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Flying insects: model systems in exercise physiology

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Abstract. Insect flight is the most energy-demanding exercise known. It requires very effective coupling of adenosine triphosphate (ATP) hydrolysis and regeneration in the working flight muscles. ^{31}P nuclear magnetic resonance (NMR) spectroscopy of locust flight muscle in vivo has shown that flight causes only a small decrease in the content of ATP, whereas the free concentrations of inorganic phosphate (P_i), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) were estimated to increase by about 3-, 5- and 27-fold, respectively. These metabolites are potent activators of glycogen phosphorylase and phosphofructokinase (PFK). Activation of glycolysis by AMP and P_i is reinforced synergistically by fructose 2,6-bisphosphate ($F2,6P_2$), a very potent activator of PFK. During prolonged flight locusts gradually change from using carbohydrate to lipids as their main fuel. This requires a decrease in glycolytic flux which is brought about, at least in part, by a marked decrease in the content of $F2,6P_2$ in flight muscle (by 80% within 15 min of flight). The synthesis of $F2,6P_2$ in flight muscle can be stimulated by the nervous system via the biogenic amine octopamine. Octopamine and $F2,6P_2$ seem to be part of a mechanism to control the rate of carbohydrate oxidation in flight muscle and thus function in the metabolic integration of insect flight.

Key words. Insect flight; aerobic exercise; glycogen phosphorylase; regulation of glycolysis; ^{31}P NMR spectroscopy; fructose 2,6-bisphosphate; phosphofructokinase; mitochondrial metabolism.

Introduction

Insects are a highly successful class of animals. Together with mammals they dominate the land habitats of our planet. There are more species of insects than in all other groups of extant organisms (plants and bacteria included). Insects have remarkable physiological capabilities, which can often be interpreted as adaptations for flight. This most sophisticated form of exercise has been mastered only by insects, birds and some mammals, but insects have developed the capacity to the extreme. Insects possess acute and sensitive senses (vision, olfaction) and the extremely rapid information processing required for flight manoeuvres. The most efficient muscles are also found in insects; flight muscles in various species bring about wing-beat frequencies of several hundred, sometimes even more than a thousand, per second. Insect flight muscles sustain the highest metabolic rates of all animal tissues. Most insects are engaged in short-range flight, but some can stay airborne for many hours (migrating locusts and moths), thus covering hundreds of miles non-stop.

Insect flight is extremely dependent on oxygen, and flight muscle metabolism is fully aerobic. Substrates are completely oxidised to CO_2 and H_2O so that no anaerobic products, such as lactate and H^+ ions, are accumulated during flight[2, 10, 30]. Insect flight muscles are resistant to fatigue as long as they do not run out of fuel.

The aerobic scope (the ratio of maximum to basal rate of respiration) of insects is unrivalled in the animal kingdom. Locusts, for instance, may increase oxygen consumption 70-fold when going from rest to flight[36], and they are outdone by bees and flies[15]. In humans, exercise can increase oxygen consumption maximally 20-fold, and small mammals or birds running or flying at maximum speed will increase their oxygen consumption by not more than 7- to 14-fold[15]. The figures reflecting insect performance become even more impressive when working flight muscles are considered, because these account for less than 20% of body mass in a locust[36, 30] but consume most of the respiratory oxygen during flight. In flight muscle, respiration and hence turnover of ATP may increase several hundredfold upon flight.

The amazing aerobic capabilities of insects are based on a highly efficient mode of oxygen provision. Oxygen is transported to the tissues in gaseous form by a system of delicate tubules (tracheae), the finest ramifications of which (tracheoles) end in direct proximity of mitochondria. Transporting air directly to the tissues has several advantages: (1) Oxygen content in air is high (21% compared with less than 1% in aqueous fluids); (2) the diffusion of O_2 and CO_2 in air is much faster than in fluids; (3) transport of gas requires less weight than transport with oxygen carriers in body fluids.

Because of their fully aerobic energy metabolism insects are able to maintain rather stable metabolic conditions

Dedicated to Dr. Ernst Zebe, Emeritus Professor of Zoology (University of Münster) on the occasion of his 70th birthday.

Table 1. Possible regulatory functions of ATP and its hydrolysis products.

ATP	Inhibitor of	• Glycogen phosphorylase • Phosphofructokinase • Ketoglutarate dehydrogenase
Inorganic phosphate	Substrate of	• Glycogen phosphorylase • Glyceraldehydephosphate DH • ATP Synthase
	Activator of	• Hexokinase • Phosphofructokinase
ADP	Substrate of	• Glycolytic phosphorylation • Oxidative phosphorylation
	Activator of	• Isocitrate dehydrogenase • Ketoglutarate dehydrogenase
AMP	Activator of	• Glycogen phosphorylase • Phosphofructokinase

despite rapidly changing and very high metabolic rates. They can hence be regarded as apt model systems for both high-intensity and endurance aerobic exercise.

Energy-rich phosphates and related compounds in resting and working flight muscles

Muscle contraction is powered by the free energy released when ATP is hydrolysed to ADP and inorganic phosphate (P_i)[16, 17]. The rate of ATP hydrolysis in working flight muscle can be very high. A locust, for instance, when flying at maximum speed may turn over the ATP in its flight muscle about three times per second, and all the energy-rich phosphate (ATP plus the phosphagen phosphoarginine) would be equivalent for less than one second of flight[31, 35]. For comparison, the phosphocreatine in human quadriceps muscle is sufficient for five to six seconds of maximal exercise, and a frog swimming vigorously can rely almost exclusively on muscle phosphagen for five seconds[16, 17, 34].

Contraction (ATPase)

$$ATP \xrightarrow[\oplus\uparrow\ Ca^{++}]{} ADP + P_i + H^+ (+ \text{work})$$

Arginine phosphokinase

$$ADP + PArg + H^+ \rightleftharpoons ATP + \text{arginine}$$

$$K_{eq} = \frac{[ATP] \cdot [\text{arginine}]}{[ADP] \cdot [PArg]}$$

$$[ADP] = \frac{[ATP] \cdot [\text{arginine}]}{K_{eq} \cdot [PArg]} \qquad 13.2 \leq K_{eq} \leq 21.1$$

Adenylate kinase

$$ADP + ADP \rightleftharpoons ATP + AMP$$

$$K_{eq} = \frac{[ATP] \cdot [AMP]}{[ADP] \cdot [ADP]}$$

$$[AMP] = \frac{[ADP]^2}{K_{eq} \cdot [ATP]} \qquad K_{eq} \approx 1$$

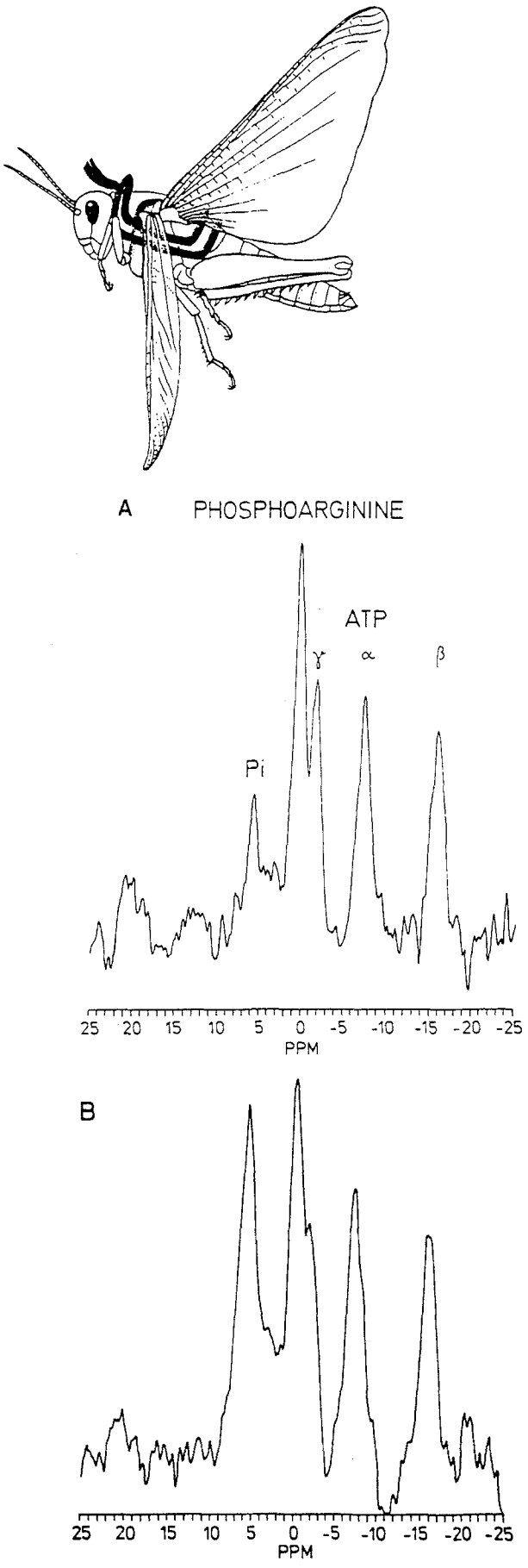
Scheme. Muscle contraction and reversible reactions involved in the equilibrium of energy-rich phosphates and related compounds in locust flight muscle.

Table 2. Contents of ATP and its hydrolysis products in locust flight muscle at rest and during flight as determined by conventional biochemical (enzymic) methods. Contents are given in μmol/g muscle[23, 40].

	Rest	Flight	Change rest to flight
ATP	5.05–5.67	4.48–5.23	not significant
P_i	9.32	11.9–13.5	not significant
ADP	0.43–1.67	1.10–1.67	significant[23] not significant[40]
AMP	0.06–0.53	0.12–0.66	significant[23] not significant[40]

To support the high rate of ATP turnover in flight, ATP-regenerating pathways must be activated almost instantaneously upon flight, and ATP hydrolysis and ATP synthesis must be exactly balanced because otherwise flight could not be sustained. How is this achieved, that is, how are muscle work and energy metabolism coordinated?

ATP is an inhibitor of key regulatory enzymes of ATP-producing pathways (table 1), so catabolic activities are suppressed if ATP demand is low, as is the case in resting muscle. If muscle starts contracting, catabolism must be activated to compensate for the increased rate of ATP hydrolysis. To activate catabolic enzymes, a marked decrease in the concentration of the inhibitor ATP is not feasible, because this would interfere with muscle work[16, 17]. Indeed, ATP content in the flight muscle of the locust appears not to be decreased by more than 10% at the initiation of flight[23, 35]. Nevertheless, the flight-induced small decrease in [ATP] reflects a massive increase in the turnover of ATP in muscle and will lead to changes in the steady-state concentrations of phosphoarginine, ADP, AMP and P_i. As the latter three metabolites are substrates or potent activators of regulatory enzymes (see scheme), it is important to know how much their concentrations change during the transition from rest to flight. This information is not easily available from biochemical analyses for the following reasons: (1) The rapid activation and potentially very high rates of ATP turnover make it difficult, if not impossible, to stop catabolic processes while the muscle is fully at rest; (2) large fractions of some metabolites are not in solution in the cytosol, but are bound to cell



structures or otherwise compartmentalised, and hence not available as enzyme effectors. Conventional biochemical methods require tissue destruction, homogenisation and dilution which will solubilise bound metabolites. Both types of artefacts would lead to an underestimation of the changes that occur in vivo in the free concentrations of metabolites. The problem is illustrated in table 2 by comparing reports of changes in metabolites in locust flight muscle during flight and their statistical significance.

^{31}P NMR spectroscopy can be used as a non-destructive and non-invasive method for the analysis of phosphorus compounds in tissue in intact animals and man. We have adopted the method for resting and flying locusts. In the spectra of flight muscle of resting locusts the area of the P_i peak was significantly smaller than the peaks produced by the phosphorus atoms of ATP (fig. 1), whereas much more P_i than ATP had been measured biochemically in flight muscle from non-flying locusts (table 2). The discrepancy indicates that most of the P_i in resting flight muscle is not free, and hence not available as substrate or effector for cystolic enzymes.

Flight brought about a marked decrease in [phosphoarginine] and a concomitant increase in the free content of P_i while no change in pH was observed. At the initiation of flight a new steady state of ATP, P_i and phosphoarginine was rapidly established, and minimal changes occurred after the first two seconds of flight[35], irrespective of whether the flight lasted for a few seconds, some minutes or more than an hour. After a flight, the metabolites returned immediately to their pre-flight levels, indicating that metabolic recovery from flight was rapid (fig. 2).

From the free contents (concentrations) of ATP and phosphoarginine, the concentrations of both ADP and AMP can be estimated, according to the formulas shown in the scheme. The calculations are based on the assumptions that ATP, phosphoarginine and arginine are in solution in the cytosol, that the reactions catalysed by arginine phosphokinase and adenylate kinase are maintained near their thermodynamic equilibria and that the equilibrium constants are known and apply to the reactions in vivo. Biochemical studies and in vivo NMR spectroscopy suggest these conditions to hold (for a discussion see ref. 35).

The changes in the free contents of ADP, AMP and P_i thus calculated are strikingly different from the total

Figure 1. ^{31}P NMR spectroscopy in vivo of a locust during rest and flight. A surface coil with a saddle shape was fixed to the thorax with beeswax such that it supported the locust in the magnet while allowing free wing movements (above). *A*) Spectrum of flight muscle of a resting locust, collected over 17 min. *B*) Spectrum of flight muscle of a flying locust, collected over 77 min of continuous flight. For details, see text and ref. 35.

Table 3. Energy-rich phosphates and related compounds in locust flight muscle at rest and during flight.

	Rest	Flight	Change
ATP	5.06	≥4.5	−10%
ADP	0.1	0.5	+400%
AMP	0.002	0.055	+2640%
PArg	8.91	4.30	−52%
Inorganic phosphate	3.00	7.83	+161%

Data are the estimated free intracellular contents based on ^{31}P NMR spectroscopy in vivo and are given in μmol/g muscle[35].

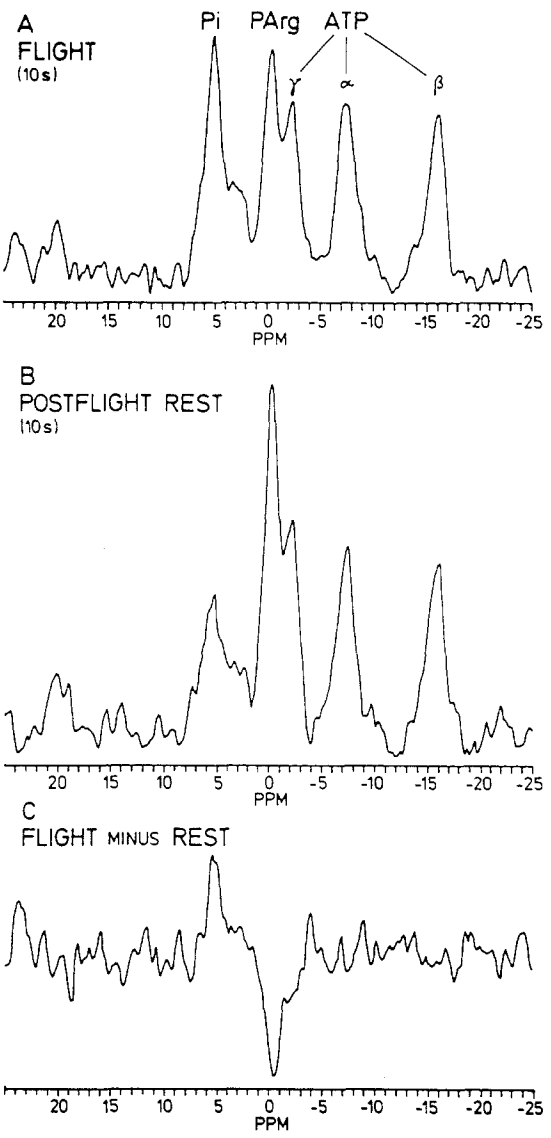


Figure 2. ^{31}P NMR spectra demonstrating metabolic changes in the flight muscle of a locust during short-time flight and short-time rest (10 s each). The content of ATP in flight muscle does not noticeably change, but phosphoarginine (PArg) is significantly decreased while inorganic phosphate (P_i) is increased during flight (for details, see text and ref. 35).

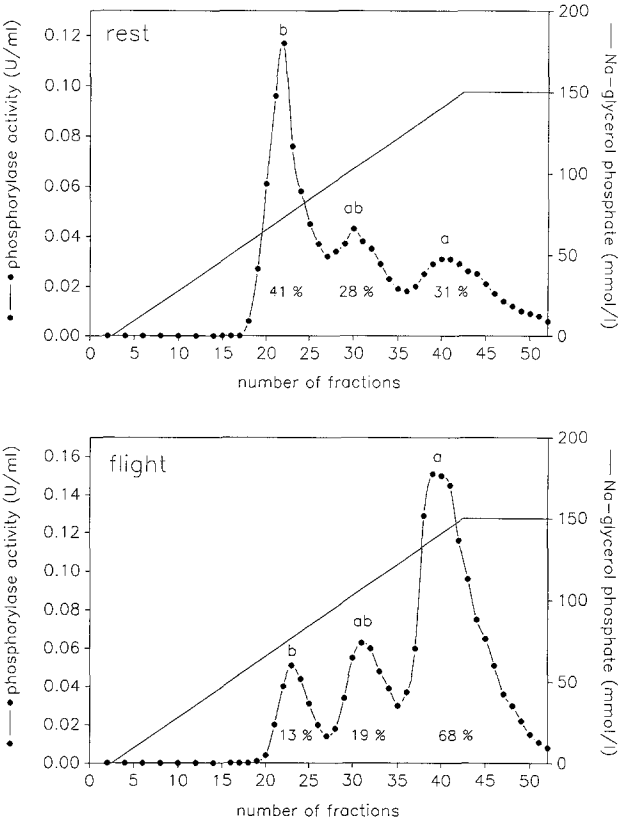


Figure 3. Effect of a brief flight (up to 30 s) on the distribution of interconvertible forms of glycogen phosphorylase in the flight muscle of the hawk moth *Manduca sexta*. Phosphorylase forms were separated by ion-exchange chromatography on DEAE Sephacel. Flight caused a decrease in the unphosphorylated *b* form (inactive form), while the fully phosphorylated *a* form (active form) was increased (for details, see text and ref. 6).

changes as measured biochemically (compare tables 2 and 3). So whereas changes in total contents of ADP and AMP did not exceed a factor of 2 to 3 the free contents appear to change by factors of 5 and 27 for ADP and AMP, respectively. Hence, a small fractional decrease in the cystolic concentration of ATP can bring about a relatively large fractional increase in [ADP], and this increase in [ADP] is again potentiated in the fractional increase in [AMP], making AMP a potent signal of the exercise-induced increase in ATP turnover[35]. Compared with working insect flight muscle even larger fractional increases in [ADP] and [AMP] have been estimated to occur in white vertebrate muscle during high-intensity exercise[16,17] which reflects the fact that the vertebrate muscle phosphagen is phosphocreatine, which buffers [ATP] at a higher [ATP]/[ADP] ratio than phosphoarginine[11]. The synthesis of ATP from phosphagen consumes protons and hence is favoured when pH decreases, as is the case when working vertebrate muscle produces lactic acid. Because flight muscle pH in insects does not decrease during flight, phosphoarginine cannot be exploited to the same extent as phosphocreatine in vertebrate muscle (cf. ref. 16).

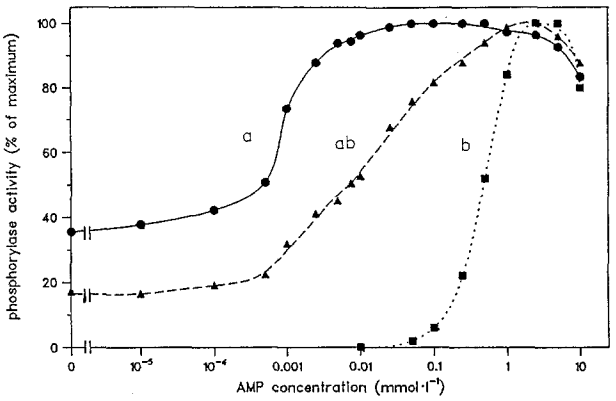


Figure 4. The three interconvertible forms of glycogen phosphorylase differ greatly in their sensitivity to the allosteric activator AMP. Half maximal activation requires 1 µM, 15.4 µM and 466 µM in the case of phosphorylase forms *a*, *ab* and *b*, respectively. For details, see text and ref. 6.

Substrates and metabolic pathways in insect flight muscle

Insects can use various fuels for flight, such as carbohydrate (glycogen, trehalose and other sugars), lipids and the amino acid proline (for reviews, see refs. 2, 9, 38). In this review I shall focus on insects which use carbohydrate during the initial stage of a flight and change over gradually to oxidising lipids if the flight is to be prolonged. This metabolic pattern is characteristic of locusts but is also found in lepidoptera such as the hawk moth *Manduca sexta*[41]. The balanced use of fuels requires sophisticated regulatory mechanisms for the mobilisation, transport and oxidation of fuels (fuel selection). Similar problems exist for mammals and humans during prolonged exercise.

Mobilisation of muscle glycogen

Some glycogen is stored in flight muscle. It is hence readily available, and several insect species are known to use muscle glycogen preferentially in the very early stage of a flight. Glycogen mobilisation is directly synchronised with muscle work via a common signal, a rise in cytosolic Ca^{++}, which triggers contraction and the interconversion of phosphorylase into an active form.

In the flight muscle of locusts[27,28] and moths[6] glycogen phosphorylase is a dimeric molecule that exists in three interconvertible forms. In addition to the unphosphorylated *b* form and the fully phosphorylated *a* form (active form), there is a hybrid of the form *ab* in which one subunit is phosphorylated while the other is not. In flight muscle of moths that were rapidly freeze-clamped while at rest, phosphorylase was found mainly in the *b* form (41%), while 28% and 31% were *ab* and *a* forms, respectively. A brief flight (up to 30 seconds) changed this distribution in favour of the active *a* form (68%), with only 13% in the *b* form and 19% in the *ab* form (fig. 3).

The three forms of glycogen phosphorylase differ markedly in their kinetic properties, particularly with respect to ligands that modulate their catalytic activity. AMP is a very potent allosteric activator of phosphorylase[25], but the three forms respond to different concentration ranges of this activator (fig. 4).

The effect of AMP on the activity of phosphorylase can be attenuated by [ATP] and [glucose 6-phosphate][6]. The actual activity of glycogen phosphorylase in insect flight muscle is therefore an effect not only of the distribution of interconvertible phosphorylase forms but also of the concentration of metabolites that act as allosteric effectors. From the kinetic properties in vitro it appears very likely that the unphosphorylated *b* form of phosphorylase will never be active under physiological conditions in vivo. The phosphorylated forms also will show very little if any activity at concentrations of the substrate P_i and the effectors AMP (activator) and ATP (inhibitor) likely to prevail in resting muscle. Although ^{31}P NMR spectroscopy on moths has not been performed in vivo, it seems reasonable to assume similar changes in metabolites as seen in locusts, since both insects have flight muscles of similar structural and metabolic organisation.

The co-existence of three interconvertible forms of glycogen phosphorylase seems to be widespread among animals, although it has unequivocally been demonstrated in only a few species of hemimetabolic[27,28] and holometabolic insects[6] and lower vertebrates[24]. The insertion of a partially phosphorylated form of phosphorylase, with properties intermediate between those of the inactive *b* and active *a* form, is thought to provide the system with greater flexibility and sensitivity towards enzyme modulators that reflect the metabolic demands of tissues[6,19,24]. As a consequence, fine-tuning of phosphorylase activity may be improved.

The regulation of glycolysis in insect flight

Like muscle contraction, mobilisation of muscle glycogen is triggered by Ca^{++}, and this signal is complemented by the contraction-induced generation of allosteric activators of glycogen phosphorylase in muscle. Thus, coordinated with muscle work, glycogenolysis produces glucose phosphate as a substrate for glycolysis. However, none of the glycolytic enzymes is activated by Ca^{++}, and how muscle work and glycolytic flux are coordinated is not fully understood either for insect or vertebrate muscle[16,18,34], although the problem has been studied for many decades.

The main regulatory enzymes of glycolysis are hexokinase and particularly phosphofructokinase (PFK). Hexokinase is strongly inhibited by its product glucose 6-phosphate, but this inhibition can be relieved by inorganic phosphate[26].

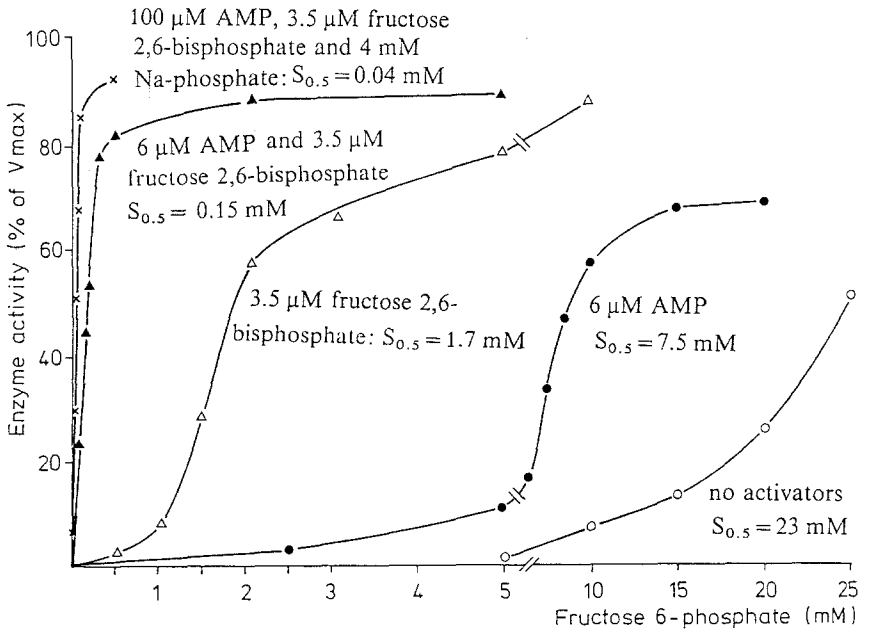


Figure 5. Regulatory properties of phosphofructokinase purified from locust flight muscle. Enzyme activity is strongly inhibited by 7.5 mM MgATP (at pH 7.2). AMP and $F2,6P_2$ show strong synergistic effects in activating (deinhibiting) the enzyme. $S_{0.5}$ = concentration of fructose 6-phosphate at which enzyme activity is half maximum. For details, see text and ref. 33.

PFK catalyses the first committed step of glycolysis and is regarded as the key regulatory enzyme of this pathway (see refs. 18, 33). PFK is a prototype of a multimodulated enzyme. The basis of its modulation is the allosteric inhibition of the enzyme by physiological concentrations of ATP, which reduces the affinity for the second substrate, fructose 6-phosphate. In PFK from vertebrates the inhibition by ATP is reinforced by H^+ and citrate; but neither compound plays a role in the regulation of PFK activity in insect flight muscle, because the pH in flight muscle is not significantly changed under physiological conditions[35], and PFK from insect flight muscle is not sensitive to citrate[21, 32, 33]. While [ATP] does not decrease much during flight, [inorganic phosphate] and particularly [AMP] show large fractional increases. Both metabolites are activators (deinhibitors) that can counteract the inhibition of PFK by ATP. However, unphysiologically high concentrations of AMP and P_i are required to activate PFK from locust flight muscle in vitro. Both activators show strong synergistic effects, but even the combined effects can hardly account for the PFK activity that will be required in vivo in working flight muscle. This suggests the existence of an additional activator of PFK in locust flight muscle, and this appears to be fructose 2,6-bisphosphate ($F2,6P_2$).

$F2,6P_2$ was discovered in rat liver, where it is present in micromolar concentrations and is a decisive factor in directing glucose metabolism (the liver changes from glycolysis to gluconeogenesis when [$F2,6P_2$] is decreased due to hormone action[29]). $F2,6P_2$ has subsequently been found in all animal cells studied so far[13], but its function in vertebrate muscle is not fully understood[34, 16, 18].

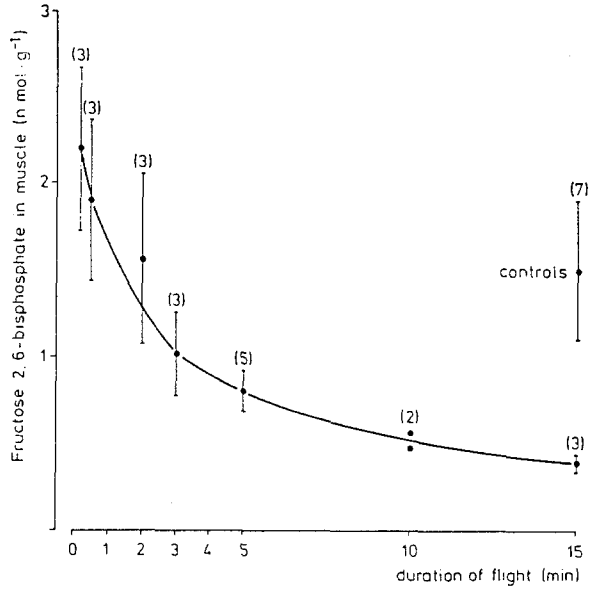
$F2,6P_2$ is present in insect flight muscle. It is a very potent activator of PFK, particularly in combination with micromolar concentrations of AMP, with which strong synergistic effects can be observed (fig. 5). $F2,6P_2$, AMP and P_i seem to be the key modulators of PFK activity in locust flight muscle in vivo (see next section).

Integration of metabolism in insect flight

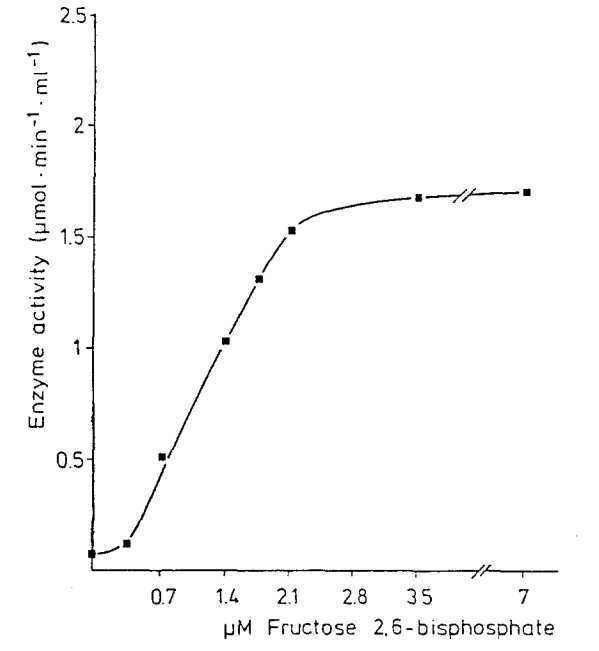
Some insects can sustain flight for many hours, thus covering hundreds of miles non-stop. Prolonged flight requires fat as the main fuel, because of all biological fuels, fat is the most efficient on an energy-to-mass ratio – approximately eight times as efficient as carbohydrate[37].

Locusts use both carbohydrate and fat in flight, but in very different proportions depending on the duration of the flight. Carbohydrate is the major fuel for the initial stage, and fat is the major fuel during prolonged flight[14, 36]. This use of fuels is similar to that of migrating birds, migrating mammals or humans during endurance exercise such as running a marathon.

In most insects fat is stored exclusively in the fat body, which, in this function, is analogous to white adipose tissue in mammals. Lipid mobilisation during flight is controlled by hormones, neuropeptides (adipokinetic hormones) from the corpora cardiaca, a hormone gland connected to the brain (see ref. 38). The smooth transi-



A)



B)

Figure 6. *A)* Effect of flight on the content of $F2,6P_2$ in flight muscle of the locust *Locusta migratoria*. *B)* Effect of $F2,6P_2$ on the activity of PFK from locust flight muscle at near-physiological concentrations of substrates and effectors (50 µM fructose 6-phosphate, 50 µM fructose 1,6-bisphosphate, 7.5 mM MgATP, 40 µM AMP, pH 7.3)[32].

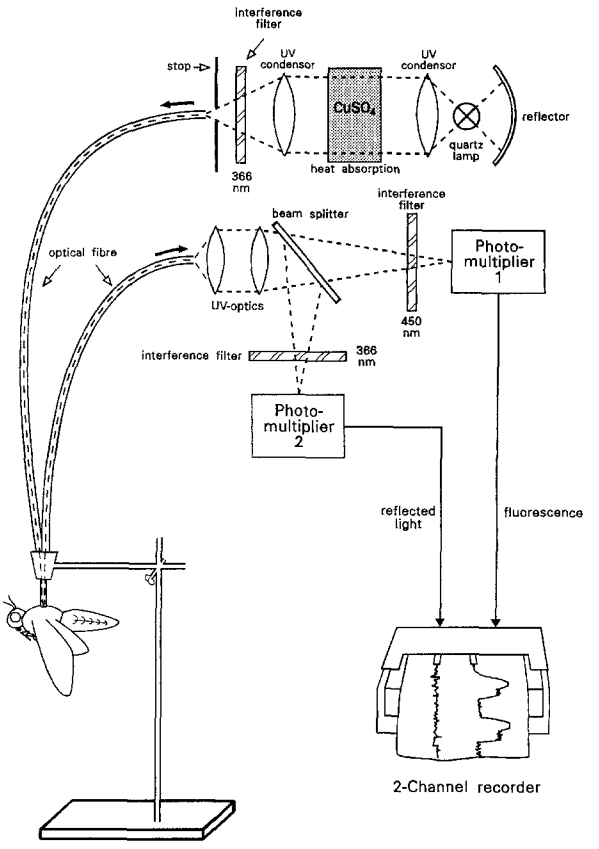


Figure 7. Experimental set-up for monitoring changes in the mitochondrial NADH content in *Manduca sexta* flight muscle in vivo by means of fluorescence (Burkhardt, Paul and Wegener, unpubl. results).

tion from carbohydrate to fat requires a decrease in glycolysis, while fatty acid oxidation is increased. A specific mechanism for decreasing glycolytic flux during prolonged flight must exist in locust flight muscle because there is still considerable carbohydrate present, and glycolytic intermediates in muscle are maintained or even elevated after 30 min flight[40], when the rate of carbohydrate oxidation accounts for less than 10% of the energy required for flight[14]. ^{31}P NMR of locusts indicates that the concentrations of the PFK activators P_i and AMP do not decline during prolonged flight (see previous section). However, a marked decrease (80%) in the content of $F2,6P_2$ in locust flight muscle occurs within 15 min flight (fig. 6A). If assayed at near-physiological concentrations of its substrates and effectors, PFK activity is very sensitive to changes in the concentration of $F2,6P_2$ in the same range as observed in flight muscle (Fig. 6B and refs. 32, 33). This suggests that the decrease in the concentration of $F2,6P_2$ that occurs in locust flight muscle in the early stages of flight is, in part, responsible for the decrease in PFK activity and thus for the conservation of carbohydrate during prolonged flight, when lipid is a major fuel.

Conserving carbohydrate is also all-important during prolonged exercise in humans and other mammals because the limited carbohydrate stores of the body are required as fuel for the brain, which is, under normal physiological conditions, totally dependent on blood glucose as the only fuel. In vertebrates, citrate is thought to be involved in the decrease in glycolytic flux when muscle preferentially oxidizes fatty acids during

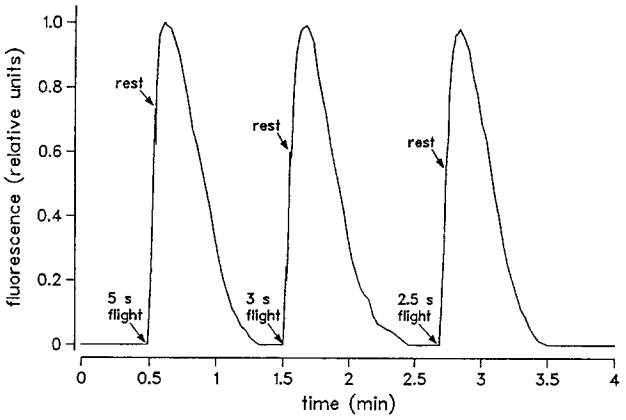


Figure 8. Fluorescence of mitochondrial NADH in *Manduca* flight muscle at rest, during a brief interval of flight and during post-flight recovery. Flight caused a rapid increase in NADH fluorescence, indicating a rapid activation of mitochondrial dehydrogenases. When moths stopped flying, fluorescence showed a further transient increase before it returned to the pre-flight level (Burkhardt, Paul and Wegener, unpubl. results).

prolonged exercise[21]. Citrate is a potent inhibitor of phosphofructokinase from vertebrate muscle but has no effect on insect phosphofructokinase[21]. Whether, in addition to citrate, $F2,6P_2$ also plays a role in fuel selection in mammalian muscle is an interesting question that has not yet been studied.

Neuronal effects on flight muscle metabolism

Insect flight is controlled by the central nervous system, and octopamine seems to be intimately involved in the coordination and integration of flight performance and metabolism. Octopamine is a biogenic amine, structurally very similar to noradrenaline, that acts as a neurotransmitter, neurohormone or neuromodulator in insects[22]. Octopamine has been reported to stimulate the oxidation of carbohydrate and also to increase the force and efficiency of flight muscle contractions in locust flight muscles[8,39]. Recent studies suggest that $F2,6P_2$ is a mediator for some of the metabolic effects of octopamine. Injection of octopamine into the haemolymph of resting locusts caused the content of $F2,6P_2$ in the flight muscles to increase. When injected into flying locusts, octopamine reversed the flight-induced decrease in $F2,6P_2$ [3]. Octopamine acts directly upon flight muscle tissue, because similar effects as in intact locusts were seen when a perfused locust thoracic muscle preparation (resting or electrically stimulated to work[7]) was exposed to octopamine in vitro[4]. Locust flight muscles contain octopaminergic nerve fibres originating in the thoracic ganglia. It is hence likely that locusts have at their command a neural mechanism for the modulation and integration of muscle metabolism and performance. The signalling pathway from octopamine to $F2,6P_2$ has not yet been elucidated, nor is it known whether analogous mechanisms exist in vertebrates.

Mitochondrial metabolism in insect flight

Mitochondrial metabolism, particularly the regulation of Krebs cycle activity, has been an active field of research (for recent reviews see refs. 1, 5, 12). Many studies have been performed on isolated cells or isolated mitochondria from various tissues and organs such as the heart, liver, kidney and muscle, and several factors have been proposed as important for regulating Krebs cycle activity, mainly the absolute and relative concentrations of ADP, P_i, ATP, reduced nicotinamide adenine dinucleotide (NADH) and Ca^{++}. An agreement has not yet been reached, and different regulatory mechanisms might be involved in different organs. Studies in vivo under physiological conditions would be particularly valuable, as mitochondrial metabolism appears to be very sensitive to experimental conditions such as the supply of substrates and oxygen (hypoxia).

Insect flight is, like no other physiological activity, dependent on mitochondrial ATP generation. Flight muscle is essentially a hydrogen engine, burning hydrogen delivered mainly by the Krebs cycle as NADH. Of special interest would be experiments recording changes in the mitochondrial NADH redox state in vivo during exercise. Monitoring of mitochondrial NADH in flight muscle of intact insects during rest and flight seems feasible. NADH, but not NAD^+, gives rise to fluorescence. This can be exploited using an experimental set-up as shown in figure 7 in which an optical fibre was glued to the bare dorsal thorax of a hawk moth. When the moth was induced to fly, NADH fluorescence was rapidly increased (fig. 8). This was a surprising result, because in excised skeletal muscle from vertebrates the mitochondrial NADH content decreases rather than increases with work[1]. The insect preparation could hold much promise and might qualify in the sense of the Krogh principle, according to which 'for many problems there is an animal on which it can be most conveniently studied'[20].

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