Control of Citric Acid Cycle Activity in Rat Heart Mitochondria*

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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 and α-ketoglutarate, but not succinate, reached a constant maximum within 8 min. Both total accumulation and loss of citrate to the medium were lower 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. Accumulation 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...
Deproteinized extracts were neutralized to pH 6.0 with 6 N HCl to stop the reaction after the required interval of time. Mitochondria (2 to 4 mg of protein) were preincubated in 1 ml of oxygenated medium for 30 min before starting the reaction by adding ADP. 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 × 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-dihydroxyazoxole and 1 mg of dimethyl 1,4-bis-2-(5-phenylazoxy)-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, acetate, 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 × 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—**[3-14C]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-[\(^{14}\)C]malic acid (23 mCi per mmole), DL-[\(^{3}\)H\(^{14}\)C]sodium 3-hydroxybutyrate (12 mCi per mmole), [5-\(^{14}\)C]sodium 2-ketoglutarate (17 mCi per mmole), and [1,4-\(^{14}\)C]fumaric acid (12 mCi per mmole) were purchased from Nuclear-Chicago. L-[\(^{1}\)H\(^{14}\)C]Glutamic acid (20 mCi per mmole) was obtained from Calbiochem. The scintillators used in the radioactivity measurements and the Triton X-100 were obtained from Nuclear-Chicago. L-[\(^{1}\)W\(^{14}\)C]Glutamic acid (20 mCi per mmole) was purchased from Nuclear-Chicago. L-[\(^{3}\)H\(^{14}\)C]sodium 3-hydroxybutyrate (12 mCi per mmole) was obtained from Calbiochem. The scintillators used in the radioactivity measurements and the Triton X-100 were obtained from Nuclear-Chicago. L-[\(^{1}\)H\(^{14}\)C]Glutamic acid (20 mCi per mmole) was obtained from Calbiochem. The scintillators used in the radioactivity measurements and the Triton X-100 were obtained from Nuclear-Chicago.

Some batches of the commercial enzyme were 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—[\(^{3}\)H\(^{14}\)C]Pyruvate (1 mm) was oxidized rapidly by rat heart mitochondria in the absence of added malate. The oxygen consumption was constant at 350 nanomoles 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 \(\beta\)-hydroxybutyrate and acetoacetate were formed, particularly in state 3, during the course of the experiment (Table I). The ratio of \(\beta\)-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 nmoles 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 \([\(^{3}\)H\(^{14}\)C]pyruvate into citrate, \(\alpha\)-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, \(\alpha\)-ketoglutarate, and malate suggested by the \(^{14}\)C 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

| Intermediate | State 3 | State 4 |
|--------------|--------|--------|
| Citrate      | 2.3 ± 0.4 | 1.7 ± 0.4 |
| \(\alpha\)-Ketoglutarate | 1.2 ± 0.1 | 2.2 ± 0.7 |
| Succinate    | 8.5 ± 1.0 | 13.8 ± 0.6 |
| Malate       | 1.5 ± 0.3 | 2.3 ± 0.9 |

* Results are expressed as nanomoles of \(^{14}\)C-pyruvate incorporated into each of the intermediates per mg of mitochondrial protein.
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 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-keto-glutarate 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 acetocetate 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 "¹⁴CO₂ from 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. Further sup-
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 μm) 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 ¹⁴C-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 [3-¹⁴C]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 oxaloacetate 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 n moles per mg of protein. In state 4, however, citrate accumulated at the rate of 3 to 4 n moles 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 n moles per mg of protein after 4 min, i.e., a value similar to the total citrate accumulation in state 3. When [1,5-¹⁴C]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
cr-ketoglutarate present in the mitochondrial suspension reached a maximum. Filtration studies showed that more than 95% of the cr-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-

**FIG. 9 (upper).** Relationship between concentration of malate in the medium and the steady state concentration of α-ketoglutarate in rat heart mitochondrial suspensions respiring in state 4 in the presence of 2 mM pyruvate. See legend to Fig. 2 for other experimental details.

**FIG. 10 (lower).** Accumulation of succinate 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.

**TABLE IV**

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

| Time (min) | Pyruvate uptake | Citrate | α-Ketoglutarate | Succinate | Malate | Fumarate |
|-----------|-----------------|---------|----------------|-----------|--------|---------|
|           | Assay [14C] | Assay [14C] | Assay [14C] | Assay [14C] | Assay [14C] | Assay [14C] |
| 0         | 0               | 0       | 0.5            | 0.9       | 0      | 1430    |
| 0.5       | 45              | 6       | 4.4            | 23.3      | 22.1   | 1430    |
| 1.0       | 107             | 98      | 5.0            | 45.9      | 44.5   | 1430    |
| 2.0       | 189             | 195     | 4.7            | 61.2      | 64.0   | 1410    |
| 4.0       | 463             | 415     | 5.7            | 72.1      | 76.7   | 1390    |
| 6.0       | 668             | 684     | 6.7            | 71.8      | 70.3   | 1370    |
| 8.0       | 848             | 880     | 7.4            | 71.8      | 70.3   | 1370    |

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.
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 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

The total malate concentration did not decrease appreciably from 5 mM during the course of the experiments. The incorporation of counts 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 70 to 80 nmoles per min per mg of protein in state 3 and 10 nmoles per min per mg of protein in state 4.

Comparison of Content 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 α-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, α-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 acetoacetate, β-hydroxybutyrate, or acetate. Less than 1 mmole 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 formation was not detectable during the 4-min experiment.

### TABLE V

Comparison of enzymic and isotopic methods of determining accumulations of intermediates of citric acid cycle: state 4 conditions

In incubation conditions were similar to those of Table IV with the exception that hexokinase and ADP were omitted.

| Time (min) | Pyruvate uptake | Citrate | α-Ketoglutarate | Succinate | Malate | Fumarate |
|-----------|----------------|--------|----------------|-----------|--------|----------|
| 0         | 14             | 2.2    | 7.5            | 13.2      | 13.1   | 7.0      |
| 0.5       | 75             | 74     | 14.8           | 15.8      | 22.8   | 23.7     |
| 1.0       | 88             | 7.5    | 7.4            | 13.2      | 13.1   | 7.0      |
| 2.0       | 117            | 19.7   | 24.0           | 37.1      | 34.6   | 15.1     |
| 6.0       | 14             | 2.2    | 7.5            | 13.2      | 13.1   | 7.0      |
| 8.0       | 173            | 25.9   | 57.8           | 55.6      | 23.0   | 25.0     |

### 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 net 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).
production from \(^{14}\text{C}\)-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 108 nmoles per min per mg of carbon flux through pyruvate dehydrogenase appeared as a-ketoglutarate. However, because of a-ketoglutarate accumulation, and loss of carbon into glutamate, flux through the a-ketoglutarate step was decreased to 49 nmoles per min per mg. Most of the succinate formed was lost from the mitochondria, so that finally only 18 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 a-ketoglutarate and succinate. After 6 min of incubation in state 3, loss of carbon as a-ketoglutarate ceased, but loss of succinate continued. In state 4, the losses of carbon as citrate, a-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, a-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, a-ketoglutarate penetration is stimulated by malate or malonate (22). Harris and Manger (13) have suggested that those activation phenomena essentially represent exchange reactions, so that those metabolites which can exchange for each other across the membrane may compete for entry into the mitochondria. In heart mitochondria, entry of malate requires the efflux of either phosphate, succinate, or a-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, a-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 a-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 \(\mu\text{M}\) with a-ketoglutarate but was greater than 1 \(\text{mM}\) with succinate, suggesting that the affinity of the respective permease for a-ketoglutarate is higher than that of the succinate permease for its substrate. Measurement of the \(K_w\) values for a-ketoglutarate and succinate oxidations in intact mitochondria respiring in state 3 showed, in fact, that the \(K_w\) for a-ketoglutarate was 30-fold less than that of succinate (Table VII). Malate was a competitive inhibitor of both a-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 a-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

**Kinetic parameters of succinate and a-ketoglutarate oxidation by rat heart mitochondria**

Rat heart mitochondria were incubated as described in Table I. Rotenone was also present in experiments with succinate as substrate to inhibit endogenous NAD-linked respiration.

| Table VII | Kinetic parameters of succinate and a-ketoglutarate oxidation by rat heart mitochondria |
|-----------|---------------------------------------------|
| Substrate | \(K_w\) | \(K_w\) malate | \(V_{\text{max}}\) |
| Succinate | 3.7 | 330 | |
| Succinate + 5 mM malate | 12.5 | 1.5 | 330 |
| a-Ketoglutarate | 0.12 | 192 | |
| a-Ketoglutarate + 5 mM malate | 1.25 | <0.5 | 192 |
Control of Citric Acid Cycle

Control Sites in Citric Acid Cycle—The major sites in the citric acid cycle where feedback control from the respiratory chain may be exerted are at pyruvate dehydrogenase, citrate synthase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase. Flux from pyruvate to acetyl-CoA is 4 to 5 times greater in state 3 than in state 4 (Table VI). This change of flux was not associated with appreciable changes of CoA or acetyl-CoA contents, but it did coincide with an oxidation of NADH. Garland and Randle (40) first demonstrated that heart pyruvate dehydrogenase is inhibited by both NADH and acetyl-CoA. More recently, Wieland et al. (41) have described the properties of purified pyruvate dehydrogenase from pig heart. Inhibition by acetyl-CoA (Km = 29 μM) and NADH were confirmed. Bremer (42) in studies with partially purified pyruvate dehydrogenase from heart and kidney concluded that the enzyme was strongly inhibited by a high NADH:NAD ratio but was relatively more weakly inhibited by a high acetyl-CoA:CoA ratio. However, by analogy with studies on α-ketoglutarate dehydrogenase (43), it would appear that the inhibitory effects of acetyl-CoA are greater when the NAD system is more reduced. It may be concluded, therefore, that in the present experiments, flux through pyruvate dehydrogenase in state 4 is controlled directly or indirectly by NADH.

Ketone body formation and fatty acid synthesis are very low in heart mitochondria; hence, most of the acetyl-CoA produced from pyruvate is converted to citrate. The source of oxalacetate required for citrate synthesis appears to be partly from endogenous aspartate. The early rapid fall of aspartate in both state 3 and state 4 on addition of substrate indicates a low intramitochondrial oxalacetate pool. Presumably, the increase of the NADH:NAD ratio after addition of pyruvate caused a fall of intramitochondrial oxalacetate, thereby shifting the glutamate-oxalacetate transaminase equilibrium in favor of a higher glutamate to aspartate ratio.

Heart citrate synthase, like that of liver, has a low Km for oxalacetate, being in the order of 2.5 μM (44, 45). The Km for acetyl-CoA is similarly small, although it can be increased 10-fold by 5 mM ATP (45). The lack of change of the intramitochondrial acetyl-CoA content (1.2 nmol per mg of protein) between states 3 and 4 suggests that citrate synthase is controlled by the concentration of oxalacetate. Presumably, in heart, as in liver (46, 47), the intramitochondrial oxalacetate is below the Km for citrate synthase. The observed 5-fold increase of flux through the citrate synthase step in the state 4 to 3 transition is probably a reflection of an increased oxalacetate concentration as a result not only of the decreased ratio of NADH to NAD, but also of an increase in the malate concentration. Thus, McElroy et al. (20), using heart mitochondria incubated with pyruvate, found a 6-fold higher intramitochondrial malate concentration in state 3 than in state 4. Because the ratio of β-hydroxybutyrate to acetocetate decreased 4-fold (Table I), the intramitochondrial oxalacetate concentration may increase by as much as 24-fold during state 3 respiration. Evidently, the oxalacetate concentration is of key importance in determining the flux of the whole cycle. Because control of citrate synthase by the ratio of NADH to NAD manifests itself by influencing oxalacetate concentrations, the increase of the NADH level which occurs after malate addition, is accompanied by an increase of flux, presumably attributable to an increase of intramitochondrial malate.

A crossover plot, showing the accumulation of intermediates after 6 min of incubation in state 3 compared with corresponding incubations in state 4 as control, is presented in Fig. 12. Although this treatment is limited by lack of accurate estimates of the intramitochondrial concentrations of the intermediates, a site of activation is observed between citrate and α-ketoglutarate, representing the isocitrate dehydrogenase site. The studies of McElroy and Plaut (8), Plaut and Aogaichi (9), and others (48, 49) have established that ADP is an allosteric activator of NAD-specific isocitrate dehydrogenase. In addition, this enzyme is inhibited by NADH and NADPH (9). Hence, the restricted enzyme activity in state 4 could be caused by a combination of high ratios of ATP to ADP and NADH to NAD. However, studies with oligomycin and atractylloside inhibited mitochondria indicate that NADH provides a more effective control than ADP. The contribution of the NADP-specific isocitrate dehydrogenase to the total isocitrate dehydrogenase activity is difficult to assess. The constancy of the NADPH levels and its lack of allosteric properties suggests that its activity may not be very different in states 3 and 4.

The present data do not indicate any specific control interactions in the citric acid cycle reactions between α-ketoglutarate and malate. However, the enhancement of succinyl-CoA inhibition of α-ketoglutarate dehydrogenase by NADH (43) provides a mechanism whereby flux from α-ketoglutarate to succinate could be controlled directly by the NAD oxidation reduction state and via 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...
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.

We postulate that, in rat heart mitochondria, feedback between the states of phosphorylation of the adenine nucleotides and the NAD dehydrogenases of the citric acid cycle is achieved principally through alterations of the oxidation-reduction state of the pyridine nucleotide systems. In the steady state, there is remarkably good coordination of control at the pyruvate dehydrogenase, citrate synthase, and isocitrate dehydrogenase sites, as befits an organ with a low cytoplasmic biosynthetic capacity. At very high NADH levels, however, isocitrate dehydrogenase may not coordinate with earlier steps in the cycle thereby resulting in an accumulation of citrate. The roles of reversed electron transfer and substrate level phosphorylation on the control of citric acid cycle activity and its interaction with oxidation require further elucidation.

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REFERENCES

1. Reed, N., and Paine, J. N., J. Biol. Chem., 245, 6077 (1966).
2. Prusiner, S. B., Cannon, B., Ching, T. M., and Lindberg, O., Eur. J. Biochem., 7, 51 (1968).
3. Williamson, J. R., Prusiner, S., Olson, M., and Furami, M., Lipids, in press.
4. Chance, B., Williamson, J. R., Jameson, D., and Srooener, B., Biochem. Z., 341, 557 (1965).
5. Williamson, J. R., Scholz, R., and Browning, E. T., J. Biol. Chem., 244, 4617 (1969).
6. Atkinson, D. E., in T. W. Goodwin (Editor), Metabolic roles of citrate, Academic Press, New York, 1968, p. 23.
7. Garland, P. B., in T. W. Goodwin (Editor), Metabolic roles of citrate, Academic Press, New York, 1968, p. 41.
8. Chen, R. A., and Plaut, G. W. E., Biochemistry, 2, 1023 (1963).
9. Plaut, G. W. E., and Aochachi, T., J. Biol. Chem., 243, 5572 (1968).
10. Williamson, J. R., Olson, M. S., Herceg, B. E., and Coles, H. S., Biochem. Biophys. Res. Commun., 27, 555 (1967).
11. Williamson, J. R., Olson, M. S., Browning, E. T., and Scholz, R., in S. P. Papp, J. M. Tager, E. Quagliariello, and E. C. Slater (Editors), Energy level and metabolic control in mitochondria, Adriatica Editrice, Bari, Italy, 1969, p. 97.
12. Portenhaus, R., and Schaffer, G., Fed. Eur. Biochem. Soc. Lett., 2, 281 (1969).
13. Harris, E. J., and Manas, J. R., Biochem. J., 109, 399 (1968).
14. Chappell, J. B., Brit. Med. Bull., 24, 150 (1968).
15. Bottger, I., Wieland, O., Brandza, D., and Pette, D., Eur. J. Biochem., 8, 113 (1969).
16. Von Korff, R. W., J. Biol. Chem., 240, 1551 (1965).
17. Von Korff, R. W., Nature, 211, 253 (1966).
18. Mehman, M. A., J. Biol. Chem., 243, 5229 (1968).
19. Mecklenburg, F. A., and Williams, G. R., Arch. Biochem. Biophys., 126, 492 (1968).
20. McCleod, F. A., Wong, G. S., and Williams, G. R., Arch. Biochem. Biophys., 126, 563 (1968).
21. Davis, E. J., Biochim. Biophys. Acta, 143, 28 (1967).
22. De Haan, E. J., and Tager, J. M., Biochim. Biophys. Acta, 153, 96 (1968).
23. LaNoue, K., Nicklas, W. J., Williamson, J. R., and Garfinkel, D., Fed. Proc., 28, 471 (1969).
24. Chance, B., and Hauhara, B., Biochem. Biophys. Res. Commun., 3, 1 (1960).
25. Williamson, J. R., and Cooke, B. E., in J. M. Lowenstein (Editor), Methods of enzymology, Vol. 15, Academic Press, New York, 1969, p. 434.
26. Gornall, A. G., Bardawill, C. S., and David, M. M., J. Biol. Chem., 177, 741 (1949).
27. Busch, H., Hulser, M. R., and Potter, V. R., J. Biol. Chem., 195, 717 (1952).
28. Bell, S., Nicklas, W. J., and Clarke, D. J., J. Neurochem., 15, 131 (1968).
29. Davis, E. J., Biochim. Biophys. Acta, 96, 217 (1965).
30. Klingenberg, M., Slenczka, W., and Ritt, E., Biochem. Z., 322, 47 (1969).
31. Freedman, A. D., and Graff, S., J. Biol. Chem., 232, 292 (1958).
32. Klingenberg, M., and Pfeff, E., in J. M. Tager, S. Papp, E. Quagliariello, and E. C. Slater (Editors), Regulation of metabolic processes in mitochondria, Elsevier Publishing Company, Amsterdam, 1966, p. 189.
33. Greiville, G. D., in J. M. Tager, S. Papp, E. Quagliariello, and E. C. Slater (Editors), Regulation of metabolic processes in mitochondria, Elsevier Publishing Company, Amsterdam, 1966, p. 86.
34. Greiville, G. D., in F. Dickens, W. J. Whelan, and P. J. Randle (Editors), Carbohydrate metabolism and its disorders, Vol. I, Academic Press, New York, 1968, p. 207; in J. M. Lowenstein (Editor), Citric acid cycle, control and compartmentation, Marcel Dekker, New York, 1969, p. 1.
35. DuFouurque, D., and Kus, E., Eur. J. Biochem., 6, 151 (1966).
36. Chappell, J. B., and Haahoff, K. N., in E. C. Slater, Z. Kanyigu, and L. Woitczak (Editors), Biochemistry of mitochondria, Academic Press, New York, 1967, p. 75.
37. England, P. J., and Robinson, B. H., Biochem. J., 123, 88 (1969).
38. Williamson, J. R., J. Biol. Chem., 240, 2308 (1965).
39. Bowman, R. H., J. Biol. Chem., 241, 3041 (1966).
40. Garland, P. B., and Randle, P. J., Biochem. J., 91, 6C (1964).
41. Wieland, O., Jagow-Westermann, B. von, and Stukowski, B., Hoppe-Seyler's Z. Physiol. Chem., 350, 329 (1969).
42. Bremser, J., Eur. J. Biochem., 6, 539 (1969).
43. Garland, P. B., Biochem. J., 92, 10C (1964).
44. Kosciuk, G. W., and Shree, P. A., J. Biol. Chem., 236, 2500 (1961).
45. Jangaard, N. O., Unkeless, J., and Atkinson, D. E., Biochim. Biophys. Acta, 151, 225 (1968).
46. Williamson, D. H., Lund, E., and Krebs, H. A., Biochem. J., 103, 514 (1967).
47. Williamson, J. R., Browning, E. T., and Olson, M. S., Advan. Enzyme Regul., 6, 67 (1968).
48. Goeberl, H., and Klingenberg, M., Biochem. Z., 340, 441 (1964).
49. Stein, A. M., Stein, J. H., and Kirkman, S. K., Biochemistry, 6, 1370 (1967).
50. Jones, E. A., and Gutfreund, H., Biochem. J., 92, 1C (1964).
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