Effect of Citrate on the Activities of 6-Phosphofructokinase from Nervous and Muscle Tissues from Different Animals and its Relationship to the Regulation of Glycolysis

By ERIC A. NEWSHOLME, PETER H. SUGDEN* and THELMA WILLIAMS
Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

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1. Citrate inhibits the activities of phosphofructokinase from muscles and nervous tissues from different animals across the Animal Kingdom except for the insects. The enzymes from the flight muscle of nine different insects and the cerebral ganglion of the locust were investigated: no inhibition by citrate was observed. Inhibition was observed with the enzymes from both aerobic (e.g. pectoral muscle of pigeon) and anaerobic (e.g. fish muscle, pectoral muscle of the game birds) muscles. It is suggested that this inhibition is of physiological importance in decreasing the rate of glucose utilization in skeletal muscle of animals during starvation and/or prolonged exercise. 2. The rates of glucose utilization by the sartorius and gastrocnemius muscles of the frog were markedly decreased by ketone bodies. The latter elevated the glucose 6-phosphate and citrate contents of the gastrocnemius muscle, indicating that citrate inhibition of phosphofructokinase could be, in part, responsible for the decreased rate of glycolysis. 3. These findings provide evidence that the concept of the glucose–fatty acid–ketone-body cycle involves both aerobic and anaerobic skeletal muscle and nervous tissue from a wide range of animals except the insects. In the latter the concept of the cycle may not be applicable.

The glucose–fatty acid cycle (Randle *et al.*, 1963) and its extension, the glucose–fatty acid–ketone-body cycle (Newsholme & Start, 1973; Newsholme, 1976) provide an explanation for the control of the concentrations of glucose, fatty acid and ketone bodies in the blood. The hypothesis explains the decreased rate of glucose utilization in muscle and brain during conditions such as starvation, despite little change in the blood glucose concentration. The experimental evidence for the hypothesis includes the observations that glucose utilization is inhibited by fatty acid and/or ketone-body oxidation in brain *in vivo*, in skeletal muscle *in vitro* and in heart muscle both *in vivo* and *in vitro* (Newsholme & Randle, 1964; Owen *et al.*, 1967; Lassers *et al.*, 1971; Ruderman *et al.*, 1974; Cuendet *et al.*, 1975; Maizels *et al.*, 1977; see also the Results section).

The conversion of fructose 6-phosphate into fructose bisphosphate, catalysed by phosphofructokinase (EC 2.7.1.11), is an important regulatory reaction in glycolysis. Since this enzyme is inhibited by citrate *in vitro* and as the citrate content in heart, brain and skeletal muscle is increased under these conditions (see Garland & Randle, 1964; Ruderman *et al.*, 1974; Maizels *et al.*, 1977; see Table 2), it is considered that the control of glucose utilization in these tissues *in vivo* is due, in part, to effects of citrate

on the catalytic activity of phosphofructokinase. Consequently, the latter property forms an integral part of the biochemical evidence for the glucose–fatty acid–ketone-body cycle. Nonetheless, the inhibition by citrate has been demonstrated with the enzyme from only two muscle tissues (i.e. rat heart and rabbit skeletal muscle; see Garland *et al.*, 1963; Parmeggiani & Bowman, 1963; Pogson & Randle, 1966) and from one brain, that of the sheep (Passonneau & Lowry, 1963).

The glucose–fatty acid cycle has been criticized on the basis that perfusion of the isolated hindquarter of the rat with fatty acids or ketone bodies does not decrease glucose utilization, nor does starvation of the donor animal (Jefferson *et al.*, 1972; Berger *et al.*, 1975). This has led to doubts as to whether the glucose–fatty acid cycle is applicable to the quantitatively important tissue (i.e. skeletal muscle) *in vivo* (see Gordon, 1970). However, one limitation with the perfused hindquarter is that sufficient endogenous fatty acids may be available in the muscle to cause inhibition of glucose utilization in the control perfusions. The trauma of preparation may cause release of fatty acids from the adipose tissue present in the hindquarter of the rat. Since this skeletal muscle has only a low capacity of the citric acid cycle (see Crabtree & Newsholme, 1975), it may take a long time for the fatty acids to be oxidized. It has been reported that fatty acids or

* Present address: Department of Clinical Biochemistry, Radcliffe Infirmary, Oxford, OX2 6HE, U.K.

ketone bodies could decrease glucose utilization in certain isolated incubated skeletal-muscle preparations of the rat and that this could be due, in part, to the effect of citrate on phosphofructokinase (Cuendet *et al.*, 1975; Maizels *et al.*, 1977). These findings suggest that the concept of the glucose-fatty acid-ketone-body cycle may apply to most, if not all, of the musculature in the intact animal.

It is known that skeletal muscles from a wide range of animals have the capacity to oxidize fatty acids (Crabtree & Newsholme, 1972*b*, 1975) and/or ketone bodies (A. Beis & E. A. Newsholme, unpublished work) and that nervous tissues from these animals have the capacity to oxidize ketone bodies (Sugden & Newsholme, 1973). These findings suggest that the concept of the glucose-fatty acid-ketone-body cycle may apply to skeletal muscles and nervous tissues of a wide range of animals, i.e. the oxidation of alternative fuels to glucose will decrease glucose utilization by these tissues. Since this decrease should depend on citrate inhibition of phosphofructokinase activity, this effect has been investigated with the enzyme from skeletal muscles and nervous tissues from a wide range of animals. These animals include the insects, since it has been reported that phosphofructokinase from the locust flight muscle is not inhibited by citrate (Walker & Bailey, 1969).

Materials and Methods

Chemicals and enzymes

All chemicals and enzymes were obtained from Boehringer Corp. (London), London W5 2TZ, U.K., except for the following: 2-heptyl-4-hydroxyquinoline *N*-oxide was obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K.; sodium citrate and all inorganic chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset BH12 4NN, U.K. All chemicals were of the highest purity available.

Sources of animals

Animals were obtained from the sources given by Newsholme & Taylor (1969) and Sugden & Newsholme (1973).

Preparation of homogenates for enzyme assay

Animals were killed and muscle or nervous tissue was removed by careful dissection as quickly as possible. (Mice and rats were anaesthetized with diethyl ether before cervical section.) Tissue was homogenized in ground-glass homogenizers with 10–20 vol. of extraction medium (50 mM-Tris/HCl/1 mM-EDTA/5 mM-MgCl₂, pH 8.2) at 0°C.

Dialysis of coupling enzymes

The coupling enzymes (aldolase, triose phosphate isomerase and glycerol 1-phosphate dehydrogenase) used in the assay were dialysed against a 400-fold excess of buffer (10 mM-Tris/HCl/1 mM-EDTA, pH 7.5) at 4°C for 5 h (with at least six changes of buffer) to remove (NH₄)₂SO₄, which activates phosphofructokinase (Underwood & Newsholme, 1965; Sugden & Newsholme, 1975).

Assay of phosphofructokinase

The assay medium consisted of 50 mM-Tris/HCl, 13.4 mM-MgCl₂, 100 mM-KCl, 0.13 mM-NADH, 5 µg of 2-heptyl-4-hydroxyquinoline *N*-oxide and 1 mM-fructose 6-phosphate. Dialysed glycerol 1-phosphate dehydrogenase/triose phosphate isomerase (not less than 10 µg) and aldolase (not less than 100 µg) were added to each cuvette. Suitable volumes of ATP (neutralized with NaHCO₃) and sodium citrate were added to give final concentrations of 1–7.5 mM and 2 mM respectively. A volume (5–10 µl) of homogenate (suitably diluted with extraction buffer if necessary) was also added. The final volume in the cuvette was 2 ml and the pH was 7.5. The assay was initiated by the addition of fructose 6-phosphate in 50 µl. The change in *A*₃₄₀ was measured in a Gilford recording spectrophotometer (model 240) at 25°C. Fructose 6-phosphate was omitted from control assays. In some assays, an ATP-regenerating system (5 mM-phosphocreatine and 100 µg of creatine kinase per cuvette) was added: the system was added in an attempt to decrease the AMP concentration (which lessens ATP inhibition) in the cuvette, but it made little difference to the enzyme activity at either ATP concentration under these conditions.

Incubation procedures

Adult frogs were killed by decapitation, gastrocnemius and sartorius muscles were rapidly dissected, placed in 5 ml of incubation medium (artificial frog plasma; see Özand & Narahara, 1964), gassed with O₂/CO₂ (19:1) and preincubated for 17–20 h at 4°C to decrease the intracellular contents of hexose monophosphates (Narahara *et al.*, 1960). The muscles were transferred to 5 ml of fresh incubation medium, gassed with O₂/CO₂ (19:1) and incubated for 2 h at 25°C. At the end of the incubation, the muscles were removed and rapidly freeze-clamped. The frozen muscles were extracted with HClO₄ (6%, w/v) after powdering of the frozen muscle at –70°C in a percussion mortar (see Beis & Newsholme, 1975). After centrifugation (3000*g* for 10 min) of the precipitated protein, the supernatant was neutralized with 5 M-KHCO₃/KCl. A 0.25 ml volume of 60% (w/v) HClO₄ was added to the

incubation medium, which was then centrifuged (1000g for 5 min) and the supernatant was neutralized with KHCO_3/KCl .

Determinations

Glucose concentration was measured by the method of Bergmeyer (1974). Adenine nucleotides and glucose 6-phosphate concentrations were measured as described by Beis & Newsholme (1975) and citrate was measured as described by Start & Newsholme (1968).

Presentation of results of citrate effects on phosphofructokinase

The effect of citrate has been investigated at two concentrations (1 and 7.5 mM) of ATP. In each animal, preliminary experiments were carried out at several concentrations of ATP to demonstrate that there was little inhibition at 1 mM-ATP, but that there was marked inhibition at 7.5 mM. The results are obtained with the enzyme from muscle or nervous tissue from different animals, so that the values of the

S.E.M. give an indication of the biological variation. In addition, the effects of citrate at several ATP concentrations with extracts from insect muscles and other muscles are presented in Figs. 1(a) and 1(b) respectively. These plots are representative of three similar experiments, and the insect species chosen in Fig. 1(a) are representative of seven other insects; the plots in Fig. 1(b) are representative of 13 non-insect species. Since, in this work, crude extracts of tissues were used, the concentrations of various de-inhibitors (e.g. AMP, P_i , NH_4^+) in the cuvette will be variable, precise quantitative interpretations on the concentration of ATP that causes inhibition of phosphofructokinase should not be made.

Results

Effect of citrate on activity of phosphofructokinase

The aim of the work was to investigate whether citrate inhibited the activity of the phosphofructokinases from tissues from various animals. It is known that citrate inhibits phosphofructokinase only

Table 1. *Effect of citrate on the activities of phosphofructokinase from muscle and nervous tissue of different animals*
The effect of citrate was tested at 1 and 7.5 mM-ATP. Activities are presented as means and S.E.M. with the number of tissues from different animals investigated given in parentheses. The statistical significance (Student's *t* test) of the difference in activity from the control without citrate is indicated by † ($P < 0.05$) and * ($P < 0.01$).

Animal	Tissue	Concn. of added citrate (mM)	Phosphofructokinase activity ($\mu\text{mol}/\text{min}$ per g fresh wt. of tissue)	
			1 mM-ATP	7.5 mM-ATP
Locust (<i>Schistocerca gregaria</i>)	Flight muscle	0	11.1 $\pm$ 0.8 (12)	8.7 $\pm$ 1.26 (11)
		2	10.5 $\pm$ 1.0 (12)	9.0 $\pm$ 1.21 (11)
	Cerebral ganglion	0	1.0 $\pm$ 0.39 (7)	0.6 $\pm$ 0.14 (7)
		2	1.0 $\pm$ 0.39 (7)	0.5 $\pm$ 0.14 (7)
Rosebeetle (<i>Pachnoda ephippiata</i>)	Flight muscle	0	9.1 $\pm$ 2.3 (6)	9.7 $\pm$ 3.3 (6)
		2	8.5 $\pm$ 2.1 (6)	8.9 $\pm$ 2.9 (6)
Dogfish (<i>Scylliorhinus canicula</i>)	White muscle	0	18.2 $\pm$ 2.2 (6)	15.3 $\pm$ 6.0 (6)
		2	13.5 $\pm$ 2.1 (6)†	2.6 $\pm$ 1.4 (6)†
Trout (<i>Salmo gairdneri</i>)	White muscle	0	23.2 $\pm$ 1.5 (5)	22.9 $\pm$ 4.2 (5)
		2	17.6 $\pm$ 1.8 (5)†	6.4 $\pm$ 1.6 (5)*
Frog (<i>Rana temporaria</i>)	Gastrocnemius muscle	0	11.6 $\pm$ 0.91 (6)	12.8 $\pm$ 0.97 (6)
		2	8.2 $\pm$ 0.96 (6)†	5.7 $\pm$ 1.8 (6)*
Pigeon (<i>Columba livia</i>)	Pectoral muscle	0	25.1 $\pm$ 3.9 (5)	30.8 $\pm$ 2.9 (5)
		2	14.7 $\pm$ 1.7 (5)†	5.0 $\pm$ 2.8 (5)*
	Brain	0	5.4 $\pm$ 0.72 (7)	3.2 $\pm$ 0.43 (7)
		2	4.4 $\pm$ 0.39 (7)	1.3 $\pm$ 0.23 (7)*
Domestic fowl (<i>Gallus gallus</i>)	Pectoral muscle	0	54.1 $\pm$ 5.9 (5)	65.5 $\pm$ 7.3 (5)
		2	32.6 $\pm$ 2.9 (5)*	32.8 $\pm$ 4.5 (5)*
Laboratory mouse	Gastrocnemius muscle	0	28.6 $\pm$ 1.1 (4)	28.8 $\pm$ 0.7 (4)
		2	18.3 $\pm$ 0.6 (4)*	1.1 $\pm$ 0.2 (4)*
Laboratory rat	Gastrocnemius muscle	0	11.8 $\pm$ 1.3 (5)	11.1 $\pm$ 2.1 (5)
		2	5.6 $\pm$ 1.8 (5)†	1.1 $\pm$ 0.4 (5)*
	Brain	0	6.0 $\pm$ 0.5 (5)	4.2 $\pm$ 0.34 (3)
		2	3.7 $\pm$ 0.3 (5)*	0.5 $\pm$ 0.04 (3)*

Fig. 1. Effect of ATP concentration in the presence and absence of citrate on the activities of phosphofructokinase from (a) insect flight muscles and (b) non-insect skeletal muscles

Enzyme activities were assayed as described in the Materials and Methods section. Open symbols represent activities in the presence of 2 mM-citrate and solid symbols represent activities in the absence of added citrate. □, ■, Yellow underwing moth (*Noctua pronuba*); ○, ●, silver-Y moth (*Plusia gamma*); △, ▲, striated 'fast' muscle of the scallop (*Pecten maximus*); ▽, ▼, white muscle of the dogfish (*Scylliorhinus canicula*).

when ATP is inhibitory (see Underwood & Newsholme, 1965; Pogson & Randle, 1966). Consequently, the effects of citrate have been studied at two concentrations (1 and 7.5 mM) of ATP to ensure that the effect of citrate was investigated at an inhibitory concentration of ATP. In some cases, the activity of phosphofructokinase was higher at the higher ATP concentration (see Table 1); this does not indicate lack of inhibition of phosphofructokinase by ATP but that, at both ATP concentrations, the activities are below the optimum. However, although the activity at 1 mM-ATP is below optimal, this low concentration of ATP appears to inhibit the enzyme, since in many cases citrate inhibition is observed at 1 mM-ATP (see Table 1). This is explained if the binding of ATP occurs at both the catalytic and inhibitory sites before saturation of the former site with ATP.

The inhibition of phosphofructokinase by citrate was most pronounced at 7.5 mM-ATP in all the vertebrate muscles investigated (see Table 1 and Fig. 1b). The inhibition was observed for both anaerobic (e.g. fish muscle, pectoral muscle of the domestic fowl, frog muscle) and aerobic (e.g. pectoral muscle of the pigeon) muscle. The inhibition by citrate was also observed with the enzyme from brain of the pigeon and rat. However, no inhibition by citrate was observed with the enzyme from muscle or nervous tissue of the insects (see Table 1, and compare the plots in Fig. 1a with those in Fig. 1b). In addition to the results with the insects reported in Table 1 and Fig. 1, similar observations have been

made with the enzyme from the flight muscles of the dung beetle (*Heliocopris* sp.), bumble bee (*Bombus lucorum* and *Bombus terrestris*) and the wasp (*Vespa vulgaris*). Walker & Bailey (1969) have shown that phosphofructokinase purified from locust flight muscle is not inhibited by citrate.

Effect of ketone bodies on glucose utilization and metabolic intermediates in isolated incubated frog muscle

Ketone bodies (either acetoacetate or 3-hydroxybutyrate) decreased glucose utilization in the isolated incubated sartorius and gastrocnemius muscles of the frog (Table 2). The metabolic intermediates were measured in the freeze-clamped gastrocnemius muscle (since a greater quantity of muscle was available). Ketone bodies did not cause any change in the contents of adenine nucleotides (except that 3-hydroxybutyrate increased that of AMP), but increased the contents of glucose 6-phosphate and citrate. It is assumed that glucose phosphate isomerase catalyses a near-equilibrium reaction in these muscles, so that changes in the contents of glucose 6-phosphate reflect changes in those of fructose 6-phosphate. Thus the increased content of glucose 6-phosphate under conditions of decreased glycolytic flux (indicated by a decreased rate of glucose utilization) suggests that the activity of phosphofructokinase has been decreased in the muscle (see Newsholme & Start, 1973). Since citrate is a potent inhibitor of phosphofructokinase from this

Table 2. Effects of acetoacetate and 3-hydroxybutyrate on the rates of glucose uptake and contents of some metabolic intermediates in isolated incubated leg muscles of the frog

Sartorius and gastrocnemius muscles were incubated, and glucose uptake and metabolic intermediates were measured, as described in the Materials and Methods section. Rates and contents are presented as means $\pm$ s.e.m. for the numbers of separate experiments given in parentheses. Results that are statistically different (Student's *t* test) from control values are shown by * ($P < 0.002$), ** ($P < 0.01$), † ($P < 0.025$).

Muscle	Additions to incubation medium	Glucose uptake ($\mu\text{mol/h}$ per g of fresh muscle)	Contents of intermediates ($\mu\text{mol/g}$ of fresh muscle)			
			ATP	ADP	AMP	Glucose 6-phosphate
Sartorius	None	10.9 $\pm$ 1.1 (6)	—	—	—	—
	Acetoacetate (5 mm)	3.7 $\pm$ 0.8* (5)	—	—	—	—
	3-Hydroxybutyrate (5 mm)	3.3 $\pm$ 1.6** (4)	—	—	—	—
Gastrocnemius	None	9.1 $\pm$ 1.2 (9)	3.3 $\pm$ 0.24 (12)	0.60 $\pm$ 0.066 (12)	0.06 $\pm$ 0.017 (9)	0.069 $\pm$ 0.012 (10)
	Acetoacetate (5 mm)	2.6 $\pm$ 0.4* (10)	3.1 $\pm$ 0.17 (13)	0.68 $\pm$ 0.11 (12)	0.09 $\pm$ 0.021 (9)	0.28 $\pm$ 0.088† (8)
	3-Hydroxybutyrate (5 mm)	2.6 $\pm$ 0.6* (7)	3.6 $\pm$ 0.39 (13)	0.72 $\pm$ 0.12 (13)	0.13 $\pm$ 0.017** (10)	0.27 $\pm$ 0.073** (10)
						0.057 $\pm$ 0.008 (6)
						0.22 $\pm$ 0.023* (7)
						0.24 $\pm$ 0.044* (7)

muscle (see Table 1) and as its content is increased in the presence of ketone bodies (Table 2), it is concluded that the increase in citrate may be responsible for the observed inhibition of phosphofructokinase and hence of glycolysis.

Discussion

Recently, the effects of fatty acids and/or ketone bodies on the rate of glucose utilization by isolated, incubated skeletal-muscle preparations of the rat have been investigated. Glucose utilization was decreased and evidence was presented that this could be partly explained by inhibition of the activity of phosphofructokinase by citrate in the soleus muscle (Cuendet *et al.*, 1975; Maizels *et al.*, 1977). However, there was no effect of ketone bodies on glucose utilization in the extensor digitorum longus (Maizels *et al.*, 1977). It was suggested that this difference reflected the greater dependence on anaerobic metabolism for energy generation in the extensor digitorum longus. In other words, citrate is only an important regulator of glycolysis in the more aerobic muscles. However, the present work (Table 1) has shown that citrate is a similarly potent inhibitor of phosphofructokinase from the anaerobic muscles (e.g. white muscle of the trout, pectoral muscle of the domestic fowl) as well as the aerobic muscles (e.g. pectoral muscle of the pigeon). The sartorius and gastrocnemius muscles of the frog are considerably less aerobic than (for example) the pectoral muscles of the pigeon (see Alp *et al.*, 1976; Crabtree & Newsholme, 1975). Nonetheless, glucose utilization is markedly decreased in both muscles incubated *in vitro* by either acetoacetate or 3-hydroxybutyrate. In the muscle that was investigated further (i.e. gastrocnemius), there was evidence that ketone bodies caused inhibition of phosphofructokinase activity (see the Results section), which could have been due to the increased concentration of citrate (see Table 2). It is concluded therefore that fatty acid and/or ketone-body oxidation can decrease the rate of glycolysis and hence glucose utilization via citrate inhibition of phosphofructokinase in skeletal muscle.

Our work, together with that of Cuendet *et al.* (1975) and Maizels *et al.* (1977), provides evidence that the concept of regulation via the glucose-fatty acid-ketone-body cycle is applicable to skeletal muscle (at least in non-insect animals; see below) as well as to cardiac muscle.

The inhibition of the activity of phosphofructokinase by citrate could be important in the aerobic muscles during prolonged exercise in the fed or starved state and at rest in the starved state. During prolonged starvation, fatty acids and/or ketone bodies will be used as a fuel largely to replace glucose (for review see Newsholme, 1976). During prolonged

exercise, fatty acids will be used in addition to blood glucose and muscle glycogen as fuels for the muscle. However, the rate of degradation of the carbohydrate fuels must be controlled in relation to the rate of energy production from fatty acid oxidation in the muscle. It is suggested that the effect of citrate on phosphofructokinase provides such a control mechanism. This ensures that carbohydrate stores are not depleted too rapidly during prolonged exercise, and this may be important in prevention or delay of physical exhaustion (see Newsholme, 1977). However, in anaerobic muscles, the inhibition of phosphofructokinase by citrate would be important only during starvation at rest.

Citrate inhibition of phosphofructokinase could not be demonstrated with the enzyme from the flight muscles of a number of insects, nor with the enzyme from the leg muscle or cerebral ganglion of the locust. This absence of effect appears to be restricted to the order Insecta, since inhibition by citrate is observed with the enzyme from tissue of the lobster (results not shown) and the scallop (see Fig. 1b). It is known that the flight muscles of the locust and some moths (e.g. yellow underwing, silver-Y) oxidize both carbohydrate and fat during flight (Weis-Fogh, 1952; Sacktor, 1970; Crabtree & Newsholme, 1972a, 1975). The lack of citrate inhibition suggests that oxidation of fat does not regulate the utilization of carbohydrate in insects as in other animals. Indeed, it is known that the control of the blood sugar content is different in insects compared with other animals. Thus in the locust, the blood sugar (i.e. trehalose) decreases by 90% during flight (Tietz, 1967), whereas in the rat the blood glucose decreases by only 30% after 4 days starvation (Hawkins *et al.*, 1971) and in man it is decreased by only 6% after 90 min running to exhaustion (Hermansen *et al.*, 1967). It is possible that the rate of carbohydrate utilization by insect flight muscles is decreased not by intracellular control of glycolysis, as in higher animals, but simply by the decrease in the concentration of sugar in the haemolymph.

The results of the present work indicate that the phosphofructokinase activities from brain of the rat and pigeon are inhibited by citrate, which could explain the decreased rate of glycolysis in rat (and human) brain when ketone bodies are oxidized (Owen *et al.*, 1967; Ruderman *et al.*, 1974). Since nervous tissue from many different animals contains the enzymes for utilizing ketone bodies (Sugden & Newsholme, 1973), it is suggested that ketone-body oxidation will restrict the rate of glucose utilization through citrate inhibition of phosphofructokinase during periods of carbohydrate deprivation or starvation. The insects appear to be an exception. It is possible that the haemolymph contains sufficient blood sugar to satisfy the requirements of the nervous tissue during even prolonged periods of

starvation or exercise, or that this tissue can utilize fat fuels exclusively.

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References

- Alp, P. R., Newsholme, E. A. & Zammit, V. A. (1976) *Biochem. J.* **154**, 689–700
- Beis, I. & Newsholme, E. A. (1975) *Biochem. J.* **152**, 23–32
- Berger, M., Hagg, S. & Ruderman, N. B. (1975) *Biochem. J.* **146**, 231–238
- Bergmeyer, H. U. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), pp. 1196–1201, Academic Press, London and New York
- Crabtree, B. & Newsholme, E. A. (1972a) *Biochem. J.* **126**, 49–58
- Crabtree, B. & Newsholme, E. A. (1972b) *Biochem. J.* **130**, 697–705
- Crabtree, B. & Newsholme, E. A. (1975) in *Insect Muscle* (Usherwood, P. N. R., ed.), pp. 405–491, Academic Press, London and New York
- Cuendet, G. S., Loten, E. G. & Renold, A. E. (1975) *Diabetologia* **11**, 336
- Garland, P. B. & Randle, P. J. (1964) *Biochem. J.* **93**, 678–687
- Garland, P. B., Randle, P. J. & Newsholme, E. A. (1963) *Nature (London)* **200**, 169–170
- Gordon, E. S. (1970) *Adv. Metab. Disord.* **4**, 243–245
- Hawkins, R. A., Williamson, D. H. & Krebs, H. A. (1971) *Biochem. J.* **122**, 13–18
- Hermansen, H., Hultman, E. & Saltin, B. (1967) *Acta Physiol. Scand.* **71**, 129–139
- Jefferson, L. S., Koehler, J. O. & Morgan, H. E. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 816–820
- Lassus, B. W., Wahlqvist, M. L., Kaijser, L. & Carlson, L. A. (1971) *Lancet* **ii**, 448–452
- Maizels, E. Z., Ruderman, N. B., Goodman, M. N. & Lau, D. (1977) *Biochem. J.* **162**, 557–568
- Narahara, H. T., Özand, P. & Cori, H. T. (1960) *J. Biol. Chem.* **235**, 3370–3378
- Newsholme, E. A. (1976) *Clin. Endocrinol. Metab.* **5**, 543–578
- Newsholme, E. A. (1977) *Ann. NY Acad. Sci.* in the press
- Newsholme, E. A. & Randle, P. J. (1964) *Biochem. J.* **93**, 641–651
- Newsholme, E. A. & Start, C. (1973) *Regulation in Metabolism*, pp. 312–313, Wiley-Interscience, London, New York, Sydney and Toronto
- Newsholme, E. A. & Taylor, K. (1969) *Biochem. J.* **112**, 465–474
- Owen, O. E., Morgan, A. P., Kemp, H. G., Sullivan, J. M., Herrera, M. G. & Cahill, G. F. (1967) *J. Clin. Invest.* **46**, 1589–1595
- Özand, P. & Narahara, H. T. (1964) *J. Biol. Chem.* **239**, 3146–3152
- Parmeggiani, A. & Bowman, R. H. (1963) *Biochem. Biophys. Res. Commun.* **12**, 268–273

- Passonneau, J. V. & Lowry, O. H. (1963) *Biochem. Biophys. Res. Commun.* **13**, 372-379
- Pogson, C. I. & Randle, P. J. (1966) *Biochem. J.* **100**, 683-693
- Randle, P. J., Garland, P. B., Hales, C. N. & Newsholme, E. A. (1963) *Lancet* **i**, 785-789
- Ruderman, N. B., Ross, P. S., Berger, M. & Goodman, M. N. (1974) *Biochem. J.* **138**, 1-10
- Sacktor, B. (1970) *Adv. Insect Physiol.* **7**, 268-347
- Start, C. & Newsholme, E. A. (1968) *Biochem. J.* **107**, 411-415
- Sugden, P. H. & Newsholme, E. A. (1973) *Biochem. J.* **134**, 97-101
- Sugden, P. H. & Newsholme, E. A. (1975) *Biochem. J.* **150**, 113-122
- Tietz, A. (1967) *Eur. J. Biochem.* **2**, 236-242
- Underwood, A. H. & Newsholme, E. A. (1965) *Biochem. J.* **95**, 868-975
- Walker, P. R. & Bailey, E. (1969) *Biochem. J.* **111**, 365-369
- Weis-Fogh, T. (1952) *Phil. Trans. R. Soc. London Ser. B* **237**, 1-36