpha \)-ketoglutarate and of aspartate with glutamate is required to carry into the mitochondria reducing equivalents formed during the cytoplasmic conversion of glucose to pyruvate. Citrate accumulation, when NADH levels are very high, may turn off the accumulation of NADH in the cytoplasm by inhibiting glycolysis (38, 39). The implication of a succinate carrier in mammalian mitochondria is less easy to understand because there are no known pathways for succinate metabolism in the extramitochondrial space.

### Table VII

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( K_m ) (mM)</th>
<th>( V_{max} ) (nmoles/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate</td>
<td>3.7</td>
<td>330</td>
</tr>
<tr>
<td>Succinate + 5 mM malate</td>
<td>12.5</td>
<td>330</td>
</tr>
<tr>
<td>( \alpha )-Ketoglutarate</td>
<td>0.12</td>
<td>192</td>
</tr>
<tr>
<td>( \alpha )-Ketoglutarate + 5 mM malate</td>
<td>1.25</td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>
The present data do not indicate any specific control interactions in the citric acid cycle reactions between $\alpha$-ketoglutarate and malate. However, the enhancement of succinyl-CoA inhibition of $\alpha$-ketoglutarate dehydrogenase by NADH (43) provides a mechanism whereby flux from $\alpha$-ketoglutarate to succinate could be controlled directly by the NADH oxidation state and succinyl-CoA by the phosphorylation state of the mitochondrial nucleotides. It seems unlikely that inhibition of succinate dehydrogenase by oxalacetate and malate (33) is responsible for the accumulation of succinate in the presence of malate. The small accumulation of succinate in state 3 in the absence of malate indicates that succinate dehydrogenase activity is controlled primarily by the intramitochondrial succinate concentration. In the absence of a competing malate-succinate exchange reaction, a relatively high intramitochondrial succinate concentration can be maintained (50). The slower rate of succinate accumulation in state 4 compared with state 3 presumably

Unpublished observations.
represents a lower intramitochondrial succinate concentration. A relatively weak feedback control at the succinate dehydrogenase site by the state of phosphorylation of the adenine nucleotides is indicated by the fact that at low external succinate concentrations (below the $K_m$ of 3.7 mM observed in the presence of rotenone), added ADP did not increase the rate of succinate oxidation. Only when the concentration of succinate exceeded 5 mM was it possible to obtain respiratory control. Also, maximum flux through succinate dehydrogenase in state 3 (in the presence of rotenone), added ADP did not increase the rate of succinate oxidation. The roles of reversed electron transfer and substrate level phosphorylation on the control of citric acid cycle activity and its interaction with β-oxidation require further elucidation.

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Control of Citric Acid Cycle Activity in Rat Heart Mitochondria
Kathryn LaNoue, William J. Nicklas and John R. Williamson


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