Changes in the Contents of Adenine Nucleotides and Intermediates of Glycolysis and the Citric Acid Cycle in Flight Muscle of the Locust upon Flight and their Relationship to the Control of the Cycle

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(Received 22 August 1978)

1. The contents of some intermediates of glycolysis, the citric acid cycle and adenine nucleotides have been measured in the freeze-clamped locust flight muscle at rest and after 10 s and 3 min flight. The contents of glucose 6-phosphate, pyruvate, alanine and especially fructose biphosphate and triose phosphates increased markedly upon flight. The content of acetyl-CoA is decreased after 3 min flight whereas that of acetylcarnitine is decreased markedly after 10 s flight, but returns towards the resting value after 3 min flight. The content of citrate is markedly decreased after both 10 s and 3 min flight, whereas that of isocitrate is changed very little after 10 s and is increased by 50 % after 3 min. The content of oxaloacetate is very low in insect flight muscle and hence it was measured by a sensitive radiochemical assay. The content of oxaloacetate increased about 2-fold after 3 min flight. A similar change was observed in the content of malate. The content of ATP decreased about 15 %, whereas those of ADP and AMP increased about 2-fold after 3 min flight. 2. Calculations based on O_2 uptake of the intact insect indicate that the rate of the citric acid cycle must be increased >100-fold during flight. Consequently, if citrate synthase catalyses a non-equilibrium reaction, the activity of the enzyme must increase >100-fold during flight. However, changes in the concentrations of possible regulators of citrate synthase, oxaloacetate, acetyl-CoA and citrate (which is an allosteric inhibitor), are not sufficient to account for this change in activity. It is concluded that there may be much larger changes in the free concentration of oxaloacetate than are indicated by the changes in the total content of this metabolite or that other unknown factors must play an additional role in the regulation of citrate synthase activity. 3. The increased content of oxaloacetate could be produced via pyruvate carboxylase, which may be stimulated during the early stages of flight by the increased concentration of pyruvate. 4. The decreases in the concentrations of citrate and α -oxoglutarate indicate that isocitrate dehydrogenase and oxoglutarate dehydrogenase may be stimulated by factors other than their pathway substrates during the early stages of flight. 5. Calculated mitochondrial and cytosolic $NAD^+/NADH$ ratios are both increased upon flight. The change in the mitochondrial ratio indicates the importance of the intramitochondrial ATP/ADP concentration ratio in the regulation of the rate of electron transfer in this muscle.

The citric acid cycle is the most important metabolic pathway for energy production under aerobic conditions. In muscle, the energy that is formed in the cycle (from the oxidation of glucose and/or fatty acids) supports sustained mechanical activity (Alp *et al.*, 1976). Consequently, the rate of energy formation and thus the rate of the cycle must be increased when the mechanical activity of muscle is increased. However, in contrast to glycolysis, in which a detailed theory of control in relation

to energy demand can be proposed (see Sugden & Newsholme, 1975; Newsholme, 1976), much less information is available on the control of the citric acid cycle in muscle. Studies on the isolated perfused rat heart suggest that citrate synthase and 2-oxoglutarate dehydrogenase may catalyse important regulatory reactions (England & Randle, 1967). On the other hand, studies with insect flight muscle and isolated mitochondria from these muscles have indicated that isocitrate dehydrogenase may catalyse an important reaction (Hansford, 1974; Johnson & Hansford, 1975).

Insect flight muscle offers several advantages in the study of the control of the citric acid cycle:

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

there is a very large increase in the rate of the cycle when flight is initiated (>100-fold; see Chance & Sacktor, 1958: the increase in the rate of the cycle in the isolated perfused rat heart when the work load is increased is only 4-fold); mitochondria from insect flight muscle are impermeable to most, if not all, of the intermediates of the cycle, so that changes in the concentrations of the latter should be restricted to the mitochondrial compartment in these muscles and this facilitates the problem of interpretation of changes in metabolite concentrations; the flight muscle, which is almost instantaneously frozen *in situ*, is readily available since it is squeezed out from the thorax in the frozen state by freeze clamping (see Beis & Newsholme, 1975). To provide a better understanding of the factors that are involved in the control of the citric acid cycle in insect flight muscle, the concentrations of acetyl-CoA, CoA, oxaloacetate, malate, citrate, isocitrate, oxoglutarate, glutamate, NH_3 and the adenine nucleotides in particular have been measured in the freeze-clamped muscle of resting and flying insects. Changes in the concentrations of these intermediates have provided information about the regulation of citrate synthase, about the importance of the isocitrate and oxoglutarate dehydrogenase reactions in regulation of the rate of flux of residues through the cycle and about changes in the mitochondrial and cytoplasmic redox state upon flight. In addition, the concentrations of some of the glycolytic intermediates have been measured. The concentrations of metabolic intermediates in locust flight muscle at rest and during flight are presented and discussed in this paper.

Materials and Methods

Chemicals and enzymes

All chemicals and enzymes were obtained from Boehringer Corp. (London), London W5 2TZ, U.K., except for the following: antimycin A and oxaloacetate were obtained from Calbiochem, London W1H 1AS, U.K. The scintillants, and also malate and 2-mercaptoethanol, were obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K.; radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks. HP7 9LL, U.K.; EDTA, toluene, alanine, aspartate, Hepes and all inorganic compounds were obtained from BDH Chemicals, Poole, Dorset BH12 4NN, U.K.

Sources of animals

Locusts (*Schistocerca gregaria*) were obtained from stocks maintained in this Department, or from the Department of Zoology, University of Cambridge.

Experimental locust flight

Locusts were induced to fly by suspending them facing into a wind tunnel through which a current of air, at about 28°C, was passed at about 3 m/sec. The locusts were suspended from a clamp in front of the wind tunnel by means of a thin piece of wire, which was attached to the dorsal part of the thorax by beeswax (Weis-Fogh, 1952). Sexually mature adults (14 days after the final moult) of both sexes were used.

The insects were freeze-clamped at rest, after 10 s and 3 min of flight. In the case of the resting insects, the locusts were caught by hand and then clamped. No marked differences in metabolite concentrations were observed if the locust was anaesthetized with CO_2 before freeze-clamping (see Beis & Newsholme, 1975).

Extraction of frozen flight muscle

Freeze-clamping of the whole insect caused the flight muscle to be squeezed out of the thorax. Any fat-body squeezed out of the thorax appeared as bright yellow against the buff colour of the muscle and was chipped away before further treatment of the freeze-clamped muscle. The latter was powdered in a percussion mortar and extracted with HClO_4 as described by Beis & Newsholme (1975). In some cases a slightly different procedure was used when less than 200 mg of muscle was extracted: HClO_4 was frozen in a porcelain mortar pre-cooled in liquid N_2 and ground to a powder. A known weight of frozen HClO_4 powder was placed in a plastic centrifuge tube cooled in liquid N_2 . A weighed quantity of muscle powder was added to the tube and the tissue and HClO_4 mixed thoroughly by using a thin metal seeker (at liquid N_2 temperatures). A further quantity of powdered HClO_4 was added to give an HClO_4 /tissue ratio of 3:1 (w/w). The tube was then removed from the liquid N_2 bath and allowed to thaw at 0°C. The mixing was continued during the thawing. After the extract had thawed, the protein precipitate was removed by centrifugation and the remainder of the procedure was as described by Beis & Newsholme (1975).

Collection of haemolymph

Haemolymph was collected either directly by inserting a hypodermic needle into the thorax or indirectly by the method of Walker *et al.* (1970). In the latter method a diagonal cut was made across the head of the locust and the insect was then placed head down in a conical centrifuge tube that had been pre-weighed. The animal was spun gently for 2 min at about 25g. The insect was then removed and the tube plus haemolymph weighed (if the contents were contaminated with vomit, the sample was discarded).

When haemolymph was removed directly by means of a syringe, the insect was anaesthetized with CO₂. Three vol. of 6% (w/v) HClO₄ was added to the sample, and the protein precipitate removed by centrifugation. The extract was then neutralized as described above.

Assay of metabolic intermediates

Except where otherwise stated all measurements were carried out at 340nm (with a Gilford 240 recording spectrophotometer). The concentrations of the metabolic intermediates given in Table 1 were measured by the methods given by Bergmeyer (1963) except for the following. Alanine was assayed kinetically in 2.0ml of medium consisting of 75mM-triethanolamine, 0.17mM-NADH, 5mM-oxoglutarate, 10 μ l of extract and 20 μ g of lactate dehydrogenase, at pH7.6. Known quantities of alanine were used to construct a standard curve; corrections were made for the inhibitory effect of the extract. The blank rate was measured and then 20 μ g of glutamate-pyruvate transaminase was added to obtain the experimental rate. P_i was determined by the method of Atkinson *et al.* (1973).

Glycogen was extracted from the muscle as follows. Approx. 50mg of frozen muscle powder was homogenized in 1.0ml of 2% HClO₄. The homogenate was left to stand overnight at room temperature, after which the precipitate was removed by centrifugation. The supernatant was neutralized with 3M-KHCO₃ and the glycogen determined by the method of Krebs *et al.* (1963). Acetyl-CoA and CoA were assayed by the method of Allred & Guy (1969) as modified by Hansford (1974). Acetylcarnitine was assayed by modification of a method described for measurement of acetyl-CoA (Decker, 1963). The assay medium consisted of 100mM-Hepes, 20mM-malate and 0.5mM-NAD⁺ at pH8.2. To 1ml of this medium in a cuvette, 100–400 μ l of extract was added and then 20 μ g of malate dehydrogenase and 20 μ g of citrate synthase. The reaction was initiated by addition of 50 μ l of 10mM-CoA and 20 μ g of acetylcarnitine transferase. Citrate was assayed by the method of Moellering & Grüber (1966). Proline was assayed by the method of Troll & Lindsley (1955), except that it was found unnecessary to shake with Permutit.

Oxaloacetate was assayed by the method of Löffler & Wieland (1963) as modified by Baird *et al.* (1968). However, since considerable difficulty was experienced in obtaining a satisfactory assay for oxaloacetate by the method described by these workers, precise details of the method used in the present work are given. After extraction with HClO₄, 2M-NaOH was added to the solution to produce a pH of 11–12, and it was then centrifuged at 3000g for 5min in a bench centrifuge. The total

supernatant was removed and incubated at 30°C for 90min to hydrolyse endogenous acetyl-CoA. A volume (0.5ml) of this extract was added to 1.0ml of 200mM-Tris/HNO₃, which contained 25nmol of [¹⁴C]acetyl-CoA (sp. radioactivity 8214 c.p.m./nmol) and 10 μ g of citrate synthase, at pH7.4 (at this stage the pH was tested to ensure that it was below 8.5), and the mixture incubated at 30°C for 30min. The pH was taken to 11–12 by addition of 2M-KOH and the solution incubated at 30°C for 90min to hydrolyse acetyl-CoA. The pH was taken down to 1–2 by addition of 2M-HNO₃ and the solution evaporated to complete dryness at 65°C under N₂ (this procedure removed ¹⁴C-labelled acetate). The N₂ was heated before passing over the solution to accelerate the evaporation. The residue was taken up in 1.0ml of cold water and, if necessary, the pH was adjusted to about 5.5 with 2M-KOH. A volume (20 μ l) of 10% potassium citrate was added, and then 2.0ml of 5% AgNO₃ in 0.5M-Tris/HNO₃ at pH5.5. The silver citrate was then precipitated by the addition of 4.0ml of a solution of acetone/ethanol (1:1, v/v). The suspension was centrifuged and the precipitate washed twice with 5.0ml of a solution of acetone/ethanol/AgNO₃ (1:1:1, by vol.). The precipitate was taken up in 1.2ml of 0.1M-HNO₃ and the silver precipitated with 0.3ml of 2M-HCl. The mixture was centrifuged and 1.0ml of the supernatant was placed in a scintillation vial. After addition of 9ml of scintillant, which consisted of a mixture of sulphur-free toluene and Triton X-100 (1:0.5, v/v) containing 2,5-diphenyloxazole plus 1,4-bis-(5-phenyloxazol-2-yl)benzene (6g and 0.1g per 1.5 litres of toluene plus Triton X-100 respectively; see Patterson & Greene, 1965), the radioactivity in the sample was measured in a Beckman liquid-scintillation counter (model LS 2B). A standard curve was constructed by using oxaloacetate dissolved in 0.01M-KOH. It is important that the standard solution be made up fresh each time in 2M-KOH at 0°C and diluted as required just before use. A range of 0.25–10nmol of oxaloacetate was used to construct the standard curve. [The oxaloacetate concentration in the standard solution was checked by using a spectrophotometric assay for oxaloacetate (Hohorst & Reim, 1963)]. The minimal concentration of oxaloacetate measured by this method was 1.0nmol/g of flight muscle (i.e. about 0.2–0.3nmol/ml of extract).

NH₃ was determined with glutamate dehydrogenase in the following way. An assay mixture was made up consisting of 100mM-sodium phosphate buffer, 1mM-ADP, 0.34mM-NADH, 5mM-2-oxoglutarate at pH7.0, and 100 μ g of glutamate dehydrogenase in glycerol in a final volume of 2.0ml. The pH of the mixture was 7.6. (All solutions were made up with doubly distilled water in thoroughly rinsed containers.) The cuvettes were then allowed to

equilibrate. (If there was an excessive decrease in the absorbance, presumably due to NH_3 contamination of the solution, the solutions were discarded and fresh ones prepared.) The change in A_{340} on addition of a volume (100 μl) of extract was equivalent to the concentration of NH_3 . The extract was pretreated by shaking with activated charcoal to remove flavins and nucleotides. The method is adapted from that of Williamson *et al.* (1967).

Extracellular space

The extracellular space was determined in the freeze-clamped flight muscle by using [^3H]inulin by the method of Hill *et al.* (1968). The inulin space of the flight muscle constitutes about 30% of the total space in the muscle. The following compounds were found to be present at sufficient concentrations in the extracellular space to warrant correction for the presentation in Table 1; alanine (1.9 $\mu\text{mol/ml}$ of haemolymph), citrate (0.64 $\mu\text{mol/ml}$), proline (6.0 $\mu\text{mol/ml}$), NH_3 (1.0 $\mu\text{mol/ml}$) and glutamate (0.18 $\mu\text{mol/ml}$). It should be emphasized that metabolite concentrations were measured only in haemolymph obtained from resting insects, but these

concentrations were used to correct concentrations in flying insects. It proved impossible to obtain haemolymph from the flying locusts.

Results

The content of glycogen decreased from 7.2 ± 0.98 to $2.6 \pm 0.59 \mu\text{mol}$ of glucose equivalent/g of muscle (means $\pm$ S.E.M. for seven individual insects) during the first 10 s of flight, but it was not further decreased after 3 min flight. It is likely, therefore, that endogenous glycogen is the first fuel to be utilized by the muscle in flight, during which time muscle and haemolymph trehalase is probably activated to provide glucose for utilization by the muscle (see Downer & Matthews, 1977). The rapid and large increase in the content of glucose 6-phosphate (Table 1) is consistent with this increased rate of glycogenolysis. [It is noteworthy that the rate of glycogenolysis measured by the change in glycogen content during the first 10 s of flight is considerably greater than the maximum phosphorylase activity (Crabtree & Newsholme, 1972). This suggests either that enzymes other than phosphorylase are involved in catalysing glycogen breakdown or that conditions that fully

Table 1. Contents of adenine nucleotides, P_i , some intermediates of the citric acid cycle and some amino acids in freeze-clamped locust flight muscle at rest and during flight

The contents of the metabolites were measured as described in the Materials and Methods section. The values have been adjusted for the concentration of metabolite present in the extracellular fluid (see the text). Results are presented as means $\pm$ S.E.M. with the numbers of animals given in parentheses. The asterisks indicate that the difference from the value at rest is statistically significant (* $P < 0.01$; ** $P < 0.001$).

Metabolite	Metabolite content ($\mu\text{mol/g}$ of frozen muscle)		
	Rest	10 s flight	3 min flight
Glucose 6-phosphate	0.099 ± 0.005 (6)	0.245 ± 0.016 (8)**	0.222 ± 0.029 (9)**
Fructose 6-phosphate	0.026 ± 0.001 (8)	0.022 ± 0.003 (7)	0.044 ± 0.007 (9)
Fructose biphosphate	0.052 ± 0.007 (8)	0.640 ± 0.060 (7)**	0.122 ± 0.012 (5)**
Triose phosphate	0.045 ± 0.003 (6)	0.116 ± 0.008 (12)**	0.054 ± 0.005 (9)
Glycerol 3-phosphate	0.29 ± 0.03 (8)	0.26 ± 0.03 (7)	0.17 ± 0.03 (8)*
Pyruvate	0.064 ± 0.009 (9)	0.361 ± 0.031 (15)**	0.238 ± 0.040 (9)**
Lactate	0.61 ± 0.06 (8)	0.36 ± 0.03 (7)*	0.35 ± 0.03 (7)*
Aspartate	0.93 ± 0.08 (7)	1.19 ± 0.13 (8)	2.80 ± 0.08 (6)**
Glutamate	4.94 ± 0.19 (7)	4.29 ± 0.24 (9)	3.36 ± 0.13 (6)**
Proline	7.94 ± 0.72 (6)	5.85 ± 0.94 (6)	6.05 ± 0.62 (6)
Alanine	1.40 ± 0.17 (6)	5.27 ± 0.18 (6)**	4.56 ± 8.72 (6)**
Acetylcarnitine	0.265 ± 0.011 (5)	0.081 ± 0.009 (6)**	0.237 ± 0.011 (12)
Acetyl-CoA	0.061 ± 0.002 (6)	0.055 ± 0.003 (6)	0.031 ± 0.003 (5)**
CoA	0.064 ± 0.002 (6)	0.057 ± 0.006 (6)	0.079 ± 0.009 (5)
Citrate	0.788 ± 0.076 (6)	0.174 ± 0.035 (8)**	0.156 ± 0.033 (7)**
Isocitrate	0.025 ± 0.002 (6)	0.023 ± 0.004 (6)	0.036 ± 0.002 (7)*
2-Oxoglutarate	0.104 ± 0.024 (8)	0.087 ± 0.008 (13)	0.070 ± 0.016 (7)
Malate	0.214 ± 0.031 (7)	0.305 ± 0.018 (9)	0.398 ± 0.050 (9)*
Oxaloacetate	0.0020 ± 0.0002 (10)	0.0034 ± 0.0005 (10)*	0.0038 ± 0.0004 (11)*
NH_3	0.77 ± 0.08 (8)	0.90 ± 0.07 (7)	1.21 ± 0.10 (6)*
ATP	5.06 ± 0.20 (13)	4.58 ± 0.13 (19)	4.32 ± 0.10 (10)*
ADP	0.427 ± 0.028 (8)	0.961 ± 0.046 (12)**	1.10 ± 0.09 (10)**
AMP	0.059 ± 0.010 (7)	0.120 ± 0.009 (10)**	0.120 ± 0.016 (12)**
Phosphate	9.32 ± 1.16 (9)	13.50 ± 1.24 (10)	11.90 ± 0.71 (9)

activate mammalian muscle phosphorylase do not maximally activate insect flight-muscle phosphorylase.] The other marked change in the contents of glycolytic intermediates is that of fructose biphosphate and triose phosphates. There is a 10-fold increase in the content of fructose biphosphate in the first 10s of flight (Table 1); despite this, there is no evidence that fructose biphosphate functions as an allosteric regulator of either locust phosphofructokinase (it does not de-inhibit the enzyme; see Rowan, 1975) or pyruvate kinase (Zammit *et al.*, 1978).

The increases in contents of alanine and pyruvate (Table 1), which are in general agreement with previous studies on the locust (Kirsten *et al.*, 1963b) and the blowfly (Sacktor & Wormser-Shavit, 1966), are consistent with the increase in glycolytic flux. The content of lactate decreases upon flight, which suggests that the rate of conversion of pyruvate into lactate is low during flight in comparison with the rate of glycolysis: this is consistent with the low activities of lactate dehydrogenase in insect flight muscle (Crabtree & Newsholme, 1972) and it suggests that lactate might be used by another tissue (e.g. fat-body). The contents of acetyl-CoA and CoA are unchanged after 10s of flight, whereas after 3 min that of acetyl-CoA decreases and that of CoA increases (Table 1). Hansford (1974) did not detect any changes in the contents of acetyl-CoA and CoA in the flight muscle of the blowfly after 2 min flight. The content of acetylcarnitine is markedly decreased after 10s flight, but it returns towards the resting concentration after 3 min flight (Table 1). This contrasts with the change in content of acetylcarnitine reported by Sacktor & Childress (1967) in the blowfly flight muscle, in which it increased initially, but then fell to a new steady-state value. Acetylcarnitine concentrations may be controlled differently in the blowfly and locust flight muscles.

The content of citrate measured in the muscle is markedly decreased after 10s and 3 min flight. Johnson & Hansford (1975) stimulated the respiration of blowfly mitochondria by adding ADP and found a decrease in the content of citrate. However, the content of isocitrate changes very little after 10s, but it is increased by about 50% after 3 min flight (Table 1). The contents of oxaloacetate and malate increase about 2-fold after 3 min flight, but that of 2-oxoglutarate is decreased slightly. The content of aspartate is increased after 3 min, whereas that of glutamate decreases (these findings are similar to those of Kirsten *et al.*, 1963a). The content of NH_3 is increased by about 50% after 3 min flight, but the changes in the content of proline are not statistically significant.

The content of ATP is decreased about 15%, whereas those of ADP and AMP are approximately doubled after 3 min flight. There was little change in the content of P_i (Table 1).

Discussion

The citric acid cycle oxidizes acetate (in the form of acetyl-CoA) to CO_2 and water. The important reactions in the control of the early stages of the cycle are probably citrate synthase, isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase. It can be shown that the flux through the cycle increases at least 100-fold during flight (Chance & Sacktor, 1958), so that the flux of intermediates through these key reactions must also increase 100-fold, but the contents, and presumably the concentrations of the pathway substrates for these reactions (i.e. acetyl-CoA, isocitrate and oxoglutarate), do not increase by a 100-fold; the largest increase is that of isocitrate, which is approximately 50%, whereas those of acetyl-CoA and 2-oxoglutarate decrease (see Table 1). Thus it is concluded that the activities of these enzymes must be regulated by factors additional to changes in the concentrations of the pathway substrates (i.e. by co-substrates or allosteric effectors). Additional factors involved in the control of citrate synthase activity are discussed below.

Changes in the concentration of oxaloacetate and regulation of citrate synthase activity

Oxaloacetate occupies a key position in relation to the regulation of citrate synthase activity and hence in the regulation of the early stages of the citric acid cycle. The flux of residues into the cycle for oxidation are derived only from acetyl-CoA (originally derived from glucose, fat or amino acids), so that oxaloacetate plays a role as 'co-substrate' for the citric acid cycle. Consequently, any change in the concentration of oxaloacetate could change the rate of acetyl-CoA oxidation (provided that citrate synthase is not saturated with oxaloacetate; see below). Furthermore, insect flight muscle possesses high activities of pyruvate carboxylase, whose properties are consistent with the view that this reaction is important in the generation of oxaloacetate for the control of the cycle (Crabtree *et al.*, 1972; Rowan *et al.*, 1978). However, since the concentration of oxaloacetate is very low and below the detectability of the usual spectrophotometric or fluorimetric methods, few reliable measurements of its concentration have been reported. Consequently, in the present work the concentration of oxaloacetate was measured by using a sensitive radiochemical assay (see the Materials and Methods section).

The content of oxaloacetate found in resting locust flight muscle was $0.002 \mu\text{mol/g}$ of frozen muscle and this is almost doubled after 3 min flight (Table 1). Similarly, the content of malate, which is expected to be in near-equilibrium with oxaloacetate (since the activity of malate dehydrogenase is high in locust flight muscle), increases approx. 2-fold. However,

the concentration of oxaloacetate that might be present intramitochondrially was calculated by the method and assumptions described in detail by Gumaa *et al.* (1971). The calculated mitochondrial concentration of oxaloacetate increases by only 3-fold after 3 min flight, which is only slightly greater than the increase in the total content. The concentration of oxaloacetate that is either measured in the whole muscle or calculated to be present in the mitochondrial compartment in the resting muscle ($0.16\mu\text{M}$; Table 2) is considerably lower than the K_m of citrate synthase for oxaloacetate ($5\mu\text{M}$; see Alp *et al.*, 1976). Since the response of citrate synthase activity to oxaloacetate concentration obeys Michaelis-Menten kinetics (Alp, 1973) and since the enzyme probably catalyses a non-equilibrium reaction, a 3-fold increase in oxaloacetate concentration would be expected to increase the activity of citrate synthase by approx. 3-fold; but the flux through the cycle increases > 100-fold when the locust flies (see the introduction section). [If, however, citrate synthase catalyses a near-equilibrium reaction (see Alp *et al.*, 1976, for discussion), a small change in oxaloacetate concentration could produce a large change in the flux through this reaction (see Newsholme & Crabtree, 1973, 1976; Crabtree & Newsholme, 1978, for theory).]

Citrate is an allosteric inhibitor of citrate synthase from rat liver and blowfly flight muscle (Smith & Williamson, 1971; Johnson & Hansford, 1975), so that the decrease in citrate concentration upon flight could cause an increase in the activity of this enzyme. Nonetheless, the change in content of citrate is about 5-fold. It is possible that the change in the citrate concentration in the mitochondrial compartment is greater than 5-fold, but it is not possible at the present time to measure the mitochondrial and cytosolic concentrations of such intermediates in muscle. Nonetheless, the inner membrane of insect flight-muscle mitochondria is not permeable to citrate, so that it seems unlikely that any greater change in citrate concentration occurs within the mitochondria. Thus from the results of the present investigation it seems unlikely that the marked increase in activity of citrate synthase that occurs during the transition from rest to flight in the locust flight muscle can be explained by the changes in concentrations of oxaloacetate and citrate. This suggests that some additional factor(s) must also modulate the activity of citrate synthase from locust flight muscle. The activity is inhibited *in vitro* by ATP (ADP and AMP) and succinyl-CoA, but Hansford (1974) has provided evidence that these factors may not be very important in control of this enzyme *in vivo*. However, a considerable amount of oxaloacetate may be bound to protein, so that the free mitochondrial concentration of oxaloacetate at rest could be even lower than reported in

Table 1 and the change in this concentration upon flight could be greater than indicated from the results in Table 1. Such a large increase in the concentration of oxaloacetate could account for the marked increase in the activity of citrate synthase.

The increase in the contents of malate and oxaloacetate upon flight in the locust flight muscle could occur in at least two ways. First, increased activity of pyruvate carboxylase, which could be due to the increased concentration of pyruvate in the early stages of flight (see Table 1 and the work of Rowan *et al.*, 1978). Secondly, metabolites of the citric acid cycle could be redistributed from citrate towards oxaloacetate (i.e. from C_6 to C_4 intermediates). Such a redistribution could be caused by stimulation of the activities of isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase.

Changes in the cytosolic and mitochondrial redox states

The method and the assumptions implicit in the method for measuring the redox states (i.e. the NAD^+/NADH concentration ratio) in the cytosolic and mitochondrial compartments have been described in detail by Williamson *et al.* (1967). It should, however, be emphasized that there is less evidence in insects that these assumptions are justified than there is in mammalian tissues. The cytosolic ratio was obtained from the cytosolic glycerol phosphate dehydrogenase reaction [see Tables 1 and 2; the concentration of dihydroxyacetone phosphate was measured (results not given) for this calculation]. The ratio at rest was 1084 (which is similar to that reported for mammalian liver and heart) and this was increased to 2173 after 3 min flight (Table 2). The increase in the cytosolic ratio upon flight will play a role in the stimulation of the glyceraldehyde 3-phosphate dehydrogenase reaction during the raised demand for energy upon the initiation of flight. Indeed, if this reaction is close to equilibrium, small changes in the redox state could produce a large change in the flux through the reaction (see Newsholme & Crabtree, 1976; Crabtree & Newsholme, 1978).

The mitochondrial ratio was calculated from the glutamate dehydrogenase reaction (see Tables 1 and 2; the enzyme has an activity of $16\mu\text{mol/min per g}$ of fresh locust muscle, which is considered to be much greater than the flux through the reaction). The ratio at rest was 5.8 (which is similar to that reported for rat liver and heart) and this was increased to 8.1 after 3 min flight (Table 2). A change in the same direction was observed by Chance & Connelly (1957) with insect flight muscle and Jöbsis & Duffield (1967) with frog skeletal muscle. However, Hansford (1975) has shown that, in blowfly flight-muscle mitochondria, the NAD^+/NADH ratio decreases when the rate of the cycle was increased. If the processes of electron

Table 2. Calculated concentrations of oxaloacetate and malate and $NAD^+/NADH$ ratios in the cytoplasmic and mitochondrial compartments of the locust flight muscle

The concentrations have been calculated assuming 0.27 ml of cytoplasmic water and 0.15 ml of matrix water.

Metabolite	Calculated concentrations			
	Cytoplasmic compartment		Mitochondrial compartment	
	(nmol/g)	(μM)	(nmol/g)	(μM)
Resting muscle				
Malate	66	240	148	980
Oxaloacetate	2.0	7.4	0.02	0.16
$NAD^+/NADH$ ratio		1084		5.8
After 3 min flight				
Malate	64	230	334	2220
Oxaloacetate	3.8	14.0	0.07	0.50
$NAD^+/NADH$ ratio		2173		8.1

transport and oxidative phosphorylation are close to equilibrium in insect flight muscle as in liver cells and pigeon pectoral muscle mitochondria (see Wilson *et al.*, 1974; Erecińska *et al.*, 1974) the direction of change in the $NAD^+/NADH$ ratio in the mitochondria will depend on whether the stimulation of the rate of cycle is greater or less than the stimulation of the rate of electron transport. Nonetheless, whatever the direction of change in the mitochondrial $NAD^+/NADH$ ratio, the steady-state rate of electron transport (and oxidative phosphorylation) will depend on the rate of the citric acid cycle, which is the flux-generating process for electron transport (see Newsholme & Crabtree, 1973; Crabtree & Newsholme, 1975) and which is controlled by factors affecting the activity of citrate synthase (see above) and other reactions in the cycle.

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