REGULATORY PROPERTIES OF CHANGES IN THE CONTENTS OF COENZYME A, CARNITINE AND THEIR ACYL DERIVATIVES IN FLIGHT MUSCLE METABOLISM OF *LOCUSTA MIGRATORIA*

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Abstract—The concentrations of coenzyme A, carnitine and their acyl derivatives in flight muscles of the locust were determined during a two hours flight The concentration of acetyl-CoA fell sharply immediately after the onset of flight, whereas coenzyme A level remained relatively constant

Acetylcarnitine showed a gradual increase during the first half an hour of flight after which it remained at the same level

Long chain acyl-carnitines showed a steady increase throughout the entire flight period

The carnitine level decreased gradually during the first fifteen minutes of flight and then stabilized

The plot of the acetyl-CoA to CoA and acetylcarnitine to carnitine ratios shows that carnitine acetyltransferase is not at equilibrium in the resting locust, but is close to equilibrium shortly after the initiation and during maintenance of flight

A small pool of the acetyl-CoA appeared not to be in equilibrium with acetylcarnitine

A buffer function of carnitine acetyltransferase and regulatory properties of acetyl-CoA with respect to citrate synthase and pyruvate dehydrogenase are discussed

Key Word Index Locusta migratoria, coenzyme A/carnitine acyl derivatives, regulatory properties, flight muscle

INTRODUCTION

FATS, which have obvious body weight advantages over carbohydrates, provide the energy for cross-continental migration flights of locusts. In the early part of the flight of the migratory locust the haemolymph diglyceride level is elevated by mobilization from fat body lipid stores (TIETZ, 1967; JUTSUM and GOLDWORTHY, 1976), under the influence of an adipokinetic hormone released from the corpora cardiaca (MAYER and CANDY, 1969a; BEENAKKERS, 1969; GOLDSWORTHY *et al.*, 1972); concomitantly there is a rapid decrease in the carbohydrate level, stabilizing at about half the value found at rest (MAYER and CANDY, 1969b; JUTSUM and GOLDSWORTHY, 1976).

VAN DER HORST *et al* (1978) showed that the oxidation of diglycerides supplies the major portion of the energy requirements of locust flight muscles during prolonged flight, the turnover rate of the haemolymph diglyceride pool during flight is 3 4 mg diglyceride/insect/hr against a rate of 0 3–0.5 mg at rest.

There are several reports showing that an enhanced rate of fatty acid utilization can reduce the carbohydrate consumption; for instance, in mammalian heart muscle carbohydrate consumption is inhibited in the presence of long chain fatty acids (NEWSHOLME *et al.*, 1962) In the flight muscle of *Schistocerca gregaria* ROBINSON and GOLDSWORTHY (1977) reported that the adipokinetic hormone both stimulates the utilization of diglycerides and reduces

trehalose catabolism, however, CANDY (1978) could not confirm this observation.

An intimate relation between the processes of fatty acid β-oxidation, leading to the formation of acetyl-CoA, and the citrate cycle activity in flight muscle of *Locusta migratoria* can be inferred from developmental studies; during differentiation of the muscles there is a marked rise in the activity of enzymes of both processes with a concomitant decrease in the anaerobic glycolytic capacity of the tissue (BEENAKKERS *et al.*, 1975).

Short term control between fatty acid metabolism and glycolysis in insect flight muscle has scarcely been studied. The reciprocal control between glycolysis and fatty acid oxidation present in mammalian heart muscle acting via inhibition of phosphofructokinase by citrate is absent in flight muscles of *S gregaria* (WALKER and BAILEY, 1969). It has been recognized for some years that oxidation of long chain fatty acids is carnitine dependent and this was demonstrated also in flight muscle mitochondria of *Manduca sexta* (HANSFORD and JOHNSON, 1976) and *L. migratoria* (BEENAKKERS, 1963; BODE and KLINGENBERG, 1965)

An approach to the study of control mechanisms is determination of the steady state concentrations of intermediates and their changes upon alterations of the flux through the metabolic pathway (see ROLLESTON, 1972, for a review of this approach). Therefore, the present paper describes the changes in acetylcarnitine, long chain acyl-carnitine and acetyl-CoA, together with changes in the pools of free carnitine and CoA in locust flight muscle during

exercise. A regulatory role of these intermediates is discussed

MATERIALS AND METHODS

Adult males of *Locusta migratoria migratorioides*, twelve days after imaginal ecdysis, were used The locusts were reared and flown under conditions described previously, termination of flight and dissection of the flight muscles (WORM and BEENAKKERS, 1980) were also carried out as before

For each extract, used for determination of intermediates, the dorsolongitudinal flight muscles of ten locusts were pooled Preparation of the extracts was based on methods described in BERGMEYER (1974)

Carnitine and acetylcarnitine

The tissue was powdered under liquid nitrogen, briefly mixed by means of a vortex mixer with 2 ml 0 2 M $HClO_4$ in a pre-weighed centrifuge tube, and reweighed, the difference in weight was taken as the muscle fresh weight After standing for 10 min in an ice-bath the tubes were centrifuged at 10,000 *g* for 15 min at 0°C, after which the supernatant was buffered with 0 5 M phosphate buffer, pH 7 5 (100 μl/g muscle), subsequently the pH was adjusted to 6 5–7 0 with 1 M KOH

Long chain acyl-carnitines

The pellet from the 10,000 *g* centrifugation was washed three times with 0 2 M $HClO_4$, with centrifugation at 10,000 *g* The final pellet was resuspended in 0 2 M KOH (4 ml/g muscle) The suspension was then incubated for 2 hr in a water bath at 55°C After hydrolysis, long chain acylcarnitines were analysed for free carnitine

Coenzyme A and acetyl-coenzyme A

The frozen tissue was weighed quickly and then powdered together with 4 ml 0 6 M $HClO_4$ /g muscle, frozen at the temperature of liquid nitrogen After thawing the extract was sonicated for 30 sec and centrifuged for 5 min at 2000 g, 0°C The pellet was re-extracted with 0 5 ml 0 6 M $HClO_4$ and after a second centrifugation the supernatants were combined The pH was adjusted to 5 5 with 1 M KOH and DTT was added to give a final concentration of 1 mM CoA and acetyl-CoA were assayed within 24 hr No evidence was obtained to suggest that DTT reduced the acetyl-CoA values using this procedure

Assay of metabolic intermediates

Carnitine was assayed by means of the DTNB-method of PEARSON *et al* (given in BERGMEYER, 1974) and acetylcarnitine was determined as described by PEARSON and TUBBS (1964) Coenzyme A and acetyl-coenzyme A were assayed with the catalytic assay of ALLRED and GUY (1969) The content of the standard CoA was determined by the end point method of STADTMAN (1952) and plotted against the rate of change in optical density in the catalytic assay to obtain a standard curve (Fig 1) All changes in optical density were recorded with a Zeiss PM–6 spectrophotometer equipped with a recorder

Statistical treatment of results

Each value represents the mean and standard deviation of the results of two to eight extracts (the number of determinations is given in parenthesis in the Figures) The significance of the apparent changes was computed by means of analysis of variance, the contrasts were judged according to the method of SCHEFFE (1953) P-values, size of contrast and its interval at 0 95 probability level are given in parenthesis

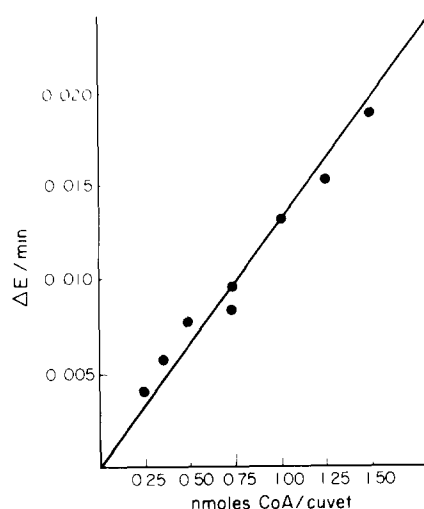### Enzymes and chemicals

All auxiliary enzymes, coenzyme A, DTT and

Fig 1 Standard curve of coenzyme A The rate of change in optical density in the catalytic assay was plotted against the exact content of standard CoA determined by the end point method

2-mercaptoethanol were obtained from Boehringer (Mannheim, FRG) DTNB and N-ethylmaleimide were obtained from BDH (Poole, United Kingdom) All other reagents were of analytical grade

Abbreviations used

Carnitine acetyltransferase (acetyl-CoA carnitine O-acetyltransferase, E C 2 3 1 7) Carnitine palmitoyltransferase (palmitoyl-CoA carnitine O-palmitoyl-transferase, E C 2 3 1 21) DTT dithiothreitol DTNB 5,5′-dithiobis-(2-nitrobenzoic acid)

RESULTS

The concentration of acetyl-CoA falls sharply immediately after the onset of flight (Fig 2) Within one minute a value of 0.023 ± 0.008 μmoles/g fresh weight is reached, which is significantly lower than the resting value of 0.062 ± 0.005 ($P < 0 001$ 0.039, 0 009–0.070). During prolonged flight the acetyl-CoA concentration remains relatively constant The concentration of free CoA, which is of the same magnitude as the resting concentration of acetyl-CoA, does not change significantly during flight (Fig. 2) Acetylcarnitine concentration shows a gradual increase during the first half hour of flight ($P < 0 001·$ 0 231, 0 003–0.459) and remains at an elevated level during continuation of flight activity (Fig 3)

Long chain acyl carnitines show a steady increase during flight, only after 30 min is there a significant increase ($P < 0.001·$ 0.097, 0 014–0.181) (Fig. 3) The concentration of carnitine, $0 919 \pm 0.121$ μmoles/g fresh weight in the resting state, decreases during the first 15 min of flight ($P < 0.001$: 0.307, 0 038–0 576) (Fig. 3), stabilizing at a level of $0 586 \pm 0 055$ μmoles/g fresh weight

Figure 4 shows the effect of flight on the mass action ratio (m) of carnitine acetyltransferase The mass action ratio is defined by.
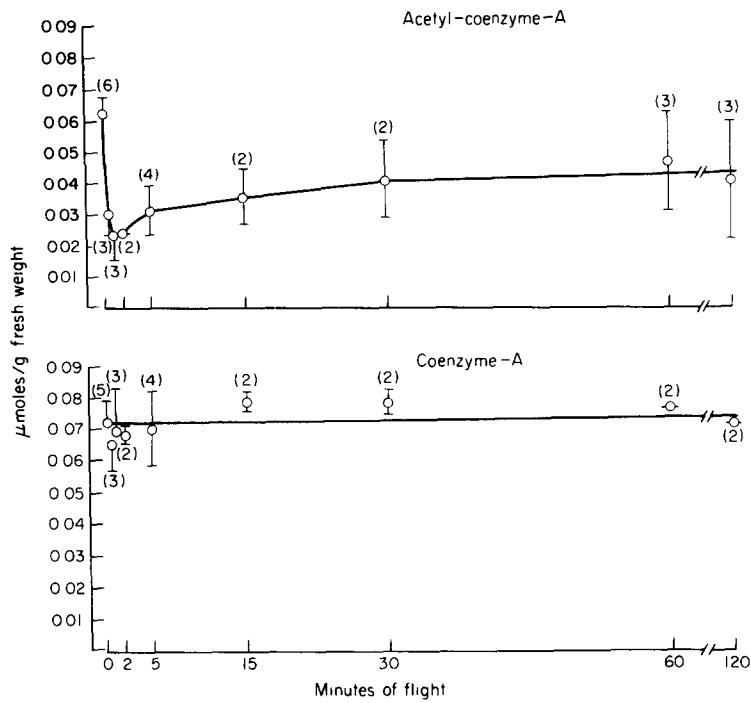
$$\frac{[AcCoA]\,[Cn]}{[AcCn]\,[CoA]}$$

Fig 2 Effect of flight of *Locusta migratoria* on the tissue levels of acetyl-CoA and CoA in the flight muscles The values represent the mean values ± standard deviation (number of extracts given in parenthesis)
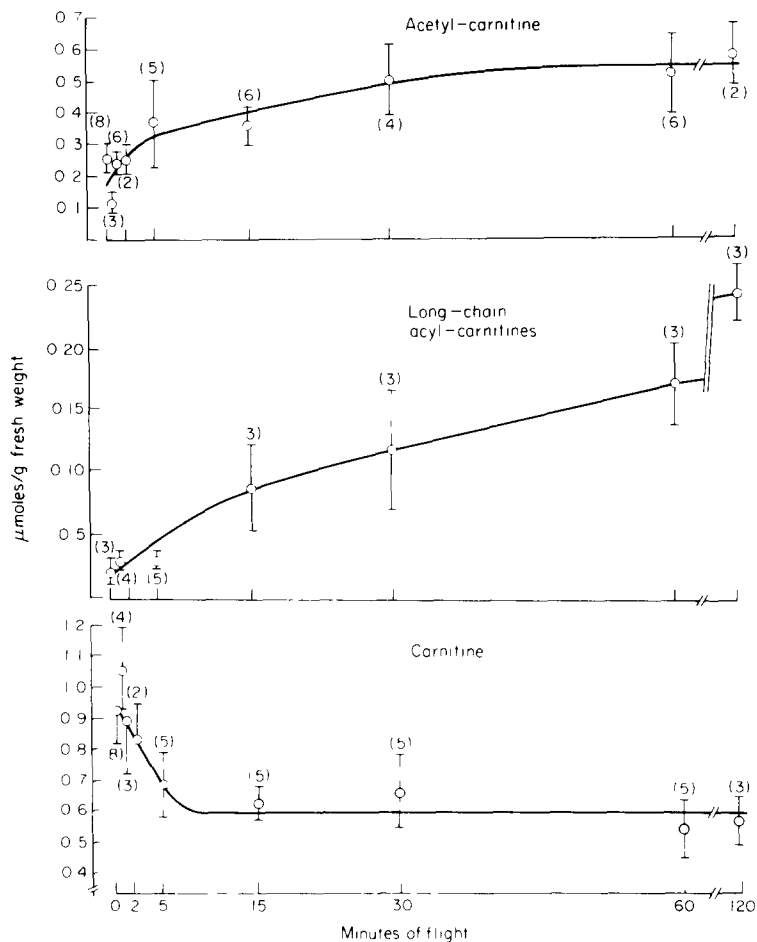

Fig 3 Effect of flight of *Locusta migratoria* on the tissue levels of acetylcarnitine, long chain acyl carnitine and free carnitine in the flight muscles The values represent the mean values ± standard deviation (number of extracts given in parenthesis)
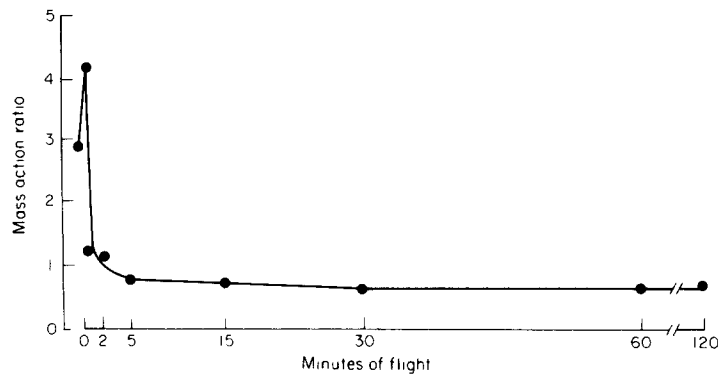
Fig 4 Effect of flight of *Locusta migratoria* on the mass action ratio of carnitine acetyltransferase The ratios were calculated from Figs 2 and 3 (Error bars omitted for clarity)

for the forward reaction AcCn + CoA⇌AcCoA + Cn, and is equal to the equilibrium constant if the reactants are at equilibrium. According to FRITZ *et al.* (1963) the equilibrium constant is 0.6. The mass action ratio reached after two minutes of flight equals 0.6 (within experimental error), and remains constant during prolonged flight. So after two minutes of flight the enzyme seems to operate near the equilibrium position of the reaction. At rest the reactants are not at equilibrium and in the first 0.5 min of flight even tend to be displaced from equilibrium still more Between 0.5 min of flight and 2 min of flight the mass action ratio decreases to the value of the equilibrium constant. The displacement from equilibrium at rest and up to 2 min of flight results in a net flow in the direction of acetylcarnitine formation, but is insufficient to consider the situation as non-equilibrium (ROLLESTON, 1972).

In Fig. 5 the substrate–product ratio at various flight times for the acetyltransferase was plotted according to the equation

$$\frac{AcCoA}{CoA} = m\ \frac{AcCn}{Cn}\quad (m = mass\ action\ ratio)$$

two regression curves occur with regression equations

of $y = 2.17\ x + 0.23$ and $y = 0.39\ x + 0\ 23$, respectively Both curves intercept the Y-axis at an acetyl-CoA to CoA ratio greater than zero (0.23), which suggests that acetyl-CoA and CoA are present in two different pools, in one of which they are not equilibrated with acetylcarnitine

Because long chain acyl-CoA concentrations were too low for accurate measurement with the method for CoA, after alkaline hydrolysis of the acid insoluble precipitate according to the method of GARLAND (in BERGMEYER, 1974, p. 2015), no such calculations could be made for the carnitine palmitoyltransferase system

DISCUSSION

Interpretation of the data for whole tissue contents of CoA derivatives is complicated by the fact that these derivatives exist in at least two pools. For mammalian heart muscle mitochondria FRITZ and YUE (1964) postulated that both the carnitine palmitoyltransferase and the carnitine acetyltransferase systems consist of two enzymes arranged in series associated with the inner mitochondrial membrane For the latter enzyme, however, both forms were shown to be interconvertible and to have identical kinetic
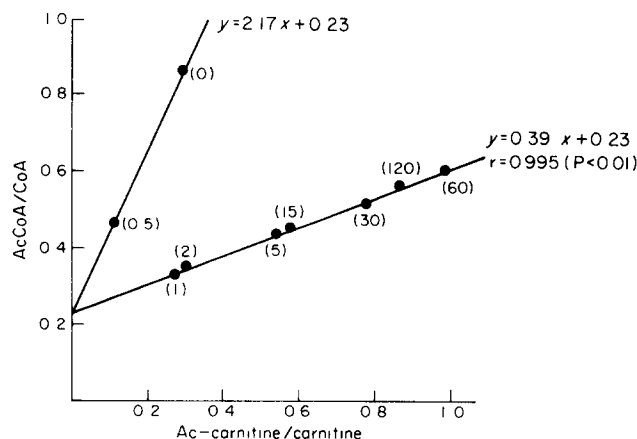

Fig 5 Effect of flight on the mass action ratio of carnitine acetyltransferase in the flight muscles of *Locusta migratoria* The acetyl-CoA to CoA and acetylcarnitine to carnitine ratios were calculated from the curves in Figs 2 and 3 for each flight period (given in minutes in parenthesis) Regression equations are shown For $y = 0\ 39\ x + 0\ 23$ the correlation coefficient is 0 995, $P < 0\ 01$

properties suggesting that the two acetyltransferase activities resulted from a single enzyme partly dissociated and partly non-dissociated from membrane material (EDWARDS et al., 1974).

Although formerly carnitine and its derivatives were thought to be located exclusively outside the inner membrane, whereas CoA and its acyl derivatives would be compartmentalized both in the cytosol and in the mitochondrial matrix, it has been shown now that an acyl-carnitine : carnitine exchange step occurs between the two carnitine acyltransferase reactions and that carnitine and its acyl derivatives are also present in both compartments (RAMSAY and TUBBS, 1975)

Oxidation of long chain fatty acids by heart mitochondria is completely carnitine dependent (FRITZ et al , 1963) and this was shown to be true also for flight muscle mitochondria of *Manduca sexta* (HANSFORD and JOHNSON, 1976) and *Locusta migratoria* (BEENAKKERS, 1963; BODE and KLINGENBERG, 1965). So it is plausible that the findings for mammalian heart muscle are valid also for insect flight muscle

In the present study the concentration of long chain acyl-carnitine shows a gradual increase within two hours of flight, amounting at that time to $0\ 244 \pm 0.033$ μmoles/g muscle; this suggests that the rate of translocation of acyl units across the inner mitochondrial membrane limited the rate of long chain acyl-carnitine oxidation; however, the activity of carnitine palmitoyltransferase in flight muscle homogenates of *Schistocerca gregaria* is 3.6 μmoles/min/g fresh weight (CRABTREE and NEWSHOLME, 1972). This would be a sufficient rate to cope with the supply of fatty acids in this period, which approximates 20 μmoles, taking into account that the major diglycerides mobilized from the fat body contain C16 and C18 long chain fatty acids (JUTSUM and GOLDSWORTHY, 1976) and that the turnover rate of diglycerides used for flight muscle metabolism is 3.0 mg/insect/hr (VAN DER HORST et al., 1978). Moreover, the change in long chain acyl-carnitine is relatively small compared to the amount of lipid utilized, and this suggests that in spite of the increase in long chain acyl-carnitines the overall process is closely regulated.

The changes in acetyl-CoA and acetylcarnitine are reflected in a change of the mass action ratio of the carnitine acetyltransferase. It appears that in the resting situation and immediately after the beginning of flight the enzyme is not at equilibrium, and there is a net flow in the direction of acetylcarnitine formation During sustained flight the enzyme remains close to equilibrium.

No other enzyme than carnitine acetyltransferase has been found that forms or breaks the acetylcarnitine bond. Moreover, PEARSON and TUBBS (1967) found the enzyme to operate under equilibrium conditions in several mammalian tissues. They supposed acetylcarnitine to be a "metabolic backwater" acting as an acetyl-CoA buffer. It appears that the enzyme in locust flight muscle also performs a buffer role A function of this buffer system could be to prevent large changes in the acetyl-CoA to CoA ratio Insect flight muscles operate completely aerobic, therefore in these muscles the citrate cycle can be regarded as the extension of the pathway from

trehalose to pyruvate. As well as the extension of the pathway from lipid to acetyl-CoA. This suggests that the rate of the citrate cycle is regulated by the rate of formation of acetyl-CoA from either lipid or carbohydrate. The enzymes which are involved in the transmission of the flux from glycolysis into the citrate cycle are pyruvate dehydrogenase and citrate synthase. ALP et al. (1976) suggested that in insect muscle citrate synthase catalyzes a near equilibrium reaction and NAD^+-linked isocitrate dehydrogenase a non-equilibrium reaction. The latter finding differs from the properties of this enzyme in all other muscles investigated. The interpretation of the maximal activities of these enzymes, however, is complicated considerably by the fact that their substrates and products are present in both cytosolic and mitochondrial compartments.

The reciprocal control between glycolysis and fatty acid oxidation present in mammalian heart muscle, which is thought to work via inhibition of phosphofructokinase by citrate is absent in flight muscles of *S. gregaria* (WALKER and BAILEY, 1969). Also the observed changes in citrate concentration in insect flight muscles during flight are not uniform. ROWAN and NEWSHOLME (1979) measured a five-fold decrease in citrate concentration in *S. gregaria*, whereas in *Phormia regina* (SACKTOR and WORMSER-SHAVIT, 1966) and *L. migratoria* (WORM and BEENAKKERS, 1980) no change in the concentration of this intermediate was found during exercise.

There have been little or no investigations of factors which can modify the activity of pyruvate dehydrogenase in insect flight muscles. In mammalian tissues the enzyme exists in two interconvertible forms, which are involved in the regulation of the activity of this enzyme. Phosphorylation catalyzed by pyruvate dehydrogenase kinase inactivates the enzyme The phosphoenzyme is reactivated by pyruvate dehydrogenase phosphatase. The degree of phosphorylation of the complex has been shown to depend on a number of effectors, one of them being the acetyl-CoA to CoA ratio (PETTIT et al , 1975). CoA inhibits the kinase, whereas acetyl-CoA activates the enzyme. A decrease in acetyl-CoA as described in the present paper, therefore, could result in a decreased activation of the kinase, thus indirectly in a reduced inactivation of the pyruvate dehydrogenase itself This, however, is at variance with the observed reduction of the flux through the glycolytic pathway so other effectors must be involved or the control must yet be exerted upon an earlier step in the pathway

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