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Activities of Citrate Synthase and NAD$^+$-Linked and NADP$^+$-Linked Isocitrate Dehydrogenase in Muscle from Vertebrates and Invertebrates

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1. The activities of citrate synthase, NAD$^+$-linked and NADP$^+$-linked isocitrate dehydrogenase were measured in muscles from a large number of animals, in order to provide some indication of the importance of the citric acid cycle in these muscles. According to the differences in enzyme activities, the muscles can be divided into three classes. First, in a number of both vertebrate and invertebrate muscles, the activities of all three enzymes are very low. It is suggested that either the muscles use energy at a very low rate or they rely largely on anaerobic glycolysis for higher rates of energy formation. Second, most insect flight muscles contain high activities of citrate synthase and NAD$^+$-linked isocitrate dehydrogenase, but the activities of the NADP$^+$-linked enzyme are very low. The high activities indicate the dependence of insect flight on energy generated via the citric acid cycle. The flight muscles of the beetles investigated contain high activities of both isocitrate dehydrogenases. Third, other muscles of both vertebrates and invertebrates contain high activities of citrate synthase and NADP$^+$-linked isocitrate dehydrogenase. Many, if not all, of these muscles are capable of sustained periods of mechanical activity (e.g. heart muscle, pectoral muscles of some birds). Consequently, to support this activity fuel must be supplied continually to the muscle via the circulatory system which, in most animals, also transports oxygen so that energy can be generated by complete oxidation of the fuel. It is suggested that the low activities of NAD$^+$-linked isocitrate dehydrogenase in these muscles may be involved in oxidation of isocitrate in the cycle when the muscles are at rest. 2. A comparison of the maximal activities of the enzymes with the maximal flux through the cycle suggests that, in insect flight muscle, NAD$^+$-linked isocitrate dehydrogenase catalyses a non-equilibrium reaction and citrate synthase catalyses a near-equilibrium reaction. In other muscles, the enzyme-activity data suggest that both citrate synthase and the isocitrate dehydrogenase reactions are near-equilibrium.

The citric acid cycle is the most important metabolic pathway for energy production under aerobic conditions. The hydrogens removed during the cycle are transferred to the electron-transport chain, and the energy released during electron transport is conserved by the formation of ATP. The energy that is formed during the oxidation of glucose or fatty acids in the cycle is important in muscle to support sustained mechanical activity. Nonetheless, a detailed investigation into the maximum activities of the enzymes that catalyse key reactions in the cycle in muscle has not been undertaken. This paper reports the results of a comparative investigation into the activities of enzymes that catalyse reactions early in the cycle, citrate synthase (EC 4.1.3.7), NAD$^+$-linked isocitrate dehydrogenase (EC 1.1.1.41) and NADP$^+$-linked isocitrate dehydrogenase (EC 1.1.1.42). It is considered that this study is important in providing information on the maximum rate of operation of the cycle in relation to other studies on the maximal rates of glycolysis and fatty acid oxidation (Crabtree & Newsholme, 1972*a*,*b*).

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Materials and Methods

Chemicals and enzymes

All chemicals and enzymes were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K., except for the following: acetyl-CoA (grade II), 5,5′-dithiobis-(2-nitrobenzoic acid), *threo*-D$_s$(+)-isocitrate and DL-isocitrate (trisodium salt) were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K.; antimycin A (B grade) and oxaloacetic acid (A grade) were obtained from Calbiochem, London W1H 1AS, U.K.; sodium citrate and all inorganic chemicals were obtained from BDH Chemicals, Poole, Dorset BH12 4NN, U.K.

Sources of animals

Most marine invertebrates were obtained from the University Marine Biology Station, Millport, Isle of Cumbrae, U.K., or from the Marine Biological Association, Citadel Hill, Plymouth, Devon, U.K. Lobsters were obtained from Fisher Bros., 12 Billingsgate Street, London E.C.3, U.K. *Lepas antifera* were obtained from Malta. Waterbugs and rhinoceros beetles were sent from Uganda or Trinidad and kept in the tropical room before use. Locusts, cockroaches and blowflies were obtained from stocks maintained in the Department. All other insects were caught locally. Pig, sheep and ox muscle were obtained from a local slaughter-house. All other animals were obtained from sources given by Sugden & Newsholme (1973).

Preparation of homogenates

Muscles were dissected from vertebrates after they had been killed by decapitation or cervical fracture. Insects were pre-cooled for about 30min in a refrigerator at 4°C until they were sufficiently immobile for dissection and removal of muscle. Other invertebrates were treated as follows. Samples of *Metridium* basalar muscle were obtained by cutting the basal part of the column while the animals were attached to a smooth surface. The muscle obtained was washed with sea water and then distilled water to remove any contaminating non-muscle tissue. Polychaetes were anaesthetized with Sandoz MS-222, which was dissolved in the sea water; muscles were dissected from the animals under water. Crustacea were usually cooled in ice for about 15min before dissection. Bivalve shells were forced open, and muscles dissected. In prosobranchs, the muscle was dissected after crushing the shell of the animal, and exposing the soft parts. Radular muscles were obtained by dissecting out the muscle from the proboscis or buccal mass of the animals. Echinoids were cut in half horizontally, and the lantern retractor muscles dissected. *Thyone* pharyngeal retractors were obtained by irritating the animal either mechanically or by using Sandoz MS-222 in solution until the pharynx was everted with the muscles attached. The muscles were then dissected off the pharyngeal structure.

Muscles were dissected as rapidly as possible after death, and were cut into small pieces before homogenization. The muscle was homogenized in ground-glass homogenizers with 10–100vol. (insect muscles) or 5–20vol. (other muscles) of extraction medium. The extraction medium for citrate synthase consisted of 25mM-Tris/HCl/1mM-EDTA at pH7.4. For muscle from vertebrates and insects, the extraction medium for isocitrate dehydrogenase consisted of 50mM-Pipes* buffer; 10mM-$MgCl_2$, 5mM-mercaptoethanol, 1mM-EDTA, 5mM-$MnCl_2$, 2mM-ADP and 5M-glycerol, at pH7.8. For muscle from other invertebrates, the extraction medium consisted of 50mM-triethanolamine, 1mM-EDTA, 3mM-$MgCl_2$, 30mM-mercaptoethanol and 2mM-ADP, at pH7.4. All homogenates were sonicated for two 15s periods with the microprobe of an MSE sonicator operating at an amplitude of 6μm; the homogenate was cooled in ice/water during the sonication. Enzyme assays were carried out as soon as possible after sonication. Homogenates were centrifuged briefly at low speed (600*g*) to sediment cell debris, in order to minimize turbidity in the cuvette.

* Abbreviation: Pipes, piperazine-*NN'*-bis-(2-ethanesulphonic acid).

Assay of enzyme activities

Citrate synthase was assayed by following the rate of change of E_{412}, and other enzymes by following the change in E_{340} in a Gilford recording spectrophotometer (model 240) at 25°C.

Citrate synthase was assayed by a method similar to that described by Srere *et al.* (1963) (see Sugden & Newsholme, 1975). The assay medium contained 50mM-Tris/HCl, 0.2mM-5,5'-dithiobis-(2-nitrobenzoic acid), 0.1mM-acetyl-CoA and 0.5mM-oxaloacetate to which 2–50μl of homogenate was added. The final volume in the cuvette was 2ml. The enzyme from vertebrate muscles and insects was assayed at pH8.1, whereas that from other invertebrates was assayed at pH7.5. The assay was initiated by addition of oxaloacetate. Controls, from which oxaloacetate was omitted, were run concurrently; the rates of increase in extinction were less than 5% of the experimental rates.

The assay medium for NAD^+-linked isocitrate dehydrogenase consisted of 70mM-Tris/HCl, 2mM-NAD^+, 2mM-ADP, 1mM-$MnCl_2$, 8mM-$MgCl_2$, 1.5mM-*threo*-D_s(+)-isocitrate, 10mM-citrate (or 3.0mM-DL-isocitrate, 20mM-citrate), 5μg of antimycin A (added as 10μl of a 5%, w/v, solution in ethanol) and 10–25μl of homogenate. The final pH was 7.1, and the reaction volume was 2.0 ml. The reaction was initiated by the addition of citrate plus isocitrate. Controls, from which citrate plus isocitrate were omitted, were run concurrently.

The assay medium for $NADP^+$-linked isocitrate dehydrogenase was the same as that for NAD^+-linked enzyme, except for the replacement of NAD^+ by 0.5mM-$NADP^+$, omission of ADP and antimycin A, and the final pH was 7.5. The reaction was started by addition of citrate plus isocitrate. Controls, from which citrate plus isocitrate were omitted, were run concurrently.

Expression of results

All activities are expressed as μmol of substrate utilized/min per g fresh wt. of muscle, at 25°C. The

values reported represent the means of a number of determinations (on tissue from different animals), which are given, together with the range in parentheses, in Table 1. Since a systematic study of factors such as season, diet, sex or age of the animals was not attempted, precise quantitative interpretations based on these activities must be made with caution.

Results and Discussion

Control experiments on conditions of extraction and assay

The aim of this comparative study on the enzymes citrate synthase, NAD^+- and $NADP^+$-linked isocitrate dehydrogenase is to provide reliable information on the maximal activities of these enzymes from muscles of a large number of animals throughout the Animal Kingdom. One difficulty in such an analysis is the possibility of variation in the properties of the enzyme from one animal to another. To overcome totally such difficulties, a detailed analysis of the properties of the enzymes from each animal should be carried out. However, the amount of work involved would restrict the accumulation of data to a very few animals. In the present work, the effect of pH, temperature, extraction conditions and known activators have been investigated with the enzyme from muscles from selected animals representing the major phyla investigated. The muscles investigated in this control study were as follows: radular muscle of the whelk, leg muscle of swimming crab, flight muscle of the locust, flight muscle of the rosechafer (or other insect), red muscle of the dogfish, pectoral muscle of the pigeon and heart muscle of the rat (or rabbit) (see Tables 1 and 2 for systematic names).

Extraction of muscle

For citrate synthase assays, muscles were extracted in the simple medium described in the Materials and Methods section, and it was established that the activities in these extracts were stable, for up to 6h at 4°C. It was further established that use of other buffers (triethanolamine or Pipes) at pH7.0, 7.5 or 8.0 made no difference to the activity, and there was no effect of 10mM-$MgCl_2$, 5mM-2-mercaptoethanol, 5mM-$MnCl_2$, 2mM-ADP or 5M-glycerol in the extraction medium.

The activities of $NADP^+$-linked isocitrate dehydrogenase from the above tissues were found to be stable for up to 8h in the extraction media described in the Materials and Methods section. Although it was established that the enzyme could be extracted in a simple medium without loss of activity, both isocitrate dehydrogenases were extracted with the same medium, since this simplified the extraction and assay procedures. Goebell & Klingenberg (1964) reported that $NADP^+$-linked isocitrate dehydrogenase was stable for up to 40h in homogenate of locust flight muscle.

In contrast with the above results, the stability of NAD^+-linked isocitrate dehydrogenase was found to be variable. Goebell & Klingenberg (1964) reported that the enzyme is stable in medium containing 2mM-ADP for up to 20h. This was not observed in the present work. Cox & Davies (1967) reported that the enzyme from pea-seedling mitochondria was stable in 5M-glycerol. In the present work, it was established that the enzymes from the rosechafer, whelk and swimming crab were stable in the extraction medium in the absence of any compounds reported to stabilize the enzyme. Similarly, the enzyme from rat heart lost only 16% of activity after 10min at 4°C. The enzyme from the other muscles lost considerable activity over this period, and the addition of either ADP or isocitrate made little difference. However, the addition of 5M-glycerol prevented any loss of activity in extracts of these muscles for periods up to 60min. Glycerol had no effect on the activities of the enzymes from the heart of the rat or flight muscle of the rosechafer. In the present work both glycerol and ADP were included in the extraction buffer (see the Materials and Methods section) for all vertebrate and insect muscles. ADP was included in the extraction medium for marine invertebrate muscles, since it is an activator of the enzyme (see below). In addition, activities of NAD^+-linked isocitrate dehydrogenase were always measured within 4min of extraction and sonication.

Preliminary experiments established that sonication for 30s produced maximal activities of all three enzymes in the above muscles. Sonication for a further 15 or 30s of either a crude muscle extract or the supernatant from a high-speed centrifugation (30min at 26000*g*) of sonicated mitochondria from these muscles had no effect on the enzyme activities. Other procedures for breaking the mitochondria (use of Triton and freeze–thawing procedures) gave lower activities than sonication.

Assay conditions

To obtain enzyme activities that were directly comparable, it was decided to measure the activities of the enzymes from all muscles at 25°C, despite the fact that the temperature may be greater than the normal temperature for poikilotherms and lower than that of homoiotherms (although it should be pointed out that the sea temperature can be as high as 21–22°C in the summer and the body temperature of marine invertebrates may be higher than this if they are present in pools on the beach at low tide). The temperature of the thorax of many insects during flight is approximately 30°C. The effect of temperature between 10° and 38°C has been investigated on

Table 1. *Effect of temperature on the activities of citrate synthase and NAD⁺- and NADP⁺-linked isocitrate dehydrogenases in muscle from various animals*

Enzyme activities were measured as described in the Materials and Methods section. Activities are presented as means of measurements on extracts from four different animals, and the ranges of activities are given in parentheses. * indicates that the activities were measured at 25°C rather than 30°C.

Animal	Muscle	Enzyme activities (μmol/min per g of fresh muscle)											
		Citrate synthase				NAD⁺–isocitrate dehydrogenase				NADP⁺–isocitrate dehydrogenase			
		10°C	20°C	30°C	38°C	10°C	20°C	30°C	38°C	10°C	20°C	30°C	38°C
Common whelk (*Buccinum undatum*)	Radular retractor	9.4 (8.6–9.7)	12.9 (11.5–13.9)	19.8 (18.6–20.8)	—	1.4 (1.3–1.6)	3.8 (3.0–4.4)	8.0 (7.6–8.2)	—	1.5 (1.2–1.7)	5.4 (5.2–5.5)	16.2 (15.6–17.7)	—
Swimming crab (*Portunus puber*)	Leg	2.6 (2.3–2.8)	3.8 (3.6–4.1)	4.8* (4.5–5.1)	—	0.2 (0.2)	0.5 (0.4–0.5)	0.9* (0.8–1.0)	—	1.4 (1.2–1.5)	3.9 (2.9–4.9)	6.2* (5.5–6.5)	—
Locust (*Schistocerca gregaria*)	Flight	—	191 (93–270)	223 (107–324)	239 (142–363)	—	5.1 (2.7–9.5)	11.6 (7.2–19.2)	18.2 (9.3–28.5)	—	2.8 (1.7–3.9)	7.4 (5.5–12.3)	11.4 (4.3–20.3)
Rosechafer (*Pachnoda* sp.)	Flight	—	109 (61–159)	131 (81–183)	132 (96–200)	—	27.0 (17.6–39.2)	40.8 (28.7–58.1)	67.6 (53.6–81.3)	—	3.4 (1.9–5.4)	5.8 (3.8–8.1)	10.5 (5.7–16.9)
Dogfish (*Scyliorhinus canicula*)	Heart	14.1 (11.3–15.6)	20.3 (18.3–21.7)	30.1 (28.7–32.4)	—	<0.05	<0.05	<0.05	—	8.7 (8.0–9.3)	25.1 (24.1–26.3)	62.3 (56.1–68.4)	—
Pigeon (*Columba livia*)	Pectoral	—	95 (67–123)	116 (81–187)	205 (115–392)	—	1.1 (0.6–1.7)	2.1 (1.0–2.9)	3.8 (1.8–5.9)	—	32.1 (15.6–51.1)	48.9 (30.4–61.6)	77.9 (61.2–109)
Laboratory rat	Heart	—	105 (95–109)	124 (91–152)	143 (103–188)	—	3.1 (2.8–3.3)	6.4 (5.0–8.1)	9.9 (8.7–11.8)	—	27.1 (23.2–31.4)	56.2 (43.8–63.8)	83.1 (67.8–92.2)

the enzyme activities for the muscles indicated above. The results are reported in Table 1. The values of the Q_{10} for each enzyme from the different muscles investigated were similar. Thus for citrate synthase the values of the Q_{10} for the poikilotherms between 10–20°C and 20–30°C are about 1.5 and for the insects and homoiotherms between 20–30°C and 30–38°C are about 1.2; for NAD⁺-linked isocitrate dehydrogenase the values of the Q_{10} for poikilotherms are about 2.4 and for insects and homoiotherms the values are about 1.8; for NADP⁺-linked isocitrate dehydrogenase the values of the Q_{10} for poikilotherms are about 2.7, but the values for insects and the homoiotherms are somewhat lower (the range is 1.5–2.6). These results suggest that, in these animals, there is no unusual effect of temperature, so that comparison of activities at 25°C is satisfactory.

The pH optimum for citrate synthase from pig heart is reported to be 8.5 (Kosicki & Srere, 1961). The pH optimum for locust and fleshfly flight-muscle citrate synthase was found to be 8.1 (results not given), whereas the pH optimum for the enzyme from the vertebrate muscles varied between 8.1 and 8.5. The pH optimum for the enzyme from the non-insect invertebrates varied between 7.5 and 8.0. In the comparative work, the activities of citrate synthase from vertebrate and insect muscles have been measured at pH8.1. The activities for the marine invertebrates have been measured at pH7.5. The largest difference in activity between pH7.5 and pH8.0 for the marine invertebrates and between pH8.0 and 8.5 for the vertebrates and insects in the animals investigated was no more than 15%.

The pH optimum of NAD⁺-linked isocitrate dehydrogenase from locust flight muscle is 7.1 in the presence of ADP (Klingenberg *et al.*, 1965). The pH optimum for the enzyme from other insect flight muscles in the presence of ADP is 6.9 (Ku & Cochran, 1971). In the present work, the pH optimum for the enzyme from the muscles studied varied between 7.0 and 7.2. Consequently, the pH of 7.1 was selected for assay of this enzyme.

The NADP⁺-linked isocitrate dehydrogenase from heart has been reported to have a rather broad pH optimum around 7.8 (Plaut & Sung, 1955). The pH optimum for the enzyme from the above muscles varied between 7.5 and 7.8. The pH of 7.5 was selected for the assay of this enzyme.

Activators and inhibitors of the enzymes

Citrate synthase is inhibited by ATP (Hathaway & Atkinson, 1965; Kosicki & Lee, 1966), but the concentrations of ATP in the extract added to the cuvette would produce a concentration of 2 μM, which is well below any reported K_i values.

NAD⁺-linked isocitrate dehydrogenase is inhibited by low concentrations of Ca^{2+}, and activated by ADP.

However, these inhibitory and activating effects are removed by high concentrations of the isocitrate (see Chen & Plaut, 1963; Goebell & Klingenberg, 1964; Vaughan & Newsholme, 1969; Zammit & Newsholme, 1976). Consequently 2mM-ADP was included in the assay medium, and the concentration of isocitrate, 1.5mM, was sufficient to overcome any effect of Ca^{2+} [see Zammit & Newsholme (1976) for detailed comparative studies of the properties of NAD^+-linked isocitrate dehydrogenase].

There are no known activators or inhibitors of $NADP^+$-linked isocitrate dehydrogenase.

Substrate concentrations in the assays

The K_m values of citrate synthase from different muscles for the two substrates have been measured by the secondary-plot procedure, and the results are as follows: the K_m values for oxaloacetate and acetyl-CoA for the enzyme from locust flight muscle are 12 and 5μM respectively; the values for the enzyme from the blowfly (*Sarcophaga barbata*) flight muscle are 4 and 1μM; and the values for the enzyme from pigeon pectoral muscle are 3 and 5μM [see Alp (1973) for details]. It seems likely, therefore, that the concentrations of 0.1mM-acetyl-CoA and 0.5mM-oxaloacetate, which are present in the assay medium, will provide maximal enzyme activities.

The responses of NAD^+-linked isocitrate dehydrogenase to isocitrate and NAD^+ concentrations from vertebrate and invertebrate muscles (e.g. rat heart, pigeon pectoral, locust and waterbug flight and whelk radular muscles) have been studied in detail (see Vaughan, 1967; Vaughan & Newsholme, 1969; Zammit & Newsholme, 1976). The results of these studies show that the concentrations of substrates used in the present work (2mM-NAD^+ and 1.5mM-isocitrate) are sufficient to provide maximal activities of this enzyme. Similar studies with the $NADP^+$-linked enzyme from rat heart, pigeon pectoral, cockchafer flight and whelk radular muscles indicate that the concentrations of $NADP^+$ and isocitrate used in the present work (0.5mM-$NADP^+$ and 1.5mM-isocitrate) are sufficient to provide maximal activities of the enzyme.

Comparative studies

For 54 species of animals from several phyla, the activities of citrate synthase, NAD^+- and $NADP^+$-linked isocitrate dehydrogenase show considerable variation (Table 2). Activities of citrate synthase range from 0.3 to 561μmol/min per g fresh wt. (catch muscle of the great scallop and flight muscle of the rosechafer respectively). Activities of NAD^+-linked isocitrate dehydrogenase range from not detectable (i.e. <0.1μmol/min per g) in many muscles to 169μmol/min per g (blowfly flight muscle). Activities of the $NADP^+$-linked enzyme range from 0.2 to 143μmol/min per g (catch muscle of the common oyster and flight muscle of the cockchafer respectively). The results indicate that certain muscles can be classified into groups that have similar enzyme activities. These groups are presented below with possible explanations for these similarities (and differences from other groups).

(1) *Very low activities of the three enzymes.* The activities of all three enzymes are very low (about 1μmol/min per g) in a number of marine invertebrate muscles (Table 2). This indicates that the muscles obtain little energy from aerobic metabolism, and that the muscles either utilize energy at a low rate and/or they depend on anaerobic metabolism of glycogen for high rates of energy production. The adductor muscles of bivalves (e.g. scallops) may utilize both processes for energy formation; glycolysis may be used for rapid energy production during closure of the shells, whereas only a low rate of energy production is required by the catch muscles for maintaining closure of the shells (Rüegg, 1971). The abdominal flexor muscle of the lobster is known to use anaerobic glycolysis and arginine phosphate breakdown to provide energy for the short, but violent, bursts of contraction, characteristic of these muscles (see Zammit, 1974). Low activities of these enzymes are also observed in a number of vertebrate muscles (e.g. white muscles of the fish, the pectoral muscles of the domestic fowl and pheasant), although the individual activities may be slightly higher than those of the marine invertebrates. These muscles have high activities of phosphorylase and the glycolytic enzymes, and appear to depend on the conversion of glycogen into lactate for energy production for contraction (see Crabtree & Newsholme, 1972*a*). It is possible that the low activities of enzymes of the cycle are required for energy formation during rest.

(2) *High activities of citrate synthase and NAD^+-linked isocitrate dehydrogenase.* In most of the insect flight muscles, the activities of both citrate synthase and NAD^+-linked isocitrate dehydrogenase are high (usually >100μmol/min per g of muscle). However, the activities of the $NADP^+$-linked enzyme are low [<20μmol/min per g (see Table 2)]. The high activities indicate that insect flight muscles depend on aerobic metabolism for energy production to support flight. The power output by the insect during flight is extremely high (Weis-Fogh, 1952), and, in order to provide sufficient energy for sustained flight, the substrates used by the muscles must be totally oxidized. The low activities of $NADP^+$-linked isocitrate dehydrogenase suggest that, in insect flight muscles, most of the isocitrate dehydrogenation is catalysed by the NAD^+-linked enzyme. However, this situation is not characteristic of all insect flight muscles nor of all insect tissues. Thus flight muscles of the beetles investigated (i.e.

Table 2. *Activities of citrate synthase, NAD$^+$-linked and NADP$^+$-linked isocitrate dehydrogenase in muscles from vertebrates and invertebrates*

Enzyme activities are presented as means, and the ranges of activities are given in parentheses. The numbers of animals used for each mean activity value are given in parentheses below the citrate synthase data. Details of enzyme assays are given in the Materials and Methods section. The maximum flux through the citric acid cycle has been estimated from oxygen-uptake data, as explained by Crabtree & Newsholme for glycolysis (1972*a*) or fatty acid oxidation (1972*b*). Fluxes have also been estimated from hexokinase activities reported by Crabtree & Newsholme (1972*a*) (Zammit, 1974).; it is assumed that, in any given muscle, hexokinase activity will not greatly exceed the maximum rate at which glucose can be provided via the circulatory system, so that, if glucose is provided, oxygen will also be provided. Consequently it would be expected that the total glucose removed by the muscle should be oxidized, i.e. the maximum rate of glucose phosphorylation should indicate the maximum rate of the cycle. This latter method of estimation is denoted by an asterisk.

			Enzyme activities (μmol/min per g fresh weight of muscle at 25°C)		
				Isocitrate dehydrogenase	
Animal	Muscle	Maximal flux through the cycle	Citrate synthase	NAD$^+$-linked	NADP$^+$-linked
Coelenterata (Anthozoa) Sea anemone, *Metridium senile*	Basilar	—	1.2 (1.2, 1.2) (2)	<0.1	0.7 (0.5, 0.8)
Annelida (Polychaeta) Sea mouse, *Aphrodite aculeata*	Longitudinal	—	0.4 (0.4, 0.4) (2)	<0.1	1.7 (1.7, 1.7)
Ragworm, *Nereis viriens*	Dorsal longitudinal	—	4.5 (4.1–5.1) (3)	1.1 (1.0–1.2)	1.9 (1.2–2.8)
Mollusca (Bivalvia) Great scallop, *Pecten maximus*	Phasic adductor	—	1.5 (1.2–2.1) (5)	<0.1	3.3 (2.8–3.8)
	Catch adductor	—	0.3 (0.2–0.5) (4)	<0.1	0.3 (0.3–0.5)
Variegated scallop, *Chlamys varius*	Phasic adductor	—	1.7 (1.1–2.7) (3)	<0.1	2.8 (2.3–3.4)
	Catch adductor	—	1.6 (0.8–2.0) (4)	<0.1	2.2 (1.8–2.6)
Venus striatula	Phasic adductor	—	7.6 (5.5–9.7) (3)	0.8 (0.6–1.2)	1.4 (1.3–1.5)
Common oyster, *Ostrea edulis*	Phasic adductor	—	1.8 (1.6–1.9) (3)	<0.1	1.0 (0.6–1.5)
	Catch adductor	—	1.0 (0.8–1.1) (3)	<0.1	0.2 (0.1–0.2)
Sand gaper, *Mya arenaria*	Adductor	—	6.9 (5.5–9.1) (5)	0.2 (0.2)	3.2 (2.1–4.7)
Razor clam, *Ensis ensis*	Pedal retractor	—	0.5 (0.4–0.6) (3)	<0.1	1.2 (0.7–1.9)
Periwinkle, *Littorina littorea*	Pedal retractor	—	1.3 (1.2–1.4) (3)	0.1 (0.1)	3.1 (2.5–5.0)
Thick top-shell, *Monodonta lineata*	Pedal retractor	—	0.9 (0.7–1.1) (3)	0.8 (0.3–0.8)	1.1 (0.6–1.6)

Table 2—*continued*

Animal	Muscle	Maximal flux through the cycle	Enzyme activities (μmol/min per g fresh weight of muscle at 25°C)		
				Isocitrate dehydrogenase	
			Citrate synthase	NAD^+-linked	$NADP^+$-linked
Common whelk, *Buccinum undatum*	Radular retractor	7.4*	24.8 (21.7–26.8) (3)	9.8 (7.2–11.5)	18.8 (15.1–23.0)
Common limpet, *Patella vulgata*	Radular retractor	6.2*	26.8 (24.5–29.1) (3)	1.5 (1.0–2.0)	108.0 (81.0–126.2)
Winkle, *Murex trunculus*	Radular retractor	31.0*	39.0 (35.7–42.2) (3)	18.4 (12.4–25.1)	18.1 (12.5–20.7)
Arthropoda (Crustaceae) Lobster, *Homarus vulgaris*	Deep abdominal flexor	—	1.0 (0.9–1.4) (5)	<0.1	1.1 (0.8–1.6)
	Claw adductor	—	1.1 (0.8–1.3) (3)	<0.1	1.1 (0.9–1.3)
Squat lobster, *Galathea squamifera*	Abdominal	—	3.4 (3.2–3.5) (3)	<0.1	3.5 (2.8–4.3)
Swimming crab, *Portunus puber*	Leg	—	7.0 (5.8–7.3) (3)	1.0 (0.9–1.1)	6.5 (4.8–6.9)
Edible crab, *Cancer pagarus*	Claw adductor	—	2.4 (1.7–3.2) (3)	<0.1	3.0 (2.4–3.4)
Pedunculate barnacle, *Lepas antifera*	Closer	—	1.2 (0.7–2.2) (5)	<0.1	2.3 (1.3–3.0)
Horse-shoe crab (Chelicerata), *Limulus polyphemus*	Leg	—	17.4 (15.0–21.3) (3)	0.6 (0.6)	10.2 (7.5–12.4)
(Insecta)					
Eyed hawk moth, *Smerinthus ocellata*	Flight	—	338 (262–465) (3)	108 (82–133)	18 (13–20)
Poplar hawk moth, *Laothoe populi*	Flight	—	430 (324–492) (3)	92 (65–110)	17 (17–17)
Yellow underwing moth, *Noctua pronuba*	Flight	—	396 (376–416) (3)	167 (127–207)	12 (11–12)
Silver-Y moth, *Plusia gamma*	Flight	26	352 (338–365) (3)	118 (114–121)	11 (10–12)
Peacock butterfly, *Vanessa io*	Flight	38	390 (379–401) (3)	110 (101–120)	9.2 (8.5–9.8)
Small tortoise-shell butterfly, *Vanessa urticae*	Flight	—	204 (186–219) (3)	72 (69–74)	7.3 (6.1–8.4)

Table 2—*continued*

Animal	Muscle	Maximal flux through the cycle	Enzyme activities (μmol/min per g fresh weight of muscle at 25°C)		
				Isocitrate dehydrogenase	
			Citrate synthase	NAD^+-linked	$NADP^+$-linked
Queen wasp, *Vespula vulgaris*	Flight	—	332 (320–344) (3)	109 (68–143)	7.4 (5.0–9.9)
Honey bee, worker, *Apis mellifera*	Flight	—	346 (344–347) (3)	94 (87–100)	4.0 (3.4–4.5)
Bumble bee, *Bombus hortorum* queen	Flight	—	325 (286–384) (4)	102 (82–122)	4.9 (4.5–5.9)
worker	Flight	—	382 (357–390) (4)	147 (128–167)	6.1 (5.0–8.0)
male	Flight	—	349 (332–376) (4)	111 (101–121)	4.7 (4.1–5.2)
Cuckoo bumble bee, *Psithyrus* sp.	Flight	—	447 (251–623) (4)	134 (103–203)	6.4 (4.7–7.6)
Giant water bug, *Lethocerus cordofanus*	Flight	—	244 (210–278) (3)	86 (76–95)	18.3 (16.9–19.7)
Cockroach, *Periplaneta americana* (male)	Flight	30	185 (151–219) (3)	74 (73–74)	3.1 (2.5–3.6)
	Femoral	—	59 (43–64) (3)	18 (15–20)	1.9 (1.8–2.0)
Locust, *Schistocerca gregaria* (male)	Flight	28	242 (223–261) (3)	58 (47–65)	4.0 (3.6–4.4)
	Femoral	—	46 (45–46) (3)	11 (8.7–14)	1.1 (1.0–1.2)
Blowfly, *Calliphora erythrocephala*	Flight	118	418 (382–444) (3)	169 (161–177)	7.9 (7.6–8.1)
Fleshfly, *Sarcophaga barbata*	Flight	—	316 (298–333) (3)	139 (130–147)	6.0 (4.2–7.7)
Cockchafer, *Melolontha melolontha*	Flight	—	243 (236–250) (3)	40 (37–47)	143 (131–154)
Rosechafer, *Pachnoda* sp.	Flight	—	561 (480–641) (3)	52 (45–59)	31 (26–36)
Dung beetle, *Heliocopris* sp.	Flight	—	294 (238–350) (3)	31 (24–38)	41 (39–54)
Echinodermata (Echinoidea) Sea urchin, *Echinus esculentus*	Lantern retractor	—	2.6 (2.3–3.2) (3)	0.2 (0.1–0.3)	5.4 (4.4–6.2)

Table 2—*continued*

Animal	Muscle	Maximal flux through the cycle	Enzyme activities (μmol/min per g fresh weight of muscle at 25°C)		
				Isocitrate dehydrogenase	
			Citrate synthase	NAD^+-linked	$NADP^+$-linked
Sea cucumber, *Holothuroidea thyone* sp.			0.5 (0.5) (3)	<0.1	3.2 (1.0–1.7)
Pisces					
Dogfish, *Scylliorhinus canicula*	White	—	1.7 (0.9–2.4) (3)	<0.1	0.6 (0.6–0.6)
	Red	3.8*	35 (32–38) (3)	0.4 (0.4–0.4)	31 (30–32)
	Heart	—	46 (36–56) (3)	0.4 (0.3–0.4)	53 (50–56)
Trout, *Salmo gairdneri*	White	—	5.2 (3.6–7.5) (3)	<0.1	9.2 (7.6–10.4)
	Red	3.2	50 (45–52) (3)	0.3 (0.1–0.4)	125 (121–132)
	Heart	—	38 (36–40) (3)	1.2 (0.6–1.8)	100 (95–106)
Amphibia					
Frog, *Rana temporaria*	Sartorius	2.6*	10 (9.9–11) (3)	0.5 (0.7–0.4)	14 (10–17)
	Heart	—	40 (37–43) (3)	5.2 (4.4–6.0)	70 (63–76)
Toad, *Xenopus laevis*	Sartorius	—	7.0 (6.7–7.2) (3)	0.5 (0.4–0.5)	5.0 (4.9–5.0)
	Gastrocnemius	—	12 (11–12) (3)	0.8 (0.7–0.9)	11 (9.0–12)
Aves					
Chicken, *Gallus gallus*	Pectoral	—	6.8 (4.1–9.4) (3)	1.0 (0.8–1.1)	2.3 (1.9–2.6)
	Heart	—	51 (50–52) (3)	5.7 (5.1–6.2)	54 (50–58)
Pigeon, *Columba livia*	Pectoral	4.8	115 (84–145) (5)	3.8 (2.2–5.8)	51 (38–81)
	Heart	—	127 (132–144) (3)	12 (10–14)	62 (59–65)

Table 2—*continued*

Animal	Muscle	Maximal flux through the cycle	Enzyme activities (μmol/min per g fresh weight of muscle at 25°C)		
				Isocitrate dehydrogenase	
			Citrate synthase	NAD^+-linked	$NADP^+$-linked
Sparrow, *Passer domesticus*	Pectoral	—	112 (69–202) (4)	1.3 (0.1–8)	43 (32–59)
	Heart	—	120 (99–128) (4)	16 (10–19)	22 (11–31)
Pheasant, *Phasianus colchicas*	Pectoral	—	14.4 (12.8–16.3) (4)	1.3 (0.7–1.6)	2.2 (0.7–3.9)
	Heart	—	99 (98–100) (3)	3.7 (2.7–4.7)	45 (39–51)
Mammalia					
Laboratory mouse	Heart	9.0*	146 (136–155) (3)	15 (13.9–17.3)	91 (79–104)
Laboratory rat	Heart	2.7	96 (89–103) (3)	5.0 (2.7–7.0)	72 (68–75)
Rabbit	Heart	—	69 (51–104) (3)	2.2 (1.7–2.5)	32 (26–46)
Sheep	Heart	—	58 (36–77) (3)	2.6 (0.7–5.7)	19 (8.8–33)
Pig	Heart	—	50 (44–55) (3)	1.6 (1.3–1.9)	12 (10–13)
Ox	Heart	—	54 (48–60) (3)	2.9 (1.6–4.2)	23 (19–26)

Coleoptera) possess activities of $NADP^+$-linked isocitrate dehydrogenase that are similar to those of the NAD^+-linked enzyme, and, in the cockchafer, the activity of $NADP^+$-linked enzyme is threefold greater. In the nervous tissue of any given insect species that has been investigated, the activities of the two isocitrate dehydrogenases are similar (Sugden & Newsholme, 1975). The reason for the emphasis on the NAD^+-linked enzyme in most of the insect flight muscles is not known (see below for comparison with vertebrate muscles), nor is it known why the beetles should be exceptional in containing high activities of $NADP^+$-linked isocitrate dehydrogenase.

(3) *High activities of citrate synthase and $NADP^+$-linked isocitrate dehydrogenase.* Except for the activities in the insects and those marine invertebrate muscles that have low activities, the activities of citrate synthase or $NADP^+$-linked isocitrate dehydrogenase are considerably greater than those of the NAD^+-linked enzyme (Table 2). If it is assumed that $NADP^+$-linked isocitrate dehydrogenase is involved in the citric acid cycle, the high activities of this enzyme and citrate synthase in vertebrate and invertebrate muscles indicate that the citric acid cycle is important for energy production. Most, if not all, of these particular muscles are mechanically active for long periods of time: vertebrate heart muscle is continually active during the life of the animal; fish red muscles are involved in the movement that produces sustained swimming (see Bone, 1966); pectoral muscles of the pigeon and sparrow are adapted structurally to enable the birds to fly long distances

(see George & Berger, 1966); the radular retractor muscles of the Gastropoda are involved in the continual rasping action of the radula, which is important in feeding in these animals (Hyman, 1967). In order to provide energy to support these sustained mechanical activities, a source of fuel must be continually supplied to the muscle via the circulatory system. Consequently, a source of oxygen is always available, and therefore the muscle can make the most efficient use of the substrate by complete oxidation via the citric acid cycle.

In the muscles described in this section the activities of citrate synthase and/or $NADP^+$-linked isocitrate dehydrogenase are considerably greater than those of NAD^+-linked isocitrate dehydrogenase. In the heart muscles of all animals investigated except the fish, sparrow and pigeon, the $NADP^+$-linked isocitrate dehydrogenase activities are at least tenfold greater than those of the NAD^+-linked enzyme; this is also the case for the pectoral muscles of the sparrow and the pigeon. However, in the hearts of these latter animals (but not those of the pheasant or the chicken) the difference between the activities of the two isocitrate dehydrogenases is smaller. In the red or heart muscle of the fish, this difference is considerably greater than in other muscles (it is about 100-fold). The radular retractor muscles of the Gastropoda demonstrate considerable variation in the difference between the two isocitrate dehydrogenase enzymes. The reason for these differences between muscles in relation to NAD^+- and $NADP^+$-linked isocitrate dehydrogenase activities is not known.

Relationship of the maximal enzyme activities to the maximal flux through the cycle

The maximal flux through the cycle in the muscles *in vivo* has been estimated either from oxygen uptake of the mechanically active tissue (or animal) or from the maximal activities of hexokinase in the muscle; justification for these methods of estimation are given by Crabtree & Newsholme (1972*a*, 1975) and in the legend to Table 2.

It has been suggested that the dehydrogenation of isocitrate in the operation of the citric acid cycle for energy formation is catalysed only by the NAD^+-linked isocitrate dehydrogenase (Goebell & Klingenberg, 1964). However, the present work demonstrates that, at least in some vertebrate and invertebrate muscles, the measured maximal activities of this enzyme cannot account for the estimated maximal flux through the cycle. These muscles include the radular retractor muscles of the common limpet and the winkle, the red muscles of the trout and dogfish, the sartorius muscle of the frog and the pectoral muscles of the pigeon (see Table 2). It is suggested, therefore, that in vertebrate and invertebrate muscles, except for most insect muscles, both isocitrate dehydrogenases are involved in the dehydrogenation of isocitrate for energy production in the cycle, at least when the flux through the cycle approaches the maximal. It is possible that, in these muscles, the role of the NAD^+-linked enzyme is to catalyse the dehydrogenation of isocitrate when the rate of the cycle is well below the maximal (e.g. when the muscle is at rest).

In most insects, the activities of $NADP^+$-linked isocitrate dehydrogenase in the flight muscles are not sufficient to account for the estimated maximal flux through the cycle during flight; in these muscles the NAD^+-linked enzyme must be responsible for most of the dehydrogenation of isocitrate during the operation of the cycle for energy production during flight. At the present time, it is not possible to put forward a biochemical explanation for this difference between insect and other muscles.

A comparison of the maximal activities of enzymes in a pathway can be used to indicate whether they catalyse near-equilibrium or non-equilibrium reactions (see Newsholme & Start, 1973). In addition, a comparison of maximal activities with the maximal fluxes through the cycle *in vivo* can be used in a similar manner (see Crabtree & Newsholme, 1975). In all the muscles investigated the activities of citrate synthase are considerably greater than the maximal estimated flux through the cycle (see Table 1). Except for most of the insect muscles, the sum of the two isocitrate dehydrogenase activities is considerably greater than the estimated flux through the cycle. In insect flight muscles, the NAD^+-linked isocitrate dehydrogenase activities are similar to the flux through the cycle. These findings suggest that citrate synthase and the isocitrate dehydrogenases catalyse near-equilibrium reactions in all muscles except those of the insect. In insect muscles, the results suggest that citrate synthase catalyses a near-equilibrium reaction, and NAD^+-linked isocitrate dehydrogenase catalyses a non-equilibrium reaction. The suggestion that the citrate synthase reaction is near equilibrium is at variance with the conclusion of Randle *et al.* (1970), who studied the distribution of ^{14}C from [^{14}C]acetate between acetyl-CoA and citrate in the perfused rat heart. It is possible that activity of citrate synthase *in vivo* is much lower than that measured *in vitro* because of inhibition by ATP or citrate (see Smith & Williamson, 1971) or very low concentrations of oxaloacetate and acetyl-CoA (see Rowan, 1975). However, the interpretation of the radioactive-isotope data by Randle *et al.* (1970) may be invalid if two pools of citrate, which equilibrate only very slowly, exist in muscle. The problem of the equilibrium nature of reactions such as citrate synthase will be resolved only when the contents of metabolic intermediates can be measured in both the cytoplasmic and mitochondrial compartments of rapidly frozen muscle so that meaningful mass-action ratios can be calculated.

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