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The Control of Tricarboxylate-Cycle Oxidations in Blowfly Flight Muscle

THE STEADY-STATE CONCENTRATIONS OF CITRATE, ISOCITRATE, 2-OXOGLUTARATE AND MALATE IN FLIGHT MUSCLE AND ISOLATED MITOCHONDRIA

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1. Blowfly (*Phormia regina*) flight-muscle mitochondria were allowed to oxidize pyruvate under a variety of experimental conditions, and determinations of the citrate, isocitrate, 2-oxoglutarate and malate contents of both the mitochondria and the incubation medium were made. For each intermediate a substantial portion of the total was present within the mitochondria. 2. Activation of respiration by either ADP or uncoupling agent resulted in a decreased content of citrate and isocitrate and an increased content of 2-oxoglutarate and malate when the substrate was pyruvate, ATP and HCO_3^-. Such a decrease in citrate content was obscured when the substrate was pyruvate and proline owing to a large rise in the total content of tricarboxylate-cycle intermediates in the presence of proline and ADP. 3. An experiment involving oligomycin and uncoupling agent demonstrated that the ATP/ADP ratio is the main determinant of flux through the tricarboxylate cycle, with the redox state of nicotinamide nucleotide being of lesser importance. 4. Addition of ADP and Ca^{2+} to activate the oxidation of both glycerol 3-phosphate and pyruvate, simulating conditions on initiation of flight, gave a decrease in citrate and isocitrate and an increase in 2-oxoglutarate and malate content. 5. There was a good correlation between these results with isolated flight-muscle mitochondria and the changes found in fly thoraces after 30s and 2min of flight except that there was no significant change in 2-oxoglutarate content in the thorax. 6. It is concluded that NAD–isocitrate dehydrogenase (EC 1.1.1.41) controls the rate of pyruvate oxidation in both resting fly flight muscle *in vivo* and isolated mitochondria in state 4 (nomenclature of Chance & Williams, 1955).

A recent report on the steady-state concentrations of CoA and its thioesters in blowfly flight-muscle mitochondria has suggested that the oxidation of pyruvate by mitochondria from this tissue is controlled at the level of the tricarboxylate cycle rather than at pyruvate dehydrogenase (Hansford, 1974). The present work seeks to establish the site of control within the reactions of the cycle by studying the response of the mitochondrial content of citrate, isocitrate, 2-oxoglutarate and malate to large changes in flux through the pathway. Fly flight-muscle mitochondria offer two advantages in this respect: (i) they are essentially impermeable to tricarboxylate-cycle intermediates (Van den Bergh & Slater, 1962), which are therefore retained within the matrix compartment; (ii) they show high respiratory control ratios and hence are capable of large changes in flux through the tricarboxylate cycle [see Sacktor (1970) for a review]. The results demonstrate a control at NAD–isocitrate dehydrogenase (EC 1.1.1.41), consistent with a suggestion made previously (Hansford, 1972*a*) on indirect evidence. Further, measurements of the fluorescence of intramitochondrial NADH allow comment to be made on the relative importance of NADH/NAD^+ and ATP/ADP ratios in the control of this enzyme. The measurements of tricarboxylate-cycle intermediates in extracts of whole thoraces which were made by Sacktor & Wormser-Shavit (1966) were extended to include isocitrate and demonstrate control at NAD–isocitrate dehydrogenase in the resting insect.

A preliminary account of a part of this work has been published (Hansford & Johnson, 1974).

Experimental

Methods

Preparation of flight-muscle extracts. The procedure used was basically that of Hansford (1974) with the following modifications: a minimum of eight, but more usually ten, thoraces per extract were used; the deproteinized extracts were adjusted to pH5.8 with KOH, with 0.05M-2-(*N*-morpholino)-ethanesulphonate (Mes) (final concentration) as a buffer; the dithiothreitol was omitted.

Assay of tricarboxylate-cycle intermediates. The fluorimetric methods of Williamson & Corkey (1969) were followed although the citrate content of flight muscle was sufficiently high to permit the use of a recording spectrophotometer. A Farrand filter fluorimeter was used as described by Hansford (1975).

Preparation of mitochondria. Flight-muscle mitochondria were prepared from blowflies (7–9-day-old *Phormia regina* of either sex) by the method of Chappell & Hansford (1969) incorporating the minor modifications of Hansford (1974).

Incubation and extraction of mitochondria. Mitochondria were incubated in a vessel fitted with a water-jacket so as to maintain a temperature of 21°C. The medium was 0.12M-KCl–20mM-potassium *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonate, pH7.2, O_2-saturated and containing additions as detailed in the legends to the Figures. A Clark-type O_2 electrode monitored the O_2 tension in all experiments. The O_2 tension shown in the Figures is approximate and based on the assumption of 2.5μg-atoms of oxygen per ml of medium when saturated at 21°C. Two portions, withdrawn by calibrated syringes, were taken for each sample noted in the text in order to make a correction for intermediate escaping from the mitochondrial matrix; one, of 1.4ml, was centrifuged in a microcentrifuge (Beckman model 152 adapted to take larger tubes) and the other, of 2ml, was expelled directly into 1.3ml of 16% (v/v) $HClO_4$ (stirred magnetically) 10s after the centrifuge was started, since it was estimated that 50% of the mitochondria were sedimented in this time. Centrifugation was for 1min, after which time 1.2ml of supernatant fraction was rapidly removed and expelled into 0.18ml of ice-cold 62% $HClO_4$. The $HClO_4$ extracts were neutralized in the same way as the flight-muscle $HClO_4$ extracts, the final volume being 5ml for the 2ml portion and 3ml for the supernatant sample. The 2ml portion was used to measure the intermediate in the total incubation medium (referred to in the text as 'Total') and the supernatant fractions gave an estimate of the intermediate external to the mitochondria. Subtraction of the latter value from the total measurement gave the amount of intermediate associated with the mitochondria and is referred to in the text as 'Mitochondrial'. In spite of performing duplicate experiments on different mitochondrial preparations, this subtraction procedure occasionally led to negative results for the mean mitochondrial intermediate content. Such values have been presented without alteration in the Tables. The times of sampling noted in the Figures indicate when the 'Total' sample was expelled into $HClO_4$. When duplicate experiments were performed, they were matched carefully with respect to protein concentration and times of sampling; where included, the O_2 electrode trace was from one of the pair of duplicates. Fluorimeter traces of intramitochondrial nicotinamide nucleotide reduction were made in parallel experiments in a volume of 2ml and the instrument used for the intermediate assays.

Assay of flight-muscle citrate synthase. Flight-muscle mitochondria (about 40mg of protein) were suspended in 3ml of 50mM-potassium phosphate, pH7.2, and subjected to 3×20s bursts of sonication in a Branson sonifier, setting 2, 15s elapsing between each burst. They were cooled during sonication by an ice–NH_4Cl freezing mixture. Submitochondrial particles were sedimented at 100000*g* for 40min, and after dilution with 20vol. of 50mM-potassium phosphate, pH7.2, containing 2mg of bovine serum albumin per ml, the supernatant fraction was used for the assay of citrate synthase activity by the fluorimetric method of Shepherd & Garland (1969).

Materials

All enzymes and coenzymes were from Boehringer–Mannheim Corp. (New York, N.Y., U.S.A.). Other reagents were of the highest grade commercially available and were dissolved in twice-glass-distilled water.

Contents of tricarboxylate-cycle intermediates in thoraces of rested and flown flies

In an earlier study (Sacktor & Wormser-Shavit, 1966) it was shown that the malate content of the thorax of the fly rose on flight. No significant changes in the concentrations of citrate and 2-oxoglutarate, the other citrate-cycle intermediates examined, were detected. In the present work the previously assayed intermediates were reinvestigated together with isocitrate, making use of the greater sensitivity of fluorimetric measurements. Flies were suspended in flight for periods of 30s and 2min since, from the data of Sacktor & Wormser-Shavit (1966), these times were judged to be the most informative. The results (Table 1) indicated that at rest the citrate content was more than 1μmol/g wet wt. of thorax, about 30-fold higher than the content of isocitrate. On flight these intermediates were diminished in concentration by about 30%, indicating a control at isocitrate dehydrogenase. The data are also consistent with the maintenance of equilibrium by aconitase and a free Mg^{2+} concentration of approx. 1.5mg-ions/litre (Veloso *et al.*, 1973) in the mitochondrial compartment. No significant differences between the values for 30s and 2min were obtained with these or the other intermediates examined. The present results indicate a citrate content far higher than that found by Sacktor & Wormser-Shavit (1966). However, the values for 2-oxoglutarate and malate were in fair agreement with those reported in the earlier study (Sacktor &

Table 1. *Concentrations of tricarboxylate-cycle intermediates in rested and flown flies*

Flies were treated and thoraces extracted as described in the Experimental section. The values are means ± S.E.M. and the numbers of extracts, containing at least eight thoraces each, are given in parentheses.

	Concentration (nmol/g wet wt. of thorax)		
	At rest	After 30s flight	After 2min flight
Citrate	1092 ±57 (10)	732 ±73 (9)†	686 ±75 (8)*
Isocitrate	30.3± 1.3 (8)	21.5± 1.7 (6)†	22.4± 1.7 (7)†
2-Oxoglutarate	89.5±11.8 (8)	102.0± 6.4 (6)	105.6±14.4 (7)
Malate	504 ±54 (10)	804 ±21 (9)*	834 ±32 (8)*

* The value is significantly different from that in the muscle at rest, $P<0.001$.
† The value is significantly different from that in the muscle at rest, $P<0.005$.

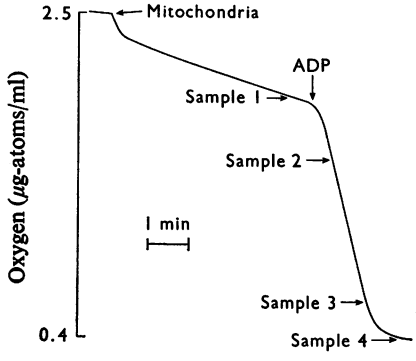

Fig. 1. *Concentrations of tricarboxylate-cycle intermediates during a state 4→3→4 transition*

Mitochondria (15mg of protein) were incubated in 19ml of a medium (see under 'Methods') containing 2.5mM-pyruvate, 0.5mM-ATP, 2.5mM-HCO_3^- and 20mM-P_i; 70μmol of ADP was added where indicated and the times of sampling were as shown on the O_2 electrode trace. The values shown below are nmol/mg of mitochondrial protein.

	Concentration			
Sample ...	1	2	3	4
Citrate				
Total	25.2	15.6	23.2	28.6
Mitochondrial	15.7	8.6	14.8	18.2
Isocitrate				
Total	1.13	0.15	0.58	1.02
Mitochondrial	0.78	0.09	0.44	0.63
2-Oxoglutarate				
Total	0.29	0.71	0.67	0.27
Mitochondrial	0.10	0.55	0.53	0.24
Malate				
Total	1.75	10.00	3.61	1.95
Mitochondrial	0.64	7.46	2.17	0.36

Wormser-Shavit, 1966), no significant change in 2-oxoglutarate content, but a marked rise on flight in the concentration of malate being observed.

Concentrations of tricarboxylate-cycle intermediates in isolated mitochondria

Fig. 1 illustrates an experiment in which flight-muscle mitochondria were incubated with pyruvate as substrate and in the presence of ATP and HCO_3^- to generate intramitochondrial oxaloacetate (Hansford, 1972*a*). A high concentration (20mM) of P_i was used to ensure maximal isocitrate dehydrogenase activity (Hansford, 1972*a*) and to prevent significant changes in P_i concentration during the experiment. Sampling as described in the Experimental section was carried out at the points indicated so that samples 1 and 4 corresponded to state 4 (nomenclature of Chance & Williams, 1955), and samples 2 and 3, immediately after ADP addition, were taken during state 3. The concentrations of intermediates in samples 1–4 are shown in the legend of Fig. 1. The addition of ADP caused a fall in the concentrations of citrate and isocitrate, and a rise in the concentrations of 2-oxoglutarate and malate. When all the ADP was phosphorylated, the changes in concentration were completely reversed so that the contents of sample 4 resembled those of sample 1. It was noted in these experiments that the precise time of sampling was an important variable. This is illustrated by a comparison of samples 2 and 3: although both samples were taken during the phase of maximal respiration it is clear that the intermediates were showing a return to state 4 concentrations in sample 3, a late state 3. This phenomenon was more clearly demonstrated in the experiments of Hansford (1974) when measuring concentrations of CoA and its thioesters in experiments of similar design.

This experiment was repeated with different results in terms of absolute concentrations of intermediates, although the pattern of change appeared to be the same. Since a mitochondrial estimate was not obtained in every case, the results for total incubation contents were normalized and the difference between a mean state 4 and a mean state 3 value was examined statistically. Although this procedure tends to minimize the differences since some late-state 3

Table 2. *Percentage change in total tricarboxylate-cycle intermediate concentrations between state 4 and state 3*

Mitochondria were incubated exactly as in Fig. 1 and the concentrations of intermediates in the total incubation were measured. The values were normalized (mean state 4 = 100%) and the difference between a mean state 4 (e.g. samples 1 and 4 of Fig. 1) and a mean state 3 (e.g. samples 2 and 3 of Fig. 1) was tested for significance by Student's *t* test. The values shown are mean percentage changes ± S.E.M. and the number of incubations sampled is given in parentheses.

Decrease in citrate concentration	23.8± 4.0 (6)	$P<0.005$
Decrease in isocitrate concentration	65.4± 8.2 (5)	$P<0.005$
Increase in 2-oxoglutarate concentration	67.8±22.0 (5)	$P<0.05$
Increase in malate concentration	152.0±38.4 (5)	$P<0.025$

Table 3. *Concentrations of tricarboxylate-cycle intermediates during uncoupler-stimulated respiration*

The conditions were as in Fig. 1 with the single exception that 0.6 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone replaced the addition of ADP. The uncoupling agent was added between samples 1 and 2, and sample 4 was taken in anaerobiosis. The values are means from duplicate experiments, and are nmol/mg of mitochondrial protein.

	Concentration			
Sample ...	1	2	3	4
Citrate				
Total	10.6	6.8	6.5	4.5
Mitochondrial	6.6	3.0	2.5	0.4
Isocitrate				
Total	0.61	0.40	0.38	0.40
Mitochondrial	0.33	0.08	0.05	0.07
2-Oxoglutarate				
Total	0.14	0.26	0.28	0.08
Mitochondrial	0.14	0.23	0.21	−0.04
Malate				
Total	0.81	1.46	1.27	0.92
Mitochondrial	0.23	0.82	0.70	0.34

contents were very like those of state 4 (e.g. see Fig. 1), the results (Table 2) indicated significant changes for all the intermediates.

Table 3 shows the results of a similar experiment with the uncoupling agent carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to elicit a rapid rate of respiration. The uncoupling agent, added between samples 1 and 2, caused changes similar to those observed with ADP addition, which were maintained in sample 3. The mitochondria were allowed to respire until anaerobiosis, when sample 4 was taken. Anaerobiosis produces a state in which the ATP/ADP ratio is low and the nicotinamide nucleotide is totally reduced; it caused a fall in concentration of all the measured intermediates, very marked for citrate and 2-oxoglutarate. It is possible that the concentration of succinate was increased under these conditions although direct measurements of succinate were not made owing to the presence of pyruvate and, presumably, ADP, which interfere with the fluorimetric succinate assay procedure (see Williamson & Corkey, 1969).

In Fig. 2 is shown an experiment in which the ATP/ADP ratio was manipulated independently of flux through the tricarboxylate cycle. This was achieved by the inclusion of oligomycin, an inhibitor of the mitochondrial adenosine triphosphatase, before the addition of ADP. Although substrate-level phosphorylation would tend slowly to increase the concentration of ATP present at the expense of ADP, it is probable that the intramitochondrial ATP/ADP ratio was much lower than the added ratio of 1:8 since coupled mitochondria selectively accumulate ADP by an obligatory exchange with ATP (Klingenberg, 1970). The effect of ADP addition was as follows: the concentrations of citrate and isocitrate were diminished, the former to a slightly greater extent; malate rose in concentration reflecting the increased nicotinamide nucleotide reduction and consequent inhibition by NADH of malate oxidation under these circumstances. Although isocitrate dehydrogenase is sensitive to inhibition by NADH (Hansford, 1968), on account of the lowered isocitrate concentration it appeared that this enzyme was more responsive towards the low ATP/ADP ratio. On stimulating respiration by addition of uncoupling agent, the nicotinamide nucleotide was reoxidized, causing a rise in citrate content. In view of the decrease in malate content it is suggested that the more oxidized nicotinamide nucleotide permitted a higher activity of malate dehydrogenase, with a consequent increase in availability of oxaloacetate to citrate synthase. It is also possible that NADH was inhibiting pyruvate dehydrogenase before the addition of uncoupler although data on this point are lacking. Isocitrate content remained low throughout, indicating the inability of aconitase to maintain equilibrium in the condition of elevated flux. Unfortunately, 2-oxoglutarate contents of the samples were so small that reliable measurements could not be made.

Although the mitochondrial experiments described up to this point provided clear evidence of the dominant role of isocitrate dehydrogenase in controlling tricarboxylate-cycle activity, they did not

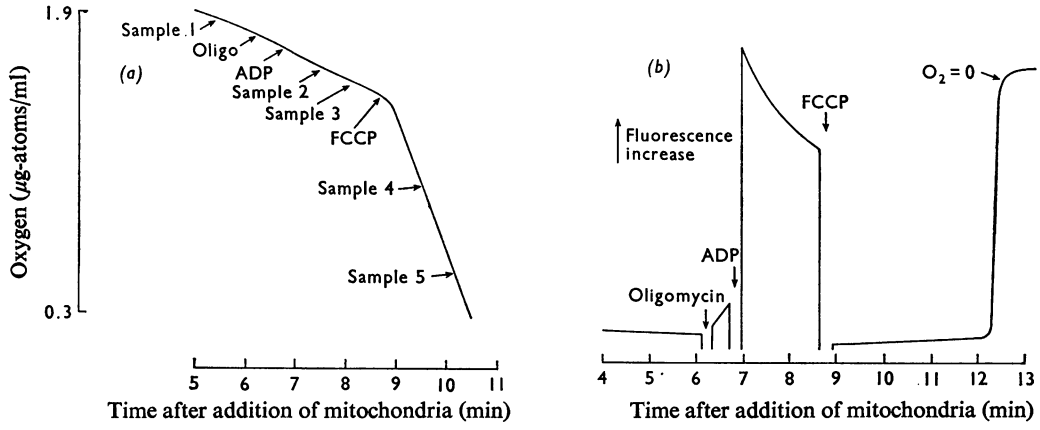

Fig. 2. *Concentrations of tricarboxylate-cycle intermediates and the redox state of NAD with oligomycin and ADP*

(*a*) Mitochondria (20mg of protein) were incubated under the conditions of Fig. 1. Where indicated on the O_2 electrode trace, oligomycin (Oligo) (25μg) and ADP (70μmol) were added, followed by 0.6μM-carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP) (final concentration). The total volume was initially 21ml. The sample contents given below are nmol/mg of mitochondrial protein, and are means from duplicate experiments. (*b*) Fluorimeter trace of intramitochondrial NADH on about 0.7mg of protein, 2.5μg of oligomycin and 7μmol of ADP but otherwise the conditions of (*a*). The total volume was 2ml. Different preparations of mitochondria were used to obtain these traces.

	Concentration				
Sample ...	1	2	3	4	5
Citrate					
Total	12.5	8.8	9.0	11.4	11.1
Mitochondrial	7.7	2.9	2.8	4.4	4.3
Isocitrate					
Total	0.70	0.58	0.59	0.65	0.67
Mitochondrial	0.41	0.20	0.18	0.14	0.18
Malate					
Total	0.72	5.74	5.70	2.54	2.30
Mitochondrial	0.19	4.80	4.60	1.22	0.91

closely mimic the physiological situation found in the fly. In the course of glycolysis equimolar quantities of pyruvate and glycerol 3-phosphate are produced (Zebe & McShan, 1957; Chefurka, 1958) and subsequently oxidized by the mitochondria [see Sacktor (1970) for review]. In addition, proline, present in the fly at high concentrations (Price, 1961), falls dramatically on flight (Sacktor & Wormser-Shavit, 1966) and is a substrate for mitochondrial oxidation (Hansford & Sacktor, 1970). Therefore, as an approximation of the biochemical events involved when a fly takes to flight, mitochondria were incubated with 5mM-glycerol 3-phosphate and 10mM-proline in addition to pyruvate; rapid respiration was initiated by the simultaneous addition of ADP and a low concentration (buffered to 4×10^{-7}g-ions/litre) of Ca^{2+} to reproduce the conditions of active muscle contraction. Both ADP and Ca^{2+} act as allosteric effectors in this situation: ADP activates proline dehydrogenase (Hansford & Sacktor, 1970) as well as isocitrate dehydrogenase (Hansford, 1972*a*) and 2-oxoglutarate dehydrogenase (Hansford, 1972*b*), and Ca^{2+} at low concentrations is required for the full activity of glycerol 3-phosphate dehydrogenase (Hansford & Chappell, 1967). The results are shown in Fig. 3. ADP and Ca^{2+} caused a substantial fall in the concentrations of citrate and isocitrate, and a rise in the concentrations of 2-oxoglutarate and malate, these changes becoming reversed in sample 3, a late state 3. Sample 4 represented a state presumably not found in the fly, namely the presence of an active (Ca^{2+}-stimulated) glycerol 3-phosphate oxidation in the absence of ADP. This caused extensive nicotinamide nucleotide reduction by reversed electron transport (Chance & Hollunger, 1961; Klingenberg & Bucher, 1961), and, principally, a resultant increase in malate content as found in the experiment of Fig. 2.

Finally, the effect of proline was considered in an experiment in which ATP and HCO_3^- were omitted so that proline alone provided tricarboxylate-cycle

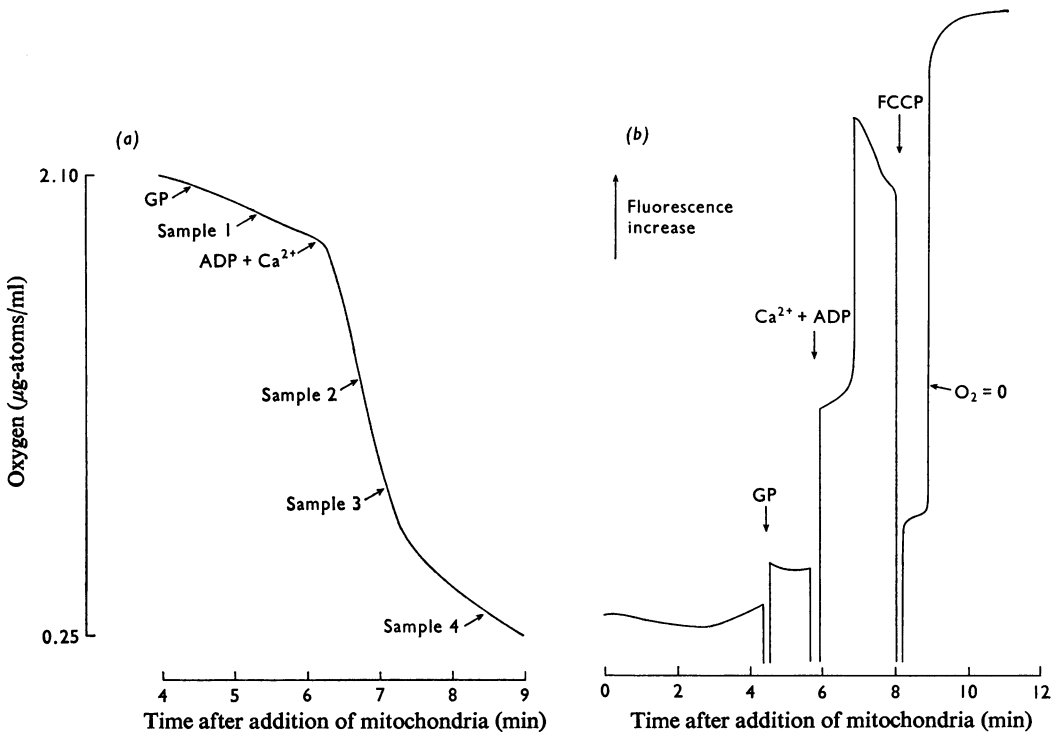

Fig. 3. *Concentrations of tricarboxylate-cycle intermediates and the redox state of NAD during a simulation of flight*

(*a*) Mitochondria (15mg of protein) were incubated in 19ml of medium (see under 'Methods') containing 2.5mM-pyruvate, 0.5mM-ATP, 2.5mM-HCO_3^-, 20mM-P_i and 10mM-proline. Where indicated on the O_2 electrode trace 5mM-DL-glycerol 3-phosphate (GP) was added followed by the simultaneous addition of ADP (30μmol) and Ca^{2+} (buffered to 0.4μM, final concentration, with a molar ratio of $CaCl_2$/ethanedioxybis(ethylamine)tetra-acetate of 3:4 at pH7.2). The sample contents given below are nmol/mg of mitochondrial protein, and are means from duplicate experiments. (*b*) Fluorimeter trace of intramitochondrial NADH on about 0.7mg of protein and 4μmol of ADP but otherwise the conditions of (*a*). Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added to 0.6μM to establish anaerobiosis. The total volume was 2ml. Different preparations of mitochondria were used to obtain these traces.

	Concentration			
Sample ...	1	2	3	4
Citrate				
Total	26.9	19.7	25.1	28.9
Mitochondrial	16.7	9.2	14.3	17.3
Isocitrate				
Total	1.48	0.68	1.13	1.26
Mitochondrial	0.80	−0.03	0.29	0.39
2-Oxoglutarate				
Total	1.48	1.74	1.28	1.20
Mitochondrial	0.34	0.72	0.24	0.30
Malate				
Total	1.12	6.48	4.38	5.26
Mitochondrial	0.07	5.54	3.36	3.91

intermediate for the complete oxidation of pyruvate. The results are shown in Table 4, ADP being added after sample 1. The effect of ADP addition was to increase the concentrations of citrate, 2-oxoglutarate and malate but to decrease isocitrate content. This result is probably explained by the activation of proline dehydrogenase by ADP (Hansford & Sacktor, 1970), which causes an increase in the overall con-

Table 4. *Concentrations of tricarboxylate-cycle intermediates during oxidation of pyruvate and proline*

The incubation medium (19ml total volume) contained 2.5mM-pyruvate, 20mM-P_i and 10mM-proline. Sample 1 was taken 4min after the mitochondrial addition (18mg of protein), and ADP (50μmol) followed 30s later. Samples 2 and 3 were taken in state 3 and sample 4 in state 4. The values are nmol/mg of mitochondrial protein, and are means from duplicate experiments.

	Concentration			
Sample ...	1	2	3	4
Citrate				
Total	10.1	13.8	15.1	24.4
Mitochondrial	5.7	8.1	9.6	17.1
Isocitrate				
Total	0.84	0.72	0.75	1.58
Mitochondrial	0.41	0.24	0.25	1.00
2-Oxoglutarate				
Total	2.19	2.67	2.62	2.47
Mitochondrial	0.28	0.75	0.69	0.19
Malate				
Total	0.76	1.77	2.60	0.73
Mitochondrial	0.33	1.21	2.04	0.07

centration of tricarboxylate-cycle intermediate. Some indication that this is true is found by comparison of sample 4, taken in state 4, and sample 1: the total concentration of tricarboxylate-cycle intermediate measured in sample 4 was 29.2nmol/mg of protein and in sample 1 was 13.9nmol/mg of protein. Thus it appeared that although isocitrate dehydrogenase was activated and the isocitrate concentration fell as a result, the stimulated proline oxidation caused an increased concentration of tricarboxylate-cycle intermediate sufficient to obscure any diminution in citrate concentration. A corollary is that aconitase was unable to maintain equilibrium under these conditions. After the ADP was phosphorylated (sample 4) a large rise in the concentrations of citrate and isocitrate was found, together with a diminished content of 2-oxoglutarate and malate, more typical of the state 3$\rightarrow$state 4 transition when ATP and HCO_3^- are used to provide tricarboxylate-cycle intermediate (see Fig. 1).

Kinetics of citrate synthase

In view of the high concentrations of citrate found in mitochondria in state 4, a kinetic study of a crude citrate synthase preparation was undertaken to determine whether citrate was an effector of this enzyme. Fig. 4 illustrates the competitive nature of citrate and oxaloacetate: the K_m for oxaloacetate was raised from 1.7 to 6.6μM in the presence of 5mM-

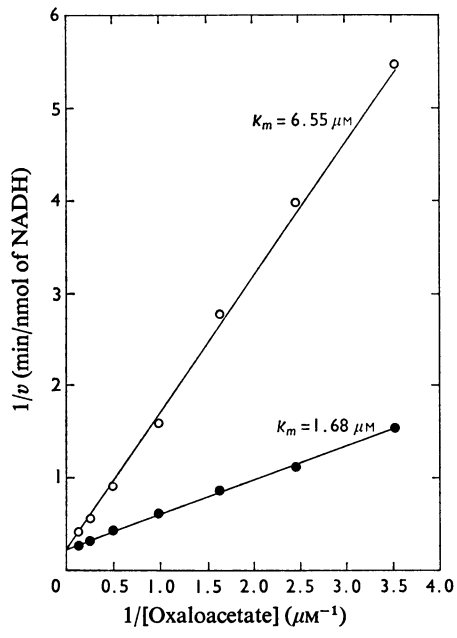

Fig. 4. *Inhibition of a crude citrate synthase by citrate*

Each determination was made in 1.8ml of 50mM-KCl–50mM-2-(*N*-2-hydroxyethylpiperazin-*N*'-yl)ethanesulphonate, pH7.2, containing 50μg of malate dehydrogenase (Boehringer Mannheim Corp.) and 10μl of crude citrate synthase solution (see under 'Methods'). Various volumes of 0.1M-malate–5mM-NAD^+ were added to give the oxaloacetate concentrations indicated and the reaction was started by the addition of acetyl-CoA to a concentration of 42μM (●). When added, citrate was present at a concentration of 5mM (○). v is the initial rate of change of NADH concentration measured fluorimetrically.

citrate, which indicates a K_i value for citrate of 1.7mM, almost identical with that obtained for the bovine heart enzyme (Smith & Williamson, 1971). This is a significant finding since the intramitochondrial citrate concentration is at least 5mM (based on a mitochondrial matrix volume of 2.5μl per mg of protein) in the resting state when malate, and hence oxaloacetate, are limitingly low. Control of citrate synthase by oxaloacetate availability is made more probable therefore.

Discussion

In the assessment of the results presented here it is of interest to use as a basis for comparison the extensive work of LaNoue *et al.* (1970, 1972) on the control of the tricarboxylate cycle in rat heart,

another predominantly catabolic tissue. The fly mitochondrion is more easily amenable to experimentation: rapid separation methods for the differentiation of intra- and extra-mitochondrial intermediates were unnecessary in view of the low permeability of flight-muscle mitochondria to citrate-cycle intermediates (Van den Bergh & Slater, 1962), and very high respiratory control ratios (20 or more) gave correspondingly high changes in flux through the tricarboxylate cycle. This permitted a more direct experimental design in which the flux was altered, often more than once, in a particular incubation. Also it allowed most of the changes noted in the text to be discerned in the total incubation mixture, indicating that most of the intermediates were within the matrix compartment and responsive to metabolic change. Although more than a minute elapsed between deproteinization of the sample of total incubation mixture and that of supernatant fluid in any given sample, it was expected that further metabolism of extramitochondrial intermediates in the supernatant sample would have been limited by the availability of suitable enzymes and cofactors.

Inspection of the results led to the following generalization: whenever tricarboxylate-cycle flux was increased, in either mitochondrial experiments or the fly, isocitrate concentration was diminished. Since isocitrate dehydrogenase catalyses a non-equilibrium reaction [according to the definition of Rolleston, 1972, i.e. ΔG is less than -12.7 kJ (-2.8 kcal)], this observation permitted the conclusion that isocitrate dehydrogenase was the major control point in the state of lower flux. There was one notable exception to the generalization outlined above: in the experiment of Fig. 2 uncoupling agent was used to stimulate respiration after a prior addition of oligomycin and ADP. Under these conditions no fall in isocitrate concentration was observed with increased flux. However, the addition of oligomycin and ADP considerably lowered the isocitrate concentration, in spite of extensive nicotinamide nucleotide reduction, with the result that no further decrease was detectable when the flux increased. This important finding demonstrated the dominance of the ATP/ADP ratio over nicotinamide nucleotide reduction in regulating isocitrate dehydrogenase.

Citrate responded in a similar fashion to isocitrate as would be expected if the aconitase reaction was close to equilibrium. In the intact fly there was a parallel fall in thoracic citrate and isocitrate content, indicating a strict maintenance of equilibrium, although in some mitochondrial experiments, for example those of Figs. 1 and 3, there was a pronounced increase in the citrate/isocitrate ratio in the state of higher flux. It is not apparent why this displacement from equilibrium should be seen in isolated mitochondria and not in the intact insect. When proline was used to generate tricarboxylate-cycle intermediate (Table 4) the concentration of citrate was elevated in state 3. This was explained by the activation of proline dehydrogenase by ADP (Hansford & Sacktor, 1970) causing a large increase in total tricarboxylate-cycle intermediate. It is probably significant that locust flight-muscle mitochondria as prepared contain a high concentration of citrate (Bellamy, 1962), although no information is available on its response to metabolic state.

In general, 2-oxoglutarate was present in low concentration although elevated in the presence of proline. Increasing flux tended to be associated with increased 2-oxoglutarate concentration although the changes were small, probably reflecting the adenine nucleotide control of both isocitrate dehydrogenase (Hansford, 1972*a*) and 2-oxoglutarate dehydrogenase (Hansford, 1972*b*). It was not possible to find a significant change of 2-oxoglutarate content in the fly: 2-oxoglutarate content was extremely variable so that any small change was not discernible.

Malate concentration was strongly influenced by two factors: flux through the tricarboxylate cycle and nicotinamide nucleotide redox state. In experiments where the nicotinamide nucleotide was extensively oxidized, the concentration of malate increased as the tricarboxylate-cycle flux increased. However, when the nicotinamide nucleotide was extensively reduced in the state of low flux, as with ADP and oligomycin, malate concentration was high, and was slightly diminished as the flux increased and the nicotinamide nucleotide became more oxidized. The response of malate concentration to nicotinamide nucleotide redox state is most easily explained by an inhibition of malate dehydrogenase under conditions of high reduction. This accounts for the very large increases in malate content which cannot be due simply to a modification of the malate/oxaloacetate ratio since there is insufficient oxaloacetate available to accommodate such large changes. One unexplained result was the finding of a low malate concentration in anaerobiosis. In being a state in which the ATP/ADP ratio is low and the nicotinamide nucleotide is highly reduced, it resembled the oligomycin-plus-ADP state, and changes in the other intermediate contents were consistent with this supposition. The values obtained for malate content in the two states were quite discrepant, however.

It is concluded that the tricarboxylate cycle in fly flight muscle is controlled primarily by isocitrate dehydrogenase and that the ATP/ADP ratio within the mitochondrial matrix is the major effector of this enzyme. In as much as ATP and ADP contents vary in a reciprocal fashion, the relative importance of the two adenine nucleotides cannot be determined in experiments involving intact mitochondria. Consistent with this proposal, the concentration of ADP in mitochondria in state 4 is vanishingly small (Hansford, 1975); this would suggest that the ADP assayed

by Sacktor & Hurlbut (1966) in the resting fly was not accessible to the mitochondria. A small increase in ADP concentration was found on flight (Sacktor & Hurlbut, 1966) and it is expected that this change would be magnified within the mitochondria since coupled mitochondria accumulate ADP in preference to ATP which is displaced (Klingenberg, 1970). That modulation by NADH is insignificant *in vivo* is supported by the finding (Hansford, 1975) that on flight nicotinamide nucleotide of the thorax showed increased reduction. It is also significant that a similar observation was made with mitochondria during a state 4 → state 3 transition (Hansford, 1972*a*) although the conditions used are critical (Hansford, 1975).

The following scheme is proposed on the basis of this work and that of Hansford (1974). In the resting state, the ATP/ADP ratio is high and this serves to limit primarily the activity of isocitrate dehydrogenase. Hence, an accumulation of acetyl-CoA, citrate and isocitrate is observed together with very low concentrations of 2-oxoglutarate, succinyl-CoA and malate. The concentration of malate is significant in that it reflects the amount of oxaloacetate available to citrate synthase, which is presumed to be limitingly low in the resting state. In addition, the competition of citrate and oxaloacetate at citrate synthase will further decrease the activity of this enzyme. When the fly takes to flight, the increased concentration of ADP signals an activation of isocitrate dehydrogenase and the concentrations of 2-oxoglutarate, perhaps succinyl-CoA, and malate are increased at the expense of acetyl-CoA, citrate and isocitrate. In this way the malate-plus-oxaloacetate pool is increased in size so as to more than offset any increase in nicotinamide nucleotide reduction and to increase the provision of oxaloacetate for citrate synthase. Competition of citrate with oxaloacetate is also diminished under these conditions since citrate is lowered in concentration as the oxaloacetate concentration is raised.

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