The Control of Tricarboxylate-Cycle Oxidations in Blowfly Flight Muscle

THE STEADY-STATE CONCENTRATIONS OF COENZYME A, ACETYL-COENZYME A AND SUCCINYL-COENZYME A IN FLIGHT MUSCLE AND ISOLATED MITOCHONDRIA

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(1) A 'cycling' method involving citrate synthase (EC 4.1.3.7) and malate dehydrogenase (EC 1.1.1.37) was modified by the inclusion of succinyl-CoA synthetase (EC 6.2.1.5) and hexokinase (EC 2.7.1.1) to permit the determination of very small amounts of succinyl-CoA in addition to CoA and acetyl-CoA. (2) Application of this technique to blowfly (*Phormia regina*) flight-muscle extracts reveals no change in acetyl-CoA concentration, a slight fall in CoA concentration and a rise in succinyl-CoA concentration during flight. (3) Extraction of isolated mitochondria during controlled (state 4) pyruvate oxidation reveals essentially only acetyl-CoA. Activation of respiration by ADP (state 3) or uncoupling agents leads to a fall in acetyl-CoA and a rise in CoA and succinyl-CoA content. (4) The presence of glycerol phosphate in addition to pyruvate results in a lower acetyl-CoA content in state 4. (5) It is contended that these results are consistent with a primary control of one of the reactions of the tricarboxylate cycle, rather than of pyruvate dehydrogenase, during the state 4 oxidation of pyruvate by isolated mitochondria, and that the modulation of citrate synthase activity by the ratio of acetyl-CoA/succinyl-CoA is unimportant under these conditions.

The oxidation of pyruvate by blowfly (*Phormia regina*) flight-muscle mitochondria presents a unique opportunity for the study of the tricarboxylate cycle because of the great degree of control exercised in the resting state [see Sacktor (1970) for a review] and because of the simplicity imposed by the lack of mitochondrial permeability to cycle intermediates (Van den Bergh & Slater, 1962). Previous work has suggested a control at NAD-isocitrate dehydrogenase (EC 1.1.1.41) (Hansford, 1972a) but this has to be defended in view of the existence of two prior reactions, those catalysed by pyruvate dehydrogenase and citrate synthase, both associated with large negative standard free-energy changes, and both shown to be subject to control in other animal tissues (pyruvate dehydrogenase, Garland & Randle, 1964; Von Jagow *et al.*, 1968; Bremer, 1969; Wieland & Siess, 1970; Martin *et al.*, 1972; Portenhauser & Wieland, 1972; citrate synthase, Smith & Williamson, 1971; LaNoue *et al.*, 1972). The study reported here attempts to differentiate between control at each of these loci by establishing the steady-state concentrations of CoA, acetyl-CoA and succinyl-CoA (3-carboxypropionyl-CoA). The content of succinyl-CoA also allows comment to be made on the original suggestion of Van den Bergh (1964) that control is exercised at succinyl-CoA synthet-

ase, operating in the direction of succinyl-CoA cleavage.

Methods and Materials

Preparation of flight-muscle extracts

Blowflies (7–9-day-old *Phormia regina* of either sex) were plunged into liquid N₂ after either 2 min of tethered flight or 2 h at 4°C to enforce inactivity. Thoraces were separated while still frozen, rapidly weighed and ground with 2 ml of 8% (v/v) HClO₄ in 40% (v/v) ethanol, either 10 or 20 thoraces being used per extract. The extracts were allowed to thaw and ground exhaustively in a mortar. They were then centrifuged to remove precipitated protein, and the pellet was washed with 1 ml of cold aq. 8% HClO₄. The two supernatants were combined and neutralized to pH 5.5 with 3 M-K₂CO₃ containing 0.05 M-2-(N-morpholino)ethanesulphonic acid (Mes). Precipitated KClO₄ was removed by centrifugation, and the pellet was washed with 1 ml of cold water. Supernatants were combined and made up to a final volume of 10 ml. Dithiothreitol was added to a final concentration of 1 mM immediately after neutralization, to minimize oxidation of CoA, and extracts were kept on ice and invariably assayed within 4 h to minimize loss of succinyl-CoA.

'Cycling' method for the determination of CoA, acetyl-CoA and succinyl-CoA

This is the method of Allred & Guy (1969) and involves the following sequence of reactions.

To these were added the following reactions.

Each day a standard CoA solution was made up as described (P-L Biochemicals Inc. information sheet, 1970) and was assayed by the method of Tubbs & Garland (1969) except that pyruvate dehydrogenase was used instead of 2-oxoglutarate dehydrogenase. A standard curve was then constructed (Fig. 1) by adding various amounts of the CoA standard to cuvettes containing 0.5ml of a mixture ('premix') comprising 0.25M-potassium *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulphonic acid (Tes), pH7.2, 0.1M-KCl, 0.02M-sodium malate, pH7.2, 8mM-acetyl phosphate, 2mM-NAD⁺, 1mM-potassium phosphate, pH7.2, 5mM-ADP, 1mM MgCl₂, 10mM-glucose, 1.67 units of malate dehydrogenase/ml, 3.5 units of citrate synthase/ml and 7 units of hexokinase/ml where 1 unit is 1 μmol/min. Final volume in each cuvette was 0.85ml and all cuvettes were incubated in water at 25°C for 3min after adding the premix to achieve temperature equilibration. Addition of 15 units of phosphotransacetylase (EC 2.3.1.8) then elicited a rate of NAD⁺ reduction which was recorded with a Beckman-Gilford spectrophotometer. When applied to the extracts this procedure gave a rate proportional to the CoA plus acetyl-CoA content. This total was determined by comparison with the standard curve and by using a 'calibration factor' obtained for each extract by adding authentic CoA. This is necessary as the extracts are slightly inhibitory.

Incubation of 0.25ml of extract with 0.02ml of 0.2M-*N*-ethylmaleimide for 5min, followed by incubation with 0.08ml of 0.1M-dithiothreitol for 5min and then addition of the premix gave a rate which was due to acetyl-CoA alone. It was confirmed that over 98% of added authentic CoA was removed by this procedure. When 3 μl of succinyl-CoA synthetase (50 units/ml), prepared from fly flight muscle (Hansford, 1972c), was added with the premix, then the rate reflected both acetyl-CoA and succinyl-CoA concentrations, and the latter could be obtained by

subtraction. It was found that inclusion of the succinyl-CoA synthetase slightly decreased the rate given by authentic CoA added with the premix, and therefore succinyl-CoA contents were determined by comparison with CoA standards in the *N*-ethylmaleimide-succinyl-CoA synthetase medium. Fig. 1 shows a typical standard curve and illustrates the extreme sensitivity of the assay, the slight inhibition in the media containing succinyl-CoA synthetase and the almost complete conversion of authentic succinyl-CoA into CoA. The authentic succinyl-CoA was assayed by incubating with succinyl-CoA synthetase in 0.1M-potassium phosphate, pH7.2, 0.5mM-ADP, allowing the reaction to reach equilibrium and assuming a millimolar extinction coefficient of 4cm⁻¹ at 235nm (Cha & Parks, 1964). It is considered that although it is tedious because of the number of controls required, this kinetic assay makes possible the determination of succinyl-CoA at concentrations not amenable to stoichiometric assay.

Incubation and extraction of mitochondria

Mitochondria were prepared as described by Chappell & Hansford (1969) with the modification that proteolytic digestion of the myofibrils was less complete. The isolation medium was more nearly neutral, pH7.2, maintained by 10mM-Tes buffer (potassium salt). Nagarse was decreased to 2.5mg per 60 flight muscles and digestion to 6min at 0°C. An upper layer of partially digested myofibrils was

Fig. 1. Calibration curve for the 'cycling' assay of CoA and succinyl-CoA

The amounts of CoA indicated were assayed in the standard incubation of premix plus water (see the Methods and Materials section) (○), and in an extract of mitochondria treated with *N*-ethylmaleimide followed by dithiothreitol (□). In the latter case the rate due to the mitochondrial extract alone was determined separately and subtracted. In addition, the rates measured with authentic succinyl-CoA, assayed in extracts treated with *N*-ethylmaleimide, dithiothreitol and then succinyl-CoA synthetase are presented (●).

shaken loose from the pellet after the second centrifugation.

All incubations were in media based on 0.12M-KCl, 20mM-Tes buffer (potassium salt), pH7.2, and containing additions as detailed in the legends to Figures. All solutions were adjusted to pH7.2. Media were saturated with O₂ and experiments were at room temperature (20–22°C) to allow higher suspension densities. In general, the initial volume was 13ml and up to six 2ml samples were withdrawn during the course of the experiment by syringe and expelled into stirred 1.3ml portions of 16% (v/v) HClO₄ in 40% (v/v) ethanol. The progress of the reaction was always followed by O₂ electrode and these traces are presented when especially informative. HClO₄ extracts were treated as described above, except that the final volume was 5ml.

The interpretation of the results obtained with mitochondrial suspensions involves the assumption that all of the CoA measured was within the mitochondria. This seems likely in view of the retention of CoA during the mitochondrial preparation, and the large and reversible changes in acylation noted during these experiments. If a portion of the CoA had leaked out of the mitochondria then it would be expected to be unaffected by changes in mitochondrial energy state.

Enzymes and reagents

Citrate synthase, malate dehydrogenase, phosphotransacetylase and hexokinase were purchased from Boehringer Mannheim Corp., New York, N. Y., U.S.A., and Nagarse was purchased from Enzyme Development Corp., New York, N.Y., U.S.A., CoA and succinyl-CoA were obtained from P-L Biochemicals, Milwaukee, Wis., U.S.A. The succinyl-CoA was made up in water each day and kept on ice. An assay of free thiol by the method of Ellman (1959) indicated less than 5% CoA in the freshly prepared solution. Other reagents were of the highest purity available commercially and were made up in twice-glass-distilled water.

Results

State of acylation of CoA in fly thoraces

Freezing flies in liquid N₂ and extracting the thoraces as described in the Methods and Materials section gave the concentrations of CoA and thioester indicated in Table 1. A flight of 2min duration was selected for this study, as most metabolic intermediates previously assayed have attained a concentration at this time which is then maintained during much longer flights (Sacktor & Wormser-Shavit, 1966). The total of CoA, acetyl-CoA and succinyl-CoA is 56nmol/g wet wt. of thorax, and does not differ appreciably in the states of rest and

flight. Moreover, the acylation pattern also differs remarkably little. Thus there is no significant change in acetyl-CoA concentration, and a barely significant ($0.05 < P < 0.10$) fall in CoA concentration on flight. Succinyl-CoA concentration, however, rises significantly ($0.001 < P < 0.01$). These results are discussed in a later section with reference to the changes found in isolated mitochondria.

Table 1. Concentration of CoA, acetyl-CoA and succinyl-CoA in the flight muscle of resting and flying flies

Results are given as means $\pm$ S.D. with the numbers of extracts in parentheses. Each extract contained at least 10 thoraces. 'Total CoA' is the sum of the three quantities measured.

State of muscle	Content (nmol/g wet wt. of thorax)			
	CoA	Acetyl-CoA	Succinyl-CoA	Total CoA
Resting	27 $\pm$ 1 (4)	19 $\pm$ 2 (4)	9.2 $\pm$ 0.7 (3)	55 $\pm$ 1 (5)
After 2min flight	24 $\pm$ 1 (3)	19 $\pm$ 2 (3)	15 $\pm$ 1 (3)	58 $\pm$ 1 (3)

Fig. 2. Mitochondrial content of CoA and acetyl-CoA during state 4 pyruvate oxidation

Mitochondria were added to a medium (see the Methods and Materials section) containing 10mM-potassium phosphate, 0.5mM-ATP and 2.5mM-KHCO₃ to initiate the experiment. The concentration of mitochondrial protein was 1.49mg/ml. Where indicated pyruvate was added to 2.5mM, proline to 5mM and ADP to 9mM. Samples were taken, extracted and assayed as described in the Methods and Materials section. CoA, ○; acetyl-CoA, □.

State of acylation of CoA in isolated flight-muscle mitochondria

Fig. 2 presents the results of an experiment in which pyruvate and proline were together added to mitochondria suspended in a buffered, oxygen-saturated medium. Samples were taken at the times indicated, and deproteinized with HClO_4 , as described in the Methods and Materials section. It is clear that mitochondrial content of acetyl-CoA is very low in the absence of added substrate and rises during the controlled oxidation of pyruvate, until the free CoA content is below the limits of detection. Addition of ADP during this state initiates a sharp fall in acetyl-CoA content, and a corresponding rise in CoA. These results suggest that the activity of the tricarboxylate cycle rather than that of pyruvate dehydrogenase limits the rate of the overall oxidation of pyruvate in the absence of ADP. Addition of ADP activates the oxidation of acetyl groups by the tricarboxylate cycle to a greater extent than the provision of acetyl groups by the pyruvate dehydrogenase reaction, and thus leads to a fall in the steady-state con-

Fig. 3. Mitochondrial content of CoA and acetyl-CoA during state 3 pyruvate oxidation and anaerobiosis

The experiment was initiated by the addition of mitochondria to a basal medium containing 10 mM-potassium phosphate, 2.5 mM-pyruvate, 5 mM-proline, 0.5 mM-ATP and 2.5 mM- KHCO_3 to give a final protein concentration of 1.45 mg/ml. ADP was added to give a concentration of 12.5 mM, and oxygen was depleted at the time indicated by the arrow. CoA, $\circ$; acetyl-CoA, $\square$.

centration of acetyl-CoA. Succinyl-CoA content was not determined in this experiment.

Fig. 3 presents the results of an experiment in which ADP was added to a concentration (12 mM) sufficient to sustain prolonged state 3 respiration, allowing more than one sampling. The acetyl-CoA content continues to fall during 1 min of activated respiration, and then changes rather little as oxygen becomes depleted. By contrast, the addition of ADP to such a concentration (3.5 mM) as to allow a state 4 $\rightarrow$ 3 $\rightarrow$ 4 transition reveals a more complex pattern of acylation (Fig. 4). There is a decline in acetyl-CoA content, with a concomitant rise in CoA during the early part of the state 3 respiration, which then reverses well before the stimulated rate of oxygen uptake begins to decline. It is tentatively suggested that the increase in the steady-state concentration of acetyl-CoA during the latter part of the state 3 phase betrays a progressive inhibition of the rate-limiting enzyme of the tricarboxylate cycle by the progressively increasing energy charge of the adenylate system. This enzyme may be the NAD-linked isocitrate dehydrogenase (EC 1.1.1.41) (Hansford, 1972a). Such an inhibition need not give rise initially to an appreciable decline in the rate of oxygen uptake if the isocitrate dehydrogenase is not rate-limiting at high ADP concentrations, but only becomes so with the rise in energy charge. Changes in succinyl-CoA content during this experiment are less clear, though there seems to be a decrease towards the end of the state 3. When respiration is limited by the availability of P_i , in the presence of added ADP (Fig. 5) there is, however, a marked accumulation of succinyl-CoA, which is then reversed on adding P_i . In this experiment, sampling was deliberately delayed for 2 min after the addition of ADP, to ensure the removal of endogenous P_i by phosphorylation. In this state of phosphate depletion, the activity of succinyl-CoA synthetase in the direction of succinyl-CoA cleavage would be expected to be very low, which is consistent with this result.

The stimulation of respiration by the uncoupling agent carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone also gives rise to a pronounced fall in acetyl-CoA, with a concomitant rise in CoA content (Fig. 6). The gradual decrease in acetyl-CoA after the first 10 s of enhanced respiration coincides with an increasing respiratory activity over the same time-period, evident from the oxygen-electrode trace. The apparent reversal of this tendency revealed in the last sample is not understood. Succinyl-CoA content appears to rise on the addition of the uncoupling agent, which is consistent with a marked activation of NAD-linked isocitrate dehydrogenase (Hansford, 1972a) and 2-oxoglutarate dehydrogenase (Hansford, 1972b) by a decrease in the energy charge of the adenylate system. However,

Fig. 4. Mitochondrial content of CoA, acetyl-CoA and succinyl-CoA during the transitions state 4 $\rightarrow$ 3 $\rightarrow$ 4

Mitochondria were added to a medium containing 15mM-potassium phosphate, 2mM-pyruvate, 4mM-proline, 0.4mM-ATP and 2mM-KHCO₃ to give a protein concentration of 0.98mg/ml. ADP was added to give 3.2mM, as indicated. (b) is a recording from an oxygen electrode in the same mitochondrial suspension. The numbers represent the samples shown in (a). CoA, $\circ$; acetyl-CoA, $\square$; succinyl-CoA, $\bullet$.

this increase is clearly not consistent with the suggested role of succinyl-CoA in the modulation of citrate synthase activity (LaNoue *et al.*, 1972), as such a control would demand a more elevated succinyl-CoA/acetyl-CoA ratio in the resting (controlled) state.

The results presented thus far fail to support the analogy between resting flight muscle and state 4 mitochondrial respiration in that CoA is far more highly acetylated in the mitochondrial extracts. Pyruvate concentration was one factor which was thought might be responsible for the difference. Accordingly, an experiment involving a state 4 $\rightarrow$ 3 $\rightarrow$ 4 transition was carried out at a pyruvate concentration of (initially) 0.44mM, which approximates to the concentration found in active flight muscles (Sacktor & Wormser-Shavit, 1966). Fig. 7 shows that CoA is still essentially all acetylated in state 4, even in the latter phase of the experiment, where the pyruvate concentration presumably fell to approx. 0.25mM. However, acetylation during state 3 is much decreased when compared with the experiment in Fig. 4, which differed only in pyruvate concentration. Thus it is noticeable that sample 4 contained a minimum of acetyl-CoA, whereas a

sample taken during the analogous portion of the state 3 rate in the experiment of Fig. 4 revealed a tendency towards increased acetylation. These differences are reproducible and suggest that the pyruvate dehydrogenase complex is considerably more active at 2.5mM-pyruvate than at 0.44mM-pyruvate, which is somewhat surprising in view of the K_m of 0.1mM obtained for the solubilized and partially purified enzyme from this tissue (R. G. Hansford, unpublished work). Conceivably, the pyruvate functions to protect the complex against phosphorylation (Hucho *et al.*, 1972) though it is equally possible that the substrate affinity of the enzyme is altered by removal from its mitochondrial environment. Repetition of this experiment at a lower phosphate concentration gave the results shown in Fig. 8. Although the pattern of change of CoA and acetyl-CoA is very similar, the succinyl-CoA content is very low in state 4, and rises in state 3. In this context it is noted that the succinyl-CoA content in the experiment of Fig. 6 was also low before the addition of the uncoupling agent, and that this experiment was performed at the same low phosphate concentration as was used in Fig. 8 (8mM). This result is probably consistent with the fact that

Fig. 5. Mitochondrial content of CoA, acetyl-CoA and succinyl-CoA during pyruvate respiration limited by phosphate

Mitochondria were added to a medium containing 2 mM-pyruvate and 4 mM-proline giving a protein concentration of 1.1 mg/ml. At the times indicated, ADP was added to 2.15 mM and potassium phosphate to 11 mM. The third sample was withdrawn before the addition of phosphate and added to HClO₄ at the same time that phosphate was added to the remaining suspension. CoA, ○; acetyl-CoA, □; succinyl-CoA, ●.

isocitrate dehydrogenase requires higher concentrations of phosphate as an activator (Hansford, 1972a) than succinyl-CoA synthetase does as a substrate (Hansford, 1972c; R. G. Hansford, unpublished work).

Another possible reason for a decreased state of acetylation in the resting fly is the presence in flight muscle of carnitine and carnitine acetyltransferase (Childress *et al.*, 1967). Fig. 9 shows that inclusion of carnitine in the mitochondrial incubation medium does indeed cause a decrease in acetyl-CoA content in state 4. Addition of ADP gives a further decrease. CoA content rises immediately on the activation of respiration (state 3) but then appears to fall, as succinyl-CoA concentration increases. Extrapolation of these results to the situation *in vivo* is difficult because although the concentration of carnitine used was similar to that in the fly (Childress *et al.*, 1967), the quantity was much greater, in view of the dilute mitochondrial suspension used in experimental work. Nevertheless, this experiment suggests that the activity of the acetyltransferase is significant compared with the activities of pyruvate dehydrogenase and the tricarboxylate cycle during state 4.

Another important aspect of flight-muscle physiology is the provision of an equimolar amount of pyruvate and glycerol 3-phosphate by glycolysis (Zebe & McShan, 1957; Chefurka, 1958). Inclusion of glycerol phosphate in the experiment (Fig. 10)

Fig. 6. Mitochondrial content of CoA, acetyl-CoA and succinyl-CoA during uncoupled pyruvate oxidation (a)

Mitochondria were added to a medium containing 8 mM-potassium phosphate, 2 mM-pyruvate, 4 mM-proline, 0.4 mM-ATP and 2 mM-KHCO₃ giving a suspension of 1.1 mg/ml. Simultaneously, 5 min 55 s after adding the mitochondria, the first sample was taken and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added to 1 μM. The oxygen recording (b) is from the same experiment. The numbers represent the samples shown in (a). CoA, ○; acetyl-CoA, □; succinyl-CoA, ●.

Fig. 7. Mitochondrial content of CoA, acetyl-CoA and succinyl-CoA during a state 4 → 3 → 4 transition at a low pyruvate concentration (a)

Mitochondria were added to a medium containing 15 mM-potassium phosphate, 0.44 mM-pyruvate, 4 mM-proline, 0.4 mM-ATP and 2 mM-KHCO₃, giving a final suspension of 1.26 mg of protein/ml. Where indicated, ADP was added to 3.4 mM. The oxygen electrode trace (b) is of the same experiment. The numbers represent the samples shown in (a). CoA, ○; acetyl-CoA, □; succinyl-CoA, ●.

Fig. 8. Mitochondrial content of CoA, acetyl-CoA and succinyl-CoA during a state 4 → 3 → 4 transition at a low pyruvate and low phosphate concentration

All conditions were identical with those described in the legend to Fig. 7 except that the phosphate concentration was 8 mM. CoA, ○; acetyl-CoA, □; succinyl-CoA, ●.

Fig. 9. Mitochondrial content of CoA, acetyl-CoA and succinyl-CoA during the oxidation of pyruvate in the presence of carnitine

Mitochondria were added to a medium containing 5mM-potassium phosphate, 2mM-pyruvate, 0.4mM-ATP, and 2mM-KHCO₃, giving a final protein concentration of 1.25mg/ml. Where indicated, DL-carnitine was added to 8.6mM and ADP to 1.6mM. CoA, ○; acetyl-CoA, □; succinyl-CoA, ●.

Fig. 10. Effect of glycerol phosphate on the mitochondrial content of CoA, acetyl-CoA and succinyl-CoA during the oxidation of pyruvate

Mitochondria were added to a medium containing 15mM-potassium phosphate, 0.44mM-pyruvate, 4mM-proline, 0.4mM-ATP and 2mM-KHCO₃, giving a suspension of 1.0mg of protein/ml. Immediately thereafter, 0.6μmol of CaCl₂ was added, giving a ratio of added Ca²⁺/ethanedioxybis(ethylamine)tetra-acetic acid of 0.6:1.0 and a [Ca²⁺] of 0.5μg-ion/litre. Where indicated DL-glycerol phosphate was added to 5.1mM and ADP to 2.6mM. CoA, ○; acetyl-CoA, □; succinyl-CoA, ●.

greatly lowered the acetyl-CoA content during state 4. It is suggested that the mechanism of this is the almost total reduction of mitochondrial NAD⁺ by reversed electron transfer, which occurs under those conditions (Hansford, 1968). If this is so, then the

pyruvate dehydrogenase complex must be more sensitive to inhibition by NADH than is the rate-limiting enzyme of the tricarboxylate cycle. Such an inhibition of the pyruvate dehydrogenase complex has been documented for other animal tissues (Bremer

Fig. 11. Effect of a simultaneous addition of ADP plus Ca^{2+} on the mitochondrial content of CoA, acetyl-CoA and succinyl-CoA during the oxidation of pyruvate and glycerol phosphate

Mitochondria were added to a medium identical with that described in Fig. 10, except that $1 \mu\text{mol}$ of ethanedioxybis(ethylamine)tetra-acetic acid was added instead of CaCl_2 , to give a final suspension of 1.10 mg/ml . Where indicated, DL-glycerol phosphate was added to 5.1 mM , ADP to 2.6 mM and CaCl_2 to give a final ratio of added Ca^{2+} /ethanedioxybis(ethylamine)tetra-acetic acid of $0.6:1.0$. CoA, $\circ$; acetyl-CoA, $\square$; succinyl-CoA, $\bullet$.

also activates pyruvate dehydrogenase directly. Low concentrations of free Ca^{2+} were present initially in this experiment to activate the glycerophosphate dehydrogenase, which is otherwise not saturated at physiological concentrations of glycerol phosphate (Hansford & Chappell, 1967). This then represents a situation which probably does not exist in flight muscle, where both ADP and Ca^{2+} are inferred to be limiting at rest. Accordingly, the experiment was modified to include the simultaneous addition of Ca^{2+} and ADP (Fig. 11), a simulation of the initiation of flight. It is seen that the activity of the glycerophosphate dehydrogenase is insufficient in the presence of ethanedioxybis(ethylamine)tetra-acetic acid to lower greatly the state 4 concentration of acetyl-CoA. Fluorimetric experiments (not shown) indicate only a partial reduction of nicotinamide nucleotide (by reversed electron transfer) during this early part of the experiment, compared with total reduction in the experiment of Fig. 10. Addition of ADP gave a pronounced decrease in acetyl-CoA content, and an increase in CoA and succinyl-CoA. The disparity between this result and the changes observed in flight muscle is discussed below.

Discussion

The tricarboxylate cycle in blowfly flight muscle serves a purely catabolic role, and is therefore of interest as a model system in the study of bioenergetics (Sacktor, 1970; Hansford & Sacktor, 1971). A comparison of the kinetic properties of isolated enzymes with the kinetics of the overall mitochondrial oxidation of pyruvate has been made (Hansford, 1972a). A study of the steady-state concentrations of CoA and its thioesters can provide more direct evidence on the control in the mitochondrion of those enzymes which react with these substrates. The data obtained may be compared with the results of an extensive study of heart mitochondria (LaNoue *et al.*, 1972), which are also primarily catabolic in function. The present results differ first in that the total pool of CoA and thioester is much smaller in the fly mitochondrion, approx. 0.5 nmol per mg of mitochondrial protein. The matrix volume of these mitochondria is approx. $2.5 \mu\text{l/mg}$ of protein during state 4 respiration (Hansford & Lehninger, 1972), indicating a total concentration of only 0.2 mM . It is possible that some loss has occurred during isolation, as a comparison with the content of the thorax indicates that only 30% of the total CoA and thioester is recovered in the mitochondrial fraction. As an upper limit, then, the original mitochondrial content could be not more than three times that actually recovered, if all of the mitochondria were extracted from the thorax and if the cytosol contained no CoA. There is some reason to believe that

1969) and noted for the fly (R. G. Hansford, unpublished work; S. Chiang & B. Sacktor, personal communication). On the addition of ADP, the nicotinamide nucleotide is almost totally oxidized (Hansford, 1968) and the inhibition is relieved, resulting in an even greater facilitation of flux through pyruvate dehydrogenase than through the tricarboxylate cycle. Accordingly, the acetyl-CoA concentration rises.

The kinetic properties of isolated pyruvate dehydrogenase and the fluorimetrically determined steady-state concentration of mitochondrial NADH support this proposal. It is conceivable that ADP

the latter condition may be satisfied in that an assay of a 100000g supernatant fraction from a flight-muscle homogenate revealed no CoA, acetyl-CoA or succinyl-CoA. Any amount in excess of 4nmol/g of flight muscle (or 2nmol/g of thorax) would have been detected.

The second clear finding is the very high degree of acetylation of CoA in fly muscle mitochondria, particularly during state 4 respiration. This is decreased on adding ADP, especially at low pyruvate concentrations (Fig. 7). The acetyl-CoA content of heart mitochondria represents less of the total pool, and does not respond in a clear fashion to the activation of respiration by ADP (LaNoue *et al.*, 1972). The data presented here are consistent with a tight control of one of the reactions of the tricarboxylate cycle in state 4. The acetyl-CoA which accumulates as a result would severely diminish the activity of the pyruvate dehydrogenase (Garland & Randle, 1964; Bremer, 1969), resulting in a secondary control at this stage. The data do not preclude an additional direct control on pyruvate dehydrogenase, but do impose limitations as to the magnitude of any such effect. It is noticeable, moreover, that there is no decrease in acetyl-CoA content with time in state 4, as would be expected if a phosphorylation of pyruvate dehydrogenase were to lower its activity close to that of the rate-limiting enzyme of the tricarboxylate cycle. Such a control would appear to be unnecessary in fly flight muscle in that pyruvate oxidation is the only source of acetyl-CoA, and possibly impractical in view of the speed of the rest-to-flight transition.

The values for succinyl-CoA are presented with caution as they are very low and represent the difference between two low rates of increase in absorbance (see the Methods and Materials section). Nevertheless, the general pattern is that succinyl-CoA concentration tends to be low during state 4 respiration and to rise on initiating state 3. This is especially true at lower phosphate concentrations (compare Figs. 7 and 8). Certainly, there is on no occasion a sustained fall in succinyl-CoA concentration in state 3, as would be expected if Van den Bergh (1964) were correct in proposing that the tricarboxylate cycle is controlled at succinyl-CoA synthetase in this tissue. The results equally cannot be reconciled with a role for succinyl-CoA in inhibiting citrate synthase by competing with acetyl-CoA, and thus controlling the cycle (LaNoue *et al.*, 1972). Were this control found in flight muscle, succinyl-CoA content would be expected to fall on stimulating respiration. In fact succinyl-CoA content either stays constant or rises and acetyl-CoA content falls. The decrease in the ratio acetyl-CoA/succinyl-CoA may tend to lower the degree of saturation of citrate synthase by its substrate, acetyl-CoA, but any consequent inhibition is in fact negligible, as the flux

through the cycle is a maximum. The other control proposed to apply to heart citrate synthase (LaNoue *et al.*, 1972) and based on availability of oxaloacetate, may also be inapplicable, as nicotinamide nucleotide becomes reduced in state 3 in the fly (Hansford, 1972a) thereby shifting the equilibrium of the malate dehydrogenase reaction towards malate. This could be compensated by a large increase in the pool of malate plus oxaloacetate in state 3 and this is under investigation.

It has been shown that succinyl-CoA synthetase from this tissue reacts with adenine nucleotides (Hansford, 1972c), and that ATP inhibits the enzymic cleavage of succinyl-CoA. Thus an experiment was performed (R. G. Hansford, unpublished work) in which the activity of partially purified succinyl-CoA synthetase was determined by thiol release as the composition of a mixture of ADP and ATP was varied, the total being kept constant at 5mM, to simulate conditions in the mitochondrial matrix. Other substrates were saturating (0.12mM-succinyl-CoA, 40mM-potassium phosphate and 5mM-MgCl₂). If the activity were 100 arbitrary units at 100% ADP and pH 7.4, it was 60 at 40% ADP and 34 at 20% ADP. Corresponding activities at pH 6.8 were 49 (100% ADP), 34 (40%) and 23 (20%). Thus an increase in energy charge of the mitochondrial adenine nucleotide would be expected to cause an increase in succinyl-CoA concentration. However, 2-oxoglutarate dehydrogenase from this tissue is also subject to allosteric inhibition by ATP (Hansford, 1972b), and this may explain why no such increase in succinyl-CoA is in fact seen in state 4. By contrast, there is no such direct effect of energy charge on the 2-oxoglutarate dehydrogenase of mammalian heart, and in that tissue an increased ATP content causes a rise in succinyl-CoA concentration and only secondarily an inhibition of 2-oxoglutarate dehydrogenase (LaNoue *et al.*, 1972).

The increase in the thoracic content of succinyl-CoA on flight is mirrored by an increase in mitochondrial succinyl-CoA on adding ADP (Figs. 8 and 11). However, the disparity between acetyl-CoA concentrations in mitochondria and flight muscle remains. Presumably, pyruvate dehydrogenase is subject to some control in resting flight muscle which has not been reproduced in the mitochondrial experiments. This could be the availability of pyruvate, as the concentration obtained in flight-muscle extracts (Sacktor & Wormser-Shavit, 1966) and utilized in the experiments of Figs. 7, 8, 9 and 10 may not all be accessible to the mitochondria *in vivo*.

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