Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 12616–12620, December 1996
Physiology

Energy metabolism, enzymatic flux capacities, and metabolic flux rates in flying honeybees

R. K. SUAREZ*†, J. R. B. LIGHTON‡, B. JOOS‡, S. P. ROBERTS§, AND J. F. HARRISON§

*Department of Ecology, Evolution and Marine Biology, University of California, Santa Barbara, CA 93106-9610; ‡Department of Biological Sciences, University of Nevada, Las Vegas, NV 89154-4004; and §Department of Zoology, Arizona State University, Tempe, AZ 85287-1501

Communicated by George N. Somero, Hopkins Marine Station, Pacific Grove, CA, August 14, 1996 (received for review April 22, 1996)

ABSTRACT **Honeybees rely primarily on the oxidation of hexose sugars to provide the energy required for flight. Measurement of VCO_2 (equal to VO_2, because VCO_2/VO_2 = 1.0 during carbohydrate oxidation) during flight allowed estimation of steady-state flux rates through pathways of flight muscle energy metabolism. Comparison of V_{max} values for flight muscle hexokinase, phosphofructokinase, citrate synthase, and cytochrome *c* oxidase with rates of carbon and O_2 flux during flight reveal that these enzymes operate closer to V_{max} in the flight muscles of flying honeybees than in other muscles previously studied. Possible mechanistic and evolutionary implications of these findings are discussed.**

In recent years, there has been considerable discussion and debate concerning the rules that govern the evolutionary design of functional capacities (1–7). A question seldom asked is how closely biochemical pathways are matched in their capacities for metabolic flux to the maximum physiological flux rates achieved *in vivo*. Muscles, biological machines designed for converting chemical energy into mechanical work (8), are ideal models for the examination of such relationships. The maximum rates at which muscles perform mechanical work determine the maximum rates at which ATP is hydrolyzed and, therefore, the maximum steady-state rates at which ATP synthesizing pathways must operate. Insects in flight achieve some of the highest known mass-specific metabolic rates among animals (9). Insect flight muscles generally consist of a single fiber type and account for >90% of organismal O_2 consumption under these circumstances (9–11). Hymenoptera, in particular, rely mainly on carbohydrate as their oxidative fuel (10, 11). Thus, organismal O_2 consumption rates can be used to estimate rates of flux through the relevant enzyme-catalyzed steps in carbohydrate catabolism in the flight muscles (11). In the present study, we have combined the use of flow-through respirometry with biochemical techniques to examine the relationships between enzymatic flux capacities and flux rates during flight in honeybees (*Apis mellifera*).

MATERIALS AND METHODS

Animals and Chemicals. Unladen honeybee (*A. mellifera*) workers were caught with perforated plastic bags immediately as they flew from their hives (Arizona State University) to forage. Biochemical reagents and enzymes were purchased from Sigma. Other chemicals were from various commercial sources and were of reagent grade.

Wingbeat Frequencies and Respirometry. Wingbeat frequencies were measured using an optical tachometer (12). Rates of CO_2 production (VCO_2) were determined as described (13) using a Sable Systems (Las Vegas) flow-through respirometry system. Briefly, honeybees were induced to fly in a 500 ml Plexiglas chamber through which dry, CO_2-free air was drawn at a rate of 2–4 liters/min. Ambient temperature was maintained at 22 ± 2°C by housing the chamber in a large temperature cabinet. Honeybees were flown in the chamber for about 5 min while expired CO_2 and H_2O were monitored downstream using a Li-Cor (Lincoln, NB) model 6262 CO_2/H_2O analyzer. Data acquisition and subsequent analyses were accomplished using an IBM-compatible computer and Datacan V software (Sable Systems). Some bees displayed an initial period of agitation, generally lasting less than 1 min. After this, wingbeat frequencies and rates of H_2O and CO_2 production remained constant during flight. Because it has been shown (10) that the respiratory exchange ratio (assumed to be equal to the respiratory quotient, $RQ = VCO_2/VO_2$) of honeybees in steady-state flight is 1.0, VCO_2 data will be referred to as VO_2 throughout this paper.

Tissue Homogenization. Except for cytochrome *c* oxidase, all enzyme activities were measured using same bees used for respirometry. After respirometry, the animals were anesthetized by cooling in a refrigerator. Heads, abdomen, wings, and legs were removed with scissors, and thoraxes were weighed to the nearest 0.1 mg. For assays of hexokinase, phosphoglucoisomerase, and citrate synthase, thoraxes were homogenized individually three times for 10 sec (with 1-min cooling intervals) at low speed using a Tissue Tearor homogenizer in 19 volumes of ice-cold buffer. The homogenization buffer consisted of 25 mM Hepes (pH 7.3 at 4°C), 2 mM EDTA, 5 mM DTT, and 0.5% (vol/vol) Triton X-100. Homogenates were then sonicated three times for 10 sec (with 1-min cooling intervals) using a Microson sonicator equipped with a small probe and finally centrifuged for 1 min in a Brinkmann microcentrifuge kept cold in a refrigerator. Procedures employed Triton X-100 and sonication to preclude loss of enzyme activity as a result of binding to particulate material (14). Supernatant fractions were kept in ice until assay.

Phosphofructokinase activity was found to be highly unstable in homogenization buffers of various compositions [this probably explains the low values reported previously (15)] but was stabilized by homogenization in a solution consisting of 25 mM Tris-potassium phosphate (pH 7.8 at 4°C), 2 mM EDTA, 5 mM DTT, 1 mM fructose 6-phosphate (F6P), 3.5 mM glucose 6-phosphate (G6P), and 0.5% (vol/vol) Triton X-100 (16). Extraction procedures for this enzyme were otherwise identical to those described above.

For assay of cytochrome oxidase, thoraxes were individually homogenized with ground glass homogenizers in 19 vol of cold 75 mM potassium phosphate (pH 7.3 at 4°C) containing 10 mg of Lubrol per ml (activating buffer described in ref. 17). Homogenates were used for measurements of enzyme activity without further treatment.

Enzyme Assays. Hexokinase, phosphoglucoisomerase, phosphofructokinase, and citrate synthase activities were measured in 1-ml glass cuvettes at 37°C using an LKB Novaspec II spectrophotometer equipped with a water-jacketed cuvette

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Abbreviations: F6P, fructose 6-phosphate; G6P, glucose 6-phosphate.
†e-mail: suarez@lifesci.lscf.ucsb.edu.

holder and interfaced to an IBM-compatible computer. Cytochrome oxidase activities were measured by monitoring O_2 consumption rates in a magnetically stirred 1 ml water-jacketed glass chamber using a Clark-type electrode (18). Data acquisition and analysis were conducted using DATACAN V software.

Enzymes were measured under conditions that yielded the highest V_{max} values obtainable, and not with the intention of simulating pseudo-intracellular conditions *in vitro*. This is consistent with the objective of determining V_{max} values *in vitro* under optimal (as opposed to physiological) conditions and then asking how flux capacities determined in this way compare with physiological flux rates *in vivo* at these steps in metabolism. The rationale for such an approach is well-established (19, 20). It is important to note that mimicking *in vivo* conditions (e.g., substrate, allosteric modifier, H^+ concentrations) generally results in rates representing various fractions of the true V_{max} values for the enzymes of interest; this would not be a useful way to address the questions asked in this study. (Such an approach would, however, be useful in subsequent kinetic studies addressing the question of how these enzymes might behave under simulated intracellular conditions.) In preliminary experiments, substrate and activator concentrations were varied several-fold to ensure that the rates reported here were obtained under saturating (and noninhibitory) conditions. Also consistent with the objectives outlined, enzyme assays were conducted at optimal pH values. Honeybee flight muscle phosphoglucoisomerase shows a broad pH profile with an optimum value of about 8.0 (J. F. Staples and R.K.S., unpublished results). Phosphofructokinases from hymenopteran flight muscles (16, 21) display the inhibition by high $[H^+]$ characteristic of the homologous enzymes from vertebrates as well as sensitivity to inhibition by physiological concentrations of ATP under these circumstances (e.g., ref. 22). Such considerations prompted the use of an assay pH value of 8.0 for this enzyme as well. The following assay conditions were found to be optimal.

Hexokinase was assayed with 5 mM D-glucose (omitted from control), 1 mM ATP, 10 mM $MgCl_2$, 100 mM KCl, 0.5 mM $NADP^+$, 5 mM DTT, and excess G6P dehydrogenase in 50 mM Hepes (pH 7.0 at 37°C). Addition of an ATP-regenerating system (creatine phosphate plus creatine kinase) had no effect on maximal rates. Phosphoglucoisomerase was assayed in the F6P to G6P direction. V_{max} in the glycolytic direction, G6P to F6P, is about the same as that obtained in this assay (ref. 23 and J. F. Staples and R.K.S., unpublished results). Conditions were 0.5 mM F6P (omitted from control), 0.5 mM $NADP^+$, 5 mM DTT, excess G6P dehydrogenase in 50 mM Tris·HCl (pH 8.0 at 37°C). Phosphofructokinase was assayed with 5 mM F6P (omitted from control), 2 mM ATP, 0.15 mM NADH, 0.01 mM fructose 2,6-bisphosphate, 10 mM $MgCl_2$, 100 mM KCl, 5 mM DTT, excess aldolase, triosephosphate isomerase, and α-glycerophosphate dehydrogenase in 50 mM Tris·HCl (pH 8.0 at 37°C). Addition of 2 mM AMP did not result in further increases in measured rates. Citrate synthase was assayed with 0.5 mM oxaloacetate (omitted from control), 0.3 mM acetyl coenzyme A, and 0.1 mM dithiobisnitrobenzoic acid in 50 mM Tris·HCl (pH 8.0 at 37°C). The V_{max} value for O_2 consumption by the cytochrome oxidase reaction was obtained by extrapolation from an Eadie–Hofstee plot of $v/[s]$ against v (24, 25). Assays contained 2.5 to 50 μM horse heart cytochrome *c*, 0.1 mM tetramethyl-*p*-phenylenediamine dihydrochloride, and 2.5 mM ascorbate in 10 mM potassium phosphate (pH 7.4 at 37°C). The O_2 content of air-saturated assay buffer at 37°C was assumed to be 406 nmol of O per ml (26).

RESULTS AND DISCUSSION

Bees, Wingbeat Frequencies, and Metabolic Rates. Worker honeybees used in the study (n = 19) weighed 78.25 ± 2.2 mg and possessed thoraxes weighing 28.58 ± 0.38 mg. Bees flew at wingbeat frequencies averaging 223 ± 4 (n = 13) Hz, respiring at mass-specific rates of 101.04 ± 4.1 ml of O_2/g of body mass per hr. Individual bees in flight respired at 7.88 ± 0.34 ml O_2/hr. Because most of the O_2 consumed by flying bees is used by the thorax, it can be calculated that thoracic VO_2 values in flight are 4.58 ± 0.18 ml/(g × min) or 180 ± 7 μmol O_2/(g × min). Because flight muscle mass accounts for 75% of thorax mass in honeybee workers (27), it can also be calculated that flight muscle VO_2 is 240 ± 41 μmol O_2/(g × min). Assuming a P/O ratio (ADP molecules phosphorylated per oxygen atom consumed) of 3 (28), the rate of muscle ATP turnover during flight is about 1440 μmol/(g × min). This rate of aerobic ATP turnover is about 3-fold greater than the rates estimated in hummingbird flight muscles during hovering flight (29, 30) and 30-fold greater than those in human athletes exercising at VO_2max (31). At this metabolic rate, the thoracic muscles of a single flying honeybee use about 1.39 × 10^{15} molecules of ATP per wingbeat cycle.

Hexose Sugars as a Metabolic Fuels for Flight. *RQ* values of 1.0 (10) as well as low activities of enzymes involved in fatty acid oxidation (32) indicate that carbohydrate is the main oxidative fuel used by flying honeybees. Low glycogen phosphorylase activities in the flight muscles (15) rule out the use of glycogen as a significant oxidative fuel. Honeybee hemolymph contains trehalose, glucose, and fructose (33), and there is convincing evidence of direct utilization of dietary sugars for flight (34). High activities of trehalase (35), hexokinase (Table 1; ref. 15), and α-glycerophosphate dehydrogenase (15), enzymes involved in trehalose hydrolysis, hexose sugar phosphorylation, and the maintenance of cytoplasmic redox balance (NAD^+/NADH ratio) during aerobic glycolysis, respectively, support the idea that hexose sugar oxidation provides the energy for flight.

Capacity/Load Matching at the Biochemical Level. Studies specifically aimed at determining the relationships between enzymatic flux capacities in pathways and physiological flux rates have been remarkably few in recent years despite great interest in the evolutionary design of functional capacities (e.g., refs. 1–4). Such comparisons between capacities and loads at the biochemical level can be undertaken on the basis of a sound theoretical framework (19, 20). V_{max} values (equal to $[E] \times k_{cat}$, where $[E]$ and k_{cat} are enzyme concentration and turnover number, respectively) are of mechanistic significance because they can be used, in combination with other data, to provide insights into enzyme function (e.g., ref. 36) and

Table 1. Enzyme V_{max} values (expressed in μmol substrate converted to product per g of thorax per min at 37°C) *in vitro* and fractional velocities, v/V_{max}, *in vivo* in flying honeybee workers

Enzyme	V_{max}	v/V_{max} Unladen*	v/V_{max} At VO_2max†
Hexokinase	39.6 ± 1.4	0.76	0.98
Phosphoglucoisomerase	702.4 ± 25.7	0.04	0.05
Phosphofructokinase	68.4 ± 2.2	0.44	0.57
Citrate synthase	219.5 ± 12.6	0.27	0.36
Cytochrome *c* oxidase	307.7 ± 7.7	0.58	0.76

In the case of cytochrome *c* oxidase, v represents the VO_2/g of thorax of 180 μmoles O_2/(g × min) in unladen bees, which may increase to 234 μmoles O_2/(g × min), assuming VO_2max is 30% higher than the unladen VO_2 (see text). Under steady-state conditions, v at each of the steps involving carbon flux equals the rate of net flux through glycolysis [30 μmol hexose phosphate/(g × min) in unladen bees and 39 μmol hexose phosphate/(g × min) at VO_2max] or the Krebs cycle [60 μmol acetylcoenzyme A/(g × min) in unladen bees and 78 μmol acetyl coenzyme A/(g × min) at VO_2max]. Values are means ± SEM, n = 6 individuals, except for cytochrome *c* oxidase where n = 4.

*At VO_2 values measured in the present study.

†Assumed to be 30% higher than unladen VO_2 values.

regulation (e.g., ref. 37) *in vivo*. V_{max} values are also of great physiological significance because they set upper limits to flux at specific steps in biochemical pathways and, thus, serve as measures of maximum capacities for flux at these steps (19, 20).

Enzymatic Flux Capacities and Carbon Flux Rates. Because 6 moles of O_2 are consumed per mole of hexose sugar oxidized to CO_2 and H_2O, the VO_2 values yield an estimated glycolytic flux rate of 30 ± 1.2 μmol/g of thorax/min during flight. Table 1 provides the V_{max} values for a number of the enzymes measured *in vitro* as well as the fractional velocities (v/V_{max}, where v is the rate of net forward flux, equal, under these circumstances, to the steady-state rate of pathway flux) at which they function *in vivo* during flight. Hexokinase and phosphofructokinase, enzymes that catalyze reactions thought to be far from equilibrium *in vivo* (e.g., ref. 37), function at about 76 and 44% of V_{max}, respectively.

Because two acetylcoenzyme A molecules are produced from each hexose sugar molecule, flux through the Krebs cycle occurs at about 60 μmol/(g thorax × min). Thus, citrate synthase, which occurs at V_{max} values of 219.5 ± 12.6 μmol/(g thorax × min) (n = 7), operates at 27% of V_{max} during flight. This fractional velocity is closer to V_{max} than in other muscles previously studied. In hummingbird flight muscles, which are capable of the highest rates of aerobic energy metabolism known among vertebrates (29, 30), citrate synthase functions at only 6% of V_{max} during hovering flight (29). The homologous enzyme in isolated, perfused rat hearts operates at between 2 to 5% of V_{max} (38).

Phosphoglucoisomerase in honeybee flight muscles occurs at maximal activities 23-fold in excess of glycolytic flux rates during flight. It has been known for decades that such reactions, which are maintained close to equilibrium, are catalyzed by enzymes present at V_{max} values in large excess over pathway flux rates. The reasons are well understood. Veech *et al.* (36) showed, using the Haldane equation, that the net forward flux (v) through triosephosphate isomerase can be predicted, given the V_{max} for the enzyme, its kinetic properties *in vitro*, and the near-equilibrium intracellular concentrations of substrate and product. It follows from this, given the kinetic properties of the enzyme, that V_{max} values in large excess of v are in fact required to maintain near-equilibrium [product]/[substrate] ratios at physiological rates of net forward flux. In the flowering plant *Clarkia xantiana* (Onagraceae), it has been shown that V_{max} values for phosphoglucoisomerase are not greatly in excess over those required to maintain the reaction (F6P ↔ G6P) close to equilibrium at high rates of sucrose synthesis (39). In yeast cells, the V_{max} value for this enzyme is 20-fold in excess of the rate of glycolytic flux, and the reaction is held near equilibrium (40). A yeast strain engineered to produce 11 times the normal amount of phosphoglucoisomerase activity showed glycolytic rates, as well as concentrations of G6P and F6P that were not significantly different from controls (40). It is therefore reasonable to expect that V_{max} values for phosphoglucoisomerase are actually not in excess of those required to sustain the glycolytic flux rates that occur during honeybee flight. Experiments to test this hypothesis are currently in progress.

Enzyme Fractional Velocities at VO_2max. Our estimates of organismal mass-specific VO_2 values in honeybees agree well with most data reported previously (e.g., refs. 41–43), but probably do not represent maximal metabolic rates. Wolf *et al.* (44) report mass-specific VO_2 values similar to ours using unladen honeybees flown in a wind tunnel at 0.5 m/sec. In their study, loaded bees yielded 42% higher mass specific VO_2 values than "empty" bees. However, various studies (e.g., refs. 41–43) suggest that honeybees can increase metabolic rates beyond those reported here by about 30%. This ability to further increase VO_2 during flight suggests that even better matches exist between enzymatic flux capacities and maximum physiological rates of carbon and O_2 flux (Table 1).

From a mechanistic perspective, it is noteworthy that under such circumstances, hexokinase would have to operate extremely close to, or at, V_{max}. G6P is a feedback inhibitor of hexokinase (45), and it has been shown in isolated, perfused rat hearts that rates of glucose utilization are inversely related to G6P concentrations (46). This is despite intracellular glucose and MgATP concentrations in the millimolar range (37, 46), well above the K_m of the enzyme *in vitro* for both substrates (37, 45, 46). In locust flight muscles, it has been suggested (47) that the inhibitory effect of G6P might be counteracted by increases in the concentrations of alanine and inorganic phosphate, which occur during the transition from rest to flight (48). In contrast with hexokinase, phosphofructokinase operates at close to half-maximal velocity as bees fly in either loaded or unloaded states. It would appear that this enzyme is kept optimally poised *in vivo* for regulation by allosteric modulators (16).

Fig. 1 shows an Eadie–Hofstee plot of $v/[S]$ versus v for cytochrome oxidase. The V_{max} value is estimated at 307.69 ± 7.7 μmol of O_2/g of thorax/min (mean ± SEM, n = 4). This value is 1.7-fold greater than the VO_2/g of thorax estimated *in vivo* by respirometry and reveals that the enzyme operates at about 59% of V_{max} during flight (Table 1). A further increase in VO_2 in loaded bees implies that the enzyme is capable of functioning *in vivo* at about 76% of V_{max} (Table 1). This significantly exceeds the fractional velocity at which this enzyme functions in mammalian muscles working at VO_2max (49, 50), and is consistent with the idea that insects possess higher capacities for O_2 delivery to muscle mitochondria than mammals (51–53).

Do Honeybees Have "Enough But Not Too Much" Enzyme? It has been proposed that optimal design in animals should involve a 1:1 match between functional capacities and maximum physiological requirements or loads (1). Protein synthesis costs an estimated five ATP equivalents per peptide bond (counting the cost of amino acid transport) (54). The various compartments in the cell appear to be highly crowded (55). Because biological structures take time, cost energy and materials to build, and occupy space, natural selection might be expected to eliminate excess (i.e., superfluous) capacities.

How much, in Diamond's words (56), is enough but not too much enzyme? A model of "economic design" might suggest that energetic costs and space limitations would result in the evolution of metabolic pathways consisting of reactions cata-

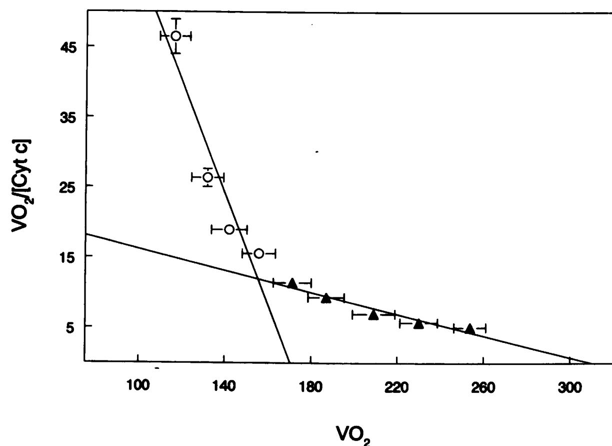

FIG. 1. Eadie–Hofstee plot of $v[S]$ against v for cytochrome c oxidase activity. Points represent mean ± SEM (n = 4 determinations). Vertical error bars, where absent, were smaller than the symbols used. Horse heart cytochrome c (M_r 12,384) concentrations ranged from 2.5 to 50 μM. Biphasic plot results from two binding sites on cytochrome c oxidase with different affinities for cytochrome c (25). VO_2 values are expressed in μmol of O_2/(g of thorax × min) and extrapolate to V_{max} = 307.7 ± 7.7 μmol of O_2/(g × min).

lyzed by enzymes whose V_{max} values simply match, but do not exceed, pathway flux rates (i.e., $v = V_{max}$). This would require having just enough $[E]$ to catalyze reactions, such that $v = V_{max} = [E] \times k_{cat}$ at each step. However, it is thought that in the evolution of enzymes, k_{cat} values have increased through decreased affinities for substrates (increased K_m values), such that $K_m >$ [substrate] (57). In addition, metabolic biochemists have known for decades that enzymes must generally operate well below their half-maximal velocities to serve their regulatory roles in pathways (58). Thus, for mechanistic reasons, V_{max} values in excess over maximum pathway flux rates are not unexpected.

The degree to which the V_{max} values of homologous enzymes exceed *in vivo* flux rates varies over a wide range in the muscles of various species. The close matches between enzymatic flux capacities and maximum physiological flux rates seen in honeybees are especially remarkable when considered in relation to data available from other animals. For example, during high-intensity aerobic exercise in rainbow trout (*Oncorhynchus mykiss*), hexokinase operates at only 0.07, 0.13, and 2.5% of V_{max} in cardiac, white, and red muscles, respectively (59, 60). In running rats, glucose phosphorylation rates (61) account for between 6 and 28% of hexokinase V_{max} values in skeletal muscles (61–63) and 6% in the heart (61, 64). Phosphofructokinase fractional velocities in running rats can be estimated to be about 1.5% in the heart (61, 64) and between 0.6 and 28% in skeletal muscles (62, 63, 65, 66).

Hymenoptera display some of the highest known mass-specific aerobic metabolic rates among animals (11, 41–43, 67). This requires mitochondrial volumes that account for up to 43% of muscle fiber volume (67) and high enzymatic capacities for flux through glycolysis, the Krebs cycle, and the electron transport chain. It is possible that the evolutionary upregulation of biochemical capacities may have reached an upper limit in hymenopteran flight muscles (52, 53, 67). Thus, their extraordinarily high metabolic flux rates may be achieved by having high (but just enough) $[E]$ in pathways of energy metabolism and by having these enzymes operate at higher v/V_{max} than the homologous enzymes in lower metabolic rate species.

R.K.S. gratefully acknowledges Prof. A. Wenner for valuable information concerning bee biology as well as assistance in bee care and beehive maintenance. Thanks also go to Drs. T. Garland and W. Watt for useful comments, particularly concerning the issue of symmorphosis. The work described here was initially supported by a Natural Sciences and Engineering Research Council of Canada operating grant, and subsequently by a U.S. National Science Foundation (NSF) Grant IBN 9507407 to R.K.S., a Packard Fellowship and NSF Grant BSR 9306537 to J.R.B.L., and NSF Grant IBN 9317784 to J.F.H.

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