Biochem. J. (1972) **128**, 89–97
Printed in Great Britain

The Activities of Fructose Diphosphatase in Flight Muscles from the Bumble-Bee and the Role of this Enzyme in Heat Generation

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(*Received* 13 *December* 1971)

1. The maximum catalytic activities of fructose diphosphatase from flight muscles of bumble-bees (*Bombus* spp.) are at least 30-fold those reported for the enzyme from other tissues. The maximum activity of fructose diphosphatase in the flight muscle of any particular bee is similar to that of phosphofructokinase in the same muscle, and the activity of hexokinase is similar to or greater than the activity of phosphofructokinase. There is no detectable activity of glucose 6-phosphatase and only a very low activity of glucose 6-phosphate dehydrogenase in these muscles. The activities of both fructose diphosphatase and phosphofructokinase vary inversely with the body weight of the bee, whereas that of hexokinase is relatively constant. 2. There is no significant hydrolysis of fructose 1-phosphate, fructose 6-phosphate, glucose 1,6-diphosphate and glycerol 3-phosphate by extracts of bumble-bee flight muscle. 3. Fructose 1,6-diphosphatase from bumble-bee flight muscle and from other muscles is inhibited by Mn^{2+} and univalent cations; the potency of inhibition by the latter varies in the order $Li^+ > Na^+ > K^+$. However, the fructose diphosphatase from bumble-bee flight muscle is different from the enzyme from other tissues in that it is not inhibited by AMP. 4. The contents of ATP, hexose monophosphates, fructose diphosphate and triose phosphates in bumble-bee flight muscle showed no significant changes between rest and flight. 5. It is proposed that both fructose diphosphatase and phosphofructokinase are simultaneously active and catalyse a cycle between fructose 6-phosphate and fructose diphosphate in resting bumble-bee flight muscle. Such a cycle would produce continuous hydrolysis of ATP, with the release of energy as heat, which would help to maintain the thoracic temperature during rest periods at a level adequate for flight.

During comparative investigations of the activities of phosphofructokinase (EC 2.7.1.11) and fructose 1,6-diphosphatase (EC 3.1.3.11) in muscle it was observed that the activities of the latter in the flight muscles of various species of the bumble-bee were extremely high and comparable with those of the former. This high activity appeared to be unique to *Bombus* species and therefore a systematic study of the activities and properties of fructose 1,6-diphosphatase from the flight muscle of bumble-bees was carried out and the results are reported in this paper.

Materials and Methods

Chemicals, enzymes and animals

All chemicals, enzymes and animals were obtained from the sources given previously (Crabtree & Newsholme, 1972) except for the following: [^{14}C]-glucose was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.; fructose 1-phosphate, glucose 1,6-diphosphate and all nucleotides were obtained from the Boehringer Corp. (London) Ltd.

Preparation of homogenates and assay of enzyme activities

The activities of fructose diphosphatase and phosphofructokinase were determined in extracts of the flight (dorsal longitudinal) muscles of individual bumble-bees that had been previously weighed on a torsion balance to determine the body weight. Pollen was always removed before weighing, but no allowance was made for the presence of eggs (in queen bees) or for the weight of the gut contents. The flight muscles from a single bee were removed by dissection, divided into two portions and weighed. One portion was homogenized at pH7.5 (for assay of fructose diphosphatase) and the other at pH8.2 (for assay of phosphofructokinase) in media which were as described by Opie & Newsholme (1967*a*), except for

the inclusion of 20mM-mercaptoethanol in the medium at pH7.5. Homogenates were prepared in ground-glass homogenizers of total capacity 0.1ml, with which a minimum of 3mg of muscle could be homogenized without frothing. Phosphofructokinase and fructose diphosphatase were assayed as described by Opie & Newsholme (1967*a*), except that Mn^{2+} was omitted from the assay for the latter; 1mM-fructose 6-phosphate replaced glucose 6-phosphate plus phosphoglucose isomerase in the assay for phosphofructokinase and muscles were extracted in no more than 10vol. of medium (see Crabtree & Newsholme, 1972). In preliminary studies with bumble-bee flight muscle homogenates it was found that EDTA and mercaptoethanol could be omitted from the assay medium without loss of fructosediphosphatase activity. When the properties of fructose diphosphatase were to be examined, the crude homogenate was centrifuged at 1500*g* for 1min in a bench centrifuge, and the supernatant was passed through a column of Sephadex G-25 (equilibrated with extraction medium) at 5°C. This procedure resulted in no significant loss of fructose diphosphatase activity. When fructose diphosphatase was assayed by measuring the release of P_i the assay conditions were as described above, except that the reaction was terminated by the addition of an equal volume of 6% (w/v) $HClO_4$. The denatured protein was removed by centrifugation and portions of the supernatant were assayed for P_i by the method of Allen (1940).

Hexokinase and mitochondrial glycerol 3-phosphate dehydrogenase activities were assayed as described by Crabtree & Newsholme (1972). Hexokinase was occasionally assayed by the radiochemical method of Newsholme *et al.* (1967) and there was always close agreement between the results of the two assays. Glucose 6-phosphate dehydrogenase was assayed by the method of Glock & McLean (1953). Glucose 6-phosphatase was assayed by the method of Harper (1963), except that the buffer was *N*-(2-acetamido)-2-aminoethanesulphonic acid and P_i was measured by the method of Allen (1940). Aldolase was assayed in an assay system similar to that for phosphofructokinase except that the final pH of the assay was 7.5, fructose 6-phosphate was replaced by fructose 1,6-diphosphate (1mM), and ATP, AMP and commercial aldolase were omitted.

Preparation of cell fractions of bumble-bee flight muscles

The medium used for the preparation of cell fractions was the same as for the extraction of fructose diphosphatase except that it contained 0.25mM-sucrose, the tris concentration was 5mM and the final pH was 8.2. The muscle was gently homogenized in a glass homogenizer with a Teflon pestle (four to six strokes of the pestle) in 10vol. of medium. After the removal of cell debris by centrifugation at 200*g* for 10min, mitochondria were sedimented by centrifugation at 15000*g* for 15min. The sarcoplasmic reticulum was sedimented by centrifugation at 100000*g* for 60min. The supernatant from this latter centrifugation was assumed to represent the soluble cytoplasm. All centrifugations were carried out at 4°C. The degree of damage to the mitochondria was determined by measuring the activity of citrate synthase in each fraction, with the method of Srere *et al.* (1963).

Freeze-clamping of bumble-bee flight muscle

Bumble-bees were collected from flowers and placed in a jar in a dark-room for 1–2h before use. With the room illuminated by a red photographic light, a bee was coaxed on to a piece of Parafilm and, when in a suitable position, the whole insect was frozen between aluminium tongs cooled in liquid nitrogen (Wollenberger *et al.*, 1960). This process resulted in much of the flight muscle being squeezed out of the thorax and the frozen muscle could then be separated easily from the cuticle and from the Parafilm. Since the bees did not attempt to fly, samples of flight muscle obtained under these conditions are assumed to represent resting muscle.

Bumble-bees were suspended by attaching a piece of thread between the thorax and the abdomen and, by gentle agitation of the insect, flight was induced and could be maintained for several minutes. After 2–3min of continuous flight the bees were freeze-clamped and the flight muscles were separated from the rest of the insect as described above.

Measurement of glycolytic intermediates and ATP

The pieces of frozen muscle were supported on a block of aluminium cooled in liquid nitrogen and cut into very small pieces with a cooled scalpel. A small quantity of this frozen muscle was transferred to a preweighed ground-glass homogenizer and pestle containing a known volume (usually 0.2ml) of 6% (w/v) perchloric acid at 0°C. The muscle was immediately homogenized with the perchloric acid and, provided that the pieces of frozen muscle were small and the total amount of muscle added to the homogenizer did not exceed 30mg, this homogenization was complete in less than 30s. The homogenizer plus pestle was reweighed to obtain the weight of frozen muscle added. The precipitated protein was removed by centrifugation at 1000*g* for 5min in a bench centrifuge, a known volume of the supernatant was adjusted to approx. pH6.5 with 3M-$KHCO_3$ at 0°C and the precipitated perchlorate was removed by centrifugation. The supernatant was used for the determination of glycolytic intermediates and ATP by using the methods described by Gevers & Krebs (1966).

Results

Activities of enzymes of bumble-bee flight muscle

The activities of fructose diphosphatase, phosphofructokinase and hexokinase in the flight muscles of various species of the bumble-bee are given in Table 1. In all bumble-bees that have been investigated the range of fructose diphosphatase activities was 4–155 μmol/min per g fresh wt. of muscle; the highest activity recorded in any other muscle is 4.4 μmol/min per g (pheasant pectoral; Newsholme & Crabtree, 1970). In many bees the activity of fructose diphosphatase is slightly higher than that of phosphofructokinase, but although the range of the mean activities of fructose diphosphatase is large, the fructose diphosphatase/phosphofructokinase ratio for all individual results given in Table 1 is 1.10±0.48 (mean±S.D.).

The variation in the mean value for phosphofructokinase is from 22 to 80, whereas for hexokinase the variation is from 70 to 112 μmol/min per g of muscle (Table 1). In general the activities of hexokinase are greater than those of phosphofructokinase, and between different castes and different species the hexokinase activities are more constant than those of phosphofructokinase. It seemed possible that the lower activity of phosphofructokinase in relation to hexokinase might be the result of an inadequate assay for the former: the presence of a high fructose diphosphatase activity in the flight-muscle homogenate could compete with aldolase for the fructose diphosphate produced during the phosphofructokinase assay. However, the presence in the assay of 2mM-Li^+ (which gives approx. 90% inhibition of fructose diphosphatase of bumble-bee flight muscle; see Table 3) did not change the measured activity of phosphofructokinase. Moreover it was found that, in six individual bees of different species, the activity of

Table 1. *Activities of fructose diphosphatase, phosphofructokinase and hexokinase in the flight muscles of various species and castes of the bumble-bee, cuckoo-bee and the honey-bee*

The results are presented as the means of the activities in a number of insects with the ranges of activities given in parentheses. All bumble-bees were caught on flowers between 11 a.m. and 3 p.m. Enzyme activities were determined within 6 h of capture. The activity of fructose diphosphatase in the flight muscles of the cuckoo-bee and honey-bee was determined as described by Newsholme & Crabtree (1970).

Species	Caste	No. of insects	Enzyme activities (μmol/min per g of fresh muscle): Fructose diphosphatase	Phospho-fructokinase	Hexokinase
Bumble-bee					
B. agrorum	Worker	15	38 (7–108)	36 (20–55)	112 (103–128)
B. agrorum	Male	2	51 (51, 51)	36 (35, 37)	—
B. agrorum	Queen	4	14 (6–18)	23 (19–23)	100 (74–119)
B. pratorum	Worker	13	73 (17–155)	56 (22–79)	109 (96–125)
B. pratorum	Male	2	77 (78, 76)	40 (37, 43)	110 (125, 96)
B. pratorum	Queen	2	25 (25, 25)	27 (37, 16)	82 (84, 80)
B. hortorum	Worker	6	22 (15–26)	29 (17–35)	83 (61–114)
B. hortorum	Male	1	27	32	100
B. hortorum	Queen	4	10 (4–16)	24 (13–39)	95 (80–110)
B. lapidarius	Worker	3	114 (96–131)	80 (68–91)	75 (51–91)
B. lapidarius	Male	1	70	68	—
B. lapidarius	Queen	2	29 (45, 12)	27 (32, 21)	70 (71, 69)
B. ruderarius	Worker	6	76 (28–115)	45 (33–74)	98 (96–100)
B. ruderarius	Queen	1	45	40	77
B. terrestris	Worker	2	65 (52, 77)	62 (46, 79)	88
B. terrestris	Queen	2	18 (20, 16)	22 (20, 24)	85
B. lucorum	Worker	5	84 (47–139)	55 (34–65)	92 (74–150)
Cuckoo-bee					
Psithyrus vestalis	Queen	2	2.2 (1.8, 2.6)	16 (16, 16)	32 (30, 34)
Psithyrus vestalis	Male	2	1.8 (1.3, 2.2)	16	31
Psithyrus campestris	Male	1	1.3	17	—
Honey-bee					
Apis mellifera	Worker	5	<0.05	20 (18–22)	29 (26–32)

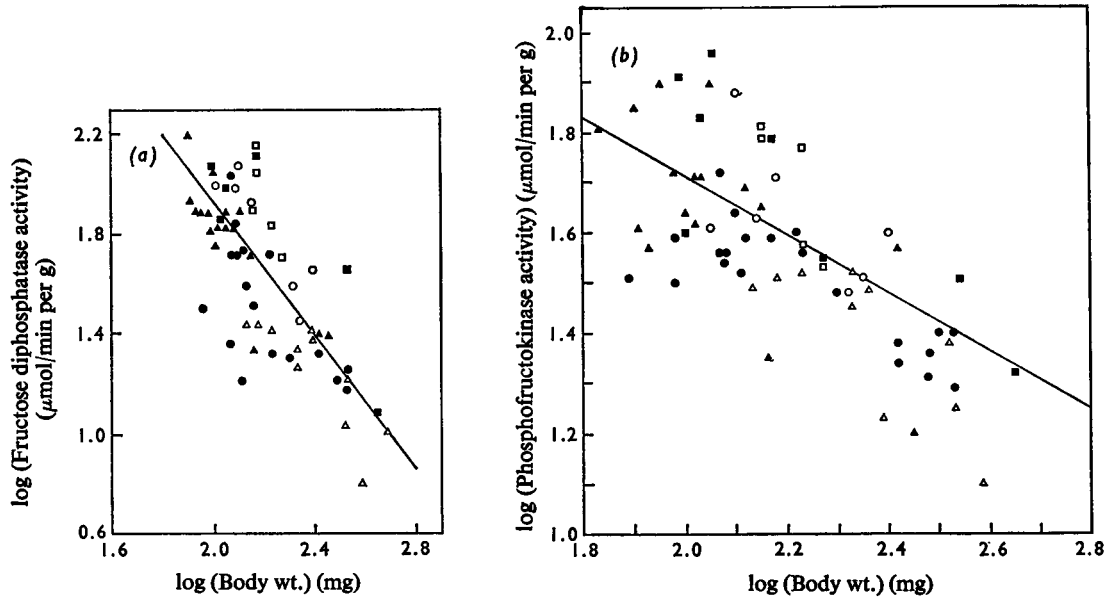

Fig. 1. *Enzyme activities in bumble-bee flight muscle as a function of body weight*

(*a*) Fructose diphosphatase; (*b*) phosphofructokinase. In both (*a*) and (*b*) the enzyme activities and body weights were determined as described in the Materials and Methods section. The regression lines were calculated by the method of least squares (Moroney, 1956) and the correlation coefficients for the relationships (Moroney, 1956) were −0.85 ($P<0.001$) for log (fructose diphosphatase) versus log (body wt.) and −0.64 ($P<0.001$) for log (phosphofructokinase) versus log (body wt.). ●, *B. agrorum*; ▲, *B. pratorum*; △, *B. hortorum*; ○, *B. ruderarius*; ■, *B. lapidarius*; □, *B. lucorum*.

aldolase in any one muscle was almost identical with that of phosphofructokinase, and the activity of the mitochondrial glycerol 3-phosphate dehydrogenase was approximately twice that of phosphofructokinase. These results suggest that the activity of phosphofructokinase and not that of hexokinase represents the maximum glycolytic flux in the bumble-bee flight muscle (see Crabtree & Newsholme, 1972).

The activity of fructose diphosphatase was always lower in queen bees than in workers of any given species of bumble-bee (Table 1), but this difference may depend upon the variation in body wt. rather than variation in caste. Fig. 1(*a*) presents a double-logarithmic plot of fructose diphosphatase activity against body wt., and from the slope of this plot it can be deduced that the activity of fructose diphosphatase in the flight muscle is proportional to the body wt. raised to the power −1.4. Similarly, Fig. 1(*b*) consists of a double-logarithmic plot of phosphofructokinase activity against body wt.; this activity is proportional to the body wt. raised to the power of −0.6.

The activity of glucose 6-phosphate dehydrogenase was 0.2 μmol/min per g and that of glucose 6-phosphatase was not detectable (<0.1 μmol/min per g) in homogenates of bumble-bee flight muscle.

Specificity of fructose diphosphatase of bumble-bee flight muscle

The activities of fructose diphosphatase in the flight muscles of many of the bumble-bees examined are at least 30-fold (on a fresh-weight basis) the activities in any other muscle so far investigated. It was therefore considered essential to demonstrate that this hydrolysis of fructose diphosphate was due to fructose diphosphatase activity, rather than that of a non-specific phosphatase or of an enzyme that hydrolysed glycolytic intermediates without absolute specificity. The release of P_i from fructose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, glucose 1-phosphate or glycerol 3-phosphate (at concentrations between 0.5 and 2 mM) was less than 5% of the activity of fructose diphosphatase in the same muscle. No detectable formation of glucose 1-phosphate or glucose 6-phosphate from glucose 1,6-diphosphate was observed with an assay system

for fructose diphosphatase that contained phosphoglucomutase and glucose diphosphate (in place of fructose diphosphate). The activity of fructose diphosphatase from the flight muscle of the bumble-bee was found to be the same whether it was assayed by measuring the formation of fructose 6-phosphate or P_I. From these results it was concluded that the enzyme under investigation was specific for the hydrolysis of phosphate from the 1-position of fructose diphosphate and could therefore be termed fructose diphosphatase. A similarity between many of the properties of this enzyme from the flight muscle of bumble-bees and from other sources reinforces this conclusion (see below).

No detectable activity of fructose diphosphatase was found in *Bombus* haemolymph, in the tergocoxal muscle of the thorax, or in the gut. Thus it is likely that the fructose diphosphatase activities reported in this paper are present in the flight muscles rather than in some other contaminating tissue.

Properties of fructose diphosphatase of bumble-bee flight muscle

Effects of pH. The fructose diphosphatase from bumble-bee flight muscle was found to have a pH optimum of approx. 7.5, in either tris–HCl or tris–maleate buffer. This pH optimum is similar to that reported for the enzyme from cat leg muscle by Krebs & Woodford (1965) and for the enzyme from liver, which was assayed under similar conditions (Wallace & Newsholme, 1967).

Effect of fructose diphosphate concentration. The optimum concentration of fructose diphosphate was approx. 0.1 mM at three different concentrations of Mg^{2+} (Table 2). Concentrations of fructose diphosphate in excess of 0.25 mM caused inhibition of fructose diphosphatase (Table 2). This response to fructose diphosphate is similar to that of the enzymes from rat liver, cat leg muscle and the pectoral muscle of the domestic fowl (Underwood & Newsholme, 1965; Krebs & Woodford, 1965; Opie & Newsholme, 1967*b*). The K_m for fructose diphosphate was approx. 12 μM; this value is somewhat higher than that of fructose diphosphatase from some other muscles, but is similar to that of the enzyme from the pectoral muscle of the domestic fowl (Opie & Newsholme, 1967*b*).

Effect of metal ions. In common with fructose diphosphatase from other sources, the enzyme from the flight muscle of the bumble-bee possessed no detectable activity in the absence of Mg^{2+}, and the optimum concentration of Mg^{2+} was approx. 5 mM (Table 2). Also, it was inhibited by low concentrations of Zn^{2+} (15 μM-Zn^{2+} caused 95% inhibition in the absence of EDTA and mercaptoethanol). This effect of Zn^{2+} is similar to that observed with the enzyme from rat liver and cat leg muscle (Underwood & Newsholme, 1965: Krebs & Woodford, 1965). In an investigation of the effects of univalent cations on fructose diphosphatase (Table 3) it was found that the enzyme from the flight muscle of the bumble-bee was strongly inhibited by Li^+ (0.2 mM-Li^+ caused approx. 50% inhibition) and to a lesser extent by Na^+, but not by K^+ or tetracyclohexylammonium ions (up to 20 mM). The same enzymes from the flight muscles of the waterbug and locust and from the leg muscles of the cockroach and frog were also inhibited by Li^+ and Na^+, and the effect of K^+ was very small (Table 3). In the presence of Mg^{2+} it was found that Mn^{2+} caused inhibition of the fructose diphosphatases from these various muscles (Table 3).

Table 2. *Effect of fructose diphosphate concentration on the activity of fructose diphosphatase from the flight muscle of the bumble-bee*

The muscles were extracted and the enzyme was assayed as described in the Materials and Methods section. The tetracyclohexylammonium salt of fructose diphosphate was used in the experiment.

Concn. of fructose diphosphate (mM)	Concn. of Mg^{2+} (mM) ...	Fructose diphosphatase activity (nmol/min per ml of extract)		
		0.5	5.0	25.0
0.02		264	356	234
0.03		260	382	267
0.05		360	436	292
0.10		382	460	347
0.25		395	375	325
0.50		345	266	295
2.0		279	202	205
5.0		170	176	167
10.0		87	173	146

Table 3. *Percentage inhibition by various metal ions of fructose diphosphatase from flight muscles of the bumble-bee, waterbug and locust, and from leg muscles of the cockroach and frog*

The muscles were extracted and the enzyme was assayed as described in the Materials and Methods section. The species used in this investigation were: bumble-bee (*Bombus* spp.), waterbug (*Lethocerus cordofanus*), locust (*Schistocerca gregaria*), cockroach (*Periplaneta americana*) and frog (*Rana temporaria*).

Concn. of metal ion (mM)	Percentage inhibition of fructose diphosphatase																			
	Bumble-bee flight muscle				Waterbug flight muscle				Locust flight muscle				Cockroach coxal muscle				Frog sartorius muscle			
Metal ion ...	Mn^{2+}	Li^+	Na^+	K^+	Mn^{2+}	Li^+	Na^+	K^+	Mn^{2+}	Li^+	Na^+	K^+	Mn^{2+}	Li^+	Na^+	K^+	Mn^{2+}	Li^+	Na^+	K^+
0.1	50	24	—	—	54	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
0.2	70	44	—	—	66	—	—	—	—	—	—	—	15	26	—	—	10			
0.5	86	80	—	—	80	10	—	—	50	35	—	—	32	44	—	—	—	18	—	—
1.0	—	88	9	—	—	24	—	—	70	45	—	—	36	60	—	—	24	11	—	—
2.0	—	91	19	—	90	—	—	—	80	—	—	—	—	—	—	—	—	—	—	—
5.0	—	95	35	0	—	60	2	—	—	83	13	—	—	78	18	10	—	50	7	3
10.0	—	—	50	0	—	—	9	—	—	—	—	0	—	—	—	—	—	—	—	—
20.0	—	—	—	0	—	74	37	6	—	—	25	0	—	—	33	3	—	—	12	3

Effect of AMP and other nucleotides. The fructose diphosphatases from the flight muscles of the waterbug, fleshfly and locust and from the leg muscles of the cockroach were inhibited by AMP (Table 4), but the sensitivity of these enzymes to AMP inhibition is less than that of the enzymes from vertebrate muscles (see Opie & Newsholme, 1967*b*). However, the fructose diphosphatase from the flight muscle of the bumble-bee was not inhibited by AMP (up to 5mM); any inhibition observed upon the addition of AMP could be explained by the effect of the Na^+ that was added together with the AMP (see Table 4). Control experiments showed that the AMP added to the assay system was not destroyed by the extract of bumble-bee flight muscle.

There was no effect on fructose diphosphatase of bumble-bee flight muscle by the following nucleotides: ATP, ADP, GTP, GDP, GMP, CTP, CDP, CMP, UTP, UDP, UMP, ITP, IDP, IMP (0.1–2mM) or 3′:5′-cyclic AMP and 3′:5′-cyclic GMP (0.1mM). Similarly there was no effect of P_i (up to 20mM), glycerol 3-phosphate (up to 10mM) or fructose 6-phosphate (up to 5mM) on the activity of this enzyme.

Fractionation of enzymes from the bumble-bee flight muscle

The results in Table 5 show that nearly all the phosphofructokinase and fructose diphosphatase activity was found in the 100000*g* supernatant. Both these enzymes are therefore likely to be present in the cytoplasm.

Content of some glycolytic intermediates and ATP in the bumble-bee flight muscle

The contents of ATP, glucose 6-phosphate, fructose 6-phosphate, fructose diphosphate and triose phosphates were measured in the flight muscle of the bumble-bee (*B. hortorum*) during rest and flight and the results are given in Table 6. No statistically significant changes in the content of any of these metabolites were observed when rest is compared with flight. Furthermore, the contents of the glycolytic intermediates are similar to those reported for the blowfly, *Phormia regina*, by Sacktor & Wormser-Shavit (1966). The ATP content of the flight muscle of the bumble-bee is, however, much lower than that of the blowfly (Sacktor & Hurlbut, 1966).

Discussion

The results in this paper demonstrate that the flight muscles of bumble-bees contain a fructose diphosphatase of unusually high maximum catalytic activity. The highest activity reported in Table 1 is approximately 30-fold (on a fresh-weight basis) the

Table 4. *Percentage inhibition by AMP and Na^+ of fructose diphosphatase from the flight muscles of the bumble-bee, waterbug, fleshfly and locust, and from leg muscles of the cockroach and frog*

The muscles were extracted and the enzyme was assayed as described in the Materials and Methods section. For the enzyme from the muscles of the waterbug, locust, fleshfly, cockroach and frog, concentrations of Na^+ below 1 mM caused no inhibition of fructose diphosphatase. The species used in this investigation were the same as those reported in Table 3. The species of the fleshfly was *Sarcophaga barbata*.

Concn. of Na_2AMP (mM)	Concn. of Na^+ (mM)	Percentage inhibition of fructose diphosphatase: Bumble-bee flight muscle Na_2AMP	Bumble-bee flight muscle Na^+	Waterbug flight muscle Na_2AMP	Locust flight muscle Na_2AMP	Fleshfly flight muscle Na_2AMP	Cockroach coxal muscle Na_2AMP	Frog sartorius muscle Na_2AMP
0.01	0.02	—	—	15	9	25	—	71
0.02	0.04	—	—	19	61	47	38	74
0.04	0.08	—	—	27	91	64	80	81
0.1	0.2	3	1	74	—	82	88	80
0.2	0.4	—	—	75	—	—	—	—
0.5	1.0	20	9	—	—	—	—	—
2.0	4.0	31	26	—	—	—	—	—
5.0	10.0	44	50	—	—	—	—	—

Table 5. *Intracellular distribution of fructose diphosphatase and phosphofructokinase in the flight muscle of the bumble-bee (B. terrestris)*

The cell fractions were prepared as described in the Materials and Methods section. The breakage of mitochondria was less than 15% (measured as described in the Materials and Methods section). The activity of each fraction has been expressed as a percentage of the activity in the crude homogenate. The activity of fructose diphosphatase in the crude homogenate was 56 and that of phosphofructokinase was 31 μmol/min per g of fresh muscle.

Cell fraction	Fructose diphosphatase	Phospho-fructokinase
Cell debris	7	4
Mitochondria	2	1
Sarcoplasmic reticulum	2	2
Soluble supernatant	96	80

activity in any other muscle or in rat liver. Furthermore, the activity of fructose diphosphatase in bumble-bee flight muscle is comparable with the activity of phosphofructokinase in the same muscle and is unaffected by AMP. Therefore in this muscle it is unlikely that the role of fructose diphosphatase is in the regulation of glycolysis at the level of fructose 6-phosphate phosphorylation, as described for this enzyme in other muscles (Newsholme & Crabtree, 1970).

It is also unlikely that this fructose diphosphatase is involved in gluconeogenesis since bumble-bee flight muscle possesses no detectable activity of phosphoenolpyruvate carboxykinase (B. Crabtree, S. J. Higgins & E. A. Newsholme, unpublished work) or glucose 6-phosphatase. The low activity of glucose 6-phosphate dehydrogenase found in this muscle makes it unlikely that fructose diphosphatase is involved in the operation of a pentose phosphate pathway. There was no significant difference in the muscle content of either fructose diphosphate or triose phosphates between rest and flight (Table 6) so that it is also unlikely that fructose diphosphatase is present to lower the content of glycolytic intermediates after a period of flight. Furthermore, assuming that the content of fructose diphosphate measured in the muscle is evenly distributed within the intracellular water, the concentration of the metabolite would be approx. 0.2 mM. Since the K_m of fructose diphosphatase for fructose diphosphate is 12 μM this suggests that the enzyme is usually saturated with substrate. If phosphofructokinase and fructose diphosphatase were simultaneously maximally active in this muscle, the resulting substrate-cycle would hydrolyse ATP at a rate of 16–70 μmol/min per g of muscle at 25°C.

Table 6. *Contents of ATP, glucose 6-phosphate, fructose 6-phosphate, fructose diphosphate and triose phosphates in the flight muscle of the bumble-bee during rest and flight*

The preparation of the tissue and assay methods are described in the Materials and Methods section. The measurements were performed on extracts obtained from the flight muscle of either one or two bees. The species used was *B. hortorum* (workers). Results are expressed as means ±S.E.M. for a minimum of four determinations on separate extracts.

Condition of bee	Content of metabolite (μmol/g fresh wt. of flight muscle)				
	ATP	Glucose 6-phosphate	Fructose 6-phosphate	Fructose diphosphate	Triose phosphates
Rest	2.69±0.18	0.11±0.03	0.06±0.01	0.16±0.02	0.26±0.06
Flight	2.10±0.28	0.22±0.09	0.03±0.01	0.16±0.01	0.28±0.08

The bumble-bee flight muscle appears to depend upon glycolysis for the provision of pyruvate for energy production during flight (see Crabtree & Newsholme, 1972), so that such a cycle could not occur during flight as it would seriously inhibit glycolysis. Therefore fructose diphosphatase must be inhibited under these conditions, but the mechanism for the regulation of its activity in bumble-bee flight muscle is unknown at the present time.

If a substrate-cycle catalysed by phosphofructokinase and fructose diphosphatase occurs at all in this muscle, it must occur when the muscle is at rest. One result of such a substrate-cycle and the consequent high rate of ATP hydrolysis would be the production of heat in the flight muscles. With maximum activities of both the enzymes the heat production could be as much as 4.2 J (1 cal)/min per g of muscle at 35°C (the approximate temperature of the thorax during flight; Sotavalta, 1954); it is assumed that the rate of ATP hydrolysis is 100 μmol/min per g of muscle at 35°C and that 1 mol of ATP hydrolysed to ADP and P_i releases 42 kJ (10 kcal) under physiological conditions. Since carbohydrate is oxidized completely by bumble-bee flight muscles, a rate of ATP hydrolysis of 100 μmol/min per g of muscle could be supported by a net glycolytic rate of 2.7 μmol of glucose/min per g of muscle, a value approx. 5% of that likely to occur during flight (see Crabtree & Newsholme, 1972).

Bees are known to be unable to fly when their thoracic temperature is less than 30°C (Krogh & Zeuthen, 1941). Since the thoracic temperature during flight is the result of a balance between the heat produced by the flight muscles and the heat lost from the thorax, it follows that as soon as the insect stops flying and settles upon a flower the thoracic temperature will fall unless there is some compensatory mechanism for the production of heat. Furthermore, this rate of temperature fall will be increased by cold, wet or windy weather. Thus any mechanism for maintaining the thoracic temperature during the collection of food will increase the efficiency of this process, since the insect will not have to spend time after food collection in restoring the thoracic temperature by processes such as abdominal pumping or wing whirring (see Krogh & Zeuthen, 1941; Sotavalta, 1954).

It is therefore proposed that the role of fructose diphosphatase in the flight muscle of bumble-bees (*Bombus* spp.) is to provide a substrate-cycle between fructose 6-phosphate and fructose diphosphate for the generation of heat during short periods of rest when food is being collected; the production of heat helps to maintain the temperature of the thorax at a level suitable for flight. This hypothesis is consistent with the fact that bumble-bees are able to collect food under inclement weather conditions, such as strong cold winds, and it is also known that the honey bee (*Apis mellifera*) does not collect food when the weather is bad (see Sladen, 1912; Free & Butler, 1959). In this connexion it is perhaps significant that the flight muscles of the honey-bee contain no detectable fructose diphosphatase activity (Table 1). Furthermore, the species of bee (i.e. *Psithyrus* sp.) that parasitizes nests of bumble-bees is known to do very little collecting of food, being very inefficient at this process (Sladen, 1912; Free & Butler, 1959). It might thus be expected that *Psithyrus* would lack this proposed mechanism for heat generation and, in support of this, it has been found that the flight muscles of the species of *Psithyrus* that have been examined contain only low activities of fructose diphosphatase (Table 1).

From the information presented in Fig. 1 it can be seen that there is an inverse relationship between the activity of fructose diphosphatase or phosphofructokinase and the body weight of bumble-bees. This relationship appears to explain the variation in the activity of these enzymes not only between different castes of any species but also between the different species themselves. Such an inverse relationship of the two enzymes with body weight is consistent with the hypothesis that they participate in the production of heat (Davson, 1959; Bernd & Bartholomew, 1971).

We thank Professor J. W. S. Pringle, F.R.S., for his interest and encouragement. We also thank Mr. C. A. O'Toole of the Hope Department of Entomology, Oxford, for assistance with the identification of species and castes, the provision of specimens and for many helpful discussions. B. C. was the recipient of a Medical Research Council Junior Research Fellowship. S. D. T. was the recipient of a Science Research Council Training Scholarship.

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