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Fructose 1,6-Diphosphatase in Striated Muscle

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1. The occurrence of fructose diphosphatase in muscle tissue was investigated with reference to the question whether lactate can be converted into glycogen in muscle, as postulated by Meyerhof (1930), fructose diphosphatase being one of the enzymes required for this conversion. 2. Fructose diphosphatase was found in skeletal muscle of man, dog, cat, rat, mouse, rabbit, guinea pig, cattle, sheep, pigeon, fowl and frog. Under the test conditions between 5 and 60 μmoles of substrate were split/g. fresh wt./hr. at 22°. 3. Like liver fructose diphosphatase, the muscle enzyme is inhibited by substrate concentrations above 0·1 mM, by AMP and by trace quantities of Zn^{2+}, Fe^{2+} and Fe^{3+}; it is 'activated' by EDTA. Inhibitions by the above agents may account for the failure of previous authors to detect the enzyme. 4. Heart muscle of several vertebrate species and the smooth muscle of pigeon and fowl gizzard had no measurable activity. 5. The presence of fructose diphosphatase and the virtual absence of the enzyme systems converting pyruvate into phosphopyruvate means that lactate and pyruvate cannot be converted into glycogen in muscle, whereas the phosphorylated C_3 compounds can. The reconversion into carbohydrate of lactate (which readily diffuses out of muscle) occurs in liver and kidney only. The reconversion of phosphorylated C_3 intermediates (which cannot diffuse out of the tissue) can occur only within the muscle. 6. α-Glycerophosphate is probably the main intermediate requiring conversion into glycogen. The possible role of α-glycerophosphate formation in vertebrate muscle, already well established in insect muscle, is discussed.

Whether lactate can be resynthesized to carbohydrate in striated muscle, as was suggested by Meyerhof (1919, 1930) and Hill (1924), is still an open question. The experimental evidence supporting this concept has never been conclusive. The starting point was a set of experiments on frog muscle that purported to show that the amounts of lactate disappearing in the muscle after exercise were much greater than could be accounted for by complete oxidation. However, the crucial test, i.e. the direct demonstration of a net synthesis of carbohydrate from lactate in muscle at a rate approaching the postulated one (1 glucose equiv. formed/mol. of oxygen used), has been lacking, as has been repeatedly pointed out (Eggleton & Evans, 1930; Janssen & Jost, 1925; Long & Horsfall, 1932; Flock, Ingle & Bollman, 1939). Peters & Van Slyke (1946), after reviewing the evidence, emphasized that part of the lactate formed in muscle is definitely not resynthesized to carbohydrate in this tissue, but escapes into the blood stream and can be converted into carbohydrate in the liver, and they add that 'it is not certain that all the lactic acid formed in muscle may not be forced to travel this circuitous course'. Since then experiments with isotopically labelled lactate (Omachi & Lifson, 1956; Cheosakul, Blide, Vanko & Knudson, 1956; Hiatt, Goldstein, Lareau & Horecker, 1958; Warnock, Inciardi & Wilson, 1963) have shown that a resynthesis of glycogen from lactate cannot be a major metabolic process in muscle tissue.

Recently it has become possible to approach the problem from a new angle. It is now known that the conversion of lactate into carbohydrate requires several special enzymes (see Krebs, 1954, 1964). These include the specific FDPase* and the enzymes that convert pyruvate into oxaloacetate and oxaloacetate into phosphopyruvate, namely pyruvate carboxylase and phosphopyruvate carboxylase. Without these enzymes lactate cannot be resynthesized to carbohydrate. The determination of the activities of these enzymes therefore supplies information relevant to the occurrence of the

* Abbreviations: FDP, fructose 1,6-diphosphate; FDPase, fructose 1,6-diphosphatase.

resynthesis. Tests for FDPase in cardiac muscle and diaphragm of rats by Newsholme & Randle (1962) and by Weber & Cantero (1959) in striated muscle of the rat gave negative results. Previous unpublished tests in this Laboratory on rat muscles seemed to confirm these findings. However, two circumstances prompted us to re-examine the activity of the phosphatase: a personal remark by Dr G. Weber of the University of Indiana Medical School that he had found some FDPase activity in human muscle, and the realization that the reported negative results could have been due to the inhibition of the enzyme by excess of substrate or the presence in the tissue extract of AMP (Newsholme, 1963; Taketa & Pogell, 1963; Krebs, 1964) and the absence of a suitable chelating agent such as EDTA. The reinvestigation reported in the present paper shows that the striated muscle of several vertebrate species in fact possesses significant amounts of the specific FDPase.

EXPERIMENTAL

Determination of fructose-diphosphatase activity. The enzyme activity was usually measured by the principle described by Taketa & Pogell (1963). The enzyme preparation was incubated with the substrate, an excess of hexose 6-phosphate isomerase, glucose 6-phosphate dehydrogenase and NADP, and the reduction of NADP was measured spectrophotometrically. For the standard assay 1·85 ml. of an aqueous solution containing the following was prepared: $NaHCO_3$, 26 mM; NADP, 0·2 mM; $MgSO_4$, 20 mM; EDTA (neutralized), 1 mM; hexose 6-phosphate isomerase (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany), 0·02 mg./10 ml.; glucose 6-phosphate dehydrogenase (C. F. Boehringer und Soehne G.m.b.H.), 0·02 mg./10 ml. This was mixed with 0·1 ml. of 2 mM-FDP (tetracyclohexylammonium salt) and 0·05 ml. of tissue preparation suitably diluted. The total volume was thus 2 ml. This mixture was placed in the 1 cm. cuvette of a Zeiss PMQ2 model spectrophotometer. The solution was gassed for 2 min. with CO_2+N_2 (5:95) through a capillary while the cell was covered with Parafilm. Changes in extinction were measured at 340 mμ over a 10 min. period, during which the rate was constant. Readings began about 3 min. after the addition of the enzyme. The temperature was 22° unless otherwise stated. The readings were corrected for a control from which FDP was omitted. It is important to treat the reaction mixture thoroughly with the CO_2-containing gas mixture before the tissue extract is added. A stream of fine bubbles was passed through the solution for about 30 min.

The enzyme preparation was the supernatant of a tissue homogenate prepared as soon as possible after removal of the tissue from the body. A weighed piece of tissue was placed in a cooled Waring Blendor or, when the quantity of tissue available was small, in an MSE top-drive homogenizer together with 9 vol. of 1 mM-EDTA neutralized to pH 7. The presence of EDTA increased the activity of the FDPase, usually by about 30%. After homogenization, which was achieved within 2 min., the material was centrifuged for 15 min. at 2500 *g*. The supernatant, which contained the total enzyme activity, was filtered through glass wool to remove fat particles floating on the surface. It was diluted for the assay if preliminary tests indicated that the FDPase activity was too high.

The control without FDP measured the formation of glucose 6-phosphate from glycogen. Owing to the high dilution of the homogenates and the low tissue concentration of FDP and hexose monophosphates, it was possible to neglect the preformed quantities of these substances in the assay system.

This procedure may not be suitable for those tissue extracts that contain glucose 6-phosphatase or other phosphatases hydrolysing sugar phosphates, because these enzymes can cause side reactions of glucose 6-phosphate or fructose 6-phosphate. The interfering enzymes are, however, absent from muscle.

A second method was used in special cases when the procedure of Taketa & Pogell (1963) was not practicable. It consisted of a determination of the FDP remaining after a fixed period of incubation by the method of Bücher & Hohorst (1963).

RESULTS

Optimum conditions for fructose diphosphatase. Preliminary experiments indicated that the nature of the buffer, the concentration of Mg^{2+} and the presence of EDTA can affect the activity of the enzyme. The effects of these agents were therefore systematically studied. Replacing the bicarbonate–carbon dioxide buffer, pH 7·4, by sodium phosphate or tris buffer did not change the reaction rate appreciably (Table 1). Since tris has a low buffering capacity at the pH optimum of the enzyme and since phosphate gives precipitates with Mg^{2+} at higher concentrations, the bicarbonate–carbon dioxide buffer was adopted for the standard procedure. Without the addition of Mg^{2+} the activity of the muscle extract was nil, and the optimum range of Mg^{2+} concentrations was found to be between 20 and 40 mM (Table 2). Without EDTA the enzyme activity was variable and very low. In the example quoted in Table 3 it was 25% of

Table 1. *Effect of buffers on the activity of fructose diphosphatase of cat leg muscle*

Standard conditions as described in the text were used, except that the buffer was varied. The pH was 7·4

Buffer (final concn.)	FDP split (μmoles/g. fresh wt./hr.)
$NaHCO_3$–CO_2 (26 mM)	27·6
Sodium phosphate (10 mM)	27·0
Sodium phosphate (20 mM)	29·3
Tris (20 mM)	30·0

Table 2. *Effect of* Mg^{2+} *on the activity of fructose diphosphatase of cat leg muscle*

Incubation was for 10 min. at 22°. The Mg^{2+} was added as $MgSO_4$. Standard conditions as described in the text were used, except that the concentration of $MgSO_4$ was varied.

Concn. of Mg^{2+} (mM)	FDP split (μmoles/g. fresh wt./hr.)
0	0
2·5	11·2
5	18·4
10	25·1
20	28·8
30	29·4
40	30·0

Table 3. *Effect of EDTA on the activity of fructose diphosphatase of cat leg muscle*

Standard conditions as described in the text were used, except that the concentration of EDTA was varied. The 2 ml. sample contained 0·05 ml. of 10% (w/v) homogenate. Incubation was at 20°.

Concn. of EDTA (mM)	FDP split in 2 ml. (μmole)		FDP split (μmoles/g. fresh wt./hr.)
	After 5 min.	After 10 min.	
0	0·0037	0·0075	9·0
0·01	0·0110	0·0218	26·2
0·1	0·0105	0·0237	28·4
1·0	0·0136	0·0301	36·1
2·0	0·0136	0·0298	35·8
5·0	0·0096	0·0198	23·8
10·0	0	0	0

Table 4. *Time-course of the action of fructose diphosphatase of cat leg muscle*

Cat leg muscle was tested under the standard conditions described in the text, with 0·05 ml. of 10% (w/v) homogenate in the 2 ml. sample to which the results refer. Incubation was at 22°.

Time (min.)	FDP split (μmole)
5	0·011
10	0·027
20	0·057
30	0·090
40	0·121
50	0·140
60	0·146
90	0·153
150	0·167
230	0·180

the optimum rate. Under the assay conditions 1 mM-EDTA was optimum; 10 mM completely inhibited the enzyme (see also McGilvery, 1964).

Time-course. The rate of the reaction was constant during the first 40 min. of the assay test (Table 4). By this time about two-thirds of the added substrate had been converted into fructose 6-phosphate. The subsequent decline of the reaction rate was mainly due to the circumstance that the enzyme preparation contained aldolase, which converted part of the substrate into triose phosphates. The very low rate at the later stages of incubation was probably connected with the fact that the reconversion of triose phosphate into FDP was rate-limiting and slow.

Effect of pH. The pH was varied by changing the concentrations of carbon dioxide, HCO_3^- and CO_3^{2-} in the medium (see Table 5). There is a broad maximum between pH 6·8 and 7·5, falling more sharply towards the acid than the alkaline region. Activity was detectable between pH 5·5 and 10·2; there was no activity at pH 5·1 and 10·8.

Effect of substrate concentration. With increased substrate concentrations the rate of reaction rose to a maximum at the very low concentration of 6·3 μM and remained about the same up to 0·1 mM (Table 6). It fell with further concentration increases and dropped to 38% of the maximum rate at 1·6 mM. Further increase of the substrate concentration did not diminish the reaction rate. The low apparent Michaelis constant (about 2 μM) and the inhibition by excess (about 0·1 mM) of substrate are quantitatively very similar to the corresponding characteristics of the liver FDPase (Underwood & Newsholme, 1965).

Effect of temperature. The standard procedure was not suitable for assay at temperatures other than room temperature because of the difficulty of keeping the temperature of the cuvette constant. The incubation was therefore carried out under standard conditions in test tubes kept in a water bath, and after short periods (20 min. at 22°, 10 min. at 30° and 5 min. at 41°) the reaction was stopped by the addition of an equal volume of 6% (w/v) perchloric acid. The remaining FDP, which amounted to about 75% of the initial amount, was determined enzymically according to the method of Bücher & Hohorst (1963). The results (Table 7) show that within the range tested Q_{10} is about 2.

Inhibition by AMP. Like the liver FDPase (Newsholme, 1963; Utter, Keech & Scrutton, 1964; Taketa & Pogell, 1963), the muscle enzyme is inhibited by very low concentrations of AMP. An inhibition of 50% required an AMP concentration of 2 μM under the test conditions (Table 8). Under the same conditions 1 mM-ATP had no effect; ADP (1 mM) was also inhibitory, but as the extract contained a highly active adenylate kinase,

Table 5. *Effect of* pH *on the activity of fructose diphosphatase of cat leg muscle*

Each sample contained 1·95 ml. of buffer solution including $MgSO_4$ (20 mM) and EDTA (1 mM), and 0·05 ml. of 10% (w/v) homogenate. The concentrations of the buffer constituents were as indicated. The pH was calculated on the assumption that pK_1 is 6·3, pK_2 10·2 and α_{CO_2} 0·82. Incubation was for 10 min. at 22°.

	Details of buffer				FDP split
Sample no.	Concn. of $NaHCO_3$ (mM)	Concn. of Na_2CO_3 (mM)	CO_2 content of gas (%)	pH	(μmoles/g. fresh wt./ 10 min.)
1	2·25	0	100	5·1	0
2	5·5	0	100	5·5	0·20
3	13	0	100	5·9	1·18
4	26	0	100	6·2	1·98
5	52	0	100	6·5	4·22
6	2·25	0	5	6·4	3·97
7	5·5	0	5	6·8	6·50
8	13	0	5	7·2	6·25
9	2·25	0	1	7·1	6·50
10	26	0	5	7·5	6·18
11	5·5	0	1	7·5	5·67
12	52	0	5	7·8	5·63
13	13	0	1	7·9	5·0
14	26	0	1	8·2	3·93
15	52	0	1	8·5	3·83
16	22·7	2·27	0	9·2	2·02
17	20	5	0	9·6	1·32
18	12·5	12·5	0	10·2	0·55
19	5	20	0	10·8	0

Table 6. *Effect of the substrate concentration on the activity of fructose diphosphatase of cat leg muscle*

Standard conditions as described in the text were used, except that the concentration of FDP was varied. The 2 ml. sample contained 0·05 ml. of 10% (w/v) homogenate. The rates were constant during the 10 min. of incubation, except in the first four samples where the rate was calculated from initial readings.

Concn. of FDP (mM)	FDP split in 10 min. (μmole)
0·0031	0·017
0·0063	0·0232
0·0125	0·0233
0·025	0·0233
0·05	0·0234
0·1	0·0234
0·2	0·0173
0·4	0·0135
0·8	0·0103
1·6	0·0088
3·2	0·0088

which rapidly generated AMP from ATP, the inhibitory action of ADP can be accounted for by the AMP formed. The sensitivity of the cat-muscle enzyme to AMP was about 100 times that of the rat-liver enzyme under the same conditions (Table 9).

Table 7. *Effect of temperature on the activity of fructose diphosphatase of cat leg muscle*

The conditions of incubation for the assay are given in the text.

Temperature	FDP split (μmoles/g. fresh wt./hr.)
22°	30·0
30	54·6
41	106

Table 8. *Effect of AMP on the activity of fructose diphosphatase of cat leg muscle*

Standard conditions as described in the text were used, except that AMP was added.

Final concn. of AMP (μM)	FDP split (μmoles/g. fresh wt./hr.)	Inhibition (%)
0	29·6	—
1	22·2	25
2·5	10·9	63
5	4·1	86
10	0	100

Inhibition by heavy-metal ions. The inhibition by heavy-metal ions (see Underwood & Newsholme, 1965) was tested in the absence of EDTA, water

being used for the extraction of the tissue. Owing to the absence of EDTA the absolute rates were lower than in other experiments. As in liver, Zn^{2+} was a very powerful inhibitor at 1 μM; Fe^{2+} and Fe^{3+} also inhibited, and the degree of the inhibition by the Fe^{2+} and Fe^{3+} depended on whether the substrate was added to the enzyme buffer mixture before or after the metal ions (Table 10). The inhibition by Zn^{2+} was independent of the order of mixing, and was abolished by a subsequent addition of EDTA and much decreased, but not completely abolished, by mercaptoethanol. The concentration of total zinc (as opposed to Zn^{2+}) in muscle and liver according to Underwood (1962) is more than 100 times as great as those that can bring about effective inhibitions (about 50 mg./kg. of human liver or muscle). It is not known, however, what fraction of the total zinc in the tissue is in the form of free Zn^{2+} ions.

Table 9. *Effect of AMP on the activity of fructose diphosphatase of rat liver*

Standard conditions as described in the text were used, except that AMP was added and the liver extract was more dilute (1:40). Each cuvette contained 0·05 ml. of the extract. No free glucose was formed on incubation, indicating the virtual absence of glucose 6-phosphatase from the extract.

Final concn. of AMP (mM)	FDP split (μmoles/g. fresh wt./hr.)	Inhibition (%)
0	220	—
0·1	174	21
0·2	75	64
0·4	24	89

Not unexpectedly, the quantitative aspects of the experiments with trace metal ions showed variability. In some samples the presence of 0·1 μM-Zn^{2+} had no effects; in these samples the activity of FDPase in the absence of a chelating agent was low and the activation on the addition of EDTA or mercaptoethanol was large. These findings are in accordance with the assumption that the original samples already contained effective concentrations of inhibiting trace metal ions.

Effect of storage. The loss of enzyme activity on storage was tested in the following way. First, fresh tissue removed from the animal immediately after death was extracted without delay and compared with samples of muscle that had been frozen in solid carbon dioxide immediately after its removal from the animal, and storage for various periods at −15°. Secondly, extracts prepared in the usual way were tested immediately and after storage at −15°. In general, the FDPase activity of muscle stored in the frozen state either as the whole tissue or as an extract was the same as in the fresh tissue.

The FDPase activity gradually disappears after death when the tissue is left *in situ*. Muscle from a dog that had been left for 3 hr. at room temperature and 21 hr. in the cold room showed 59% of the activity of fresh muscle. After another 24 hr. in the cold room it had dropped to 42%.

Effect of exercise. It was thought that physical exercise, which creates a need for a rapid reconversion of lactate into carbohydrate, might induce a formation of FDPase in striated muscle. Rats were exercised by swimming, as described by Krebs & Yoshida (1963), but no increase in enzyme activity was found.

Table 10. *Inhibition of fructose diphosphatase of cat leg muscle by heavy-metal ions*

Standard conditions as described in the text were used, except that EDTA was omitted from the extraction and incubation media. The cuvettes were first filled with the complete reaction medium without substrate and heavy-metal salts. Heavy-metal ions were added as sulphate salts either 2 min. before the substrate or immediately after the substrate.

Heavy-metal salt added (final concn.)	Timing of addition (before or after substrate)	FDP split (μmoles/g. fresh wt./hr.)	Inhibition (%)
None		16·7	
$FeSO_4$ (10 μM)	Before	2·3	86
$FeSO_4$ (10μM)	After	12·1	28
$FeSO_4$ (1 μM)	Before	9·1	46
$FeSO_4$ (1 μM)	After	14·3	13
$Fe_2(SO_4)_3$ (10 μM)	Before	8·1	51
$Fe_2(SO_4)_3$ (10 μM)	After	12·9	28
$Fe_2(SO_4)_3$ (1 μM)	Before	15·4	8
$Fe_2(SO_4)_3$ (1 μM)	After	17·0	0
$ZnSO_4$ (10 μM)	Before	5·9	65
$ZnSO_4$ (10 μM)	After	4·9	70
$ZnSO_4$ (1 μM)	Before	9·8	41
$ZnSO_4$ (1 μM)	After	10·0	40
$ZnSO_4$ (0·1 μM)	Before	14·7	12

Fructose diphosphatase in muscle and other tissues. Voluntary striated muscle of all species tested showed some enzyme activity (Table 11), varying between 5 μmoles (pigeon) and 63·5 μ-moles (rabbit) of substrate split/g. fresh wt./hr. For comparison measurements of the enzyme activity of liver and kidney cortex were made under the same conditions (Table 12). In the Wistar rat the activity of liver and kidney cortex was 40–60 times that of skeletal muscle. In the cat, dog, rabbit and guinea pig the liver was 5–6 times as active as muscle. In the tame rabbit the activity of white muscle was about two-thirds that of liver or kidney cortex. The absolute value of the muscle enzyme activity showed great variations from species to species, and no simple correlations between the FDPase activity and functional aspects of muscle physiology are evident. Relatively sluggish animals (guinea pig, tame rabbit, cattle) had activities as high as those of fast-moving species (dog, cat, wild rabbit). Rabbit white muscle was almost twice as active as rabbit red muscle. The

Table 11. *Fructose diphosphatase activity of muscle of various vertebrate species*

The activity was measured under the standard conditions described in the text. When more than three samples were tested the results are given as means ± S.E.M. with the numbers of observations in parentheses. When one or two samples were tested the results obtained are recorded.

Species	Type of muscle	FDPase activity (μmoles/g. fresh wt./hr. at 22°)
Man	Latissimus dorsi	16·3 ± 1·82 (7)
Cat	Leg	37·0 ± 2·93 (5)
Cat	Diaphragm	12·9
Dog	Leg	25·5 ± 3·08 (3)
Rat (Wistar)	Leg	6·5 ± 0·19 (6)
Rat (Wistar)	Diaphragm	9·4
Rat (Wild)	Leg	14·6
Mouse	Leg	12·2
Rabbit (tame)	Leg (white muscle)	63·5 ± 11·6 (3)
Rabbit (tame)	Leg (red muscle)	31·6
Rabbit (tame)	Diaphragm	14·2; 16·0
Rabbit (wild)	Leg	24·5
Rabbit (wild)	Diaphragm	23·8
Guinea pig	Leg	30·6; 28·5
Cattle	Leg	26·6
Sheep	Leg	16·2
Pigeon	Breast	5·4; 10·0
Fowl (white Leghorn)	Breast	40
Fowl (white Leghorn)	Leg	12
Frog (*Rana pipiens*)	Leg	23·0
Frog (*Rana temporaria*)	Leg	27·3

Table 12. *Fructose diphosphatase activity of liver and kidney cortex of various vertebrate species*

The activity was measured as described in the text for muscle. Tests for the formation of free glucose showed that glucose 6-phosphatase did not interfere with the assay. For the diets used see Krebs, Bennett, de Gasquet, Gascoyne & Yoshida (1963). The results are expressed as means ± S.E.M. with the number of observations in parentheses, or as single measurements.

Species	FDPase activity (μmoles/g. fresh wt./hr. at 22°)	
	Liver	Kidney cortex
Rat (Wistar) on standard diet	261 ± 12·2 (6)	286 ± 13·2 (6)
Rat (Wistar) on low-carbohydrate diet	354 ± 15·3 (11)	379 ± 26·8 (11)
Mouse	292 ± 19·9 (8)	247 ± 8·9 (8)
Rabbit (Chinchilla × Blue Bevron cross)	88	86
Rabbit (wild)	143	89
Pigeon	333	123
Cat	187	115
Dog	250	216

physiologically most powerful muscle among those tested, the flight muscle of pigeons, had very low activity. The enzyme was also present in all diaphragms tested, but the hearts (not recorded in Table 11), wherever tested (cat, rat, rabbit, guinea pig and pigeon), gave negative results (i.e. less than 1 μmole/g. fresh wt./hr.). The smooth muscle of the gizzard of pigeon and fowl, contrary to the statement of Bargoni, Sisini & Majorano (1964), was also inactive, as were rat brain, lung, bone marrow, spleen, submaxillary gland, adipose tissue (renal and epididymal), calf thymus and guinea-pig mammary gland. Some activity comparable with that of muscle was found in rat testis, pancreas and intestinal mucosa (see also Weber, 1964); it was inhibited by 1 mM-AMP. It remains to be investigated whether these activities were due to an unspecific phosphatase. Traces of FDPase activity (1–2 μmoles/g. fresh wt./hr.) were found in sheep and ox retina and ox cornea.

In most species the FDPase activities of liver and kidney cortex were very similar (Table 12). An exception is the pigeon, where the activity of the kidney cortex was much lower than that of liver. As already known (Mokrasch, Davidson & McGilvery, 1956), the FDPase activity of liver increases when the diet is low in carbohydrate. In the series included in Table 12 this increase was 37%. There was a parallel increase of 33% in the kidney cortex.

Specificity of muscle fructose diphosphatase. Muscle extracts that utilize FDP fail to split *p*-nitrophenyl phosphate when tested as described by Linhardt & Walter (1963). This indicates that the fission of FDP cannot be ascribed to a general phosphatase.

DISCUSSION

Occurrence of fructose diphosphatase in muscle tissues. The experiments establish the fact that the voluntary striated muscle of many vertebrate species possesses an FDPase that, wherever tested, has the same characteristic properties as the FDPase of liver and kidney: it is highly specific for FDP; it is inhibited by excess of substrate, AMP and certain heavy-metal ions; it has the same pH optimum. The enzyme is absent from the heart of all the species tested and from pigeon and fowl gizzard. The variability of FDPase activity in frog and rabbit muscle noted by Lohmann (1933), and the negative results of Weber & Cantero (1959) in rat skeletal muscle and of Newsholme & Randle (1962) in diaphragm, were probably due to the inhibition by AMP and excess of substrate. These earlier measurements were carried out before the kinetics of the enzyme were adequately known.

Magnesium effect. It is remarkable that the optimum Mg^{2+} concentration was about 20 mM under the test conditions. It is unlikely that the high concentration of Mg^{2+} is directly needed for the maximal action of the enzyme. The fact that Mg^{2+} decreases the sensitivity of the enzyme to AMP (Underwood & Newsholme, 1965) suggests that the effect of high concentrations of Mg^{2+} may be due to the formation of an AMP complex. The existence of such complexes has been established by Smith & Alberty (1956) and by Walaas (1958).

Fructose-diphosphatase activity in relation to muscle metabolism. If FDPase played a role in the resynthesis of carbohydrate from lactate on the scale envisaged by Meyerhof (1930), the FDPase activity should be of the same order as the oxygen consumption because 1 mol. of oxygen is postulated to supply the energy for the resynthesis of 1 mol. of carbohydrate. A rate of 30 μmoles of FDP split/g. fresh wt./hr. at 22° corresponds to a rate of about 100 μmoles/g./hr. at 40°. The oxygen consumption of a severely exercised mammalian muscle is somewhat higher (between 200 and 300 μmoles/g. fresh wt./hr.). Thus the activity of muscle FDPase approaches the range where it could make a significant contribution to the removal of lactate by resynthesis to carbohydrate. However, the occurrence of this resynthesis requires the presence of other enzymes, namely pyruvate carboxylase (or malic enzyme) and phosphopyruvate carboxylase.

Pyruvate carboxylase, malic enzyme and phosphopyruvate carboxylase in striated muscle. The activities of the two enzymes that catalyse the carboxylation of pyruvate are very weak in striated muscle (Utter, 1959). The values obtained hardly exceed the limits of error of the methods. Phosphopyruvate carboxylase appears to be absent altogether (Keech & Utter, 1963). Unpublished work by L. V. Eggleston in this Laboratory confirms low values for the muscle of various species, including human muscle, which gave completely negative results. It is true that lactate and pyruvate can be incorporated into muscle glycogen, but the rates are relatively low and the isotope-distribution data (no randomization of C-2 and C-3 of lactate and pyruvate) indicate that the incorporation is not due to a net synthesis but to an isotope-exchange reaction in muscle (Hiatt *et al.* 1958; Omachi & Lifson, 1956; Cheosakul *et al.* 1956; Warnock *et al.* 1963). The energy barrier that prevents a net synthesis of phosphopyruvate from pyruvate does not prevent an exchange of pyruvate carbon with phosphopyruvate carbon, and as the activity of pyruvate kinase is exceedingly high in muscle (in rat muscle more than 10 times that in liver) the transfer of some radioactivity from pyruvate to phosphopyruvate is not unexpected. Owing to the ready reversibility of the reactions catalysed by enolase, phospho-

glycerate mutase, triose phosphate dehydrogenase, triose phosphate isomerase and aldolase, the carbon atoms of pyruvate or lactate must also appear in the phosphoglyceric acids, triose phosphates and FDP. The phosphofructokinase reaction, however, is not readily reversible, and the muscle FDPase must therefore play a part in the appearance of the lactate or pyruvate carbon in glycogen.

Physiological significance of muscle fructose diphosphatase. The presence of FDPase in muscle and the virtual absence of phosphopyruvate carboxylase and pyruvate carboxylase implies that one of the two energy barriers that block the reversal of glycolysis can be overcome and that glycogen can be resynthesized in muscle from the two triose phosphates, α-glycerophosphate, the two phosphoglyceric acids and phosphopyruvate, but not from lactate or pyruvate. It is very probable that the specific FDPase and the partial capacity of muscle tissue to reverse the effects of glycolysis have a role to play in muscle metabolism, and since glucogenesis is the only established role of muscle FDPase the question arises whether any of the above six intermediates can accumulate and may require reconversion into glycogen. α-Glycerophosphate is the most likely intermediate to which this applies. It has been found in striated mammalian muscle in appreciable quantities (Klingenberg & Bücher, 1960; Peterson, Gaudin, Bocek & Beatty, 1964; H. A. Krebs & E. Holdsworth, unpublished work).

The question of the significance of FDPase in muscle is thus linked with that of the role of α-glycerophosphate. Why does this intermediate arise in vertebrate muscle, where the anaerobic degradation of carbohydrate is taken to be represented by the overall equation:

$$\text{Glucose} + 2\text{ADP} + 2\text{P}_i = 2\ \text{lactate} + 2\text{ATP} \quad (1)$$

and where α-glycerophosphate is not an intermediate in the main pathway of glycolysis? α-Glycerophosphate is known to be one of the main products of the anaerobic degradation of carbohydrate in insect muscle, where glycolysis is represented by the overall equation:

$$\text{Glucose} + \text{P}_i = \alpha\text{-glycerophosphate} + \text{pyruvate} \quad (2)$$

(Kubišta, 1958; Zebe, Delbrück & Bücher, 1959). There is, on balance, no anaerobic synthesis of ATP in insect muscle. Lactate dehydrogenase is absent from, or very weak in, this tissue (Zebe & McShan, 1957; Kubišta, 1958); this accounts for its inability to form lactate.

Since the physiological significance of the 'lactate glycolysis' (reaction 1) lies in the generation of ATP under anaerobic conditions, and since the 'α-glycerophosphate–pyruvate glycolysis' (reaction 2) does not yield ATP, the significance of this form of glycolysis cannot be the immediate supply of utilizable energy. Its function is to prepare carbohydrate for subsequent oxidative degradation (see Zebe & McShan, 1957; Klingenberg & Bücher, 1960). It is relevant that in muscle glucose as such cannot reduce NAD. To release the potential reducing power of glucose for mitochondrial ATP generation, glucose must first be broken down to α-glycerophosphate or lactate or pyruvate. Triose phosphate, it is true, can reduce NAD but not within the mitochondria. It is also of importance that the initial reactions of glucose, preparing it for oxidation in the mitochondria, take place in the cytoplasm, and that the continuous degradation of the triose phosphates in the cytoplasm depends on the reconversion in the cytoplasm of $NADH_2$, formed in the triose phosphate-dehydrogenase reaction, into NAD. In the 'lactate glycolysis' this is achieved by the reaction:

$$\text{Pyruvate} + \text{NADH}_2 \rightarrow \text{lactate} + \text{NAD} \quad (3)$$

which anaerobically is the only major reaction of pyruvate in muscle. Aerobically pyruvate can also undergo rapid oxidation in the mitochondria, into which it readily diffuses. Rapid oxidation is bound to decrease the amounts of pyruvate available for reaction (3) and to lower the concentration of NAD in the cytoplasm. This in turn is liable to slow down, and eventually bring to a standstill, the triose phosphate-dehydrogenase reaction, because the $NAD/NADH_2$ ratio will become unfavourable for this reaction, E'_0 (pH 7, 25%) being $-0{\cdot}286$ for the triose phosphate-dehydrogenase system and $-0{\cdot}320$ for the $NAD–NADH_2$ system. In these circumstances, i.e. at low pyruvate concentrations, the reduction of dihydroxyacetone phosphate to α-glycerophosphate serves as an alternative mechanism of oxidizing $NADH_2$ to NAD in the cytoplasm. Thus the 'α-glycerophosphate–pyruvate glycolysis' allows the breakdown of glucose to proceed and to maintain a supply of oxidizing material to the mitochondria when the pyruvate concentration, owing to the rapid oxidation of pyruvate, is inadequate. The crucial feature of the 'α-glycerophosphate–pyruvate glycolysis' is the independence of this mode of glucose degradation on the availability of pyruvate. This is a *raison d'être* for a pathway that alone yields no utilizable energy; it is a link in the energy transformations converting a storage form of energy into a readily oxidizable form. Unlike lactate, α-glycerophosphate cannot diffuse out of muscle cells (Peterson *et al.* 1964). Therefore it cannot enter the Cori cycle, and, if it is to be removed, it must either be oxidized or reconverted into carbohydrate within the muscle, a consideration that could also apply to other

phosphorylated intermediates, and this is a *raison d'être* for the presence of FDPase in striated muscle.

Analogous role of α-glycerophosphate in yeast. Neuberg & Reinfurth (1918) described two types of yeast fermentation that, in the light of the newer knowledge on the involvement of ATP, may be written as follows:

$$\text{Glucose} + 2\text{ADP} + 2\text{P}_i = \\ = 2\ \text{ethanol} + 2\text{CO}_2 + 2\text{ATP} \quad (4)$$

$$\text{Glucose} = \text{glycerol} + \text{acetaldehyde} + \text{CO}_2 \quad (5a)$$

or

$$\text{Glucose} + \text{P} = \alpha\text{-glycerophosphate} + \\ + \text{acetaldehyde} + \text{CO}_2 \quad (5b)$$

It is thought (Meyerhof, 1935; Holzer, Bernhardt & Schneider, 1963) that reactions (5*a*) and (5*b*) may initiate fermentation by building up a sufficient concentration of acetaldehyde to act, like pyruvate in 'lactate glycolysis', as a hydrogen acceptor. The roles of *α*-glycerophosphate in the anaerobic energy metabolism of vertebrate muscle and in yeast thus appear to be analogous.

Other roles of fructose diphosphatase. The proposition that muscle FDPase plays a part in the resynthesis of glycogen from phosphorylated C_3 intermediates arising during activity, it should be emphasized, is hypothetical, and a search for other possible functions of FDPase is not unwarranted. Since glyceraldehyde phosphate is known to be an end product of the oxidative pentose phosphate cycle, and since the complete oxidation of glucose through this cycle necessitates the formation of glucose 6-phosphate from triose phosphate with the participation of FDPase (see Krebs & Kornberg, 1957), it might be thought that the functions of muscle FDPase are related to the pentose phosphate cycle. But in normal muscle the rate of this cycle is negligible (Glock & McLean, 1954; Rossi, Zatti & Greenbaum, 1963).

Appearance of lactate and pyruvate carbon in muscle glycogen. As mentioned above, small amounts of isotopically labelled lactate and pyruvate carbon have been shown to appear in muscle glycogen (Hiatt *et al.* 1958; Warnock *et al.* 1963). In view of the virtual irreversibility of the phosphofructokinase reaction this was difficult to explain, but the presence of FDPase, together with the ready reversibility of all steps of glycolysis except the phosphofructokinase reaction, can account for the behaviour of the isotopic carbon.

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REFERENCES

Bargoni, N., Sisini, A. & Majorano, C. (1964). *Ital. J. Biochem.* **13**, 53.
Bücher, T. & Hohorst, H. J. (1963). In *Methods of Enzymatic Analysis*, p. 246. Ed. by Bergmeyer, H. U. New York and London: Academic Press Inc.
Cheosakul, P., Blide, L. W., Vanko, M. & Knudson, A. (1956). *Science*, **123**, 375.
Eggleton, M. G. & Evans, C. L. (1930). *J. Physiol.* **70**, 269.
Flock, E. V., Ingle, D. J. & Bollman, J. L. (1939). *J. biol. Chem.* **129**, 99.
Glock, G. E. & McLean, P. (1954). *Biochem. J.* **56**, 171.
Hiatt, H. H., Goldstein, M., Lareau, J. & Horecker, B. L. (1958). *J. biol. Chem.* **231**, 303.
Hill, A. V. (1924). *Science*, **60**, 505.
Holzer, H., Bernhardt, W. & Schneider, S. (1963). *Biochem. Z.* **336**, 495.
Janssen, S. & Jost, H. (1925). *Hoppe-Seyl. Z.* **148**, 41.
Keech, D. B. & Utter, M. F. (1963). *J. biol. Chem.* **238**, 2609.
Klingenberg, M. & Bücher, T. (1960). *Annu. Rev. Biochem.* **29**, 669.
Krebs, H. A. (1954). *Johns Hopk. Hosp. Bull.* **95**, 19.
Krebs, H. A. (1964). *Proc. Roy. Soc. B.*, **159**, 545.
Krebs, H. A., Bennett, D. A. H., de Gasquet, P., Gascoyne, T. & Yoshida, T. (1963). *Biochem. J.* **86**, 22.
Krebs, H. A. & Kornberg, H. L. (1957). *Ergebn. Physiol.* **49**, 212.
Krebs, H. A. & Yoshida, T. (1963). *Biochem. Z.* **338**, 241.
Kubišta, V. (1958). *Biochem. Z.* **330**, 315.
Lohmann, K. (1933). *Biochem. Z.* **262**, 137.
Long, C. N. H. & Horsfall, F. L. (1932). *J. biol. Chem.* **95**, 715.
Linhardt, K. & Walter, K. (1963). In *Methods of Enzymatic Analysis*, p. 779. Ed. by Bergmeyer, H. U. New York and London: Academic Press Inc.
McGilvery, R. M. (1964). In *Fructose-1,6-diphosphatase and its Role in Gluconeogenesis*, p. 3. Ed. by McGilvery, R. M. & Pogell, B. M. Washington, D.C.: American Institute of Biological Sciences.
Meyerhof, O. (1919). *Pflüg. Arch. ges. Physiol.* **175**, 88.
Meyerhof, O. (1930). *Die chemischen Vorgänge in Muskel*, p. 36. Berlin: J. Springer.
Meyerhof, O. (1935). *Ergebn. Enzymforsch.* **4**, 208.
Mokrasch, L. C., Davidson, W. D. & McGilvery, R. W. (1956). *J. biol. Chem.* **222**, 179.
Neuberg, C. & Reinfurth, E. (1918). *Biochem. Z.* **92**, 234.
Newsholme, E. A. (1963). *Biochem. J.* **89**, 38P.
Newsholme, E. A. & Randle, P. J. (1962). *Biochem. J.* **83**, 387.
Omachi, A. & Lifson, N. (1956). *Amer. J. Physiol.* **185**, 35.
Peters, J. P. & Van Slyke, D. D. (1946). *Quantitative Clinical Chemistry*, vol. 1, p. 44. London: Baillière, Tindall and Cox.
Peterson, R. D., Gaudin, D., Bocek, R. M. & Beatty, C. H. (1964). *Amer. J. Physiol.* **206**, 599.
Rossi, F., Zatti, M. & Greenbaum, A. L. (1963). *Biochem. J.* **87**, 43.
Smith, R. M. & Alberty, R. A. (1956). *J. Amer. chem. Soc.* **78**, 2376.
Taketa, K. & Pogell, B. M. (1963). *Biochem. biophys. Res. Commun.* **12**, 229.

Underwood, A. H. & Newsholme, E. A. (1965). *Biochem. J.* (in the Press).

Underwood, E. J. (1962). *Trace Elements in Human and Animal Nutrition*, p. 207. London and New York: Academic Press Inc.

Utter, M. F. (1959). *Ann. N.Y. Acad. Sci.* **72**, 451.

Utter, M. F., Keech, D. B. & Scrutton, M. F. (1964). In *Advances in Enzyme Regulation*, vol. **2**, p. 49. Ed. by Weber, G. New York: Pergamon Press.

Walaas, E. (1958). *Acta chem. scand.* **12**, 528.

Warnock, F. G., Inciardi, N. P. & Wilson, W. E. (1963). *Fed. Proc.* **22**, 298.

Weber, G. (1964). In *Fructose*-1,6-*diphosphatase and its Role in Gluconeogenesis*, p. 57. Ed. by McGilvery, R. M. & Pogell, B. M. Washington, D.C.: American Institute of Biological Sciences.

Weber, G. & Cantero, A. (1959). *Cancer Res.* **19**, 763.

Zebe, E., Delbrück, A. & Bücher, T. (1959). *Biochem. Z.* **331**, 254.

Zebe, E. C. & McShan, W. H. (1957). *J. gen. Physiol.* **40**, 779.