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Glycerol Kinase Activities in Muscles from Vertebrates and Invertebrates

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1. Glycerol kinase (EC 2.7.1.30) activity was measured in crude extracts of skeletal muscles by a radiochemical method. The properties of the enzyme from a number of different muscles are very similar to those of the enzyme from rat liver. Glycerol kinase from locust flight muscle was inhibited competitively by L-3-glycerophosphate with a K_i of $4{\cdot}0 \times 10^{-4}$M. 2. The activity of glycerol kinase was measured in a variety of muscles from vertebrates and invertebrates in an attempt to explain the large variation in the activity of this enzyme in different muscles. 3. In vertebrates glycerol kinase activities were generally higher in red muscle than in white muscle; the highest activities (approx. $0{\cdot}2\,\mu$mole/min./g. fresh wt.) were found in the red breast muscle of some birds (e.g. pigeon, duck, blue tit) whereas the activities in the white breast muscle of the pheasant and domestic fowl were very low (approx. $0{\cdot}02\,\mu$mole/min./g.). 4. On the basis of glycerol kinase activities, muscles from insects can be classified into three groups: muscles that have a low enzyme activity, i.e. $< 0{\cdot}3\,\mu$mole/min./g. (leg muscles of all insects studied and the flight muscles of cockroaches and the tsetse fly); muscles that have an intermediate enzyme activity, i.e. $0{\cdot}3$–$1{\cdot}5\,\mu$moles/min./g. (e.g. locusts, cockchafers, moths, water-bugs); and muscles that have a high enzyme activity, i.e. $> 1{\cdot}5\,\mu$moles/min./g. (e.g. bees, wasps, some blowflies). 5. The function of glycerol kinase in vertebrate and insect muscles that possess a low or intermediate activity is considered to be the removal of glycerol that is produced from lipolysis of triglyceride or diglyceride by the muscle. Therefore in these muscles the activity of glycerol kinase is related to the metabolism of fat, which is used to support sustained muscular activity. A possible regulatory role of glycerol kinase in the initiation of triglyceride or diglyceride lipolysis is discussed. 6. The function of glycerol kinase in the insect muscles that possess a high activity of the enzyme is considered to be related to the high rates of glycolysis that these muscles can perform. The oxidation of extramitochondrial NADH, and therefore the maintenance of glycolysis, is dependent on the functioning of the glycerophosphate cycle; if at any stage of flight (e.g. at the start) the rate of mitochondrial oxidation of L-3-glycerophosphate was less than the activity of the extramitochondrial glycerophosphate dehydrogenase, this compound would accumulate, inhibit the latter enzyme and inhibit glycolysis. It is suggested that such excessive accumulation of L-3-glycerophosphate is prevented by hydrolysis of this compound to glycerol; the latter would have to be removed from the muscle when the accumulation of L-3-glycerophosphate had stopped, and this would explain the presence of glycerol kinase in these muscles and its inhibition by L-3-glycerophosphate.

Glycerol kinase (EC 2.7.1.30) has been reported to be absent, or present only at very low catalytic activities, in muscle: Wieland & Suyter (1957) found no activity in rat heart or skeletal muscle; Zebe (1959) could not detect any activity in locust flight muscle; Sacktor (1955) observed that flight muscle of flies oxidized glycerol only at a very low rate; and Gilbert (1967) reported only a low activity of glycerol utilization in flight muscle of the American silkmoth (*Hyalophora cecropia*). However, Robinson & Newsholme (1967), using a sensitive radiochemical technique, unequivocally demonstrated the presence of a low activity of glycerol kinase in rat heart. This finding prompted a reinvestigation of the activity of this enzyme in various skeletal muscles. Preliminary investigations established that this enzyme was present in skeletal muscle, but the activity varied greatly in

different muscles. In an attempt to explain this variation in glycerol kinase activity and to relate it to the metabolic characteristics of the muscle a detailed investigation of the activities of this enzyme in various muscles from various animals was carried out, and the results are reported and discussed in this paper. Some properties of glycerol kinase from the flight muscle of the locust are also reported.

MATERIALS AND METHODS

Chemicals and enzymes. All chemicals and enzymes were obtained from the same sources as given by Robinson & Newsholme (1969).

Animals. Locusts, flies, cockroaches, silkmoths and the mammals were bred in this Department. Pigeons, ducks, pheasants and domestic fowls were obtained from Park Farm, Northmoor, Oxford. Frogs (*Rana temporaria*) were obtained from L. Haig and Co. Ltd., Bean Brook, Newdigate, Surrey, and *Xenopus laevis* was obtained from Dr J. Gurdon of this Department. The blue tits were obtained from Mr J. Krebs of this Department. The robin was taken from a family cat soon after he had caught and killed it. The marine animals were obtained from the Marine Biological Laboratory, Plymouth. The chub and trout were caught in local rivers. The water-bugs and rhinoceros beetles were sent to this Department from Uganda and Trinidad, and were kept for some days in the tropical room before use. Other insects were caught locally and were used within a few hours.

Preparation of crude extracts of muscles. Muscles, always dissected from freshly killed animals, were rapidly chilled in a glass beaker on ice, weighed rapidly and cut into small pieces. All vertebrate muscle and crab muscle was homogenized in a Silverson homogenizer (Silverson Machines Ltd., London, S.E.1); all insect muscle was homogenized with a power-driven Teflon–glass homogenizer. The extraction medium consisted of 20mM-tris, 1% (w/v) KCl, 2mM-EDTA and 10mM-mercaptoethanol at pH7·6 and tissues were diluted fourfold or tenfold with extraction medium according to the expected glycerol kinase activity. These homogenates were used for glycerol kinase assays without any further treatment.

Centrifugation of extracts of locust flight muscle. Locust flight muscle was extracted as above, except that 0·25M-sucrose replaced the KCl. The extract was centrifuged at 6000*g* at 2° for 20min. (MSE High Speed 18, 8×5ml. rotor), and the pellet, supernatant and lipid layer on top of the supernatant were separated for glycerol kinase assays. This supernatant was further centrifuged at 25000*g* at 2° for 45min. and the pellet and supernatant were separated for analysis.

The particulate fraction of locust flight muscle, which was used for investigation of the properties of glycerol kinase, was obtained by centrifuging the crude extract at 6000*g* at 2° for 20min., resuspending the pellet in 10 vol. of extraction medium and repeating the centrifugation.

Assay of glycerol kinase activity. Glycerol kinase was assayed by the radiochemical method described by Newsholme, Robinson & Taylor (1967). The incubation medium usually consisted of 62·5mM-tris, 12·5mM-mercaptoethanol, 12·5mM-NaF, 0·6mM-EDTA, 4·1mM-ATP, 8mM-creatine phosphate, 4 units of creatine phosphokinase/ml. and various concentrations of Mg^{2+} and [^{14}C]glycerol at pH7·5 (the above concentrations are final concentrations in the incubation–extract mixture). A sample (20μl.) of the extract was incubated with 100μl. of the incubation buffer for various times (1–15min., depending on the activity of glycerol kinase) and the reaction was stopped by addition of 100μl. of 98% ethanol. The incubation was carried out at room temperature (approx. 21°) for assays with fish, frog and crustacean muscle, 30° for assays with insect muscle and 37° for assays with avian and mammalian muscle. After the reaction had been stopped the procedure was almost identical with that described by Newsholme *et al.* (1967) except that the DEAE-cellulose-paper disks were counted in a Beckman model LS200 liquid-scintillation counter.

The concentrations of glycerol in the extracts of the flight muscles of a large number of insects were measured and found to be too low to interfere in the radiochemical assay. However, in the angle-shade moth (*Phlogophora meticulosa*), which was caught in the autumn, the glycerol concentration in the flight-muscle extract was 54μmoles/g. of muscle, and this was taken into account in the calculation of glycerol kinase activity.

For experiments involving assay of glycerol kinase in crude extracts of muscle, the glycerol kinase activities were obtained from progress curves constructed from the radioactivity retained on the DEAE-cellulose paper after different times of incubation (zero-time radioactivities were always subtracted). For experiments involving a study of the properties of glycerol kinase, activities are based on radioactivities retained on the paper after incubation for 3 or 5min.; preliminary experiments had established that progress curves were linear for the periods of incubation.

Recovery of L-3-*glycerophosphate.* [^{14}C]L-3-Glycerophosphate was added to a final concentration of 0·3mM to the usual incubation buffer (except that [^{14}C]glycerol was replaced by unlabelled glycerol) and incubated at 30° for 6 or 10min. with various muscle extracts [locust leg and flight muscles, blowfly (*Sarcophaga*) flight muscles, cockroach flight muscle and pigeon breast muscle] before addition of ethanol. The radioactivity was completely recovered on DEAE-cellulose paper disks after the incubation. Similar experiments in which unlabelled L-3-glycerophosphate was incubated with incubation buffer and the reaction stopped with $HClO_4$ were carried out and the recovery of L-3-glycerophosphate was measured enzymically; in this case recoveries varied in the range 65–85%. The difference between the radiochemical and enzymic recoveries may be explained by conversion of L-3-glycerophosphate into other anionic compounds, e.g. dihydroxyacetone phosphate.

Expression of results. Glycerol kinase activities are expressed either as radioactivity (c.p.m.) retained on the DEAE-cellulose paper multiplied by a factor (usually 11) representing the ratio of sample taken to the volume of inhibited incubated mixture, or as μmoles/min./g. fresh wt. of muscle. The latter can be calculated from the radioactivity (c.p.m.) incorporated into product/min. of incubation time with a knowledge of the amount of glycerol in the incubation tube, the total radioactivity (c.p.m.) in the incubation tube and the amount of tissue used (see, e.g., Newsholme, Rolleston & Taylor, 1968).

Glycerol kinase activities reported in this paper are the

means of at least two separate determinations (with two animals or two separate pools of muscle from a larger number of animals) with a variation of less than 30% between individual determinations.

RESULTS

Retention of radioactivity on DEAE-cellulose paper as a measure of glycerol kinase activity. Newsholme *et al.* (1967) showed that L-3-[^{14}C]-glycerophosphate was retained on DEAE-cellulose-paper disks whereas [^{14}C]glycerol was removed by a washing procedure that was used in the present investigation. However, conversion of [^{14}C]-glycerol into a product other than L-3-glycerophosphate, which was also retained on the DEAE-cellulose-paper, would be indistinguishable from glycerol kinase in this assay. Therefore a number of tests were carried out to provide evidence that radioactivity retained on the DEAE-cellulose-paper disks represents glycerol kinase activity. (1) Retention of radioactivity was dependent on the presence of ATP in the incubation buffer. (2) There was no retention of radioactivity when the extract was boiled for 5min. before incubation. (3) In all extracts tested glycerol kinase activity was inhibited by L-3-glycerophosphate, ADP and AMP (see Table 5), and these inhibitions are characteristic properties of the enzyme from rat liver (Robinson & Newsholme, 1969). (4) Extracts from the flight muscles of locust, water-bug, bumble bee and blowfly (*Phormia*) and from pigeon breast muscle were incubated as described above, but the incubation was stopped by the addition of 6% perchloric acid, the mixture was neutralized and L-3-glycerophosphate, triose phosphates and fructose diphosphate were measured enzymically. The results are shown in Table 1; there is very close agreement between the glycerol kinase activity as measured by the formation of the intermediates and the activity as measured radiochemically. Moreover, most of the activity can be accounted for as L-3-glycerophosphate formation, as there is little production of triose phosphate or fructose diphosphate. It is therefore concluded that retention of radioactivity on DEAE-cellulose paper under the experimental conditions used in these experiments represents glycerol kinase activity.

Glycerol kinase activities in various muscles. Glycerol kinase activities were usually measured at high (1mM) and low (0·17mM) concentrations of [^{14}C]glycerol: at the high concentration maximum activities are expected owing to substrate saturation of the enzyme and to minimization of the isotope-dilution effect from unlabelled glycerol in the tissue extract; but it seemed possible that high concentrations of glycerol could inhibit glycerol kinase and give rise to erroneously low activities, and therefore the enzyme activity was also measured at the low glycerol concentration. In general, glycerol kinase activities were two- to three-fold higher at 1mM-glycerol in comparison with the activities at the lower concentration (see Table 2). The minimum activity that could be detected with 1mM-[^{14}C]glycerol (specific radioactivity 16·7μc/μmole) was 0·0006μmole/min./g. fresh wt.

Table 1. *Formation of* L-3-*glycerophosphate, triose phosphate and fructose diphosphate from glycerol by extracts of various muscles and comparison with radiochemical assay of glycerol kinase activity*

Muscles were extracted as described in the Materials and Methods section and were diluted tenfold except for pigeon breast muscle, which was diluted fourfold. Radiochemical assays were carried out as described in the Materials and Methods section. For the spectrophotometric assays incubations were carried out with 1·0ml. of incubation buffer containing 1·2mM-glycerol and 0·2ml. of muscle extract, the mixture was incubated for 6min. at 30° and the reaction was stopped by addition of $HClO_4$. Control incubations were carried out in the absence of glycerol. The solutions were neutralized with $KHCO_3$, and L-3-glycerophosphate and triose phosphate plus fructose diphosphate were assayed by the methods of Hohorst (1965) and Bücher & Hohorst (1965).

		Formation of intermediates from 1mM-glycerol as assayed spectrophotometrically (μmole/6min./0·2ml. of muscle extract)				Radiochemical assay of glycerol kinase (μmole/6min./0·2ml. of muscle extract)
Animal	Muscle	L-3-Glycero-phosphate	Triose phosphate	Fructose phosphate	Total	
Locust (*Locusta migratoria*)	Flight	0·04	0·02	0	0·06	0·07
Water-bug (*Lethocerus cordofanus*)	Flight	0·11	0·01	0·01	0·13	0·12
Blowfly (*Phormia terranova*)	Flight	0·09	0·01	0	0·10	0·10
Bumble bee (*Bombus hortorum*)	Flight	0·17	0	0	0·17	0·19
Pigeon	Breast	0·09	0	0	0·09	0·07

Table 2. *Glycerol kinase activities in various muscles from different animals at two initial concentrations of glycerol*

Muscle extracts were prepared and glycerol kinase assays were carried out as described in the Materials and Methods section. N.D., Not detectable, i.e. activity < 0.0006 μmole/min./g. fresh wt.

Animal	Muscle	Glycerol kinase activity (μmoles/min./g. fresh wt.) 0·17 mM-Glycerol	1·0 mM-Glycerol
Crustacea			
Crab (*Carcinus maenus*)	Pincer	N.D.	—
	Leg	N.D.	—
Mollusca			
Sea mussel (*Mytilus edulis*)	Posterior adductor	0·070	0·084
Insecta			
Orthoptera			
Locust (*Locusta migratoria*)	Flight	0·092	0·520
	Hind-leg femoral	0·025	0·020
(*Schistocerca gregaria*)	Flight	0·096	0·320
	Hind-leg femoral	—	0·035
Dictyoptera			
Cockroach (*Periplaneta americana*)	Flight	0·004	0·058
	Femoral	0·030	0·122
(*Blaberus discoidalis*)	Flight	0·010	0·012
	Coxal	0·010	0·007
Hemiptera			
Water-bug (*Lethocerus cordofanus*)	Flight	0·700	1·400
(*Lethocerus maximus*)	Flight	0·174	0·834
	Tergo-coxal	0·036	0·075
	Fore-leg femoral	0·050	0·118
Coleoptera			
Cockchafer (*Melolontha melolontha*)	Flight	0·550	1·540
Rhinoceros beetle (*Oryctes rhinoceros*)	Flight	—	0·864
Hymenoptera			
Wasp (*Vespula vulgaris*)	Flight	0·310	1·920
Queen wasp (*Vespula vulgaris*)	Flight	0·940	6·400
Honey bee (*Apis mellifera*)	Flight	0·820	2·000
Bumble bee (*Bombus hortorum*)	Flight	0·380	2·400
(*Bombus lapidarius*)	Flight	0·280	1·550
(*Bombus latreillellus*)	Flight	0·659	2·930
(*Bombus helferanus*)	Flight	0·280	1·630
(*Bombus agrorum*)	Flight	0·370	1·900
Queen bumble bee (*Bombus hortorum*)	Flight	1·382	6·780
Lepidoptera			
Small tortoiseshell butterfly (*Vanessa urticae*)	Flight	0·104	0·789
Silkmoth (*Philosamia cynthia cynthia*)	Flight	0·240	0·680
Hawk moth (*Deilephila elpenor*)	Flight	0·250	0·850
Angle-shade moth (*Phlogophora meticulosa*)	Flight	—	0·600
Dusky-thorn moth (*Deuteronomos fuscantarin*)	Flight	—	0·950
Yellow-underwing moth (*Triphaena pronuba*)	Flight	0·190	0·960
Diptera			
Tsetse fly (*Glossina morsitans*)	Flight	—	0·210
Blowfly (*Sarcophaga barbata*)	Flight	0·185	0·326
(*Lucilia cuprina*)	Flight	0·380	0·780
(*Phormia terranova*)	Flight	0·624	2·840
(*Calliphora erythrocephala*)	Flight	0·318	1·740
Pisces			
Dogfish (*Scylliorhinus caniculus*)	Red	N.D.	0·003
	White	N.D.	0·002

Table 2 (*continued*)

Animal	Muscle	Glycerol kinase activity (μmoles/min./g. fresh wt.) 0·17 mM-Glycerol	1·0 mM-Glycerol
Plaice (*Pleuronectes platessa*)	White	N.D.	0·002
	Heart	N.D.	0·008
Flounder (*Pleuronectes flesus*)	White	N.D.	0·004
	Heart	N.D.	0·008
Chub (*Squalius cephalus*)	White	N.D.	0·005
	Red	N.D.	0·004
	Heart	N.D.	0·017
Trout (*Salmo gairdneri*)	Red	N.D.	N.D.
	White	N.D.	N.D.
Amphibia			
Frog (*Rana temporaria*)	Heart	N.D.	N.D.
	Various hind-leg muscles	N.D.	N.D.
	Rectus abdominus	N.D.	N.D.
(*Xenopus laevis*)	Heart	0.009	0·004
	Various hind-leg thigh muscles	0·006	0·010
	Gastrocnemius	0·005	0·015
Aves			
Pigeon	Pectoral	0·153	0·190
	Gastrocnemius	0·006	0·021
Blue tit (*Parus caeruleus*)	Pectoral	—	0·200
Robin (*Erithacus rubecula*)	Pectoral	—	0·200
Duck	Pectoral	0·024	0·150
Domestic fowl	Pectoral	—	0·016
Pheasant (*Phasianus colchicas*)	Pectoral	N.D.	0·026
	Gastrocnemius	0·010	0·040
Mammalia			
Rabbit	Semitendinosus (red)	0·011	0·020
	Pectineus (red)	0·008	0·016
	Gastrocnemius (red)	0·009	—
	Adductor longus (white)	0·002	0·007
Rat	Diaphragm	0·019	0·030
	Heart	0·012	0·016
	Thigh	0·003	0·007

There was an extremely large variation in the activities of glycerol kinase from the range of animals and muscles investigated (Table 2). With vertebrates higher activities were generally found in red muscle than in white muscle; the highest activities were found in the red breast muscles of blue tit, robin, duck and pigeon, but very low activities were found in the white breast muscle of the domestic fowl and the pheasant; in rabbit leg glycerol kinase activities were higher in red muscle than in white muscle, and in rat were higher in heart and diaphragm than in the thigh muscle. In the fish muscles studied glycerol kinase activities were very low, with little difference between red muscle and white muscle; slightly higher activities were observed in heart muscle. Glycerol kinase activities were not detected in various muscles of the terrestrial frog (*Rana*), but low activities were found in the aquatic frog (*Xenopus*).

Among the invertebrates insects were mostly studied as the variation in the activities of glycerol kinase in the flight muscles was extremely large (Table 2): the lowest activity was observed in the flight muscle of the cockroach *Blaberus discoidalis* (0·012 μmole/min./g.) and the highest was found in those of the queen bumble bee *Bombus hortorum* (6·8 μmoles/min./g.). The latter activity is the highest glycerol kinase activity reported for any animal tissue (the activity of glycerol kinase in rat liver is 2 μmoles/min./g.).

The activity of this enzyme in the flight muscle does not appear to be related to the ability of the insect to perform sustained flight: thus in locusts and moths, some of which are migratory, the activities were much lower than the activities in flies, bees and wasps, which do not normally fly long distances. In most cases glycerol kinase activities in insect leg muscles were much lower than in the flight muscles: this is very obvious with *Lethocerus maximus*, in which the activity in the flight muscles was ten times that in the tergo-coxal muscles, although both muscles are in the thorax

Table 3. *Effect of centrifugation on the glycerol kinase activity of locust flight muscle*

Whole homogenate of locust flight muscle was prepared and centrifuged as described in the Materials and Methods section.

Cell fraction	Glycerol kinase activity (μmole/min./g. fresh wt. of original muscle)		% of activity of whole homogenate (before ultrasonic treatment)
	Before ultrasonic treatment	After ultrasonic treatment	
Whole homogenate	0·54	0·59	—
6000*g* pellet	0·38	0·45	66
6000*g* supernatant	0·19	—	32
6000*g* lipid layer	0·009	—	2
25000*g* pellet	0·045	0·056	8
25000*g* supernatant	0·111	—	20

Table 4. *Glycerol kinase activity of locust flight muscle during development*

Locusts and hoppers were taken at the times indicated before and after the imaginal moult and the flight muscles removed and extracted as in the Materials and Methods section. Zero time indicates that locusts were taken less than 12hr. after the adult had emerged. Each value is the mean of at least two individual animals in which the variation was less than 20%.

Age of hopper (days after fourth moult)	Glycerol kinase activity (μmole/min./g. fresh wt.)
5	0·15
8	0·20
Age of adult (days after final moult)	
0	0·31
1	0·32
2	0·32
4	0·39
5	0·47
7	0·46
9	0·49
14	0·52

of the insect. An exception to this difference between leg-muscle and flight-muscle glycerol kinase activities is the cockroach *Periplaneta*, in which the activity in the femoral muscle was about twice that in the flight muscle (see Table 2).

There was no detectable activity in the haemolymph of the locust, bumble bee, honey bee or blowfly *Phormia terranova*. The activity of glycerol kinase in the fat body of the locust was 0·23μmole/min./g., which is about half the activity in the flight muscle.

Effect of centrifugation on locust flight-muscle glycerol kinase. As shown in Table 3 approx. 66% of the glycerol kinase was sedimented at 6000*g*. After further centrifugation at 25000*g* for 45min. approx. 20% of the original activity still remained in the supernatant. This suggests that glycerol kinase in locust muscles exists in two forms, one more easily sedimentable than the other, but more work is needed to establish the subcellular localization of the particulate glycerol kinase. The fact that the activity was slightly increased by ultrasonic treatment suggests that some activity may reside in the mitochondria. The slight increase in activity observed after ultrasonic treatment can also be obtained by extracting the muscle in 1% potassium chloride.

This particulate preparation (i.e. 6000*g* pellet) was used for a number of studies on the properties of locust flight-muscle glycerol kinase; one advantage of this preparation is that it is possible by repeated washings and centrifugations to lower the concentration of small molecules present in the crude tissue extract (e.g. glycerol, AMP).

Glycerol kinase activity during development of the locust. The activity of glycerol kinase in locust flight muscle increases with development of the muscle (Table 4). The activity at the earliest time at which the muscles were removed (5 days after the fourth moult) was 0·15μmole/min./g. fresh wt. and this increased to 0·2μmole/min./g. at 8 days: there was a 50% increase in this activity during the final moult and a further increase of approx. 70% during the later stages of development in the adult insect.

Inhibition of glycerol kinase by AMP, ADP and L-3-*glycerophosphate.* The activities of muscle glycerol kinases from a variety of animals were inhibited by L-3-glycerophosphate, AMP and ADP (Table 5). In general, the inhibitions by L-3-glycerophosphate were similar for the different animals, whereas the inhibitions by AMP varied from 16 to 75%; it is possible, however, that this variation represents different capacities of the

Table 5. *Effect of* L-*3-glycerophosphate, ADP and AMP on muscle glycerol kinase activities from various animals*

Activities were measured as described in the Materials and Methods section; in Expts. 1 and 2 the incubation was for 2 min., in Expt. 3 for 4 min. and in all other experiments for 6 min. Concentrations of [^{14}C]glycerol, ATP and Mg^{2+} were 1·0 mM, 5·0 mM and 5·0 mM respectively.

Expt. no.	Animal	Muscle	Glycerol kinase activity (c.p.m. incorporated into product/min./20 μl. of extract)				Inhibition (%) by		
			No addition	5 mM-L-3-Glycerophosphate	10 mM-AMP	10 mM-ADP	L-3-Glycerophosphate	AMP	ADP
1	Bumble bee (*Bombus hortorum*)	Flight	37300	10100	19000	22500	73	49	41
2	Blowfly (*Phormia terranova*)	Flight	14200	3100	3600	6800	79	75	53
3	Cockchafer (*Melolontha melolontha*)	Flight	68400	28500	58300	60500	58	16	11
4	Locust (*Locusta migratoria*)	Flight	15600	7800	7100	10200	51	56	37
		Femoral	2700	500	700	1600	83	75	42
5	Blowfly (*Sarcophaga barbata*)	Flight	30200	12500	8100	13700	58	72	54
6	Cockroach (*Periplaneta americana*)	Flight	8700	5600	5300	—	36	39	—

muscle extracts to break down AMP, rather than different sensitivities of glycerol kinase to AMP. The inhibitory effects of AMP were slightly greater than those observed with ADP (Table 5). The effect of other nucleotide monophosphates and adenosine were tested on locust particulate glycerol kinase: at concentrations of 5 mM they produced less than 10% inhibition of the enzyme.

The effects of increasing the concentrations of AMP and ADP on glycerol kinase of the particulate preparation from locust flight muscle are shown in Table 6. The muscle enzyme appears to be less sensitive to the effects of AMP and ADP than is the enzyme from rat liver (see Robinson & Newsholme, 1969), although the assays were not performed under identical conditions.

The sensitivity of the locust flight-muscle particulate glycerol kinase to L-3-glycerophosphate is shown in Table 7 and double-reciprocal plots are shown in Fig. 1; the inhibition by L-3-glycerophosphate is competitive with respect to glycerol (similar to the rat liver enzyme; Robinson & Newsholme, 1969) with a K_i value of $4{\cdot}0 \times 10^{-4}$M. The K_m for glycerol is $3{\cdot}7 \times 10^{-4}$M.

DISCUSSION

The high sensitivity of the radiochemical assay and the presence of an ATP-regenerating system (to lower the ADP and AMP concentrations) in the assay system have been contributing factors that have enabled glycerol kinase activity to be detected in muscle. One problem with the radiochemical assay is the possibility that the retention of radioactivity on DEAE-cellulose-paper disks does not represent glycerol kinase activity; the reasons for considering that this retention does represent glycerol kinase activity are given in the Results section. This is supported by the fact that the properties of the muscle glycerol kinase are qualitatively very similar to those of the enzyme from rat liver, and the latter enzyme has been purified and its activity assayed by several different methods. Thus AMP, ADP and L-3-glycerophosphate inhibit muscle glycerol kinase activity, and the L-3-glycerophosphate inhibition is competitive with respect to glycerol (Fig. 1) (for properties of the enzyme from rat liver see Robinson & Newsholme, 1969).

The question arises as to the metabolic significance of glycerol kinase in muscle. In vertebrates glycerol kinase activity is generally found to be higher in red muscle than in white muscle, which suggests that the enzyme activity may be related to metabolism that provides energy for sustained muscular activity. In red muscle oxidation of triglyceride (or fatty acids) appears to be more important than oxidation of carbohydrate for

Table 6. *Effects of ADP and AMP on the activity of particulate glycerol kinase from locust flight muscle*

The preparation of particulate glycerol kinase and the assay of glycerol kinase are as described in the Materials and Methods section. The Mg^{2+} concentration was 5·0mM in Expt. 1 and 25·0mM in Expt. 2.

Expt. no.	Concn. of ADP (mM)	Glycerol kinase activity (c.p.m. incorporated into product/3 min./ 20μl. of homogenate)	Inhibition of glycerol kinase (%)	Concn. of AMP (mM)	Glycerol kinase activity (c.p.m. incorporated into product/3 min./ 20μl. of homogenate)	Inhibition of glycerol kinase (%)
1	0	35800	—	0	35800	—
	1	27200	24	1	23200	35
	2	26300	26	2	22900	36
	5	23700	34	5	20100	44
	10	22500	37	10	19500	46
	20	14000	61	20	17600	51
2	0	29400	—	0	29400	—
	1	27900	5	1	22100	25
	2	27100	8	2	19800	33
	5	22400	24	5	15100	49
	10	19500	34	10	10500	64
	20	14100	52	20	8350	72

Table 7. *Effect of* L-3-*glycerophosphate on the activity of particulate glycerol kinase from locust flight muscle*

Concn. of L-3-glycerophosphate (mM)	Glycerol kinase activity (c.p.m. incorporated into product/3min./ 20μl. of homogenate)	Inhibition by L-3-glycerophosphate (%)
0	43500	—
0·2	36500	16
0·5	26700	38
1·0	19300	56
5·0	7200	83
10·0	3400	92

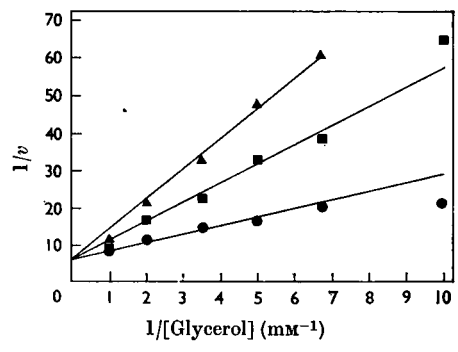

Fig. 1. Double-reciprocal plots of particulate glycerol kinase activity of locust flight muscle against glycerol concentration in the presence and absence of L-3-glycerophosphate. The method of assay and concentrations of ATP and Mg^{2+} were as described in the Materials and Methods section. The activity, v, is expressed as μmoles of L-3-glycerophosphate formed/min./g. fresh wt. of original muscle. ●, In the absence of L-3-glycerophosphate; ■, in the presence of 5·0mM-L-3-glycerophosphate; △, in the presence of 10·0mM-L-3-glycerophosphate.

energy production, whereas white muscle depends on glycolysis from glucose or glycogen (see George & Jyoti, 1955; George & Scaria, 1958; Bilinski, 1963; George & Berger, 1966; Denton & Randle, 1967). For energy formation in red muscle the triglyceride is hydrolysed to glycerol and fatty acids and the latter are oxidized by the mitochondria to provide energy. The fate of the glycerol that is produced from lipolysis is not known, but the presence of glycerol kinase in this type of muscle suggests that the function of this enzyme is to convert at least some of this glycerol into L-3-glycerophosphate.

On the basis of glycerol kinase activities, insect muscles can be divided arbitrarily into three groups (see Table 2): muscles that have a low glycerol kinase activity, i.e. $<0·3\mu$mole/min./g. (e.g. leg muscles of all insects studied and flight muscles of the cockroaches and tsetse fly); muscles that have an intermediate glycerol kinase activity, i.e. 0·3–1·5μmoles/min./g. (e.g. locusts, cockchafer, some blowflies, moths, water-bugs); and muscles that have a high glycerol kinase activity, i.e. $>1·5\mu$-

moles/min./g. (e.g. bees, wasps, some blowflies). The low activity of glycerol kinase in the 'low-activity' muscles is probably an indication that these muscles use little or no triglyceride or diglyceride for energy production: thus the flight muscles of the tsetse fly may use proline (Bursell, 1966) and those of the cockroaches may use carbohydrate (see Sacktor, 1964). It is considered that the role of this enzyme in the 'intermediate-activity' muscles is similar to that proposed for vertebrate muscles. Thus the flight muscles of locusts and moths are known to oxidize fats (see Sacktor, 1964), and more recently it has been shown that diglyceride, which is present in high concentrations in insect haemolymph, provides an important fuel for oxidation by the flight muscles of a number of insects (for review see Gilbert, 1967). This diglyceride is hydrolysed by a muscle diglyceride lipase (Gilbert, 1967; B. F. Crabtree & E. A. Newsholme, unpublished work) to fatty acids and glycerol; thus the role of glycerol kinase in some insect muscles would appear to be the removal of glycerol produced from lipolysis of muscle triglyceride or haemolymph diglyceride or both.

However, this function of glycerol kinase cannot explain the high activities of the enzyme in flight muscles of the bee, wasp and some flies. These muscles are considered to be almost exclusively dependent on carbohydrate metabolism for energy production (see Sacktor, 1964); and Childress, Sacktor & Traynor (1966) found that with isolated mitochondria from bee and blowfly (*Phormia regina*) the oxidation of palmitoylcarnitine was extremely low. It therefore seems unlikely that the high activity of glycerol kinase in these muscles is related to tri- or di-glyceride lipolysis. One common factor in the metabolism of the flight muscle of these insects is the high rate of glycolysis; as there is almost no lactate dehydrogenase in these muscles, the NADH produced from glycolysis must be oxidized via the L-3-glycerophosphate cycle (see Sacktor, 1964) and formation of L-3-glycerophosphate by action of the extramitochondrial glycerophosphate dehydrogenase is of obvious importance. If at any stage of flight (e.g. at the start of flight) the rate of mitochondrial oxidation of L-3-glycerophosphate was less than the activity of glycerophosphate dehydrogenase, then this compound would accumulate in the muscle. But, as glycerophosphate dehydrogenase is inhibited by L-3-glycerophosphate (Blanchaer, 1965), any large accumulation in muscle would inhibit the oxidation of extramitochondrial NADH and inhibit glycolysis. It is therefore suggested that under such conditions L-3-glycerophosphate would be hydrolysed to glycerol (by the action of a specific glycerophosphatase), so that glycerophosphate dehydrogenase activity and therefore the glycolytic rate would be maintained. This glycerol would eventually have to be removed from the muscles, which would explain the presence of high activities of glycerol kinase in these muscles and their inhibition by physiological concentrations of L-3-glycerophosphate. If this hypothesis is correct, then glycerol formation in these muscles would play a somewhat similar role to lactate formation in vertebrate muscles, but the glycerol would be removed by the muscle through glycerol kinase activity when the mitochondrial oxidation of L-3-glycerophosphate could take place.

In comparison with the activities of enzymes of glycolysis or the tricarboxylic acid cycle, that of glycerol kinase in either vertebrate or invertebrate muscle is low (e.g. in the locust the activities of glycerol kinase, hexokinase and isocitrate dehydrogenase are 0·5, 8 and 26 μmoles/min./g. fresh wt. respectively; B. F. Crabtree & E. A. Newsholme, unpublished work). However, the important point is that in the lipolysis of triglyceride 1 μmole of glycerol and 3 μmoles of fatty acid result, whereas from diglyceride 1 μmole of glycerol and 2 μmoles of fatty acid result. Assuming that the fatty acid is palmitic acid, 24 (triglyceride) or 16 (diglyceride) μmoles of acetyl-CoA are produced for 1 μmole of glycerol. Although the activity of glycerol kinase is low, so is the concentration of glycerol produced from tri- or di-glyceride relative to the amount of acetyl-CoA that the fatty acid molecules produce on oxidation. Thus with a diglyceride, for 1 μmole of glycerol, 2 μmoles of palmitic acid would require 46 μmoles of oxygen via β-oxidation and the tricarboxylic acid cycle. Hence a glycerol kinase activity in the muscle of 1 μmole/min./g. fresh wt. would be required to dispose of the glycerol associated with an oxygen uptake of 46 μmoles/min./g. (or 69 μmoles/min./g. from lipolysis of triglyceride). The oxygen uptake of locust (*Schistocerca gregaria*) flight muscle during flight is approx. 60 μmoles/min./g. fresh wt. of muscle (Weis-Fogh, 1952); if this was due solely to oxidation of diglyceride it would require a glycerol kinase activity of 1·3 μmoles/min./g. Similarly the oxygen uptake by the flight muscles of a moth was approx. 150 μmoles/min./g. (Zebe, 1954) and this would require a glycerokinase activity of 3 μmoles/min./g. This suggests that, if glycerol kinase activity reflects the rate of diglyceride hydrolysis, from the enzyme activities present in Table 2 this hydrolysis could account for only 30–40% of the oxygen uptake by the muscles during flight. These calculations suggest either that glycerol kinase activity is less active than diglyceride (or triglyceride) lipase and that there is a release of glycerol by these muscles during flight, or that substrates other than diglyceride (and triglyceride) are used by these muscles during flight.

It is noteworthy that glycerol kinase, which converts glycerol into L-3-glycerophosphate, provides a common link between carbohydrate and triglyceride metabolism; and it seems possible that this link could furnish the basis for a control point in the regulation of fatty acid oxidation in relation to the availability of carbohydrate. Thus depletion of carbohydrate stores might lower the intracellular concentration of L-3-glycerophosphate, which would increase the activity of glycerol kinase, and therefore lower the intracellular glycerol concentration; and this, in turn, might increase the activity of diglyceride (or triglyceride) lipase.

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