Biochem. J. (1972) **126**, 49–58
Printed in Great Britain

The Activities of Phosphorylase, Hexokinase, Phosphofructokinase, Lactate Dehydrogenase and the Glycerol 3-Phosphate Dehydrogenases in Muscles from Vertebrates and Invertebrates

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(*Received* 20 *July* 1971)

1. The maximum activities of hexokinase, phosphorylase and phosphofructokinase have been measured in extracts from a variety of muscles and they have been used to estimate the maximum rates of operation of glycolysis in muscle. These estimated rates of glycolysis are compared with those calculated for the intact muscle from such information as oxygen uptake, glycogen degradation and lactate formation. Reasonable agreement between these determinations is observed, and this suggests that such enzyme activity measurements may provide a useful method for comparative investigations into quantitative aspects of maximum glycolytic flux in muscle. 2. The enzyme activities from insect flight muscle confirm and extend much of the earlier work and indicate the type of fuel that can support insect flight. The maximum activity of hexokinase in some insect flight muscles is about tenfold higher than that in vertebrate muscles. The activity of phosphorylase is greater, in general, in vertebrate muscle (particularly white muscle) than in insect flight muscle. This is probably related to the role of glycogen breakdown in vertebrate muscle (particularly white muscle) for the provision of ATP from anaerobic glycolysis and not from complete oxidation of the glucose residues. The activity of hexokinase was found to be higher in red than in white vertebrate muscle, thus confirming and extending earlier reports. 3. The maximum activity of the mitochondrial glycerophosphate dehydrogenase was always much lower than that of the cytoplasmic enzyme, indicating that the former enzyme is rate-limiting for the glycerol 3-phosphate cycle. From the maximum activity of the mitochondrial enzyme it can be calculated that the operation of this cycle would account for the reoxidation of all the glycolytically produced NADH in insect flight muscle but it could account for only a small amount in vertebrate muscle. Other mechanisms for this NADH reoxidation in vertebrate muscle are discussed briefly.

Muscle tissue is characterized by its ability to perform mechanical work and the rate at which this work is done varies considerably according to the type of muscle and the animal from which it is derived.

To examine possible relationships between the type of fuel utilized and the physiological function of the muscle it is necessary to determine the rate of fuel utilization by the muscle during mechanical activity. For a comparative investigation the direct approach of investigating metabolism in intact muscle preparations is not feasible as there are only a small number of such preparations available for precise experimental analysis. Nonetheless, this problem can be tackled indirectly by measuring the catalytic activities *in vitro* of enzymes that are involved in the metabolism of fuels. These measurements can be performed relatively easily and quickly and therefore provide suitable results for comparative investigations. Such an approach has been used to provide information about the differences in rates of fuel utilization by various muscles (e.g., Beenakkers, 1963, 1969; Pette, 1966; Bass *et al.*, 1969). However, most of the enzymes that were selected for assay in these investigations (e.g. glyceraldehyde 3-phosphate dehydrogenase) are generally considered to catalyse near-equilibrium reactions in the cell, and consequently they possess maximum catalytic activities that are much greater than the maximum rate of operation of the metabolic pathway in the intact muscle (Krebs, 1963; Newsholme & Gevers, 1967; Scrutton & Utter, 1968). It was decided to perform a comparative investigation of the maximum catalytic activities *in vitro* of those enzymes involved in carbohydrate metabolism that catalyse reactions far-displaced from equilibrium in the cell, the activities of which are thought to limit the rate of the metabolic pathways. The assumption that the maximum catalytic activities of these enzymes might provide a reasonably accurate assessment of the maximum rate of these pathways *in vivo* has been examined and the results are presented in this paper.

The enzymes chosen to provide information about

the rate of utilization of carbohydrate in muscle were phosphorylase (EC 2.4.1.1), hexokinase (EC 2.7.1.1) and phosphofructokinase (EC 2.7.1.11). Also, the activities of lactate dehydrogenase (EC 1.1.1.27) and both mitochondrial and cytoplasmic glycerol 3-phosphate dehydrogenase (EC 1.1.1.8 and EC 1.1.99.1 respectively) have been measured in an attempt to provide some quantitative information about the role of the glycerol 3-phosphate cycle in the oxidation of cytoplasmic NADH in vertebrate and invertebrate muscles.

Materials and Methods

Chemicals and enzymes

All chemicals and enzymes were obtained from the Boehringer Corp. (London) Ltd., London W.5, U.K., except for the following: 2-mercaptoethanol was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.; bovine serum albumin (fraction V) was obtained from the Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.; antimycin A (type III), glycerol 3-phosphate (grade X) and glycerol 2-phosphate (grade I) were obtained from Sigma (London) Chemical Co., London S.W.6; glycogen (oyster), 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride, phenazine methosulphate, EDTA and all inorganic reagents were obtained from BDH Chemicals, Poole, Dorset, U.K., and were of the highest purity available. The dimethylketal derivative of dihydroxyacetone phosphate was obtained from the Boehringer Corporation and was converted into the sodium salt of dihydroxyacetone phosphate by the method of Ballou (1960).

Sources of animals

Animals were obtained from the sources given by Newsholme & Taylor (1969) and Crabtree & Newsholme (1970). Locusts were used between 7–14 days after the final moult. Flies were used between 7–14 days after emerging from pupae. All other insects were of undetermined age but, apart from cockroaches, were known to be capable of flight. Male cockroaches, locusts, cockchafers, waterbugs, ducks, fowls, pheasants, rats and mice were used. Female wasps, bees, tsetse flies and rabbits were used. All other animals were of undetermined sex.

Preparation of homogenates

Freshly dissected muscles were homogenized manually in ground-glass homogenizers with 10–50 vol. of extraction medium. For the assay of hexokinase, lactate dehydrogenase and both types of glycerol 3-phosphate dehydrogenase the extraction medium consisted of 50mM-triethanolamine, 1mM-EDTA, 2mM-$MgCl_2$ and 30mM-mercaptoethanol at pH7.5. For the assay of phosphofructokinase the extraction medium was as described by Opie & Newsholme (1967). For the assay of phosphorylase the extraction medium consisted of 35mM-glycerol 2-phosphate, 20mM-NaF, 1mM-EDTA and 30mM-mercaptoethanol at pH6.2 (based on that described by Cornblath *et al.*, 1963).

All homogenates were prepared and kept at 0°C until assayed for enzyme activity. Preliminary experiments with homogenates from rat heart muscle, rabbit leg muscle and locust flight muscle established that treatment of the homogenates before assay (e.g. centrifugation and sonication) did not change the maximum activities of glycerol 3-phosphate dehydrogenase, hexokinase and phosphofructokinase, compared with the activities obtained with crude extracts. Thus enzyme assays could be performed almost immediately after the preparation of the crude homogenate, and this was considered to be important in order to minimize loss of activity by inactivation after homogenization.

Enzyme assays

Hexokinase was assayed by measuring the rate of reduction of $NADP^+$ with glucose 6-phosphate dehydrogenase in a Gilford recording spectrophotometer (model 240) at 340nm and 25°C. The assay medium contained 75mM-tris, 7.5mM-$MgCl_2$, 0.8mM-EDTA, 1.5mM-KCl, 4mM-mercaptoethanol, 0.4mM-$NADP^+$, 2.5mM-ATP, 10mM-creatine phosphate and 1mM-glucose at pH7.5. Creatine phosphokinase (100μg), glucose 6-phosphate dehydrogenase (10μg) and 10–20μl of extract were added to 2ml of assay medium in a cuvette. Control assays contained the above medium except that either glucose or the extract was omitted; these controls corrected for any reduction of $NADP^+$ not due to hexokinase, and the contamination of the commercial glucose 6-phosphate dehydrogenase with hexokinase. The ATP-regenerating system (creatine phosphate plus creatine phosphokinase) was found to be essential for the measurement of the maximum activity of hexokinase.

Phosphorylase was assayed in the direction of glycogen synthesis and the assay conditions were adapted from those of Cornblath *et al.* (1963). Volumes (0.2ml) of a solution consisting of 32mM-glucose 1-phosphate, 0.5mM-AMP and 2% (w/v) glycogen were pipetted into polystyrene tubes, which were incubated in a Seroblock (model 13: Precision Scientific Co., Chicago, Ill., U.S.A.) at 25°C. The enzyme reaction was initiated by the addition of 0.2ml of homogenate. Glucose 1-phosphate and AMP were omitted from control incubations. After 1–6min of incubation the reactions were terminated by the addition of 0.6ml of 6% (w/v) $HClO_4$. The

acid extracts were then centrifuged for 5min at 2700*g* and 0.5ml samples of the supernatant were taken for the determination of P_i by the method of Allen (1940). The release of P_i from glucose 1-phosphate was linear with time under these conditions, and preliminary experiments established that excess of glucose 1-phosphate, AMP and glycogen were present and that there was no release of P_i from either glucose 1-phosphate or AMP in the absence of homogenate. The activity of phosphorylase obtained under these conditions was found to be similar to that obtained by using the method described by Burleigh & Schimke (1968). This latter method measures glycogen degradation as opposed to synthesis and thus represents the physiological direction of phosphorylase activity.

Phosphofructokinase was assayed by the method of Opie & Newsholme (1967). Initially this enzyme proved difficult to assay in homogenates of certain insect flight muscles as the activities were very low and variable. This was found to be due to a loss of enzyme activity during the period between preparation of the homogenate and assay of activity. This loss of activity was prevented by preparing the homogenate in no more than 10vol. of extraction medium and assaying 1–5μl samples by using a microlitre syringe. Under these conditions the enzyme was stable for up to 30min after the preparation of the homogenate.

Cytoplasmic glycerol 3-phosphate dehydrogenase was assayed by measuring the oxidation of NADH at 340nm in a Gilford recording spectrophotometer. The assay medium contained 70mM-tris, 0.08mM-NADH, 1mM-KCN and 0.4mM-dihydroxyacetone phosphate at pH7.5: 10–20μl samples of extract were added to 2ml of the assay medium to initiate the reaction.

Mitochondrial glycerol 3-phosphate dehydrogenase was assayed by measuring the reduction of the tetrazolium salt 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride. The assay conditions were the same as those given by Crabtree & Newsholme (1970) for proline dehydrogenase, except that the proline was replaced by 20mM-DL-glycerol 3-phosphate. Phenazine methosulphate was not always included in this assay because the non-enzymic reduction of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride by this substance was troublesome (Nachlas *et al.*, 1960; Pennington, 1961). However, it was found that the phenazine methosulphate increased the rate of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride reduction by approx. 2.5-fold (Table 1), and therefore many of the results reported in this paper were obtained in the absence of this compound and the measured activities have been multiplied by 2.5 for presentation in Table 3.

Lactate dehydrogenase was assayed by measuring the oxidation of NADH at 340nm in a Gilford recording spectrophotometer. The assay medium contained 60mM-tris, 0.17mM-NADH, 1mM-KCN and 0.3mM-pyruvate at pH7.5: 10–20μl samples of homogenate were added to 2ml of the assay medium to initiate the reaction.

All enzyme activities are expressed in terms of μmol of product formed/min per g fresh wt. of muscle at 25°C. The values reported represent the mean of at least two determinations (with two animals or two separate pools of muscle from a larger number of animals). The variation between individual determinations of any enzyme in any one muscle type was less than 20% of the mean value.

However, a systematic study of the effects of factors such as season, diet or sex on the activities of these enzymes has not been attempted in this work, and any precise quantitative interpretations based on these activities should be made with caution.

Table 1. *Effect of phenazine methosulphate on the rate of* 2-(*p-iodophenyl*)-3-(*p-nitrophenyl*)-5-*phenyltetrazolium chloride reduction by glycerol* 3-*phosphate*

Experimental details are given in the text. Abbreviation: INT, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride.

Animal	Muscle	Ratio of the rate of INT reduction in the presence of phenazine methosulphate to that in its absence
Bumble-bee (*Bombus hortorum*)	Flight	2.4
Locust (*Locusta migratoria*)	Flight	2.6
Wasp (*Vespa vulgaris*)	Flight	2.4
Cockchafer (*Melolontha melolontha*)	Flight	2.2
Blowfly (*Phormia terranova*)	Flight	2.7
Pigeon	Pectoral	2.2
Rat	Heart	2.7

It is assumed that the assay procedures described above provide conditions that are optimal for each enzyme from the whole range of muscles tested. In preliminary studies the conditions of assay were shown to be optimal for all the enzymes reported in this paper for the following muscles: rabbit skeletal muscle, rat heart muscle, locust flight muscle, waterbug flight muscle, bumble-bee flight muscle and fleshfly flight muscle.

Results and Discussion

Maximum activities of hexokinase, phosphorylase and phosphofructokinase in relation to the maximum rate of glycolysis in muscle

The approach used in the present investigation is based on the assumption that the maximum catalytic activity of an enzyme that catalyses a non-equilibrium reaction in a metabolic pathway may provide a means of determining the maximum rate of operation of the pathway *in vivo*. The validity of this assumption can be tested by comparing the maximum rates of utilization of carbohydrate by intact muscles with those predicted from the activities of the enzymes hexokinase, phosphorylase and phosphofructokinase.

The high rate of metabolism that occurs during the flight of an insect is primarily due to the energy demands of the flight muscle (Weis-Fogh, 1961; Sacktor, 1965), and the increased oxygen uptake of the insect observed during flight is an indication of the rate of fuel utilization by the flight muscles. Therefore the maximum rate of carbohydrate (or any fuel) utilization can be calculated from the oxygen uptake results, assuming a temperature of the thorax during flight and that the fuel is completely oxidized by the flight muscles. In Table 2 the calculated rates of carbohydrate utilization by insect flight muscles are compared with the activities of hexokinase and phosphorylase and reasonable agreement is obtained. The results chosen for this comparison were obtained under conditions when carbohydrate was the main energy source for flight.

With animals other than insects the published results about oxygen uptake during mechanical activity are relatively sparse. However, results concerning the oxygen uptake of the black duck during flight and the oxygen uptake of the trout during continuous swimming have been reported (see Table 2). Although in these two cases the type of fuel that is supporting the mechanical activity was not identified, it seems likely that during such continuous activity as swimming or flight, glucose rather than glycogen would be utilized (see below). Therefore hexokinase activity is likely to provide a better indication of the rate of fuel utilization under these conditions than either phosphorylase or phosphofructokinase; the extent of agreement between hexokinase activity and the glycolytic rate as calculated from the oxygen uptake is reasonably satisfactory (Table 2). It must be pointed out, however, that fat could supply some, if not all, of the energy requirements of these two muscles during continuous mechanical activity, although carbohydrate would have to meet the energy requirements if fat was not available. Thus, even if carbohydrate is not utilized continuously, the enzymic capacity to do so may always have to be present.

Another method for determining the rate of glycolysis in the intact muscle is to measure lactate accumulation (in muscles that have a minimal oxidative capacity), glycogen degradation, or glucose uptake (allowance being made for glycogen synthesis). Rates of lactate formation have been measured in the isolated frog sartorius muscle incubated *in vitro* under anaerobic conditions, and in the leg muscles of a mouse stimulated *in situ*: values of 13 and 30 μmol of glycogen glucose utilized/min per g of muscle (at 25°C) respectively were obtained in these experiments (Table 2). Similarly with the quadriceps muscle of the dog, the rate of glycogen disappearance on electrical stimulation *in situ* was approximately 10 μmol of glucose equivalent/min per g of muscle (Table 2). These glycogenolytic rates are similar to those predicted from the maximum phosphorylase activities measured in white mammalian skeletal muscle (approx. 40 μmol/min per g) and in frog sartorius muscle (29 μmol/min per g) (Table 3). Thus, considering that the experimental conditions may not stimulate glycolysis maximally, there is reasonable agreement between the two sets of results. However, in the case of the rat heart, the rate of glucose uptake by the working isolated heart preparation is fivefold less than the maximum activity of hexokinase. This suggests either that hexokinase is never fully active in this muscle (owing to inhibition by glucose 6-phosphate; see England & Randle, 1967), or that the heart *in vitro* may not be performing as much work as it does during extreme conditions of mechanical activity in the living animal.

Utilization of carbohydrate for energy production by various muscles

Insect flight muscles. In the above discussion evidence was presented to show that the measurement of the maximum catalytic activities of certain key enzymes of metabolism provides a simple method for determining the maximum rate of carbohydrate utilization by muscle. Because of its experimental simplicity this approach is particularly advantageous for comparative investigations.

In many insects (e.g. flies and bees), the activities of hexokinase and phosphorylase are sufficient for carbohydrate oxidation to supply the necessary energy for flight (see Tables 2 and 3). On the basis

Table 2. *Rate of carbohydrate utilization by intact muscles*

The metabolic rates given in this table have been calculated from the following values. (*a*) Weis-Fogh (1952) obtained a value of 40ml of O_2/h per g of insect; the flight muscles represent 18% of the body weight in this insect (Zebe *et al.*, 1959); the thoracic temperature during flight is assumed to be 35°C. (*b*) Polacek & Kubista (1960) obtained a value of 36ml of O_2/h per g of insect; the thoracic temperature during flight is 30°C (Hofmanova *et al.*, 1966), and the flight muscles are assumed to represent 20% of the body weight. (*c*) Jongbloed & Wiersma (1934) obtained a value of 100ml of O_2/h per g of insect; the flight muscles represent 13% of the body weight (Greenewalt, 1962) and the thoracic temperature during flight is 40°C (Krogh & Zeuthen, 1941; Sotavalta, 1954). (*d*) Davis & Fraenkel (1940) obtained a value of 187ml of O_2/h per g of insect; the flight muscles are assumed to represent 20% of the body weight; the thoracic temperature during flight is assumed to be 35°C. (*e*) P. Webb (personal communication) obtained a value of 0.45ml of O_2/h per g of fish; the metabolic activity is assumed to be solely due to the red muscles, which are taken as representing 18% of the total muscle weight, which, in turn represents 50% of the body weight (Bone, 1966); the muscle temperature is assumed to be 15°C. (*f*) Danforth (1965) obtained a value of 36μmol/min per ml of intracellular water; 1g of muscle contains 0.5ml of intracellular water (Newsholme & Randle, 1961); the temperature was 20°C. (*g*) Berger *et al.* (1970) obtained a value of 218ml of O_2/min per bird of weight 1026g; the pectoral muscle is assumed to represent 20% of the body weight; the thoracic temperature during flight is assumed to be 40°C (Welty, 1955). (*h*) Crass *et al.* (1969) obtained a value of 444μmol of glucose/h per g dry wt. at 37°C; the wet wt./dry wt. ratio for heart is assumed to be 3. (*j*) Danforth & Lyon (1964) obtained a value of 120μmol of lactate/min per g of muscle at 37°C; the rate of pyruvate oxidation is assumed to be negligible. (*k*) Corsi *et al.* (1969) obtained differences in glycogen contents of stimulated and control muscles; the rate of pyruvate oxidation is assumed to be negligible. The Q_{10} of the enzymic reactions is assumed to be 2.0 in all cases and, in the flying insects, carbohydrate is assumed to be oxidized completely with 1μmol of glucose (or glycogen glucose) being equivalent to the uptake of 6μmol of oxygen. The symbols indicate the actual species used in the assay of enzyme activities: *, *Calliphora erythrocephala*; †, mallard.

Animal	Experimental conditions	Rate of carbohydrate utilization necessary to account for the measured metabolic rate (as μmol of C_6 unit/min per g of fresh muscle at 25°C)	Enzyme activity (μmol/min per g of fresh muscle at 25°C)	
			Hexokinase	Phosphorylase
Locust (*Schistocerca gregaria*)	Oxygen uptake of flying insect[a]	14.0	11.5	7.5
Cockroach (*Periplaneta americana*)	Oxygen uptake of flying insect[b]	15.0	18.0	30.0
Honey-bee (*Apis mellifera*)	Oxygen uptake of flying insect[c]	32.0	29.0	4.0
Blowfly (*Lucilia sericata*)	Oxygen uptake of flying insect[d]	59.0	35.0*	55.0*
Trout (*Salmo gairdneri*)	Oxygen uptake during continuous swimming[e]	1.2	2.6	14.0
Frog (*Rana temporaria*)	Isolated sartorius muscle incubated anaerobically: rate of lactate production[f]	13.0	1.3	29.0
Black duck (*Anas rubripes*)	Oxygen uptake of flying bird[g]	1.5	3.8†	50.0†
Rat heart	Perfused, *in vitro*, working against 10cmH_2O ventricular pressure: glucose uptake measured[h]	1.2	6.1	12.0
Mouse 'skeletal' muscle	Electrically stimulated *in situ*: lactate production measured[j]	30.0	3.9	34
Dog quadriceps	Electrically stimulated *in situ*: decrease in glycogen content measured[k]	10.0	—	—

Table 3. *Activities of hexokinase, phosphorylase, phosphofructokinase, mitochondrial and cytoplasmic glycerol 3-phosphate dehydrogenase and lactate dehydrogenase in various muscles*

Experimental details are given in the text.

Animal	Muscle	Enzyme activities (μmol/min per g fresh wt. at 25°C)					
					Glycerol 3-phosphate dehydrogenase		
		Hexokinase	Phosphorylase	Phospho-fructokinase	Mitochondrial	Cytoplasmic	Lactate dehydrogenase
Crustacea							
Crab (*Carcinus meanus*)	Claw	1.0	5.1	6.4	0.07*	—	6.0
Mollusca							
Sea mussel (*Mytilus edulis*)	Posterior adductor	0.4	3.1	2.6	0.3*	1.8	2.6
Insecta							
Orthoptera							
Locust (*Locusta migratoria*)	Flight	8.0	8.0	13.0	33.0	124.0	1.6
	Hind leg femoral	2.3	20.0	16.0	25.0	33.0	53.0
Locust (*Schistocerca gregaria*)	Flight	11.5	7.5	17.0	43.0*	141.0	2.9
Dictyoptera							
Cockroach (*Periplaneta americana*)	Flight	18.0	30.0	19.0	48.0*	216.0	1.5
	Coxal (white)	1.3	3.0	4.0	9.0*	35.0	—
Hemiptera							
Waterbug (*Lethocerus cordofanus*)	Flight	4.0	1.0	6.0	8.0*	51.0	1.3
	Foreleg femoral	0.05	12.0	—	0.9*	13.0	59.0
Waterbug (*Lethocerus maximus*)	Flight	4.5	—	5.8	—	—	—
Coleoptera							
Cockchafer (*Melolontha melolontha*)	Flight	2.3	14.0	13.0	36.0	103.0	4.4
Hymenoptera							
Wasp (*Vespa vulgaris*)	Flight	77.0	3.0	74.0	120.0	—	—
Honey-bee (*Apis mellifera*)	Flight	29.0	4.0	20.0	44.0*	257.0	1.5
Bumble-bee (*Bombus hortorum*)	Flight	114.0	8.0	33.0	90.0	513.0	1.8
Lepidoptera							
Small tortoiseshell butterfly (*Vanessa urticae*)	Flight	4.8	8.0	14.0	24.0*	—	—
Poplar hawk moth (*Laothoe populi*)	Flight	3.2	6.0	9.0	13.0*	36.0	3.2
Silver-Y moth (*Plusia gamma*)	Flight	50.0	2.0	41.0	110.0*	—	—
Diptera							
Tsetse fly (*Glossina austeni*)	Flight	2.3	1.8	—	4.0*	29.0	1.5
Fleshfly (*Sarcophaga barbata*)	Flight	17.0	57.0	—	100.0*	270.0	2.5
Blowfly (*Phormia terranova*)	Flight	14.0	54.0	46.0	110.0	300.0	1.7
Blowfly (*Calliphora erythrocephala*)	Flight	35.0	55.0	43.0	90.0*	—	—

Table 3 (*continued*)

Pisces							
Dogfish (*Scylliorhinus canicula*)	Red	1.9	12.0	14.0	0.1*	5.0	110.0
	White	0.1	62.0	50.0	0.6*	8.0	330.0
Trout (*Salmo gairdneri*)	Red	2.6	14.0	12.2	0.1*	9.0	23.0
	White	1.6	48.0	58.3	0.1*	18.0	—
Silver eel (*Anguilla anguilla*)	Red	1.3	3.6	7.9	—	—	—
	White	0.2	13.0	18.0	—	—	—
Amphibia							
Frog (*Rana temporaria*)	Sartorius	1.3	29.0	22.0	0.4*	24.0	398.0
Aves							
Domestic pigeon (*Columba livia*)	Pectoral	3.0	18.0	24.0	1.2	33.0	314.0
Mallard (*Anas platyrhynchos*)	Pectoral	3.8	50.0	41.0	1.9*	35.0	276.0
Domestic fowl (*Gallus gallus*)	Pectoral	1.1	83.0	105.0	0.6*	76.0	870.0
Pheasant (*Phasianus colchicas*)	Pectoral	2.3	120.0	143.0	2.8*	103.0	542.0
Mammalia							
Rabbit (*Oryctolagus cuniculus*)	Semitendinosus (red)	1.9	8.0	8.0	0.2*	4.6	60.0
	Adductor longus (white)	0.3	30.0	26.0	0.8*	55.0	372.0
Laboratory rat (Wistar strain)	Heart	6.1	12.0	10.0	0.3	6.0	311.0
	Quadriceps femoris	1.9	50.0	47.0	1.2*	48.0	448.0
Bush baby (*Galago* sp.)	Sartorius	1.1	—	11.0	—	—	—
Laboratory mouse (ZO strain)	Quadriceps	3.9	34.0	28.0	—	—	—

* Signifies that the value obtained has been multiplied by 2.5 (see the Materials and Methods section).

of studies of the respiratory quotient during flight it has been established that many insects utilize fat for energy production for flight (e.g. Lepidoptera; see Zebe, 1954). In the present investigation the low activities of hexokinase and phosphofructokinase in insects such as the hawk moth and waterbug (Table 3) suggests that carbohydrate cannot supply all the energy demands of flight and that some other fuel would be required (e.g. fat). However, in the flight muscles of one moth that was examined, the silver-Y moth, the activities of hexokinase and phosphofructokinase are reasonably high, which suggests that in this species carbohydrate may be able to supply much of the energy that is required for flight. Thus it is probably incorrect to regard Lepidoptera as an order of insects that utilizes only fat for energy production during flight (see also Stevenson, 1968).

The locust is considered to utilize carbohydrate during the initial period of flight and to switch to fat utilization after about 30min (Krogh & Weis-Fogh, 1951; Weis-Fogh, 1952). The activities of the glycolytic enzymes in the flight muscles of the locust are reasonably high and could account for the known rate of oxygen uptake during the early stages of flight (see Tables 2 and 3). Thus, although the demonstration of reasonably high activities of glycolytic enzymes indicates an ability to utilize carbohydrate for energy formation, it does not exclude the possibility that fat can be used to supplement or even replace carbohydrate.

In the tsetse fly (*Glossina austeni*) the activities of the glycolytic enzymes are very low (Table 3), in contrast with the other members of the Diptera which have been currently investigated. However, it is known that in this insect the amino acid proline is an important fuel for providing energy during flight (Bursell, 1963). A similar situation may occur in the cockchafer (*Melolontha melolontha*), whose flight muscles possess a very high activity of proline dehydrogenase (Crabtree & Newsholme, 1970) and a low activity of hexokinase (Table 3).

In the Hymenoptera and Diptera (as well as in the cockroach), which are generally believed to utilize only carbohydrate during flight (Sacktor, 1965; Weis-Fogh, 1967), the activities of the glycolytic enzymes are high (Table 3). In fact the hexokinase activity in the flight muscles of the bumble-bee (*Bombus hortorum*) of 114μmol/min per g is the highest (on a fresh weight basis) that has been reported in any tissue. It is noteworthy that in three species of Diptera in which the activities of the glycolytic enzymes were high, the activity of phosphorylase was two- to three-fold higher than the activity of hexokinase (a similar case is also seen in the cockroach), whereas in all the Hymenoptera examined the reverse was the case.

Vertebrate muscle. The results given in Table 3 for the activities of glycolytic enzymes extends the work of Burleigh & Schimke (1969) in that it includes muscles from vertebrates other than mammals, and it includes the activities of phosphofructokinase. In all vertebrate muscles examined the activity of phosphofructokinase in any one muscle is very similar to that of phosphorylase (Table 3). The conclusion of Burleigh & Schimke (1968, 1969) that utilization of glycogen is more important, and utilization of glucose less important, in white than in red muscles for the provision of energy is supported and extended by the present work (Table 3). A comparison of these enzyme activities between insect flight muscles and vertebrate muscles shows at least two notable points. First, in the vertebrate muscles examined the highest activity of hexokinase is found in the rat heart (6.1μmol/min per g, Table 3) and this is approx. 20-fold lower than the maximum activity found in some insect flight muscles (114μmol/min per g, Table 3). Weis-Fogh (1961) has pointed out that a tenfold difference may exist in the maximum continuous mechanical power output between human muscle and insect flight muscle. Secondly, despite the higher metabolic rate of insect flight muscles, the activities of phosphorylase are usually less than those of vertebrate white muscles (Table 3). The reason for this may be that the latter muscles depend almost completely on anaerobic glycolysis (i.e. conversion of glycogen into lactate) for energy production during mechanical activity. Such a method for energy production is much less efficient than the complete oxidation of glucose via the tricarboxylic acid cycle, so that the maximum capacity for glycolysis must be high to provide sufficient energy to support muscular activity. Also this type of metabolism can only provide energy for very short bursts of activity as the glycogen reserves in muscle are limited (see Weis-Fogh, 1967). On the other hand, insect flight muscles possess a high aerobic metabolic capacity (see Sacktor, 1965, 1970) and glucose residues produced from glycogen can be completely oxidized to obtain the maximum release of energy from each glucose molecule. Further, anaerobic glycolysis in which net ATP formation occurs cannot take place in insect flight muscles as the activity of lactate dehydrogenase is very low (Zebe & McShan, 1957; see also Table 3). Therefore the activity of phosphorylase need not be as great as in vertebrate muscles to obtain sufficient energy for muscular activity. In vertebrate red muscles (e.g. semitendinosus of the rabbit, mammalian heart) the activity of hexokinase is much greater than in the white muscles (see also Bass *et al.*, 1969; Burleigh & Schimke, 1969). This suggests that glucose might be a more important source of energy for contraction in red than in white muscle. Nonetheless red muscle possesses higher activities of phosphorylase and phosphofructokinase than hexokinase. It is possible to speculate that these high activities enable the muscles to produce energy in excess of the rate of fuel or oxygen supply for short periods of time.

This energy would be provided by the conversion of glycogen into lactate, which also might explain the high activity of lactate dehydrogenase in these muscles (Table 3).

Oxidation of glycolytically produced NADH

The conversion of glyceraldehyde 3-phosphate into 1,3-diphosphoglycerate during glycolysis requires the conversion of NAD^+ into NADH and, to maintain the glycolytic flux, the NADH must be continuously reoxidized. In white vertebrate muscle glycolysis is stimulated during mechanical activity and NADH reoxidation is achieved by the conversion of pyruvate into lactate. However, in muscles that oxidize a considerable amount of pyruvate via the tricarboxylic acid cycle (e.g. insect flight and vertebrate red muscles) the NADH is oxidized by means of the mitochondrial electron transport chain. However, the mitochondrial membrane is impermeable to NAD^+ and NADH so that some indirect means of transporting the cytoplasmic reducing equivalents into the mitochondria must exist (Borst, 1963; Krebs, 1967; Chappell, 1968). Insect flight muscles are believed to possess a glycerol 3-phosphate cycle which, via the activities of the cytoplasmic NAD-linked glycerol 3-phosphate dehydrogenase and the mitochondrial flavoprotein-linked glycerol 3-phosphate dehydrogenase, catalyses a unidirectional net oxidation of cytoplasmic NADH by the mitochondrial electron transport chain (Klingenberg & Bücher, 1960; Sacktor, 1965, 1970). The present work (Table 3) shows that, in the flight muscles of many insects, the activity of the cytoplasmic enzyme is three- to six-fold greater than that of the mitochondrial enzyme, so that the latter enzyme is likely to be rate-limiting for the operation of the glycerol 3-phosphate cycle. Further, in most flight muscles the maximum glycolytic capacity (estimated from the activities of phosphofructokinase; Table 3) is approximately the same as the maximum capacity of the glycerol 3-phosphate cycle (estimated from the activity of the mitochondrial glycerol 3-phosphate dehydrogenase in Table 3). This suggests that the operation of this cycle in insect flight muscle could account for most, if not all, of the oxidation of NADH produced during glycolysis. Therefore, no other mechanisms for reoxidation of cytoplasmic NADH need exist in these muscles.

In vertebrate muscles the activity of the mitochondrial glycerol 3-phosphate dehydrogenase is very low so that the maximum capacity of the glycerol 3-phosphate cycle must be very low (Table 3). However, the activity of this enzyme is higher in white than in red muscle (Pette, 1966; Table 3). The activity of the cycle in white muscle is not sufficient to account for the major part of the NADH oxidation when glycolysis is maximally stimulated, but it may play a supplementary role to the conversion of pyruvate into lactate. Alternatively, the glycerol 3-phosphate cycle may function in these muscles during rest, when the rate of glycolysis will be low and when a reasonable proportion of the pyruvate that is produced by glycolysis may be oxidized by the tricarboxylic acid cycle. In red muscles a large proportion of the pyruvate produced from glucose is oxidized (e.g. up to 80% in the perfused rat heart under some conditions; Morgan *et al.*, 1965). The estimated rate of the operation of the glycerol 3-phosphate cycle suggests that it could account for only a small proportion of the reoxidation of the glycolytically produced NADH, and therefore another mechanism must be available. One suggested route is the 'malate–oxaloacetate shuttle', which functions in a similar manner to that of the glycerol 3-phosphate cycle (Chappell, 1968).

However, pigeon heart mitochondria possess an enzyme or enzyme complex which is capable of oxidizing NADH directly and which is external to the nicotinamide nucleotide barrier of the mitochondria (Griffiths & Blanchaer, 1967; Rasmussen, 1969). This direct oxidative system could provide a mechanism for the oxidation of NADH without any requirement for a cytoplasmic dehydrogenase, such as malate or glycerol phosphate dehydrogenase. Thus the cytoplasmic NADH concentration could be maintained at a very low value, so that the rate of conversion of pyruvate into lactate would be limited (Newsholme, 1970). However, further information on the distribution of this enzyme system is needed before its importance in the reoxidation of glycolytically produced NADH can be assessed.

We thank Professor J. W. S. Pringle, F.R.S., for his interest and encouragement. B. C. was the recipient of a Medical Research Council Training Scholarship.

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