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Estimation of the Fructose Diphosphatase–Phosphofructokinase Substrate Cycle in the Flight Muscle of *Bombus affinis*

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1. Substrate cycling of fructose 6-phosphate through reactions catalysed by phosphofructokinase and fructose diphosphatase was estimated in bumble-bee (*Bombus affinis*) flight muscle *in vivo*. 2. Estimations of substrate cycling of fructose 6-phosphate and of glycolysis were made from the equilibrium value of the $^3H/^{14}C$ ratio in glucose 6-phosphate as well as the rate of ^3H release to water after the metabolism of [5-^{3}H,U-^{14}C]glucose. 3. In flight, the metabolism of glucose proceeded exclusively through glycolysis (20.4μmol/min per g fresh wt.) and there was no evidence for substrate cycling. 4. In the resting bumble-bee exposed to low temperatures (5°C), the pattern of glucose metabolism in the flight muscle was altered so that substrate cycling was high (10.4μmol/min per g fresh wt.) and glycolysis was decreased (5.8μmol/min per g fresh wt.). 5. The rate of substrate cycling in the resting bumble-bee flight muscle was inversely related to the ambient temperature, since at 27°, 21° and 5°C the rates of substrate cycling were 0, 0.48 and 10.4μmol/min per g fresh wt. respectively. 6. Calcium ions inhibited fructose diphosphatase of the bumble-bee flight muscle at concentrations that were without effect on phosphofructokinase. The inhibition was reversed by the presence of a Ca^{2+}-chelating compound. It is proposed that the rate of fructose 6-phosphate substrate cycling could be regulated by changes in the sarcoplasmic Ca^{2+} concentration associated with the contractile process.

Flight muscle of various species of bumble-bees has been reported to contain high activities of fructose diphosphatase (EC 3.1.3.11) (Newsholme *et al.*, 1972). Since this tissue does not possess phosphoenolpyruvate carboxykinase (EC 4.1.1.32) or glucose 6-phosphatase (EC 3.1.3.9) (Newsholme *et al.*, 1972), it is unlikely that fructose diphosphatase is involved in gluconeogenesis but rather it may participate in alternative metabolic pathways, e.g. substrate cycling.

For a bumble-bee to fly, the thoracic flight muscle must attain a minimum temperature of 30°C (Krogh & Zeuthen, 1941). Since the thoracic temperature is maintained at 30°C (Heinrich, 1972) between periods of flight, Newsholme *et al.* (1972) have postulated that the high activities of fructose diphosphatase and phosphofructokinase (EC 2.7.1.11) in flight muscle allow the operation of a fructose diphosphatase–phosphofructokinase-mediated substrate cycle that could be responsible for heat-generation. Further, it is noted (Newsholme *et al.*, 1972) that this system has advantages over mechanical processes such as wing-whirring and abdominal pumping (Krogh & Zeuthen, 1941; Sotavalta, 1954).

Bloxham *et al.* (1973*a*,*b*) have shown that the loss of ^3H from [5-^{3}H,U-^{14}C]glucose 6-phosphate may be used to estimate the rate of substrate cycling of fructose 6-phosphate *in vivo*. In the present work, the rate of substrate cycling *in vivo* has been estimated from the equilibrium $^3H/^{14}C$ ratio of glucose 6-phosphate derived from the metabolism of [5-^{3}H,U-^{14}C]glucose. It is shown that as the temperature is decreased below 24°C, the non-flying bumble-bee exhibits a high rate of substrate cycling. In contrast, flight at all temperatures is characterized by an absence of substrate cycling and a high rate of glycolysis.

Materials and Methods

Materials

D-[5-^{3}H]- and D-[U-^{14}C]-glucose were obtained from Amersham/Searle (Des Plaines, Ill., U.S.A.). All enzymes were obtained from Boehringer Corp., New York, N.Y., U.S.A. Rabbit muscle fructose diphosphatase was obtained from the Sigma Chemical Co., St. Louis, Mo., U.S.A.

Methods

Injection of labelled glucose. Individuals of *Bombus affinis* (average weight, 0.19g) were collected from flowers and kept in the dark at 27°C until required. Bees were immobilized by exposure to −20°C for

4.5min. Immediately after the bees had been returned to 27° or 21°C, 2μl of a solution containing labelled glucose was injected into the thoracic flight muscle with a finely-honed syringe (10μl capacity; point, 0.4mm diam.; Hamilton Co., Reno, Nev., U.S.A.). The needle was inserted centrally on the ventral side of the insect at the junction of the thorax and abdomen. Injection of this volume had no apparent effect on the bumble-bee, which fully recovered normal activity within 3.0–3.5min. Insects designated as 'non-flying' were placed in small cages (8cm^3) that allowed free circulation of air but prevented flight. After the required interval of exposure of the insects to 5°, 21° or 27°C, the cage containing each bee was immersed in liquid N_2. Insects designated as 'flying' were attached to thread, one end of which was fastened about the insect's abdomen, the other secured to a rod. Bees were induced to fly by gentle agitation at either 21° or 27°C and, when necessary, flown into an environment of 5°C. After the required interval of flight the bee was immediately frozen in liquid N_2.

Metabolite concentrations in bumble-bee thorax, measured by this technique in the present work, were similar to those observed by Newsholme *et al.* (1972), who used freeze-clamping. Since all visible signs of life ceased within 0.2s upon contact with liquid N_2 it is unlikely that the bees could respond to the sudden change in temperature.

Perchlorate extracts of bumble-bee flight muscle. Flight muscles (approx. 0.2g fresh wt.) were dissected from frozen bees and homogenized in 2ml of 6% (w/v) $HClO_4$. The perchlorate extract was centrifuged at 10000*g* for 10min and the supernatant fluid was neutralized with 2M-KOH. The final volume was adjusted to 3ml and a 1ml sample was retained for the determination of metabolite concentrations. The remaining extract, together with 10μmol of authentic glucose 6-phosphate, was applied to a column (0.5cm×6.0cm) of Dowex AG 1 resin (borate form; X8). Each column was washed with 1ml of water and a 0.5ml portion of the combined eluate was counted for radioactivity. The remainder of the eluate was evaporated to dryness, reconstituted in water and counted to determine non-volatile tritiated material. The difference between each of these measurements was used to evaluate ^3H_2O released as c.p.m./g fresh wt. Material retained by the borate column at this stage included glucose 6-phosphate as well as other sugars and sugar phosphates (e.g. Williams *et al.*, 1971: see their Fig. 1). For the specific elution of glucose 6-phosphate each column was eluted successively with 2×5ml of 0.2M-ammonium tetraborate (discarded) and 2×5ml of 0.35M-ammonium tetraborate (retained). Control experiments indicated that 10ml of 0.35M-ammonium tetraborate completely eluted glucose 6-phosphate, which was free of glucose and fructose 1,6-diphosphate. Ammonium tetraborate was removed by repeated evaporations with methanol and samples of glucose 6-phosphate were further purified by using the paper-chromatographic technique of Bloxham *et al.* (1973*a*). Glucose 6-phosphate was eluted from the chromatogram and the specific radioactivity as well as the $^3H/^{14}C$ ratio determined. The glucose 6-phosphate eluted from the borate column was fairly pure since chromatography did not alter the $^3H/^{14}C$ ratio.

Calculation of the rate of phosphorylation of fructose 6-phosphate. The rate of fructose 6-phosphate phosphorylation (T) can be determined by assuming that the loss of ^3H from [5-^{3}H,U-^{14}C]fructose 1,6-diphosphate is rapid (Bloxham *et al.*, 1973*a*). Under these conditions, ^3H release is equivalent to fructose 6-phosphate phosphorylation and can be expressed in μmol/min per g fresh wt. by the use of the following equation:

$$T = \frac{^3H_2O \text{ released (c.p.m./g fresh wt.)}}{\text{Sp. radioactivity of [}^{14}\text{C]glucose 6-}P\text{ (c.p.m./}\mu\text{mol)} \times (^3H/^{14}C \text{ ratio of glucose 6-}P) \times t \text{ (min)}}$$

The values of T (cf. Table 1) are the averages of four values at each time-interval. T was found to be approximately constant over the duration of the experiment for the different conditions.

Determination of metabolites. Glucose was determined in a 0.05ml sample of the neutralized perchlorate extract by the glucose oxidase method of Werner *et al.* (1970). Glucose 6-phosphate and fructose 6-phosphate were determined by the method of Hohorst (1963) and fructose 1,6-diphosphate was determined by the method of Racker (1963).

Preparation of flight-muscle homogenates and assay of enzyme activities. The flight muscle was dissected from ether-anaesthetized bumble-bees and homogenized in 10vol. of ice-cold 50mM-Tris–HCl buffer, pH7.6, containing 5mM-$MgSO_4$ and 1mM-EDTA. Small pieces of contaminating carapace were removed by filtration through glass wool. A supernatant fraction was obtained by centrifugation at 10000*g* for 20min and used in the subsequent assays. Phosphofructokinase was assayed in a solution of 50mM-Tris–HCl buffer, pH7.6, 1mM-$MgSO_4$, 1mM-ATP, 1mM-fructose 6-phosphate, 1mM-dithiothreitol, 0.3i.u. of aldolase, 3.6i.u. of triose phosphate isomerase, 0.5i.u. of glycerol phosphate dehydrogenase and 0.2mM-NADH. The aldolase assay contained 50mM-Tris–HCl buffer, pH7.6, 1mM-dithiothreitol,

1mM-fructose 1,6-diphosphate, 3.6i.u. of triose phosphate isomerase, 0.5i.u. of glycerol phosphate dehydrogenase and 0.2mM-NADH. The triose phosphate isomerase assay contained 50mM-Tris–HCl buffer, pH7.6, 1mM-dithiothreitol, 0.2mM-glyceraldehyde 3-phosphate, 0.5i.u. of glycerol phosphate dehydrogenase and 0.2mM-NADH. The fructose diphosphatase assay contained 50mM-Tris–HCl buffer, pH7.6, 2mM-$MgSO_4$, 0.2mM-fructose 1,6-diphosphate, 3.5i.u. of glucose phosphate isomerase, 0.35i.u. of glucose 6-phosphate dehydrogenase and 0.125mM-$NADP^+$. The glucose phosphate isomerase assay contained 50mM-Tris–HCl buffer, pH7.6, 1mM-fructose 6-phosphate, 0.35i.u. of glucose 6-phosphate dehydrogenase and 0.125mM-$NADP^+$. All assays were performed in 1ml total volume at 28°C. The change in redox state of the pyridine nucleotides was followed at 340nm in a Gilford 2000 spectrophotometer. Variations in the bivalent cation composition of the assays of fructose diphosphatase and phosphofructokinase are given in the Results section.

Results

Metabolism of labelled glucose by bumble-bee flight muscle

Substrate cycling *in vitro* (Bloxham *et al.*, 1973*a*) was estimated by measurement of the $^3H/^{14}C$ ratio in [5-^{3}H,U-^{14}C]glucose 6-phosphate. Whereas labelled hexose 6-phosphate was added directly to the system *in vitro*, the introduction of radioactive label into hexose 6-phosphates *in vivo* relies upon the metabolism of [5-^{3}H,U-^{14}C]glucose by reactions of glycolysis. Therefore it was necessary to establish the time-course of the labelling of intracellular [^{14}C]glucose 6-phosphate. Fig. 1 shows that, after injection of [U-^{14}C]-glucose, the specific radioactivity of flight-muscle glucose 6-phosphate reached its maximum value 30s after the insects were returned to the higher temperatures and was approximately constant for a subsequent 7min. When either flying or non-flying bees were exposed to 5°C, a marked increase in the specific radioactivity of glucose 6-phosphate was observed.

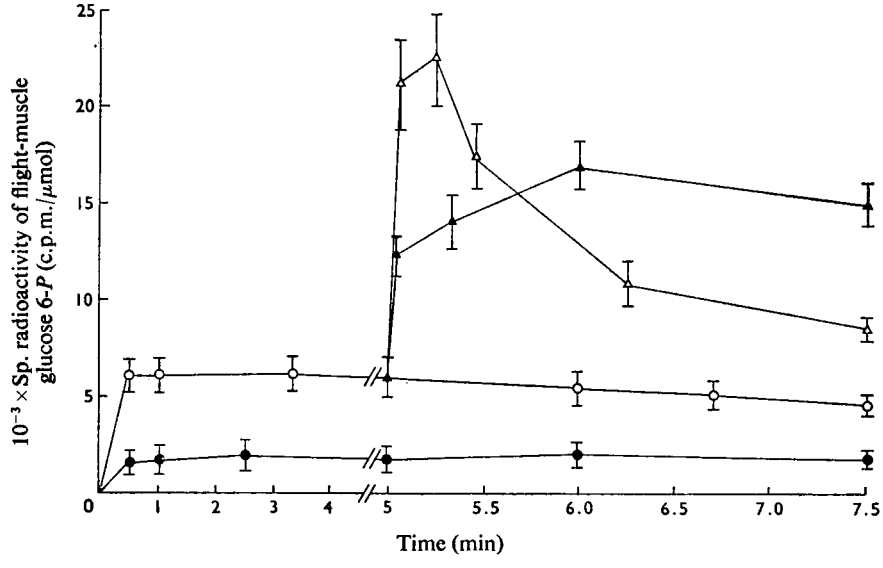

Fig. 1. *Effect of environmental temperature on the specific radioactivity of flight-muscle glucose 6-phosphate*

Bumble-bees were anaesthetized by cold-exposure (4.5min at −20°C) and removed into environments of 21° and 27°C. Injections of [U-^{14}C]glucose (0.03μCi and 0.114nmol) were immediately made into the thoracic flight muscle. The specific radioactivity of glucose 6-phosphate, isolated from the flight muscle, was measured at the time-intervals indicated. Conditions were as follows: ●, non-flying bees at 27°C; ○, non-flying bees at 21°C; △, bees induced to fly at 21°C and flown into an environment of 5°C; ▲, non-flying bees at 21°C placed in an environment of 5°C after 5min. Each result is the mean value from four insects. The bars represent the S.E.M.

The increase occurred within 3s and demonstrated the insect's capacity for rapid metabolic responses to changes in environmental temperature. This compares with previous studies, which have shown that bumble-bees can alter their thoracic temperature within a few seconds (Heinrich, 1972). The specific radioactivity of glucose 6-phosphate in flying bees was maximal within 30s and decreased progressively beyond this time, whereas the specific radioactivity of glucose 6-phosphate in the non-flying bee increased to a maximum over 1min and remained approximately constant for 6.5min. At all temperatures flight resulted in a marked increase in the specific radioactivity of glucose 6-phosphate. It is apparent that the biochemical responses to low temperatures occur within a few seconds and therefore any evidence for the operation of substrate cycling should also occur within this time.

After the injection of [5-^{3}H,U-^{14}C]glucose, the ^{3}H/^{14}C ratio of labelled glucose 6-phosphate from flight muscle was measured under a variety of conditions (Fig. 2). When the insects were maintained at 27°C (Fig. 2*a*), there was no change in the ^{3}H/^{14}C ratio of glucose 6-phosphate, indicating that substrate cycling had not occurred. At 21°C (Fig. 2*a*), the ^{3}H/^{14}C ratio did not change in the re-awakening period (3min); however, once the animal became active there was a sharp decrease in the ratio, which eventually reached equilibrium after 10min. When the environmental temperature was suddenly decreased from 21° to 5°C (Fig. 2*b*), there was a dramatic decrease in the ^{3}H/^{14}C ratio of glucose 6-phosphate within 3–6s. After this, the ratio remained constant. In contrast, if the bumble-bees were induced to fly at 21°C before the decrease in environmental temperature, the ^{3}H/^{14}C ratio of glucose 6-phosphate actually increased. This must reflect the rapid phosphorylation of unchanged [5-^{3}H,U-^{14}C]glucose. As there was no detectable decrease in the ^{3}H/^{14}C ratio of glucose 6-phosphate when bees were flown at either 21° or 27°C (Fig. 2*a*), flight was concluded to be associated with the absence of substrate cycling. Interestingly, the decrease in ^{3}H/^{14}C ratio of glucose 6-phosphate appears to be related to the insect's ability to detect changes in temperature rather than the absolute temperature. Thus when bumble-bees were injected and maintained at 5°C (a condition in which they never recovered activity) there was no change in the ^{3}H/^{14}C ratio of intracellular glucose 6-phosphate.

Fig. 3 shows the rate of ^{3}H release from [5-^{3}H,U-^{14}C]glucose under the same conditions as those described for Fig. 2. Again it is apparent that decreasing the temperature to 5°C markedly influenced metabolism and in this instance accelerated the rate of ^{3}H release in either the flying or non-flying bumble-bee. It is noteworthy that the ^{3}H release was highest in flying bumble-bees at 5°C despite the fact that no decrease in the ^{3}H/^{14}C ratio of glucose 6-phosphate was observed (cf. Fig. 2*b*).

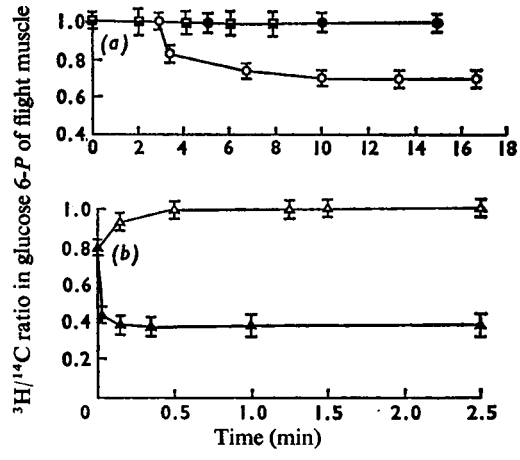

Fig. 2. *Effect of environmental temperature on the ^{3}H/^{14}C ratio of flight-muscle glucose 6-phosphate*

Bumble-bees were treated as described for Fig. 1 and injected with [5-^{3}H,U-^{14}C]glucose (1.7μCi and 1.7nmol of [5-^{3}H]glucose and 0.03μCi and 0.114nmol of [U-^{14}C]glucose). Conditions were as follows: ■, flying bees at 27°C; □, flying bees at 21°C; ●, non-flying bees at 27°C; ○, non-flying bees at 21°C; △, bees induced to fly at 21°C and flown into an environment of 5°C; ▲, non-flying bees at 21°C placed in an environment of 5°C after 5min. Methods for the isolation of glucose 6-phosphate from flight muscle and for the determination of the ^{3}H/^{14}C ratio were as described in the Materials and Methods section. Each result is the mean value from four insects. The bars represent the S.E.M.

The results of Figs. 1, 2 and 3 were used to calculate the rate of phosphorylation of fructose 6-phosphate as described in 'Methods'. The equilibrium value of ^{3}H/^{14}C ratio in flight-muscle glucose 6-phosphate was used to calculate the rate of substrate cycling (Table 1) as described by Bloxham *et al.* (1973*b*). The rate of glycolysis was estimated by subtracting the rate of substrate cycling from the rate of phosphorylation of fructose 6-phosphate. Flight at all temperatures was characterized by an unchanged ^{3}H/^{14}C ratio of glucose 6-phosphate indicative of the absence of substrate cycling. Non-flying bees at 21°C (Fig. 2*a*) exhibited a slight decrease in the ^{3}H/^{14}C ratio of glucose 6-phosphate, which corresponded to a rate of substrate cycling of 0.48μmol/min per g fresh wt. of flight muscle. At 5°C, the rate of substrate cycling in the flight muscle of non-flying bumble-bees was 10.4μmol/min per g

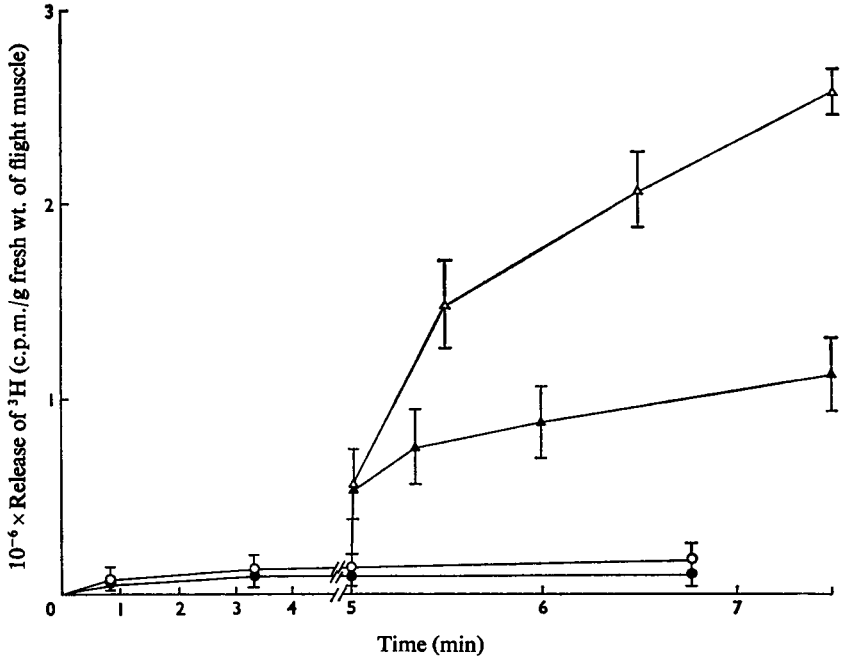

Fig. 3. *Effect of environmental temperature on the release of ^3H from [5-^3H]glucose by bumble-bee flight muscle*

Bumble-bees were treated as described for Fig. 1 and injected with [5-^{3}H,U-^{14}C]glucose (1.7μCi and 1.7nmol of [5-^{3}H]glucose and 0.03μCi and 0.114nmol of [U-^{14}C]glucose). Conditions were as follows: ●, non-flying bees at 27°C; ○, non-flying bees at 21°C; △, bees induced to fly at 21°C and flown into an environment of 5°C; ▲, non-flying bees at 21°C placed in an environment of 5°C after 5min. The concentration of ^3H_2O in flight muscle was determined as described in the Materials and Methods section. Each result is the mean value±S.D. from four insects.

fresh wt. When the values for the constants shown in Table 1 were substituted in the equations for the rate of change of the $^3H/^{14}C$ ratio in glucose 6-phosphate (i.e., b^t/b^κ; Bloxham *et al.*, 1973*b*), it was shown that for the non-flying bumble-bee at 5°C the $^3H/^{14}C$ ratio obtained an equilibrium value of 0.36 within 12s, which is in close agreement with the experimental observations (Fig. 2*b*).

Activities of enzymes in bumble-bee flight muscle

The method of analysing the rate of substrate cycling *in vivo* required that ^3H was readily lost from the C-5 position of fructose 1,6-diphosphate by equilibration through reactions catalysed by aldolase and triose phosphate isomerase. It has been shown that this criterion is satisfied *in vitro* provided that the aldolase activity exceeds 1.5i.u./ml and the triose phosphate isomerase activity exceeds 0.1i.u./ml when the activity of phosphofructokinase is 2.2i.u./ml. Measurement of the activities of these enzymes in samples of bumble-bee flight muscle indicated that they were present in excess and that they were not rate-limiting when compared with the activities of either fructose diphosphatase or phosphofructokinase (Table 2). It was also shown that high activities of glucose phosphate isomerase were present in bumble-bee flight muscle (250i.u./g fresh wt.), which should act to establish rapid isotopic equilibrium between fructose 6-phosphate and glucose 6-phosphate (cf. the reconstructed substrate cycle *in vitro* of Bloxham *et al.*, 1973*a*, their Fig. 3, where isotope equilibrium was maintained by a glucose phosphate isomerase activity of 3.5i.u./ml). This is important since in the development of the mathematical expression it is assumed that the hexose phosphates are in equilibrium (Bloxham *et al.*, 1973*b*).

Ca^{2+} regulation of fructose diphosphatase

The results presented so far indicate that as the ambient temperature was decreased, the activity of the fructose diphosphatase–phosphofructokinase substrate cycle was enhanced and that flying, irrespective

Table 1. *Effect of environmental temperature and flight on the rate constants of substrate cycling and glycolysis*

Bumble-bees were treated as described for Fig. 2. Metabolite concentrations are expressed in μmol/g fresh wt. and as means±S.D. The rate of phosphorylation of fructose 6-phosphate (T) was calculated as described in 'Methods' and is expressed in μmol/min per g fresh wt. The $^3H/^{14}C$ ratio in glucose 6-phosphate at equilibrium was extrapolated from the results of Fig. 2(*a*) and 2(*b*). The rates of substrate cycling (S_c) and glycolysis were calculated by the formulae of Bloxham *et al.* (1973*b*) and are expressed in μmol/min per g fresh wt.

$$S_c = T\left[1 - \frac{\text{equilibrium } ^3H/^{14}C \text{ ratio of glucose 6-}P}{^3H/^{14}C \text{ ratio of glucose}}\right]$$

Glycolysis was determined from the expression: Glycolysis $= T - S_c$. The rate constants k_1 (hexokinase), k_2 (phosphofructokinase), k_3 (fructose diphosphatase) and k_4 (composite rate constant for the overall conversion of fructose 1,6-diphosphate into pyruvate) were calculated from the steady-state equation for glycolysis (Bloxham *et al.*, 1973*b*):

$$k_1[\text{Glc}] = k_2[\text{Glc 6-}P + \text{Fru 6-}P] - k_3[\text{Fru 1,6-di}P] = k_4[\text{Fru 1,6-di}P] = \text{Glycolysis}$$

Temperature ...	21°C		5°C	
Mode ...	Flying	Non-flying	Flying	Non-flying
Rate of phosphorylation of fructose 6-phosphate	14.7	1.6	20.4	16.2
Equilibrium $^3H/^{14}C$ ratio in glucose 6-phosphate	1.0	0.7	1.0	0.36
Substrate-cycling rate	0	0.48	0	10.4
Glycolytic rate	14.7	1.12	20.4	5.8
Metabolites:				
Glucose	8.2 ±2.5	11.2 ±3.2	7 ±2.7	10.1 ±3.1
Glucose 6-phosphate	0.19±0.04	0.16±0.03	0.20±0.04	0.14±0.04
Fructose 6-phosphate	0.03±0.01	0.04±0.01	0.04±0.01	0.04±0.01
Fructose 1,6-diphosphate	0.16±0.02	0.17±0.03	0.16±0.02	0.17±0.03
Rate constants:				
k_1	1.79	0.1	2.91	0.59
k_2	66.8	8	85	90
k_3	0	2.82	0	61.2
k_4	91.8	6.58	127.5	34.11

Table 2. *Maximal catalytic activities of some enzymes of the flight muscle of the bumble-bee (Bombus affinis)*

Thoracic flight muscle was dissected from frozen bees and treated as described in the Materials and Methods section. The maximal catalytic activities of the enzymes were determined at 28°C as described in the text and are expressed in μmol/min per g fresh wt. as mean values±S.D. with the numbers of observations in parentheses.

	Catalytic activity
Phosphofructokinase	42.6±10.9 (6)
Fructose diphosphatase	45.3±11.5 (6)
Aldolase	92.2±23.6 (3)
Glucose phosphate isomerase	250 ±62 (3)
Triose phosphate isomerase	476 ±37 (3)

of ambient temperature, completely suppressed substrate cycling. Since the rate of glycolysis is high under these conditions, it is apparent that substrate cycling must be decreased by virtue of an inhibition of fructose diphosphatase. For most preparations of fructose diphosphatase, the control of activity is probably achieved through the regulation of AMP concentration (Pontremoli & Horecker, 1971). This method of control appears to be inoperative in bumble-bee flight muscle, since Newsholme *et al.* (1972) have shown that flight-muscle fructose diphosphatase is not inhibited by AMP. With flight-muscle fructose diphosphatase we also found that the addition of 1 mM-AMP had no significant effect whereas rabbit liver fructose diphosphatase assayed under the same conditions was completely inhibited.

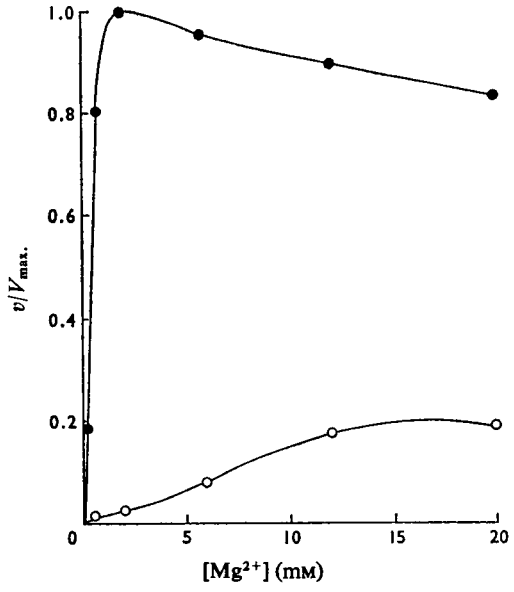

Fig. 4. *Effect of Ca^{2+} on the reaction velocity of fructose diphosphatase from the flight muscle of Bombus affinis*

Fructose diphosphatase was assayed as described, except that the concentration of Mg^{2+} was varied and the concentration of Ca^{2+} was either 0 (●) or 1 mM (○).

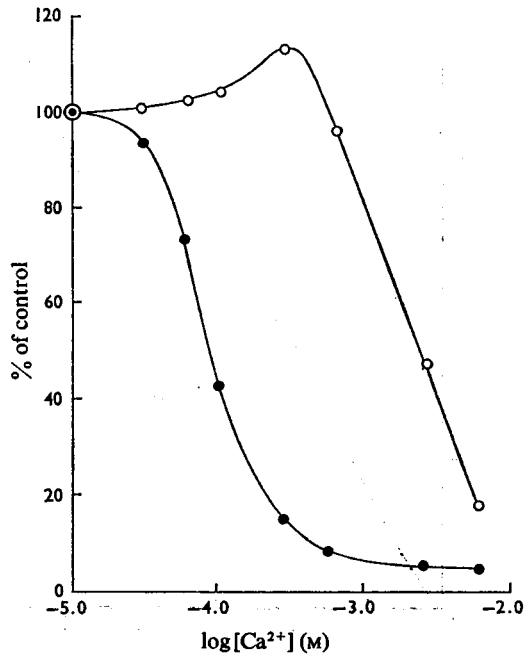

Fig. 5. *Inhibition of flight-muscle fructose diphosphatase and phosphofructokinase by Ca^{2+}*

The enzymes were assayed by using the conditions described in the Materials and Methods section in the presence of various Ca^{2+} concentrations. ○, Phosphofructokinase activity; ●, fructose diphosphatase activity.

Since it has been established that the concentration of sarcoplasmic Ca^{2+} is closely related to the contractile process (Ebashi & Endo, 1968; Tregear, 1967), the effect of this cation on the activity of bumble-bee flight-muscle fructose diphosphatase was measured. Fig. 4 shows that Ca^{2+} completely inactivated fructose diphosphatase and that this inhibition was only partially reversed by increasing the Mg^{2+} concentration.

Since some sources of phosphofructokinase are inhibited by Ca^{2+} (Lowry & Passoneau, 1966), it was necessary to compare the susceptibility of flight-muscle phosphofructokinase and fructose diphosphatase to Ca^{2+} inhibition. Fig. 5 shows that when the enzymes were assayed at optimum substrate concentrations for maximum catalytic activity, fructose diphosphatase (apparent $K_i = 9.5 \times 10^{-5}$ M) was far more sensitive to Ca^{2+} inhibition than was phosphofructokinase (apparent $K_i = 2.2 \times 10^{-3}$ M).

The inhibition of fructose diphosphatase was not the result of an irreversible inactivation of the enzyme since activity was restored to the control value when Ca^{2+} was chelated by an excess of ethanedioxybis(ethylamine)tetra-acetate (EGTA) (Fig. 6). The time-course for the inactivation of fructose diphosphatase by Ca^{2+} and the reactivation of this enzyme by EGTA showed that the changes in enzyme activity occurred within the time required for mixing.

Discussion

The present results indicate that, provided that the decrease in $^3H/^{14}C$ ratio of [5-^{3}H,U-^{14}C]glucose 6-phosphate is a measure of fructose 6-phosphate substrate cycling, decreases in the ambient temperature markedly increase the rate of substrate cycling in the resting flight muscle of bumble-bees. In contrast, the metabolism of glucose by working flight muscle is completely unidirectional and substrate cycling at the fructose 6-phosphate level is negligible at all temperatures.

The relationship between substrate cycling and the decrease of the $^3H/^{14}C$ ratio of [5-^{3}H,U-^{14}C]glucose 6-phosphate was established for a system *in vitro* where the decrease in the $^3H/^{14}C$ ratio of [5-^{3}H,U-^{14}C]glucose 6-phosphate occurred through the concerted actions of phosphofructokinase, fructose

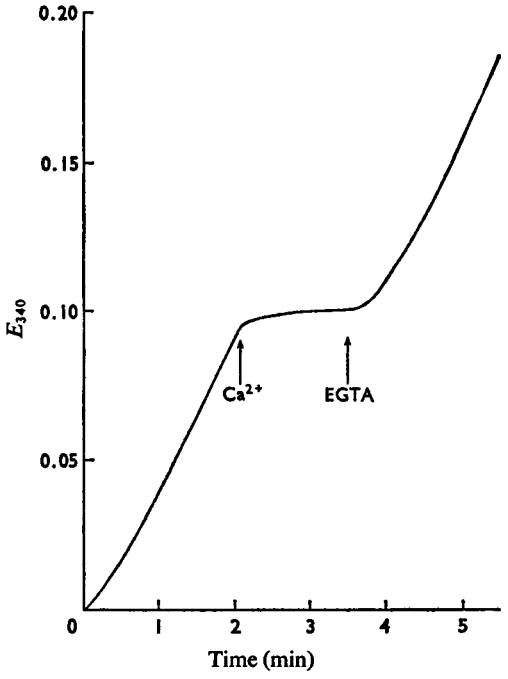

Fig. 6. *Reversal of Ca^{2+} inhibition by ethanedioxybis-(ethylamine)tetra-acetate (EGTA)*

Fructose diphosphatase was assayed as described in the Materials and Methods section and, at the times indicated on the trace, 0.5 mM-Ca^{2+} and 1 mM-EGTA were added.

diphosphatase, aldolase and triose phosphate isomerase. For more complicated systems, such as those *in vivo*, other reactions must be considered which can convert [5-^{3}H,U-^{14}C]glucose into glucose 6-phosphate with the loss of ^{3}H. Reactions of this type include (i) the Cori cycle, (ii) the oxidative and non-oxidative segments of the pentose phosphate pathway and (iii) the transaldolase exchange reaction (Ljungdahl *et al.*, 1961).

Reactions of the Cori cycle may result in the formation of [U-^{14}C]glucose 6-phosphate from [5-^{3}H,U-^{14}C]glucose by (a) reactions of glycolysis leading to the formation of [U-^{14}C]lactate in tissues other than flight muscle, followed by (b) resynthesis of glucose from [U-^{14}C]lactate in flight muscle. This pathway is unlikely as flight muscle does not contain phosphoenolpyruvate carboxykinase (Newsholme *et al.*, 1972). Alternatively, reactions of the pentose phosphate pathway could result in the conversion of [5-^{3}H,U-^{14}C]glucose 6-phosphate into [2-^{3}H,U-^{14}C]glyceraldehyde 3-phosphate without the involvement of phosphofructokinase. The contribution of this pathway to the formation of glyceraldehyde 3-phosphate is also unlikely as bumble-bee flight muscle contains only a low activity of glucose 6-phosphate dehydrogenase (Newsholme *et al.*, 1972). We have not shown whether flight muscle can catalyse a transaldolase exchange; however, the observation that either flight or high temperature completely eliminates the decrease in the ^{3}H/^{14}C ratio of glucose 6-phosphate suggests that this possibility does not contribute since these conditions would not be expected to influence an exchange reaction.

An important observation resulting from the present studies is that, provided that corrections are made for the rate of substrate cycling, the release of ^{3}H from [5-^{3}H,U-^{14}C]glucose can be used to measure glycolysis in situations where contribution of the pentose phosphate pathway is small. The loss of ^{3}H is guaranteed by reactions of glycolysis because of the high activities of triose phosphate isomerase (cf. the activity of triose phosphate isomerase at which ^{3}H loss is limited; Bloxham *et al.*, 1973*a*). Findings by Katz & Wals (1972), from the metabolism of [5-^{3}H]glucose, showed that only glucose and lactose together with cellular water contained radioactivity. These results reinforce conclusions that [5-^{3}H]glucose may be a useful substrate for the estimation of glycolytic rates *in vivo* where this method is much simpler than any previous method.

From the present data a mechanism may be formulated for temperature regulation in the bumble-bee thorax. With the insect at rest or collecting pollen, the degree of substrate cycling of fructose 6-phosphate is increased, where the increase is inversely proportional to the ambient temperature. An increased hydrolysis of ATP results in the generation of heat. When flight is commenced substrate cycling is no longer required and is suppressed. Glucose utilization occurs solely by reactions of glycolysis and gives rise to the generation of ATP required for the contractile processes of flight. During flight, the generation of heat from muscle contraction is always sufficient to maintain the thoracic temperature above the minimum required for flight even when ambient temperatures are very low. The observed rates at which the ^{3}H/^{14}C ratio of glucose 6-phosphate changes as well as the equilibrium values for this ratio are in accord with these suggestions. Our results show that the ^{3}H/^{14}C ratio of glucose 6-phosphate decreases dramatically and is at equilibrium within 3–6s at 5°C. Finally, and as might be expected, flight was found to coincide with an immediate acceleration of glycolysis and suppression of substrate cycling (Fig. 2).

Unlike other enzymes in insect flight muscle, which respond to changes in Ca^{2+} concentration in the region of 1 μM or less [myofibrillar ATPase (Maruyama *et al.*, 1968), mitochondrial glycerol phosphate dehydrogenase (Donnellan & Beechey, 1969) and phosphorylase *b* kinase (Sacktor, 1970)],

fructose diphosphatase was not inhibited until the concentration of Ca^{2+} exceeded 0.03mM (Fig. 5). As the operation of fructose diphosphatase opposes carbon flux through reactions of glycolysis, it is possible to develop a rational explanation of a control mechanism for the regulation of substrate cycling and glycolysis involving changes in Ca^{2+} concentration in the range 10^{-4} to 10^{-5}M. Thus muscle contraction is associated with the release of bound Ca^{2+} from the sarcoplasmic reticulum and an increase in the sarcoplasmic concentration of Ca^{2+} (Ebashi & Endo, 1968). This phenomenon is associated with the activation of the contractile process and it has been calculated that for most muscles the increase in cytosolic Ca^{2+} concentration is of the order of 10–100μM (Hoyle, 1969; Sandow, 1970), which is a value similar to the reported K_t (Ca^{2+}) for fructose diphosphatase (95μM), but very much lower than the K_t (Ca^{2+}) for phosphofructokinase (2.2mM). Therefore under these conditions of muscle contraction substrate cycling should be inhibited and glucose metabolism will proceed freely through glycolysis. At the end of flight the cessation of contractile processes should lead to the binding of Ca^{2+} to the sarcoplasmic reticulum and the lowering of cytosolic Ca^{2+} concentration. At this time, fructose diphosphatase should regain activity and substrate cycling will occur. At 5°C, the maximum rate of substrate cycling was 10.4μmol/min per g fresh wt., which indicates that fructose diphosphatase is almost maximally active under these conditions.

Regulation of fructose diphosphatase by Ca^{2+} does not explain all of the possible control mechanisms since it does not account for the observation that the degree of substrate cycling is inversely proportional to the ambient temperature. However, it must be appreciated that bumble-bees have temperature-sensing antennae, which means that neural or hormonal factors may also regulate this process.

It is noteworthy that Van Tol *et al.* (1972) have proposed a role for Ca^{2+} in the regulation of the activity of mammalian muscle fructose diphosphatase since Ca^{2+} is an inhibitor of rabbit muscle fructose diphosphatase. These authors propose that fructose diphosphatase is active only during relaxation.

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