Biochem. J. (1967) **103**, 39

The Activities of Fructose 1,6-Diphosphatase, Phosphofructokinase and Phosphoenolpyruvate Carboxykinase in White Muscle and Red Muscle

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(*Received* 1 *August* 1966)

1. The activities of fructose 1,6-diphosphatase were measured in extracts of muscles of various physiological function, and compared with the activities of other enzymes including phosphofructokinase, phosphoenolpyruvate carboxykinase and the lactate-dehydrogenase isoenzymes. 2. The activity of phosphofructokinase greatly exceeded that of fructose diphosphatase in all muscles tested, and it is concluded that fructose diphosphatase could not play any significant role in the regulation of fructose 6-phosphate phosphorylation in muscle. 3. Fructose-diphosphatase activity was highest in white muscle and low in red muscle. No activity was detected in heart or a deep-red skeletal muscle, rabbit semitendinosus. 4. The lactate-dehydrogenase isoenzyme ratio (activities at high and low substrate concentration) was measured in various muscles because a low ratio is characteristic of muscles that are more dependent on glycolysis for their energy production. As the ratio decreased the activity of fructose diphosphatase increased, which suggests that highest fructose-diphosphatase activity is found in muscles that depend most on glycolysis. 5. There was a good correlation between the activities of fructose diphosphatase and phosphoenolpyruvate carboxykinase in white muscle, where the activities of these enzymes were similar to those of liver and kidney cortex. However, the activities of pyruvate carboxylase and glucose 6-phosphatase were very low in white muscle, thereby excluding the possibility of gluconeogenesis from pyruvate and lactate. 6. It is suggested that the presence of fructose diphosphatase and phosphoenolpyruvate carboxykinase in white muscle may be related to operation of the α-glycerophosphate–dihydroxyacetone phosphate and malate–oxaloacetate cycles in this tissue.

The enzyme PFK‡ (ATP–D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) in muscle has been shown to be a regulatory enzyme for glycolysis, and, on the basis of the properties of this enzyme, a theory for metabolic control has been proposed (Newsholme & Randle, 1961, 1964; Karpatkin, Helmreich & Cori, 1964; Passonneau & Lowry, 1962; Mansour, 1963, 1965; Parmeggiani & Bowman, 1963; Regen, Davis, Morgan & Park, 1964). The emphasis for control was placed on PFK because it was considered that the activity of FDPase (D-fructose 1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) was too low to play any role in regulation at this step in metabolism (Gomori, 1943; Newsholme & Randle, 1962; Özand & Narahara, 1964). However, Krebs & Woodford (1965) found significant FDPase activity in some skeletal muscles, and the enzyme possessed similar properties to that found in liver and kidney cortex. These observations raised the possibility that the activities of both PFK and FDPase could contribute to the control of glycolysis in skeletal muscle, as proposed for liver and kidney cortex (Underwood & Newsholme, 1965*b*,*c*).

The activities of FDPase and PFK in muscles from a variety of species were measured to see whether the presence of FDPase was restricted to any particular class of animal or type of muscle. In most muscles the activity of PFK was very much greater than that of FDPase. The highest activities of FDPase were obtained in white muscle, in which the activity of PFK exceeded that of FDPase by 12-fold.

The presence of considerable FDPase in white

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‡ Abbreviations: PFK, phosphofructokinase; FDPase, fructose 1,6-diphosphatase.

muscle posed the problem of the source of substrate (fructose 1,6-diphosphate) for this enzyme. Therefore the activities of a number of enzymes that might possibly give rise, either directly or indirectly, to fructose diphosphate were measured. This investigation showed that phosphoenolpyruvate carboxykinase [GTP–oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32] was present in white muscle, and, moreover, that the variation in its activity between various muscles corresponded to that of FDPase. A preliminary report of some of these results has been published (Opie & Newsholme, 1965).

MATERIALS AND METHODS

Chemicals and enzymes. All chemicals and enzymes were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, with the exception of the following: 2-mercaptoethanol and IDP were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks; bovine serum albumin (fraction V) was obtained from the Armour Pharmaceutical Co., Eastbourne, Sussex; $MgSO_4$, $MgCl_2$, $MnCl_2$, KCN, KCl, $NaHCO_3$ and EDTA were AnalaR grade from British Drug Houses Ltd., Poole, Dorset. All were used without further purification.

Preparation of crude extracts. Crude extracts or high-speed supernatant fractions were prepared from animals fed *ad libitum*. Human tissue was obtained at thoracotomy. After removal, the muscle was first chilled in a glass beaker kept on ice and then weighed rapidly on a torsion balance. The muscle was finely cut with scissors and homogenized at 4° for 2min. in an MSE top-drive homogenizer with 10vol. of 50mM-tris–HCl buffer, pH8·2, containing EDTA (1mM) and $MgSO_4$ (5mM). This pH was chosen to avoid acid inactivation of PFK (see Mansour, 1965). The homogenate was centrifuged for 10min. at 600*g* at 0° and the supernatant (crude extract) was used for enzyme activity assays.

Preparation of cell fractions. The crude extract (in the ionic homogenizing medium) was centrifuged for 10min. at 10000*g* (Beckman Spinco model L preparative ultracentrifuge, 40.3 rotor; 11000 rev./min.). The precipitate is termed the 10000*g* pellet. The supernatant was centrifuged at 114000*g* for 60min. (same rotor; 40000 rev./min.), and the precipitate is termed the 114000*g* pellet. For measurement of the PFK activity, pellets were resuspended in the following medium: 10mM-tris–HCl buffer containing $MgSO_4$ (50mM), mercaptoethanol (5mM) and ATP (0·5mM), final pH8·0. The suspension was incubated at 37° for 20min. immediately before assay, to ensure maximum activity of PFK (see Mansour, 1965).

Sucrose-density-gradient ultracentrifugal analysis. One part (by vol.) of the crude extract, homogenized in the ionic medium to which sucrose was added to a final concentration of 0·25M, was layered on 50 parts of sucrose prepared by making a 10–30% (w/v) exponential gradient with the device of Salo & Kouns (1965). The sucrose solution was made up in 50mM-tris–HCl buffer containing EDTA (1mM) and $MgSO_4$ (10mM), final pH8·2 (at 4°). The homogenates were centrifuged at about 0–4° in a Beckman Spinco model L ultracentrifuge, in a swinging-bucket rotor (SW39) at 35000 rev./min. for 45min. The centrifuge brake was not used, and after centrifugation the contents of the centrifuge tube (5ml.) were collected in 30–40 fractions and assayed for PFK, aldolase and E_{260}.

Assay of fructose 1,6-diphosphatase. FDPase was usually assayed on the 114000*g* supernatant by a modification of the methods of Taketa & Pogell (1963). The usual assay medium was 50mM-tris–HCl buffer containing mercaptoethanol (20mM), $MgSO_4$ (6mM), EDTA (1mM), NADP (0·2mM) and fructose 1,6-diphosphate (0·1mM), final pH7·5. To 2ml. of assay medium were added 4μg. of each of glucose 6-phosphate dehydrogenase and phosphoglucose isomerase and 0·01–0·05ml. of undiluted tissue extract. The reduction of NADP was followed at 340mμ and at 25° in a Gilford recording spectrophotometer. A control, without fructose 1,6-diphosphate, measured formation of glucose 6-phosphate from glycogen (Krebs & Woodford, 1965).

Assay of phosphofructokinase. PFK was usually assayed on the crude extract by a modification of the method of Mansour (1965). The medium was 50mM-tris–HCl buffer containing $MgCl_2$ (5mM), KCl (200mM), glucose 6-phosphate (3mM), ATP (1mM), AMP (2mM), bovine serum albumin (0·01g./100ml.), $NADH_2$ (0·1mM) and KCN (0·3mM), final pH8·2. Aldolase (50μg.), α-glycerophosphate dehydrogenase (5μg.), phosphoglucose isomerase (5μg.) and triose phosphate isomerase (5μg.), and 10–50μl. of tissue extract (diluted if necessary in the same buffer as used for homogenization), were added to 2ml. of medium in the cuvette. The oxidation of $NADH_2$ was followed at 340mμ and at 25° in a Gilford recording spectrophotometer.

Assay of phosphoenolpyruvate carboxykinase. This was usually carried out on the crude extract or the diluted uncentrifuged muscle homogenate by a modification of the method of Stickland (1959*a*) developed by L. V. Eggleston & H. A. Krebs (unpublished work). The muscle was homogenized with 5vol. of water at 4°. The assay medium was 66mM-tris–HCl buffer containing $MnCl_2$ (1·1mM), phosphoenolpyruvate (tricyclohexylammonium salt) (1·1mM), $NADH_2$ (0·16mM), IDP (sodium salt) (1·54mM) and $NaHCO_3$ (17mM; freshly gassed with 5% CO_2 in O_2), final pH7·5. Malate dehydrogenase (0·01ml.) that had been dialysed overnight against water at 4° to remove NH_4^+ was added to 3ml. of assay medium and the rate of oxidation of $NADH_2$ followed at 340mμ and at 25° in a Gilford recording spectrophotometer. A control (blank) cuvette from which $NaHCO_3$ was omitted compensated for non-specific oxidation of $NADH_2$.

Assays of other enzymes. Lactate-dehydrogenase and glucose 6-phosphate-dehydrogenase activities were measured on the same 114000*g* supernatant used for FDPase assays. Lactate-dehydrogenase isoenzymes were measured by the pyruvate inhibition method of Wilson, Cahn & Kaplan (1963). The isoenzyme ratio was defined as the ratio between the two activities measured in the presence of low (0·33mM) and high (10mM) pyruvate concentrations. Glucose 6-phosphate dehydrogenase was assayed by the method of Glock & McLean (1953). Aldolase was measured by the method of Delbrück, Schimmassek, Bartsch & Bücher (1959).

Glycerokinase activities were assayed on a 114000*g* supernatant of a muscle homogenized with 5vol. of 1% (w/v) KCl containing EDTA (1mM) by the rate of formation of α-[^{14}C]glycerophosphate from [^{14}C]glycerol (Newsholme, Robinson & Taylor, 1967). A 1:50 dilution of muscle

Table 1. *Effects of centrifugation on phosphofructokinase activity in muscle*

Activities are expressed in μmoles of substrate transformed/g. fresh wt./hr. at 25°. Recoveries of crude-extract activity were calculated as sums of the percentages of crude-extract activity found in 10000*g* pellet, 114000*g* pellet and 114000*g* supernatant. Methods were as described in the Materials and Methods section.

		PFK activity					
			10000*g*		114000*g*		
Animal	Tissue	Crude extract	Pellet	Supernatant	Pellet	Supernatant	Recovery (%)
Frog	Leg	826	107	669	174		35
Hen	Heart	458	238	343	123	109	103
	Breast	2485	422	1863	373	323	45
	Leg (white)	1735	347	1543	330	226	52
	Leg (red)	903	172	886	226	154	51
Rat	Heart	578				400	
Hamster	Thigh	2700				673	
Rabbit	Semitendinosus	478		500		382	
	Adductor (white)	1751		1814		1735	

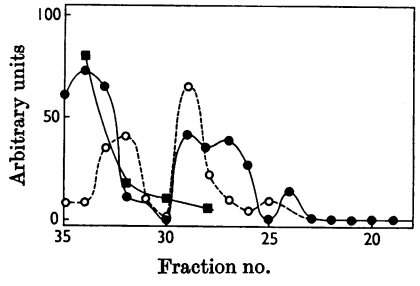

Fig. 1. Ultracentrifugal analysis in a sucrose gradient of 600*g* supernatant of frog leg-muscle homogenate. Details were as given in the text. ○, PFK activity; ■, aldolase activity; ●, E_{260}.

homogenized in 5vol. of water was used to assay 'malic enzyme' (EC 1.1.1.38) by the method of Stickland (1959*b*) as modified by L. V. Eggleston, and to assay glutamate–oxaloacetate transaminase and glutamate–pyruvate transaminase by the methods of Bergmeyer & Bernt (1963). Glucose 6-phosphatase was assayed by the method of Harper (1963) on a 600*g* supernatant of muscle homogenized in 5vol. of 100mM-potassium citrate buffer, pH6·5. All enzyme activities are expressed as μmoles of substrate transformed/g. fresh wt. of tissue/hr. at 25°.

RESULTS

Conditions for assay of phosphofructokinase and fructose 1,6-*diphosphatase.* As shown in Table 1, it was necessary to use the crude extract for assay of PFK activity because the enzyme from some muscles is partially sedimented after centrifugation at 10000*g* and 114000*g* (in the ionic extraction medium). The values presented in a preliminary communication (Opie & Newsholme, 1965) were based on assays of the 114000*g* supernatant, which explains why those activities are much lower than those now reported.

To investigate this further, a crude extract of frog leg muscle was subjected to a sucrose-density-gradient ultracentrifugal analysis. PFK activity and the extinction at 260mμ were found in two major peaks (Fig. 1).

To measure maximum PFK activities fresh tissue extracts were always used in the assay. Storage at −10° led to inactivation, which, however, differed from that described by Mansour (1965), as incubation with ADP or fructose 1,6-diphosphate did not result in reactivation. In the assay medium for PFK, 2mM-AMP and 0·3mM-potassium cyanide were present to inhibit FDPase and $NADH_2$ oxidase respectively (see Underwood & Newsholme, 1965*b*).

No loss of FDPase activity was observed after centrifugation at 114000*g* and therefore the supernatant after this centrifugation was used for measurements of FDPase activity. It was, however, necessary to decant and mix the whole 114000*g* supernatant, because FDPase activity layered out during centrifugation.

Certain experimental procedures were carried out to achieve maximal activity of FDPase. First, an alternative extraction medium (see Underwood & Newsholme, 1965*a*) consisting of EDTA (1mM), potassium chloride (154mM) and mercaptoethanol (10mM), final pH7·0, was used. FDPase activities were similar to those obtained with the usual extraction medium, which was designed to maintain maximal PFK activity. Secondly, the possibility of AMP in the muscle extract inhibiting FDPase activity (Krebs & Woodford, 1965) was

investigated by passing some extracts through a Sephadex G-25 column before assay; because there was no increase in FDPase activity, extracts were not normally passed through Sephadex. EDTA could be omitted from the assay mixture (but not the extraction medium) with a loss of only 6% of the FDPase activity. Mercaptoethanol and Mg^{2+} were required in the assay medium to ensure maximum activity. Increase of Mg^{2+} concentration from 5 to 30mM produced no significant changes in FDPase activity. A tenfold increase of glucose 6-phosphate-dehydrogenase concentration in the assay cuvette did not increase FDPase activities.

Conditions for assay of phosphoenolpyruvate carboxykinase. The activities of phosphoenolpyruvate carboxykinase were similar in the crude extract and in the 114000*g* supernatant. The following procedures did not change the phosphoenolpyruvate-carboxykinase activity of hen breast muscle: the replacement of 1mM-Mn^{2+} by 5mM-Mg^{2+} in the assay system, the addition of 10mM-mercaptoethanol to the assay cuvette, or ultrasonic treatment of the crude extract. The enzyme was not inhibited by AMP (1·7mM). The accuracy of the measurements of phosphoenolpyruvate-carboxykinase activity was impaired by the high 'blank' activity observed in the absence of sodium hydrogen carbonate. Such blank activity could be due to $NADH_2$-oxidase, pyruvate-kinase or lactate-dehydrogenase activity. If the potassium salt of phosphoenolpyruvate was used, blank activity was 75% of the total; this was decreased to 50% with the sodium or tricyclohexylammonium salt. The blank was decreased to below 50% if the manganese chloride in the assay medium was replaced by magnesium chloride (5mM). The addition of sodium cyanide (0·1mM) to the assay medium did not influence blank activity.

Comparison of fructose 1,6-diphosphatase and phosphofructokinase activities. FDPase activity was absent (less than 1 μmole/g. fresh wt./hr. at 25°) in the heart ventricle of the following animals: frog (*Rana* and *Xenopus*), domestic fowl (hen), albino rat, hamster, tame rabbit and cat, and also in the atrium of human heart (Table 2). There was a trace of activity in pigeon ventricle (about 1 μmole/g./hr.). There was also no activity in the leg and pectoral muscles of the tropical water-bug *Lethocerus*. FDPase activity was, however, absent from only one, namely rabbit semitendinosus (Tables 2–4), of the skeletal muscles of all the other species examined. In all these muscles, the activity of PFK varied between 500 and 1000 μmoles/g. fresh wt./hr., except for insect muscle, in which PFK activity was about 10000 μmoles/g. fresh wt./hr.

Highest FDPase activities were found in turtle (*Pseudemys scripta*) muscles, rabbit white muscle, frog leg and hen breast and wing (Table 3). The heart of the turtle, in contrast with that of other species, had some FDPase activity. These FDPase activities found in the 114000*g* supernatant were compared with PFK activities of the crude extracts (Table 3). For every muscle in which both activities were measured the PFK/FDPase activity ratio was greater than 10.

Activities of fructose 1,6-diphosphatase, phosphofructokinase and lactate-dehydrogenase isoenzymes in red muscle and white muscle. Initial studies sug-

Table 2. *Muscles without fructose 1,6-diphosphatase activity*

The conditions of incubation for the assays are given in the text. Activities are expressed in μmoles of substrate transformed/g. fresh wt./hr. at 25°. The numbers of homogenates analysed are given in parentheses. L.V., Left ventricle. Methods were as described in the Materials and Methods section.

Animal	Muscle	FDPase activity	PFK activity
Frog (*Rana*)	Heart	<1	—
Frog (*Xenopus*)	Heart	<1	—
Hen	Heart (L.V.)	<1	478 (2)
	Gizzard	<1	—
Rat	Heart (L.V.)	<1	578
Hamster	Heart (L.V.)	<1	932
Rabbit	Heart (L.V.)	<1	1090
	Semitendinosus	<1	478 (4)
Cat	Heart	<1	524 (2)
Human	Heart (atrium)	<1	—
Pigeon	Heart (L.V.)	<2	1052
	Gizzard	<1	105
Lethocerus	Leg	<1	11750 (2)
	Pectoral	<1	8000 (2)

Table 3. *Muscles with fructose 1,6-diphosphatase activity*

Activities are expressed in μmoles of substrate transformed/g. fresh wt./hr. at 25° with means ± S.E.M. The numbers of homogenates analysed are given in parentheses. Methods were as described in the Materials and Methods section.

Animal	Muscle	FDPase activity	PFK activity	PFK/FDPase activity ratio
Mollusca:				
Sea mussel (*Mytilus*)		49 (2)	—	—
Crustacea:				
Crab (*Maia squinado*)	Pincer	29 ± 3 (5)	—	—
Amphibia:				
Frog (*Rana*)	Thigh	47 ± 4 (10)	826 ± 48 (4)	18
	Gastrocnemius	47 ± 3 (4)	—	—
	Pectoral	35 ± 2 (3)	—	—
Frog (*Xenopus laevis*)	Thigh	74 ± 4 (7)	—	—
	Pectoral	56	—	—
Reptilia:				
Turtle (*Pseudemys scripta*)	Heart	17	—	—
	Pectoral	98 (2)	—	—
	Back	117 ± 10 (4)	—	—
	Hind limb	95 (2)	—	—
Aves:				
Pigeon	Pectoral	43 ± 6 (10)	1521	35
	Wing	41	—	—
	Thigh	36 ± 5 (3)	743	21
Hen (White Leghorn)	Pectoral	211 ± 7 (23)	2435 ± 43 (4)	12
	Wing	141 ± 18 (6)	1985 (2)	14
Mammalia:				
Rat	Pectoral	6	2320	380
	Abdominal	10	2430	243
	Front leg	8	—	—
	Hind leg	22 ± 2 (4)	2800	127
Hamster	Quadratus lumborum	34	2850	84
	Front leg	31	2620	85
	Hind leg	34 ± 5 (4)	2700	79
Rabbit	Diaphragm	32	895	28
	Quadratus lumborum	59 ± 23 (3)	2104	36
Cat	Diaphragm	36	—	—
	Pectoral	49	—	—
	Thigh	43 ± 4 (7)	—	—
Human	Latissimus dorsi	10 ± 0·3 (7)	—	—

gested that white rather than red muscle had the higher FDPase activity (Table 3). FDPase activity was therefore measured in white muscle and red muscle of the dogfish (*Squalus acanthias*), the hen and the rabbit. The activities of PFK and of the isoenzymes of lactate dehydrogenase were measured, and the PFK/FDPase activity ratios and the isoenzyme ratios were calculated (Table 4). In general, red muscle had lower PFK and FDPase activities, lower lactate-dehydrogenase activities and higher isoenzyme ratios than white muscle. In particular the skeletal muscle that had the reddest appearance, namely rabbit semitendinosus, resembled heart muscle in that FDPase activity was absent and the isoenzyme ratio was high.

Activities of phosphoenolpyruvate carboxykinase and other enzymes. The activities of pyruvate carboxylase and glucose 6-phosphatase were generally very low in the muscles that were investigated. However, activities of the malic enzyme, glutamate–oxaloacetate transaminase and glutamate–pyruvate transaminase were observed in some of these muscles (Table 5). (Although glutamate–pyruvate transaminase is absent from hen heart, it is very active in pig heart, from which source it is commercially purified.)

Table 4. *Activities of fructose 1,6-diphosphatase, phosphofructokinase and lactate dehydrogenase in red muscles and white muscles*

Activities are expressed in μmoles of substrate transformed/g. fresh wt./hr. at 25°. The numbers of homogenates analysed are given in parentheses. Muscles of rabbits were visually graded from reddest (heart) to whitest (white fibres of gastrocnemius). L.V., Left ventricle. Methods were as described in the Materials and Methods section.

Animal	Muscle	FDPase activity	PFK activity	PFK/FDPase activity ratio	Lactate-dehydrogenase activity: Pyruvate concn. 0·33mM	Lactate-dehydrogenase activity: Pyruvate concn. 10mM	Lactate-dehydrogenase isoenzyme ratio
Dogfish	Red	12 (4)	—	—	3663 (4)	1144 (4)	3·9 (4)
	White	32 (4)	—	—	20650 (2)	13450 (2)	1·6 (2)
Hen	Thigh (red)	58	947 (3)	16	1682 (2)	1012 (2)	1·7 (2)
	Thigh (white)	149	1565 (2)	11	2885 (2)	3405 (2)	0·9 (2)
Rabbit	Heart (L.V.)	0 (4)	1090	∞	2020	297	6·8
	Semitendinosus	0 (4)	478	∞	1061 (4)	208 (4)	5·3 (4)
	Pectineus	27 (2)	729	27	1474 (2)	1030 (2)	1·5 (2)
	Vastus intermedius of quadriceps	54 (2)	1422	26	1732 (2)	1682 (2)	1·1 (2)
	Gastrocnemius (red)	48 (2)	1460	30	2242 (2)	2095 (2)	1·1 (2)
	Vastus lateralis of quadriceps	55 (2)	1520	28	2570 (2)	2000 (2)	1·4 (2)
	Adductor magnus	54 (2)	1662	31	3370 (2)	4220	0·9 (2)
	Adductor longus	72 (2)	1840	26	1980 (2)	2395 (2)	0·9 (2)
	Gastrocnemius (white)	50 (2)	1710	34	1930 (2)	3142 (2)	0·6 (2)
Turtle	Heart	17	—	—	552	161	3·4
	Back	117 (4)	—	—	968 (2)	515 (2)	1·9 (2)

The presence and activity of phosphoenolpyruvate carboxykinase in muscle correlates well with FDPase activity (Table 5). Previous studies have suggested that phosphoenolpyruvate carboxykinase is absent from muscle (Utter, 1959; Keech & Utter, 1963), but the activity was measured in mixed muscle and was not investigated in white muscle.

DISCUSSION

Krebs & Woodford (1965) found FDPase activity in skeletal muscle from various animals and birds and discussed the role of this enzyme in skeletal muscle. This work did not, however, explain the marked variation in FDPase activity in various muscles, nor did it indicate the possible significance of FDPase activity in modifying the control of glycolysis at the level of phosphofructokinase.

In the present investigation the PFK/FDPase activity ratio was so high in most muscles that it seems unlikely that FDPase could play a major role in the regulation of fructose 6-phosphate phosphorylation. Therefore the presence of FDPase activity in muscle does not imply that both PFK and FDPase provide a regulatory cycle in which both enzymes participate, and through which metabolic control is exerted, as has been proposed for liver and kidney cortex (Underwood & Newsholme, 1965*b*,*c*). The potential for PFK activity suggests that this enzyme activity is dominant in muscle tissue and that control of glycolysis would be obtained through changes in the activity of this enzyme. Nevertheless, the activity of FDPase, in relation to PFK, must be controlled so that, when FDPase is active, PFK activity must be strongly inhibited.

It was observed that in some muscle extracts PFK activity was not fully recovered on high-speed centrifugation (Table 1). This could not be explained solely by inactivation of PFK, because it was observed that some PFK had sedimented during centrifugation. On sucrose-density-gradient analysis of an extract of frog leg muscle, about half the PFK activity moved well ahead of the marker enzyme aldolase (see also Mansour, 1965), and coincided with a peak of 260mμ extinction (Fig. 1). This is consistent with the finding of Pette & Hofer (1965) that a fraction of muscle PFK is a nucleic acid–protein complex.

The variation in FDPase activity of muscle cannot be explained on a phylogenetic basis (Tables

Table 5. *Activities of some enzymes related to carbohydrate or amino acid metabolism in skeletal muscle*

Activities are expressed in μmoles of substrate transformed/g. fresh wt./hr. at 25°, except for glycerokinase (30°). For red muscle of rabbit leg, (P) indicates pectineus and (S) indicates semitendinosus. FDPase values are taken from Tables 3–5. Methods were as described in the Materials and Methods section. Results of pyruvate carboxylase assay were provided by L. V. Eggleston & H. A. Krebs (personal communication).

		Enzyme activity								
Animal	Muscle	FDPase	Phosphoenol-pyruvate carboxykinase	Pyruvate carboxylase	Malic enzyme	Glutamate–oxaloacetate transaminase	Glutamate–pyruvate transaminase	Glycerokinase	Glucose 6-phosphate dehydrogenase	Glucose 6-phosphatase
Hen	Pectoral	211	160	< 2	38	86	0	0	—	1·2
	Leg (white)	149	37	—	31	1566	—	0·1	—	2·0
	Leg (red)	58	5	—	28	3600	—	—	—	3·5
	Heart	0	0	—	72	7300	0	0·5	—	—
Rabbit	Leg (white)	54	120	< 2	6	458	4	—	1·4	—
	Leg (red) (P)	27	23	—	—	840	—	—	—	—
	Leg (red) (S)	0	0	—	5	648	—	—	1·5	—
	Heart	0	—	—	5	—	—	—	2·1	—
Frog	Thigh	74	62	< 2	12	—	—	—	—	—
Pigeon	Pectoral	43	35	—	32	8060	1080	—	—	—
	Gizzard	0	12	—	25	1380	—	—	—	—
	Heart	0	0	—	12	9650	—	—	—	—

3 and 4). However, there was generally less activity in red muscle than in white (see Table 4). In particular, rabbit semitendinosus, which looked the reddest of the skeletal muscles investigated, possessed no FDPase activity. Similarly, the heart and smooth muscles tested were deep red and also contained no FDPase activity. The highest FDPase activities were found in hen breast and wing, and in turtle skeletal muscle, all of which are white. Moreover, pigeon breast muscle, with both white and red fibres (George & Naïk, 1958), had much less FDPase activity than hen breast muscle, with only white fibres. An investigation into the activity of other enzymes in white muscle that might give rise to substrate for FDPase was carried out; phosphoenolpyruvate carboxykinase was the only enzyme tested whose activity correlated with that of FDPase. The activities of FDPase and phosphoenolpyruvate carboxykinase in white muscle are similar to those in liver and kidney cortex (L. V. Eggleston, unpublished work), which are the main gluconeogenic tissues of the body. However, the other two characteristic gluconeogenic enzymes, pyruvate carboxylase and glucose 6-phosphatase, are virtually absent from white muscle, which excludes the possibility of gluconeogenesis from pyruvate and lactate in this tissue.

Therefore it is pertinent to enquire about the significance of the high activities of FDPase and phosphoenolpyruvate carboxykinase in white muscle, and how they relate to metabolic differences between white muscle and red muscle. White muscle depends more on glycolysis for its energy production than does red muscle, as shown by a comparison of the activities of the enzymes of glycolysis, and of the rates of substrate utilization (Lawrie, 1953*a*,*b*; Czok & Bücher, 1960; Bilinski, 1963; Pette & Bücher, 1963; Wirsen, 1965; Pette, 1966). Moreover, Kaplan & Goodfriend (1964) have shown that the ratio of the two lactate-dehydrogenase isoenzymes (H isoenzyme/M isoenzyme) is high in muscles that function aerobically, and low in those that depend more on anaerobic glycolysis; in the present investigation the isoenzyme ratio decreased as the activity of FDPase increased. Thus FDPase and phosphoenolpyruvate-carboxykinase activities are present in muscles that depend to a high degree on glycolysis for energy production. Heart muscle possessed no detectable FDPase activity, except in turtle, in which it has a relatively low isoenzyme ratio and can function anaerobically (Reeves, 1963). However, dependence on glycolysis for energy production does not imply that energy is obtained solely from glucose (or glycogen) conversion into lactate. Thus white muscle possesses the capacity for mitochondrial oxidation, but to a smaller extent than red muscle (see Pette, 1966). The substrate for oxidation in

white muscle is probably glucose or glycogen, rather than lipid, which is more important in red muscle (George & Jyoti, 1955; George & Scaria, 1958; Bilinski, 1963). Therefore in white muscle glycolysis will provide energy directly and also pyruvate for mitochondrial oxidation. Maintenance of rapid glycolysis necessitates the possession of an adequate system for oxidation of the $NADH_2$ that is produced extramitochondrially. Two systems for oxidation of glycolytic $NADH_2$ by the mitochondria may be present in muscle, the α-glycerophosphate–dihydroxyacetone phosphate cycle (Klingenberg & Bücher, 1960; Sacktor, 1961) and the malate–oxaloacetate cycle (Sacktor, 1961; Kaplan, 1963). Two results support the idea that oxidation of $NADH_2$ by these cycles may be more important in white muscle than in red muscle. First, the activities of the mitochondrial α-glycerophosphate oxidase and the soluble α-glycerophosphate dehydrogenase are much higher in white muscle than in red muscle (Young & Pace, 1958; Pette & Bücher, 1963; Pette, 1966). Secondly, the rate of oxygen consumption of mitochondria from white muscle is higher with α-glycerophosphate as substrate than with lactate, succinate or $NADH_2$, whereas the reverse is true for red muscle (Blanchaer, 1964).

The hypothesis that FDPase activity is present in skeletal muscle to permit glycogen formation from α-glycerophosphate, which might accumulate during exercise, was proposed by Krebs & Woodford (1965). The present work strengthens this hypothesis, as it shows that FDPase is present in muscles that contain active α-glycerophosphate–dihydroxyacetone phosphate-cycle enzymes, and in which an accumulation of intermediates of this cycle (α-glycerophosphate and dihydroxyacetone phosphate) might be expected during exercise. This hypothesis can be extended to include the malate–oxaloacetate cycle. Phosphoenolpyruvate-carboxykinase activity would ensure that malate and oxaloacetate, which might accumulate during exercise, could be converted into phosphoenolpyruvate and hence into fructose diphosphate. It is suggested that the activities of these cycles are controlled by the concentrations of α-glycerophosphate and malate, so that the function of FDPase and phosphoenolpyruvate carboxykinase in white muscle might be to decrease the concentrations of accumulated intermediates of the α-glycerophosphate–dihydroxyacetone phosphate and malate–oxaloacetate cycles, thereby inhibiting the activities of these cycles after muscular exercise. The absence of FDPase from *Lethocerus* suggests that control of this cycle in insect muscles, some of which are known to have an active α-glycerophosphate–dihydroxyacetone phosphate cycle, may differ from that proposed here for white skeletal muscle. The present proposals would explain the presence of phosphoenolpyruvate carboxykinase in white muscle despite the absence of pyruvate carboxylase, which in liver and kidney cortex functions sequentially with phosphoenolpyruvate carboxykinase and so allows gluconeogenesis from lactate and pyruvate.

We thank Professor Sir Hans Krebs, F.R.S., for his interest and encouragement. The assistance of Miss Janice Robinson, Mr L. Eggleston and Dr J. Wallace is gratefully acknowledged. L.H.O. was supported by the Wellcome Trust. This work was supported by U.S. Public Health Service Grant no. AM08715.

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