Feedback Interactions in the Control of Citric Acid Cycle Activity in Rat Heart Mitochondria*

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SUMMARY

Factors regulating the citric acid cycle have been investigated in rat heart mitochondria oxidizing pyruvate plus malate or acetyl carnitine plus malate as substrates. Effects caused by changing the NAD oxidation-reduction state, the intramitochondrial ATP:ADP ratio, and the respiratory rate were studied in five different metabolic states produced by additions of ADP, oligomycin, or uncouplers. The accumulations of cycle intermediates and the mitochondrial content of CoA derivatives and pyridine nucleotides were measured in extracts prepared from the whole incubation medium or after rapid separation of the mitochondria from the medium. These data, together with measurements of substrate utilization, were used to calculate flux through the individual steps of the citric acid cycle.

Pyruvate dehydrogenase was found not to be rate limiting for citric acid cycle activity. Flux through citrate synthase in State 3 was approximately the same (100 nmoles per min per mg) whether pyruvate or acetyl carnitine were used to generate acetyl-CoA, whereas acetyl-CoA levels were higher with pyruvate as substrate. Flux in State 4 with either substrate was diminished by 75 to 85% relative to State 3 and was associated during early incubation times with elevated levels of both NADH and acetyl-CoA, consonant with regulation of pyruvate dehydrogenase by product inhibition.

A comparison of flux through citrate synthase and changes in the levels of intramitochondrial malate, citrate, ATP, and the NAD oxidation-reduction state showed that increased flux associated with a State 4 to 3 transition could be accounted for largely by an increased availability of oxalacetate to citrate synthase rather than by ATP inhibition. On the other hand, comparisons between states in which phosphate acceptor was not rate limiting for electron transport (State 3, uncoupled or uncoupled plus oligomycin) showed that oxalacetate availability remained high because of the highly oxidized state of the pyridine nucleotides, and indicated a regulation of citrate synthase under conditions of low acetyl-CoA availability by an energy-linked process dependent on substrate level phosphorylation. Thus, a 55% inhibition of flux through citrate synthase was obtained after addition of oligomycin to the uncoupled state with acetyl carnitine plus malate as substrate, but only a 25% inhibition was achieved with pyruvate plus malate as substrate. The intramitochondrial ATP:ADP ratio increased from 0.3 to 4.6 with either substrate under these conditions. A kinetic evaluation of the data indicated that the energy-dependent inhibition was not caused by a direct effect of ATP on citrate synthase, but was due to changes of the succinyl-CoA content relative to that of acetyl-CoA. This conclusion is based (a) on the finding of an accumulation of a short chain acyl-CoA compound identifiable as succinyl-CoA by direct enzyme assay under conditions of diminished citrate synthase flux, (b) a correlation between decreased flux and lowered acetyl-CoA levels in the different experiments, and (c) the observation that succinyl-CoA is an inhibitor of citrate synthase competitive with acetyl-CoA.

A comparison of succinyl-CoA levels in mitochondria incubated in the different metabolic states indicated that its content was regulated by the phosphorylation state of the adenine nucleotides, presumably via the effect of altered GTP-GDP ratios on succinate thiokinase. Product inhibition of α-ketoglutarate dehydrogenase by succinyl-CoA was evidenced from an inverse relationship between α-ketoglutarate accumulation and succinyl-CoA levels. The conclusion is reached that feedback from the electron transport chain to the citric acid cycle is mediated by a combination of factors which include the phosphorylation state of the adenine and guanine nucleotides and the oxidation-reduction state of the pyridine nucleotides. Changes of these parameters secondarily affect the intramitochondrial concentrations of oxalacetate, acetyl-CoA, and succinyl-CoA, which are the direct regulators of citrate synthase activity.

In the previous paper of this series (1) we reported that in rat heart mitochondria oxidizing pyruvate as substrate, significant extramitochondrial accumulations of intermediates of the citric acid cycle were found only in the presence of added malate. This is in accord with the virtual lack of anaplerotic mechanisms for dicarboxylic acid synthesis in heart muscle (2-4). In the presence of malate, the permeability of the heart mito-
chloridial membrane to α-ketoglutarate and succinate was high, and that to citrate low. From a comparison of flux through individual steps of the citric acid cycle in mitochondria respiring under conditions of controlled (State 4) and active (State 3) respiration, together with a consideration of the kinetic properties of the isolated enzymes, it was concluded that feedback from the respiratory chain to individual dehydrogenases was exerted principally through alterations of the state of reduction of the pyridine nucleotides.

In the present paper, we explore in greater depth the effects of alterations of energy level and pyridine nucleotide oxidation-reduction state on the control properties of the citric acid cycle in intact heart mitochondria. Five respiratory states have been studied, which exhibited either low or high flux through the electron transport chain. State 4, with high intramitochondrial ATP, is compared with the ATP-depleted oligomycin-inhibited state, while for higher respiratory rates, State 3 is compared with the uncoupled state and the uncoupled plus oligomycin state. Variations of flux through specific enzyme steps have been correlated with changes of known modulators in order to assess the possible physiological significance of the feedback interactions. Our major conclusion is that in addition to an important influence of the pyridine nucleotide oxidation-reduction state on the relative and absolute concentrations of intramitochondrial malate and oxalacetate, feedback to the citric acid cycle from the phosphorylation state of intramitochondrial adenine nucleotides is mediated indirectly; first via the GTP: GDP ratio which influences the succinyl-CoA concentration, and secondly by succinyl-CoA inhibition of citrate synthase.

**EXPERIMENTAL PROCEDURE**

**Preparation and Incubation of Mitochondria**—Mitochondria were prepared as described previously (1) from 4 to 10 pooled hearts of fed, Holtzman rats (250 to 300 g) by a slight modification of the manganous succrose EDTA method of Chance and Hagihara (5). The basic incubation medium contained 130 mM KCl, 20 mM KH₂PO₄, 20 mM Tris chloride, 5 mM MgCl₂, and 30 mM glucose, adjusted to pH 7.2. For experiments in which samples were taken for analyses, the buffer was saturated with 100% oxygen, and oxygen was blown over the surface of the stirred reaction mixture. Mitochondrial metabolism was studied in five separate respiratory states, each of which included buffer, substrate, and 1 to 3 mg per ml of mitochondrial protein in a total volume of 6 ml contained in a water-jacketed chamber maintained at 28°C. Additional inclusions were: (a) State 3: no additions; (b) oligomycin-inhibited: hexokinase (1 mg per ml), ADP (100 μM), and oligomycin (up to 1 μg per mg of protein); (c) State 3: hexokinase (1 mg per ml) and ADP (100 μM); (d) uncoupled: FCCP, (~0.2 μM); and (e) uncoupled plus oligomycin: FCCP (~0.2 μM) and oligomycin (1 μg per mg of protein).

**Analytical Methods—Oxygen** was measured polarographically with a Clark electrode. Metabolic intermediates in aliquots of the reaction medium were extracted and assayed fluorometrically using enzymic methods (6), or isopiotically as described previously (1). CoA and acetyl-CoA were measured directly by the method of Garland et al. (7). Total acid-soluble acetyl-CoA plus CoA was measured as CoA after alkaline hydrolysis of an aliquot of the neutralized perchloric acid extract in the presence of 5 mM dithiothreitol (6). Unknown acyl-CoA was calculated by subtracting values for CoA and acetyl-CoA from the total acid-soluble CoA content. Succinyl-CoA (up to 1 μM) was measured fluorometrically by coupling succinyl-CoA transferase to β-hydroxybutyryl-CoA dehydrogenase with a reaction medium containing 0.1 mM KH₂PO₄, pH 7.0, 5 μM NADH, and 1 mM lithium acetacetate. The lithium salt of acetacetate was prepared by the method of Hall (8) and was recrystallized three times. The succinyl-CoA reaction reached completion in about 10 min, and the unknowns were compared with internal standards. The reaction ended in a slight drift due to NADH oxidase activity in the succinyl-CoA transferase. This was corrected for by subtracting the slope of the drift from the over-all NADH fluorescence decrease. Recoveries of succinyl-CoA added to mitochondrial extracts were better than 80%. Succinyl-CoA transferase was prepared from beef heart acetone powder. Mitochondrial protein was determined by the biuret method (11).

**Citrull acid cycle intermediates** were separated by Dowex-1 plus chromatography for radioactivity determinations as described previously (1). Radioactivities were measured with a Packard Tri-Carb liquid scintillation spectrometer.

**Separation of Mitochondria from Incubation Medium**—Several methods for the separation and quenching of mitochondria from the incubation medium in times fast enough to prevent them from becoming anaerobic were investigated. The basic test used was whether the content of adenine nucleotides in the supernatant and mitochondria after separation equaled the amount found in the whole mitochondrial extract. Centrifugation in the manner described by Pfaff (12), as practiced by Papa et al. (13) was unsuitable because it was too slow for conditions associated with actively respiring mitochondria (14). Filtration through Millipore filters as described by Garber and Ballard (15) was unsatisfactory for two reasons: the mitochondria became anaerobic on the filter, and the sucrose wash, designed to remove contaminating extramitochondrial fluid, also removed most of the intramitochondrial intermediates. This was shown by a drastic fall of 14C-counts on the filter and an increase in the ratio of 14C-counts to 3H-counts in the wash fluid when [14C]pyruvate was used as substrate and D-[1-3H]mannitol used as an extramitochondrial volume marker.

Two modifications, one of the microcentrifugation method (16, 17) and the other of the filtration method, gave satisfactory results within limitations. The centrifugation method relies on the mitochondria moving through a silicone oil layer to a layer

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1 Type V from Sigma Chemical Co.
2 The abbreviation used is: FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone. FCCP was generously supplied by Dr. Peter Heytler.
of perchloric acid on the bottom of the tube fast enough that the limited oxygen content of silicone oil does not become exhausted. For heart mitochondria in State 3, this time is 5 to 10 sec. The Coleman bench top centrifuge used by Harris and Van Dam (16) has a rapid acceleration time, but the capacity of the centrifuge tubes (0.35 ml) is impractical when multiple assays of metabolites in the mitochondrial matrix are required. The centrifuge head was modified to hold two tubes of 0.5 ml capacity which allowed 0.5 ml of a mitochondrial suspension containing 8% (w/v) dextran and 4 to 6 mg per ml of protein to be placed above the silicone oil layer (1.05 specific gravity), and be centrifuged into 0.1 ml of 1.5 x perchloric acid. The top layer after centrifugation was immediately transferred to perchloric acid to prevent enzyme transformation of the metabolites. The main advantage of the centrifugation method is the small volume of extramatrix fluid carried down with the mitochondria (about 4 µl per mg of protein).

A satisfactory filtration method was devised only with the use of automated time-programmed air-driven pistons connected to two syringes, one containing the mitochondrial suspension and the other 0.5 x perchloric acid. A brief pulse of oxygen was blown through the filter prior to delivery of the perchloric acid to aid removal of extramitochondrial fluid and ensure oxygenation of the mitochondria. With this instrument, the filtration cycle occurred in 1 to 2 sec, and could be repeated every 5 sec. Two 2.5-cm diameter Millipore filters (0.65 µm pore size) were used after rigorous washing with perchloric acid and water to remove detergents and other compounds which interfered with the analytical assays. The filters were able to retain up to 0 mg of mitochondrial protein, but the major disadvantage of the method was the relatively large volume of extramatrix water trapped with the mitochondria (0.2 to 0.3 ml). d-[3H]Mannitol was routinely added to the mitochondrial incubation mixture to permit calculation of the volume of extramitochondrial fluid retained with the mitochondria and subsequently extracted into the perchloric acid.

RESULTS

Correlation between Rates of Oxygen Consumption and Extent of Reduction of Pyridine Nucleotides in Various Energy States of Mitochondria—Initial rates of oxygen utilization by rat heart mitochondria metabolizing a variety of substrates in different metabolic states are shown in Table I. Linear rates were obtained under most experimental conditions. When compared with State 4 respiration, oxygen uptake was stimulated 6- to 12-fold by addition of ADP or uncoupler. The State 3 rate of oxygen utilization to the initial values.

<table>
<thead>
<tr>
<th>Condition</th>
<th>2 min Pyruvate</th>
<th>2 min Pyruvate plus 1 mM malate</th>
<th>2 min Pyruvate plus 5 mM malate</th>
<th>2 min Acetylcarnitine plus 1 mM malate</th>
</tr>
</thead>
<tbody>
<tr>
<td>State 4</td>
<td>36</td>
<td>38</td>
<td>33</td>
<td>37</td>
</tr>
<tr>
<td>Oligomycin-ADP</td>
<td>26</td>
<td>42</td>
<td>40</td>
<td>35</td>
</tr>
<tr>
<td>State 3</td>
<td>340</td>
<td>379</td>
<td>307</td>
<td>292</td>
</tr>
<tr>
<td>Uncoupled</td>
<td>313 (140)*</td>
<td>286</td>
<td>387</td>
<td>202</td>
</tr>
<tr>
<td>Uncoupled + oligomycin</td>
<td>264 (70)*</td>
<td>360</td>
<td>360</td>
<td>202 (53)*</td>
</tr>
</tbody>
</table>

* Numbers in parentheses refer to rates after 2 min and are shown only in the situations where these were different from the initial rates.

These findings suggested an involvement of ATP in the control of the citric acid cycle which was dependent on the nature of the acetyl-CoA donor, and prompted us to characterize all five metabolic states with regard to the degree of reduction of the pyridine nucleotides and the phosphorylation state of the intramitochondrial adenine nucleotides. Table II shows a summary of values obtained for the levels of NADH 2 min after addition of substrate, and the calculated NADH:NAD ratios based on a mean NADH + NAD content of 6.2 nmoles per mg of mitochondrial protein. The content of NADPH was also measured in these experiments but showed little change with the different experimental conditions varying only over a total range of 0.76 to 1.56 nmoles per mg of protein. With each substrate, the NADH content was highest in the oligomycin-ADP-inhibited state, and lowest in the presence of uncoupler. Addition of oligomycin in the uncoupled state did not appreciably affect the NADH content. Malate added together with pyruvate increased NADH levels in each of the metabolic states, and 5 mM malate had a larger effect than 1 mM. Malate also allowed a more complete reduction of NAD (up to 86%) in the presence of rotenone. In general, pyruvate in the presence of 1 mM malate produced a greater degree of NAD reduction in each metabolic state than acetylcarnitine and 1 mM malate. However, a comparison of Tables I and II shows that under conditions of active respiration, there was no proportionality between the mitochondrial NADH content and the rate of respiration.

The above results suggested that externally added malate had a limited ability to increase the malate concentration in the matrix space. Measured intramitochondrial malate concentrations (Table III) confirmed this conclusion. The concentration of added malate in these experiments was limited to 0.1.
The concentration of malate in the mitochondrial matrix space (malate in to malate out) was decreased by malate because of the difficulty of measuring the low mitochondrial matrix contents in the presence of higher extramitochondrial malate concentrations. Addition of malate increased the intramitochondrial malate concentration in all metabolic states, the greatest effect being in State 4 and the oligomycin-inhibited state, namely under conditions of high NAD reduction. The ratio of the concentrations of malate in the matrix and extra-matrix space (malate in to malate out) was decreased by malate addition to values which approached unity in the presence of uncoupler. The concentration of malate in the mitochondrial matrix space was about the same in States 3 and 4, increased 2-fold in the oligomycin-inhibited state and decreased 2- to 4-fold in the presence of uncoupler. In the absence of added malate, the intramitochondrial malate concentration became immeasurably low with longer times of incubation in the uncoupled state, namely under conditions of high NAD reduction. The ratio of the concentrations of malate in the matrix and extra-matrix space (malate in to malate out) was decreased by malate because of the difficulty of measuring the low mitochondrial matrix contents in the presence of higher extramitochondrial malate concentrations. Addition of malate increased the intramitochondrial malate concentration in all metabolic states, the greatest effect being in State 4 and the oligomycin-inhibited state, namely under conditions of high NAD reduction. The ratio of the concentrations of malate in the matrix and extra-matrix space (malate in to malate out) was decreased by malate addition to values which approached unity in the presence of uncoupler. The concentration of malate in the mitochondrial matrix space was about the same in States 3 and 4, increased 2-fold in the oligomycin-inhibited state and decreased 2- to 4-fold in the presence of uncoupler. In the absence of added malate, the intramitochondrial malate concentration became immeasurably low with longer times of incubation in the uncoupled state, in accordance with the decreased rate of respiration. The volume of the mitochondrial matrix remained relatively constant at about 1 ul per mg of protein with the different respiratory conditions. These data indicate that changes of the intramitochondrial malate concentration and the NAD oxidation-reduction state both contributed toward determining the intramitochondrial oxalacetate concentration in the different respiratory states, but provided no explanations for the inhibition of respiration observed in the presence of uncoupler and oligomycin with acetyl carnitine and malate as substrate or the increased NADH formation observed in the oligomycin-inhibited state. Changes of the pyridine nucleotide oxidation-reduction level as a function of energy state and malate availability were further investigated with a combination of direct measurements of pyridine nucleotide fluorescence and sampling of the incubation medium to determine the contents of NADH and NADPH by direct assay. Data obtained with acetyl carnitine plus 1 mM malate as substrate are shown in Fig. 1. The tracing shown on the left hand side of the figure was performed in the absence of oligomycin and that on the right hand side in its presence. In the absence of substrate the contents of both NADH and NADPH were low (<0.1 nmole per mg of protein). After the addition of substrate, the degree of reduction of the pyridine nucleotides increased (State 4 level), and exhibited a cycle of NADH formation observed in the oligomycin-inhibited state. Conditions of energy state and malate availability were further investigated with a combination of direct measurements of pyridine nucleotide fluorescence and sampling of the incubation medium to determine the contents of NADH and NADPH by direct assay. Data obtained with acetyl carnitine plus 1 mM malate as substrate are shown in Fig. 1. The tracing shown on the left hand side of the figure was performed in the absence of oligomycin and that on the right hand side in its presence. In the absence of substrate the contents of both NADH and NADPH were low (<0.1 nmole per mg of protein). After the addition of substrate, the degree of reduction of the pyridine nucleotides increased (State 4 level), and exhibited a cycle of oxidation (State 3 level) upon addition of ADP. The State 4 to 3 transition was characterized by a large decrease of the NADH level and only a small decrease of the NADPH level. Oligomycin had a small effect on the level of reduction of the pyridine nucleotides attained after addition of substrate (cf. Samples 2 and 6, Fig. 1). However, a striking increase in NADH was observed upon addition of ADP to the oligomycin-
inhibited mitochondria. Similar changes were observed with pyruvate plus malate as substrates in the presence and absence of oligomycin.

Further experiments were designed to determine which dehydrogenase step was activated upon ADP addition. When arsenite, an inhibitor of α-ketoglutarate oxidation, was included in the reaction mixture with acetyl carnitine and malate (Fig. 2), the State 4 to 3 transition was normal. However, the increased formation of NADH induced by the addition of ADP in the presence of oligomycin was abolished. Moreover, while no oligomycin-ADP-induced reduction of NADH was obtained with succinate as substrate, the largest effect was observed in the presence of α-ketoglutarate (19). These results suggest that removal of succinyl-CoA inhibition of α-ketoglutarate dehydrogenase as a result of a decreased GTP:GDP ratio is responsible for the higher state of reduction of the pyridine nucleotides upon addition of ADP in the presence of oligomycin. Presumably increased flux from α-ketoglutarate to malate also accounts for the higher intramitochondrial malate concentration observed under these conditions.

Intramitochondrial ATP:ADP Ratios—Changes of the ATP content of the mitochondrial suspensions incubated under different respiratory states in the presence of acetyl carnitine and malate are shown in Fig. 3. Similar results were obtained when other substrates were used. No data are shown for State 3 since the measured ATP in that situation (3 to 10 nmoles per mg of protein) is a function merely of the relative activity of the mitochondrial phosphorylating system and the extramitochondrial glucose-hexokinase-phosphorylating system. As shown in Fig. 3, the ATP level remained constant after the first minute in all metabolic situations studied with the exception of the uncoupled state where it appeared to rise slowly. In the presence of the low concentrations of uncoupler used in these experiments, substrate level phosphorylation presumably proceeds at a slightly faster rate than dissipation of ATP by mitochondrial ATPases, so that phosphate transfer from GTP to ADP via nucleoside diphosphate kinase causes a slow net formation of ATP. After addition of oligomycin to the uncoupled mitochondria, intramitochondrial dissipation of ATP was inhibited, and the ATP level reached higher values than with uncoupler alone. When oligomycin alone was added under State 4 conditions, a slow fall of ATP was observed, but addition of oligomycin together with 100 μM ADP and glucose-hexokinase as shown in Fig. 3 resulted in a rapid depletion of ATP to 1 n mole per mg of protein. Presumably the extramitochondrial ADP exchanged with intramitochondrial ATP on the adenine nucleotide carrier (20, 21) and was subsequently removed by the glucose-hexokinase-trapping system, as confirmed by measurements of glucose-6-P formation.

Since the matrix enzymes are affected only by the intramitochondrial concentrations of the adenine nucleotides, separation techniques were used to measure intr- and extramitochondrial contents of ATP, ADP, and AMP in the various metabolic states 2 min after the addition of substrate (Fig. 4). Acetyl carnitine plus malate was the substrate used in this study, but essentially similar results were obtained with pyruvate plus malate as substrate. An ATP:ADP ratio of about 0.7 was observed in both State 3 and the uncoupled state. The absolute intramitochondrial ATP and ADP levels, however, were slightly higher in State 3. An unexplained difference between State 3 and the uncoupled state appeared in the intramitochondrial

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**Fig. 1.** Effect of oligomycin on pyridine nucleotide reduction as measured by fluorescence changes in suspensions of rat heart mitochondria with acetyl carnitine and malate as substrate. Mitochondria (~3 mg per ml) were incubated in buffer containing 130 mM KCl, 20 mM KH2PO4, 20 mM Tris-chloride, 5 mM MgCl2, and 30 mM glucose, adjusted to pH 7.2 and saturated with 100% oxygen. Further additions to the media are shown on the figure. The initial volume in the cuvette was 2.5 ml, and 0.5-ml samples were taken for analyses at the times indicated.

**Fig. 2.** Effect of oligomycin on pyridine nucleotide reduction as measured by fluorescence changes in suspensions of rat heart mitochondria with acetyl carnitine and malate as substrate in the presence of arsenite. The incubation conditions were the same as in Fig. 1.

**Fig. 3.** Changes of the ATP content of suspensions of rat heart mitochondria incubated under different respiratory states, as described under “Experimental Procedure.” The substrate used in each state was 5 mM acetyl carnitine and 1 mM malate. The total volume of the reaction media was 5 ml. One-milliliter samples containing 1 to 2 mg of mitochondrial protein were taken for analyses at the stated intervals.

AMP levels, which were an order of magnitude higher in State 3. The total content of intramitochondrial nucleotides was also lower in the presence of uncoupler than in other states. Oligomycin plus ADP decreased the ATP:ADP ratio from 6 to 0.1
rate dehydrogenase and ɑ-ketoglutarate dehydrogenase were observed with pyruvate or acetylcarnitine and 1 mM malate. Similar effects of oligomycin in stimulating flux through isocitrate (Figs. 1 and 2), and also a stimulation of citrate utilization.

When intramitochondrial ATP levels decrease, in accordance with the conclusion reached from studies of the NADH fluorescence in suspensions of rat heart mitochondria incubated for 2 min in the presence of 5 mM acetylcarnitine and 1 mM malate. Metabolic states were produced as described under "Experimental Procedure." Mitochondria were separated from the medium by filtration through Millipore filters.

Accumulation of Cycle Intermediates: Comparison of State 4 and Oligomycin-Inhibited State—In order to identify regulatory steps of the citric acid cycle associated with changes of NADH and ATP, rates of substrate utilization and rates of accumulation of cycle intermediates were measured. With this data, flux through individual reaction steps can be calculated (1). In State 4, the respiratory chain is inhibited by lack of ADP, while in the presence of oligomycin and ADP, the respiratory chain is inhibited by an inability to use the ADP provided. With [3-14C]pyruvate and 5 mM malate as substrates, rates of pyruvate utilization were measured in conjunction with rates of accumulation of citrate, ɑ-ketoglutarate, and succinate (Fig. 5). Incorporation of isotope from pyruvate into the large pool of unlabeled malate provides an estimate of net cycle flux (1). Pyruvate utilization (30 nmoles per mg per min, not shown) was approximately the same in the two states. Oligomycin (0.35 μg per mg of protein) markedly decreased the rates of appearance of citrate and ɑ-ketoglutarate and increased those of succinate and [14C]malate. These data suggest a stimulation of ɑ-ketoglutarate utilization when intramitochondrial ATP levels decrease, in accordance with the conclusion reached from studies of the NADH fluorescence (Figs. 1 and 2), and also a stimulation of citrate utilization. Similar effects of oligomycin in stimulating flux through isocitrate dehydrogenase and ɑ-ketoglutarate dehydrogenase were observed with pyruvate or acetylcarnitine and 1 mM malate as substrates (19). These results showed that the effect of oligomycin on the rates of substrate accumulation was independent of the method of generating acetyl-CoA, but that the absolute rates depended on the malate concentration.

Data showing flux through individual steps in the citric acid cycle in State 4 and in the presence of oligomycin, with pyruvate plus malate or acetylcarnitine plus malate as substrates are summarized in Table IV. In State 4, the largest decrease in cycle flux occurred between isocitrate dehydrogenase and ɑ-ketoglutarate dehydrogenase resulting in a substantial accumulation of ɑ-ketoglutarate. This was smaller at the lower malate concentration. In the presence of oligomycin, very little accumulation of ɑ-ketoglutarate occurred, and flux through ɑ-ketoglutarate dehydrogenase became equal or greater than flux through citrate synthase. The inhibition of ɑ-ketoglutarate dehydrogenase observed in State 4 is probably caused by the accumulation of succinyl-CoA (22), since substrate level phosphorylation will be inhibited when mitochondrial GDP levels are low.

Accumulation of Cycle Intermediates: Comparison of State 3 and Uncoupled States—The accumulations of citric acid cycle intermediates in State 3, the uncoupled state, and the uncoupled plus oligomycin state are shown in Fig. 6. Pyruvate (1 mM) plus 1 mM malate were present as substrates. Pyruvate uptake was the same in State 3 as in the uncoupled state but was slightly inhibited after 2 min of incubation in the presence of uncoupler plus oligomycin. The rates of oxygen utilisation were about the same in these respiratory states, but the ATP:ADP ratios were very different, being 0.7, 0.3, and 6.5, respectively, for the State 3, uncoupled and uncoupled plus oligomycin respiratory conditions. Citrate accumulation rapidly reached its low equilibrium value (2 to 4 nmoles per mg) in all three states. Signif-
Effect of oligomycin on flux through individual steps of citric acid cycle

Rat heart mitochondria (1 to 2 mg of protein per ml) were incubated in media containing either 1 mM [3H]pyruvate and malate (1 or 5 mM) or 5 mM acetyl carnitine and 1 mM malate. Flow rates through the individual steps were calculated on the basis of the measured rates of accumulation of intermediates in the medium and the rate of substrate removal over the time interval from 3 to 6 min of incubation. The rate of acetyl carnitine oxidation was measured by the rate of carnitine appearance. Incubation conditions were similar to those described in Table I.

Table IV

<table>
<thead>
<tr>
<th>Reaction step</th>
<th>Pyruvate dehydrogenase</th>
<th>Citrate synthase</th>
<th>Isocitrate dehydrogenase</th>
<th>a-Ketoglutarate dehydrogenase</th>
<th>Succinate dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM Pyruvate + 3 mM malate</td>
<td>10.2 μmol/min/mg protein</td>
<td>38.6 μmol/min/mg protein</td>
<td>17.3 μmol/min/mg protein</td>
<td>24.3 μmol/min/mg protein</td>
<td>24.0 μmol/min/mg protein</td>
</tr>
<tr>
<td>1 mM Pyruvate + 1 mM malate</td>
<td>18.9 μmol/min/mg protein</td>
<td>38.3 μmol/min/mg protein</td>
<td>16.3 μmol/min/mg protein</td>
<td>24.0 μmol/min/mg protein</td>
<td>23.0 μmol/min/mg protein</td>
</tr>
<tr>
<td>5 mM Acetyl carnitine + 1 mM malate</td>
<td>15.9 μmol/min/mg protein</td>
<td>37.4 μmol/min/mg protein</td>
<td>15.0 μmol/min/mg protein</td>
<td>21.5 μmol/min/mg protein</td>
<td>22.0 μmol/min/mg protein</td>
</tr>
</tbody>
</table>

Fig. 6. Effect of uncoupler and uncoupler plus oligomycin on changes in the levels of citric acid cycle intermediates in rat heart mitochondria incubated in the presence of 2 mM pyruvate and 1 mM malate. Reactions were started by the addition of mitochondria (1.3 mg per ml). The initial volume was 6 ml, and 1-ml aliquots were removed for analyses at the stated times.

Fig. 7. Effect of uncoupler and uncoupler plus oligomycin on changes in the levels of citric acid cycle intermediates in rat heart mitochondria incubated in the presence of 5 mM acetyl carnitine and 1 mM malate. Reactions were started with the addition of mitochondria (1.4 mg per ml). The initial volume was 6 ml, and 1-ml aliquots were removed for analyses at the stated times.

Significant variations of the activity of α-ketoglutarate dehydrogenase are apparent from the differences in the rates of accumulation of α-ketoglutarate. Inhibition at this step in the presence of uncoupler plus oligomycin is suggested not only from the large amounts of α-ketoglutarate which accumulated, but also from the small accumulation of succinate. On the other hand with uncoupler alone, the increased accumulations of both α-ketoglutarate and succinate relative to State 3 suggest that the permeability properties of the inner mitochondrial membrane to α-ketoglutarate and succinate may be altered in the uncoupled state (23, 24).

The results of a similar experiment with 5 mM acetyl carnitine and 1 mM malate as substrates are shown in Fig. 7. In these experiments, carnitine production may be equated with flux through citrate synthase since no significant accumulations of acetyl-CoA or ketone bodies occur (1). The release of carnitine was linear in State 3 at a rate of 95 nmol/sec per mg of protein. Carnitine production decreased by 25% in the presence of uncoupler, but was inhibited by 65% when oligomycin was added together with uncoupler. This effect was also observed on the rate of oxygen utilization (Table I). α-Ketoglutarate accumulated more in the uncoupled state than in State 3, but unlike the experiment with pyruvate and malate as substrates (Fig. 6), α-ketoglutarate accumulated less in the presence of uncoupler plus oligomycin. This is a direct consequence of the decreased rate of citrate formation, as shown by the diminished rate of carnitine production and the lower citrate accumulation when oligomycin was added together with uncoupler. Likewise, succinate accumulation was also inhibited in the uncoupled plus oligomycin state, but was also lower in the uncoupled state than in State 3.

A comparison of flow rates through the individual steps of the
cycle in mitochondria incubated with pyruvate and malate or acetyl carnitine and malate under conditions of State 3, uncoupled and uncoupled plus oligomycin respiration is shown in Table V. These results show that addition of oligomycin to the uncoupled state decreased flux through citrate synthase to a greater extent with acetyl carnitine and malate as substrates than with pyruvate and malate as substrates. On the other hand, with the latter substrate pair there was a much larger inhibition at the α-ketoglutarate dehydrogenase step in this respiratory state. Since oligomycin added with uncoupler increased the intramitochondrial ATP:ADP ratio with either type of substrate, the data suggest a rather indirect relationship between the energy state of the mitochondria and the activities of citrate synthase and α-ketoglutarate dehydrogenase. This conclusion was substantiated by other experiments in which the high ATP:ADP ratio obtained in the uncoupled plus oligomycin state was decreased by the further addition of hexokinase. Thus, after 4 min of incubation, addition of hexokinase decreased the intramitochondrial ATP:ADP ratio from 6.5 to 0.8 with pyruvate and malate as substrates with little effect on respiration, and from 4.1 to 0.6 with acetyl carnitine and malate as substrates with a stimulation of respiration to the rate observed with uncoupler alone.

**Table V**

*Effect of uncoupler and uncoupler plus oligomycin on flux through individual steps of citric acid cycle*

Rat heart mitochondria (1 to 2 mg of protein per ml) were incubated under conditions described in Table I. Fluxes were calculated on the basis of the measured rates of substrate disappearance and accumulation of intermediates over the time interval from 2 to 4 min of incubation as illustrated in Figs. 6 and 7.

<table>
<thead>
<tr>
<th>Reaction step</th>
<th>2 mM Pyruvate + 1 mM Malate</th>
<th>5 mM Acetyl carnitine + 1 mM Malate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate → acetyl-CoA</td>
<td>98 100 75 95 73 33</td>
<td>98 100 75 95 73 33</td>
</tr>
<tr>
<td>Acetyl-CoA → citrate…</td>
<td>98 100 75 95 73 33</td>
<td>98 100 75 95 73 33</td>
</tr>
<tr>
<td>Citrate → α-ketoglutarate…</td>
<td>98 100 75 95 73 33</td>
<td>98 100 75 95 73 33</td>
</tr>
<tr>
<td>α-Ketoglutarate → succinate…</td>
<td>98 86 39 89 56 29</td>
<td>98 86 39 89 56 29</td>
</tr>
<tr>
<td>Succinate → malate…</td>
<td>81 46 35 70 48 25</td>
<td>81 46 35 70 48 25</td>
</tr>
<tr>
<td>Succinate → malate*…</td>
<td>71 48 26 65 50 24</td>
<td>71 48 26 65 50 24</td>
</tr>
</tbody>
</table>

* Calculated from rates of malate uptake and substrate utilization.

**Fig. 8.** Contents of CoA, acetyl-CoA, and acid-soluble acyl-CoA in rat heart mitochondria incubated in the presence of 2 mM pyruvate and 1 mM malate. The changes in State 4 are compared with those in the oligomycin-inhibited state. Zero time samples were obtained by adding mitochondria to 1 ml of medium containing 0.3 ml of 18% (w/v) perchloric acid. The initial volume was 9 ml, and contained approximately 4 mg of mitochondrial protein per ml. At the stated intervals after the addition of mitochondria, 1.5-ml samples were removed for analyses.

**Fig. 9.** Contents of CoA, acetyl-CoA, acid-soluble acyl-CoA, and succinyl-CoA in rat heart mitochondria incubated in the presence of 5 mM acetyl carnitine and 1 mM malate. The changes in State 4 are compared with those in the oligomycin-inhibited state.
gradually accumulated with time during incubation with either substrate under State 4 conditions. Direct assays showed that the unknown acyl-CoA could not be accounted for as propionyl CoA or acetoacetoyl-CoA, but that its amount agreed quantitatively with the succinyl-CoA content. Third, in the oligomycin-ADP-inhibited state, the acetyl-CoA levels tended to remain elevated for longer periods than during State 4 respiration, but accumulations of the acyl-CoA compound (Fig. 8) and succinyl-CoA (Fig. 9) were decreased. It is apparent, therefore, that the measured fall of succinyl-CoA levels in the oligomycin-inhibited state is in agreement with its postulated role in the control of α-ketoglutarate dehydrogenase.

A similar comparison of CoA and the contents of acyl-CoA derivatives in mitochondria incubated under conditions of State 3, uncoupled and uncoupled plus oligomycin respiration are shown in Fig. 10 with pyruvate plus malate as substrate, and in Fig. 11 with acetylcarnitine plus malate as substrate. The acetyl-CoA content was highest in State 3 with both substrates. The measured contents of succinyl-CoA agreed very well with the calculated contents of the acyl-CoA derivative and were the same in State 3 and in the uncoupled state, but were markedly increased in the uncoupled plus oligomycin state with both substrates. Furthermore, addition of hexokinase to the uncoupled plus oligomycin state caused a marked fall of the succinyl-CoA levels. It is apparent, therefore, that there is a correlation between the intramitochondrial ATP:ADP ratio (cf. Fig. 4) and the succinyl-CoA content. This is to be expected if the ATP-ADP couple is in equilibrium with the GTP-GDP couple via nucleoside diphosphate kinase, since the rate of succinyl-CoA utilization by substrate level phosphorylation will depend on the availability of GDP. Of particular importance in these results is the fact that in the uncoupled and uncoupled plus oligomycin states acetyl-CoA levels were about 2 fold higher with pyruvate than with acetylcarnitine as substrate, since this was the only major difference observed which could relate to the different flux through citrate synthase.

The relatively high content of CoA under all experimental conditions (0.1 to 1.4 nmoles per mg of protein) suggests that it does not become rate limiting for acetylcarnitine transferase. The concentration of acetylcarnitine producing a half-maximum stimulation of respiration under State 3 conditions was determined to be 0.6 mM. At this concentration, the acetyl CoA content was about 0.1 nmoles per mg of protein.

**Table VI**

*Measurements of intramitochondrial contents of citric acid cycle intermediates with pyruvate as substrate in absence of added malate*

<table>
<thead>
<tr>
<th>Condition</th>
<th>Citrate</th>
<th>α-Ketoglutarate</th>
<th>Succinate</th>
<th>Malate</th>
</tr>
</thead>
<tbody>
<tr>
<td>State 4</td>
<td>0.51 ± 0.09</td>
<td>0.43 ± 0.13</td>
<td>0.16</td>
<td>0.27 ± 0.03</td>
</tr>
<tr>
<td>Oligomycin ADP</td>
<td>0.47 ± 0.1</td>
<td>0.27 ± 0.06</td>
<td>0.20</td>
<td>0.40 ± 0.04</td>
</tr>
<tr>
<td>State 3</td>
<td>0.50 ± 0.11</td>
<td>0.42 ± 0.12</td>
<td>0.13</td>
<td>0.35 ± 0.05</td>
</tr>
</tbody>
</table>

Intramitochondrial Substrate Concentration: Citric Acid Cycle Intermediates—Rapid separation of mitochondria from the incubation medium with pyruvate alone as substrate showed that the contents of all the intermediates of the citric acid cycle in the matrix space of rat heart mitochondria were below 0.5 mM (Table VI). Despite large differences in respiratory rate and citric acid cycle flux between the various states, the matrix content of citrate, α-ketoglutarate, succinate, and malate remained approximately constant, indicating that substrate
control is not a primary factor in the regulation of cycle flux. Measurements of the intramitochondrial contents of citrate, \( \alpha \)-ketoglutarate, and succinate with pyruvate and 1 mm malate as substrate are shown in Table VII. A comparison of Tables VI and VII shows that the presence of 1 mm malate did not greatly change intramitochondrial \( \alpha \)-ketoglutarate levels, although citrate and succinate levels both increased. No large differences of the intramitochondrial levels of \( \alpha \)-ketoglutarate and succinate were observed between states, but citrate varied over a 6-fold range being highest in State 4 and lowest in the presence of uncoupler and oligomycin. Since large differences in the rates of anion efflux from the mitochondria exist both between different states and in the presence and absence of malate, it is evident that the measured intramitochondrial concentrations of the anions must reflect changes in the transport properties of the mitochondrial membrane in addition to changes in the activities of the intramitochondrial enzymes.

### DISCUSSION

**Regulation of Citrate Synthase**—Citrate synthase is the first committed step for oxidation of acetyl-CoA in the citric acid cycle. It is present in relatively high concentration in cardiac mitochondria (25), the reaction is associated with a large negative free energy change (26), and is responsible for allowing flux through malate dehydrogenase in the thermodynamically unfavorable direction. Both isolated heart mitochondria (1) and the perfused rat heart (27) have a limited ability to convert fatty acids to ketone bodies because of the virtual absence of \( \beta \)-hydroxy-\( \beta \)-methylbutyrate-CoA synthase (28), so that flux through citrate synthase is directly related to the over-all rate of oxygen consumption by the myocardium irrespective of the nature of the respiratory fuel. This relationship is clearly revealed by the present experiments with isolated heart mitochondria. It may be expected, therefore, that citrate synthase will be subject to regulation by a variety of different feedback influences from the phosphorylating electron transport chain, which combine to provide a coordination of flux through successive steps of the citric acid cycle so that cycle flux can be geared to the energy requirements of muscular contraction.

Contrary to earlier reports (29, 30), it has now become apparent that citrate synthase is not regulated by an allosteric interaction with modifiers, but by competitive inhibition of metabolites with either of its substrates (31). Studies with crystalline beef heart citrate synthase have shown that CoA and a variety of short chain acyl-CoA esters are strongly competitive with acetyl-CoA, while citrate was found to be strictly competitive with respect to oxalacetate (31). Several discussions of the control of the citric acid cycle at the citrate synthase step (29, 30, 32-35) have focused attention on the possibility of its regulation by ATP since free ATP was shown to be inhibitory and would provide the most direct feedback interaction between the citric acid cycle and the respiratory chain. This theory essentially became untenable when it was realized that the Mg-ATP complex was much less inhibitory than free ATP (36), and that the amount of magnesium available in the mitochondrial matrix for nucleotide binding was in excess of the ATP content (37, 38), thereby providing presumptive evidence that mitochondrial ATP was available only as the Mg-ATP chelate. An alternative theory (39), based on regulation of citrate synthase by oxalacetate as determined by the mitochondrial NAD oxidation-reduction state, has received experimental support particularly with studies utilizing isolated liver mitochondria (40-45) and perfused livers (46, 47). However, this method of regulation is insufficient from a theoretical point of view since the pyridine nucleotide oxidation-reduction state is determined by the supply and nature of the substrate as well as by the activity of the respiratory chain (48). Furthermore, in previous experiments the intramitochondrial malate concentration was not defined so that the relationship between changes of the NADH:NAD and the intramitochondrial oxalacetate concentration was indefinite.

Data presented in this paper together with studies on the kinetic properties of isolated citrate synthase (31), permit a more detailed evaluation of the various factors contributing to the regulation of citrate synthase in intact uninhibited mitochondria respiring in different metabolic states. It is now clear that citrate synthase activity is regulated through alterations of both the availability and affinity of either of its substrates to the enzyme, the latter change being mediated by competitive inhibitors. Direct estimates of the amount of oxalacetate available to citrate synthase cannot readily be made since the total oxalacetate pool will largely be bound to malate dehydrogenase, glutamate oxalacetate transaminase, as well as to citrate synthase. Assayable oxalacetate in the matrix of rat heart mitochondria incubated under State 4 conditions amounted to about 38 \( \mu \)M (38). However, calculations of the amount of free oxalacetate in equilibrium with malate dehydrogenase provided a value two orders of magnitude lower than the total oxalacetate content, which agrees better with the value of 1.6 \( \mu \)M observed for the \( K_m \) of citrate synthase for oxalacetate (31). Since citrate synthase in cardiac tissue operates far below its \( V_{\max} \) in all metabolic states (34), it is reasonable to assume that the effective oxalacetate concentration at the active site may be less than the \( K_m \) and is responsive both to changes of the malate concentration and the NADH:NAD ratio in the matrix. The present data are consistent with the proposal that under conditions of controlled respiration (State 4), when acetyl-CoA levels are high and the NADH:NAD ratio is also high, flux through citrate synthase is limited by the availability of oxalacetate. Also, under these conditions, the mitochondrial citrate concentration is high relative to its \( K_i \) for citrate synthase because of the low affinity of isocitrate dehydrogenase, so that citrate synthase activity may be further depressed due to competitive inhibition between citrate and oxalacetate.

The fine balance between these various control factors is fur-
ther emphasized from comparisons between State 4 and the oligomycin-inhibited state. Flux through citrate synthase was approximately the same in the two states, but the increase in the NADH:NAD ratio observed in the presence of oligomycin and ADP, which would tend to decrease the oxaloacetate concentration, was counteracted by an increased malate concentration and a decreased citrate concentration. These changes may be ascribed to the fall of the ATP:ADP ratio, which affected the relative concentrations of the citric acid cycle intermediates in the matrix by the activation effects of ADP and GDP at NAD-isocitrate dehydrogenase and succinate thiokinase, respectively, but not the cycle input flux through citrate synthase. Succinyl-CoA levels decreased in the oligomycin-ADP state while acetyl-CoA levels increased. However, citrate synthase activity under these conditions is probably less influenced by changes of the acetyl-CoA than the oxaloacetate concentration. The data clearly indicate the absence of a direct ATP interaction at cit-
acetyl- than the oxaloacetate concentration. The data clearly indicate the absence of a direct ATP interaction at cit-

acet-

but Ilot the cycle input flux through citrate synthase. Succinyl-CoA inhibition in intact mitochondria does not appear to be greatly changed from values obtained with the isolated enzyme since a value of 2/0 μM was determined from Dixon plots of the reciprocal of flux through citrate synthase against succinyl-CoA concentration at various acetyl-CoA ranges (38). However, it is clear that succinyl-CoA will only be an effective inhibitor when its concentration rises well above the K_i for succinyl-CoA and when the acetyl-CoA concentration approaches the K_m region. The measured succinyl-CoA concentration increased to 1.8 μM in the uncoupled plus oligomycin state compared with concentrations of 0.5 to 0.7 in State 3 or the uncoupled state, thereby fulfilling the first criterion. Furthermore, since the measured succinyl-CoA levels agreed closely with the calculated content of short chain acyl-CoA compounds other than acetyl-CoA, it is clear that no other acyl-CoA compound can be involved in citrate synthase regulation under the conditions of the experiments. A comparison of acetyl-CoA levels with pyruvate and malate or acetyl carnitine and malate as substrates suggests that variations of acetyl-CoA concentration in the region of 100 to 300 μM are sufficient to produce a large effect on flux through citrate synthase when succinyl-CoA levels are high. We conclude, therefore, that regulation of citrate synthase activity by succinyl-CoA is achieved under conditions of high oxaloacetate and low acetyl-CoA availability.

Regulation of α-Ketoglutarate Dehydrogenase—Measurements of the accumulation of citric acid cycle intermediates in the experiments reported in this paper show that flux through α-ketoglutarate dehydrogenase and rates of efflux of α-ketoglutarate from the mitochondria are also controlled by the intramitochondrial concentration of succinyl-CoA. Inhibition of the isolated enzyme by succinyl-CoA was first demonstrated by Garland (22). More complete kinetic studies with pig heart α-ketoglutarate dehydrogenase showed that the K_m for CoA was 3.6 μM and that succinyl-CoA was a competitive inhibitor with a K_i of about 10 μM (38). Regulation of α-ketoglutarate dehydrogenase activity by the intramitochondrial succinyl-CoA concentrations is shown by the inhibition of α-ketoglutarate accumulation in the oligomycin-inhibited state relative to State 4 (Fig. 5), and by the stimulation of α-ketoglutarate accumulation in the uncoupled plus oligomycin state relative to the uncoupled state with pyruvate as substrate (Fig. 6). These changes correlate well with changes of the succinyl-CoA levels, but not with changes of the NADH levels, indicating that NADH has a much weaker inhibitory effect on α-ketoglutarate dehydrogenase than succinyl-CoA. The significance of this interaction, discussed at greater length elsewhere (38), is that it will exert a strong influence on the final extramitochondrial α-ketoglutarate concentration obtained in the steady state when rates of α-ketoglutarate efflux become equal to the rates of influx, as applies in the in vivo situation. Moreover, α-ketoglutarate efflux from mitochondria is an integral feature of the malate-aspartate shuttle (19), which in cardiac tissue is the principal mechanism

* C. M. Smith, unpublished observations.
for the transport of NADH from the cytosolic to the mitochondrial space (49). Regulation of flux through α-ketoglutarate dehydrogenase also provides the basis for the control of transamination reactions in the cytosol during transient states associated with repletion or depletion of citric acid cycle intermediates in heart (49).

**Control of Succinate Thiokinase and Succinate Dehydrogenase** -
The relationship between ATP and succinyl-CoA levels shown in the present paper suggests that in the absence of acetoacetate the succinyl-CoA content is determined primarily by the intramitochondrial GTP:GDP ratio. Although this ratio is not readily measurable, it will be reflected by the ATP:ADP ratio if the activity of nucleoside diphosphate kinase in the inner membrane matrix fraction of heart mitochondria is great enough to maintain the adenine and guanine nucleotides in equilibrium (50, 51). An alternative mechanism for transphosphorylation via GTP-AMP phosphotransferase requires a source of intramitochondrial AMP, which could be supplied under some conditions by the ATP-linked fatty acyl-CoA synthetase (52). It is clear, however, that since the regulation of the succinyl-CoA content of mitochondria is energy linked, feedback inhibition of succinyl-CoA to citrate synthase will provide and energy-dependent mechanism for the control of the citric acid cycle which could readily be confused with a direct inhibitory effect of ATP (35).

It has been known for a long time that succinate dehydrogenase is activated by its substrate (53). The present data show that the intramitochondrial succinate concentration is approximately the same in State 4 as in State 3 despite a large increase of flux, indicating that substrate activation cannot be involved in the regulation of succinate dehydrogenase activity in the intact mitochondria. Two other mechanisms for the regulation of succinate dehydrogenase have recently been described (54). One is activation by reduced CoQ6 and the other by an indirect effect mediated by ATP. The physiological significance of the interaction between reduced CoQ6 and succinate dehydrogenase is difficult to understand since the enzyme apparently becomes inactivated under precisely those conditions when flux through it is increased, i.e. State 3 and uncoupled respiration. The indirect ATP effect described by Gutman et al. (54) is oligomycin insensitive, thereby suggesting a mechanism mediated via the guanine nucleotides. Succinyl-CoA itself could be the activator, thereby permitting increased flux through succinate dehydrogenase with a stabilization of the intramitochondrial succinate concentration. The present data show in fact an increased accumulation of succinate in the oligomycin-inhibited state relative to State 4 together with diminished succinyl-CoA levels, whereas succinate accumulation decreased in the uncoupled plus oligomycin state relative to State 3 in accordance with an increase of succinyl-CoA levels.

**Control of Pyruvate Dehydrogenase** - Evidence presented in this paper, based on comparisons of flux with pyruvate or acetyl-carnitine as substrate, suggests that the activity of pyruvate dehydrogenase does not become rate limiting for the entry of acetyl-CoA into the citric acid cycle. Thus, flux through citrate synthase was similar under conditions of State 3 respiration with the two substrates whereas acetyl-CoA levels were higher in all respiratory states with pyruvate as substrate. It appears, therefore, that feedback effects from the rate of utilization of acetyl-CoA serve to limit its rate of production from pyruvate.

Isolated pyruvate dehydrogenase has been shown to be inhibited by both NADH and acetyl-CoA (55–58), and regulation of pyruvate dehydrogenase by product inhibition is supported by studies with isolated mitochondria (58–60). In addition, regulation by phosphorylation and dephosphorylation of the enzyme has recently been described, the phosphorylated form being the less active species (56, 61). Notably, perfusion of rat hearts with long chain fatty acids, ketone bodies and acetate has been shown to cause a conversion of active to inactive pyruvate dehydrogenase within a period of 20 min (62). Whether a similar interconversion of the enzyme forms can be induced in isolated mitochondria has not yet been ascertained, but the kinetics of acetyl-CoA formation after addition of substrate (Figs. 8 and 10) suggests that an inactivation of pyruvate dehydrogenase may be occurring during the course of the experiment which could account for the gradual fall of acetyl-CoA after its initial rise, without a change in the rate of pyruvate uptake. With a lowered total enzyme capacity, it is clear that the same flux could be achieved at a lower concentration of acetyl-CoA.

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Feedback Interactions in the Control of Citric Acid Cycle Activity in Rat Heart Mitochondria
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