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Glucose Metabolism in Perfused Skeletal Muscle

PYRUVATE DEHYDROGENASE ACTIVITY IN STARVATION, DIABETES AND EXERCISE

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1. The interconversion of pyruvate dehydrogenase between its inactive phosphorylated and active dephosphorylated forms was studied in skeletal muscle. 2. Exercise, induced by electrical stimulation of the sciatic nerve (5/s), increased the measured activity of (active) pyruvate dehydrogenase threefold in intact anaesthetized rats within 2min. No further increase was seen after 15min of stimulation. 3. In the perfused rat hindquarter, (active) pyruvate dehydrogenase activity was decreased by 50% in muscle of starved and diabetic rats. Exercise produced a twofold increase in its activity in all groups; however, the relative differences between fed, starved and diabetic groups persisted. 4. Perfusion of muscle with acetoacetate (2mM) decreased (active) pyruvate dehydrogenase activity by 50% at rest but not during exercise. 5. Whole-tissue concentrations of pyruvate and citrate, inhibitors of (active) pyruvate dehydrogenase kinase and (inactive) pyruvate dehydrogenase phosphate phosphatase respectively, were not altered by exercise. A decrease in the ATP/ADP ratio was observed, but did not appear to be sufficient to account for the increase in (active) pyruvate dehydrogenase activity. 6. The results suggest that interconversion of the phosphorylated and dephosphorylated forms of pyruvate dehydrogenase plays a major role in the regulation of pyruvate oxidation by exercise and by variations in the hormonal and nutritional state. 7. Comparison of enzyme activity with measurements of lactate oxidation in the perfused hindquarter [see the preceding paper, Berger *et al.* (1976)] suggest that pyruvate oxidation is also modulated by the concentrations of substrates, cofactors and inhibitors of (active) pyruvate dehydrogenase activity.

In the preceding paper (Berger *et al.*, 1976), we reported that the oxidation of lactate in perfused skeletal muscle is enhanced by exercise and inhibited by starvation, diabetes and perfusion with acetoacetate. These findings prompted further study of the influence of the hormonal and nutritional state on the activity of pyruvate dehydrogenase (EC 1.2.4.1) and the role of this enzyme in muscle fuel metabolism during exercise.

In mammalian tissues, pyruvate dehydrogenase is inhibited by its reaction products NADH and acetyl-CoA (Garland, 1964; Garland & Randle, 1964*b*). In addition, Reed and his co-workers (Linn *et al.*, 1969*a*,*b*) have demonstrated that the enzyme exists in two forms, an inactive phosphorylated form and an active dephosphorylated form, and that interconversion is mediated by a kinase and a phosphatase, which are subunits of the pyruvate dehydrogenase complex. Taylor & Halperin (1973) have provided evidence that the model of Reed applies to pyruvate dehydrogenase of skeletal muscle; however, it is not known how physiological alterations affect the enzyme.

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The present paper describes the effects of starvation, diabetes and perfusion with acetoacetate on pyruvate dehydrogenase activity in skeletal muscle during rest and exercise. In general, changes in the proportion of the enzyme in the active form parallel changes in the rate of pyruvate oxidation. The results support the view that interconversion of pyruvate dehydrogenase is important in the regulation of pyruvate oxidation in skeletal muscle.

A preliminary account of some of this work has already appeared (Hagg *et al.*, 1975). While this manuscript was in preparation, Hennig *et al.* (1975) reported similar findings in rat gastrocnemius muscle.

Materials and Methods

Animals

Female Sprague–Dawley rats were used as described previously (Berger *et al.*, 1976). All studies were carried out in rats anaesthetized by intraperitoneal injection of pentobarbital (see Ruderman *et al.*, 1971). In the diabetic rats, the mean (±S.E.M.) concen-

tration of glucose in tail-vein blood was 23.6 (±0.7) mM; the 3-hydroxybutyrate concentration was 4.3 (±1.1) mM and that of acetoacetate 2.1 (±0.5) mM, at the time of the experiment.

Materials

Radiochemicals were obtained from New England Nuclear Corp., Boston, MA, U.S.A. and Ruthenium Red from Sigma Chemical Co., St. Louis, MO, U.S.A. The origin of all other materials was as described by Berger *et al.* (1976).

Perfusion procedure

Perfusion of the isolated rat hindquarter was carried out as described by Ruderman *et al.* (1971) and Berger *et al.* (1976). The tissue was perfused with 200 ml of standard medium [composed of Krebs–Henseleit solution, containing aged human erythrocytes (7–8g/100ml of haemoglobin), 6mM-glucose, 4g of albumin/100ml, 1.5–2mM-lactate and 0.15mM-pyruvate; insulin was not added]. The first 70ml of perfusate which passed through the tissue was discarded, and then the medium was recycled. After 10 min of recycled perfusion, exercise was induced in one limb by electrical stimulation of the sciatic nerve for 15min at a rate of 5/s (see Berger *et al.*, 1976). The contralateral limb was not stimulated and its sciatic nerve was severed to prevent retrograde conduction of impulses from the stimulated extremity. Right hindlimbs were stimulated in half of the rats in each group and left hindlimbs in the other half.

Preparation of tissue extracts

After 15min of perfusion, portions of the posterior thigh musculature of both the resting and exercising hindlimbs were obtained by using aluminum tongs cooled in liquid N_2 (Ruderman *et al.*, 1971). Samples were stored for 1–3 days in liquid N_2 before processing. The muscle was pulverized in a mortar under liquid N_2 and then divided for determination of active pyruvate dehydrogenase, total pyruvate dehydrogenase and tissue metabolites. For assay of active pyruvate dehydrogenase, the powdered muscle was homogenized in 20 vol. of an ice-cold buffer containing potassium phosphate (10mM, pH7.2), dithiothreitol (1mM), EDTA (2mM) and NaF (20mM), with a motor-driven Teflon pestle and Nalgene tubes. For assay of total pyruvate dehydrogenase activity, the buffer contained potassium phosphate (10mM, pH7.2), dithiothreitol (1mM), $CaCl_2$ (1mM) and $MgCl_2$ (10mM). The homogenates were centrifuged at 20000*g* at 4°C for 30min in a Beckman model L ultracentrifuge. The pellet was then resuspended in an equal volume of fresh homogenization buffer and the centrifugation was repeated. The pellet from this spin was resuspended in fresh buffer, by using a Ten-Broeck homogenizer, so as to produce a protein concentration of 8–12mg/ml. Protein was measured by the method of Lowry *et al.* (1951). Approx. 60% of the total protein of the initial sample was found in this final pellet, as was the vast majority of pyruvate dehydrogenase (see below). For assay of tissue metabolites, the powdered muscle was deproteinized in ice-cold 6% (w/v) $HClO_4$ as described previously (Ruderman *et al.*, 1971).

The muscle biopsy used for assay of pyruvate dehydrogenase and for determination of metabolite concentrations contains a mixture of muscles of the posterior thigh and leg; however, the sample is reasonably constant from one experiment to another. Further, random variations in the exact composition of the biopsy specimen would be expected to average out with a sufficiently large number of experiments.

Assay of pyruvate dehydrogenase

Pyruvate dehydrogenase was assayed by the method of Taylor *et al.* (1973) with several modifications. The time of assay was extended to 5min and the procedure was carried out in 15ml test tubes equipped with disposable plastic centre wells (Kontes Glass, Vineland, NJ, U.S.A.). The scintillation fluid for counting $^{14}CO_2$ was composed of PPO (2,5-diphenyloxazole) (5g) and POPOP [1,4-bis-(5-phenyloxazol-2-yl)-benzene] (50 mg) in toluene/ethanol (9:1, v/v). All assays were performed in triplicate and were corrected for the non-enzymic production of $^{14}CO_2$, by subtracting the $^{14}CO_2$ produced in the presence of sodium arsenite (4mM), an inhibitor of lipoic acid-containing enzymes. [In previous studies (Taylor *et al.*, 1973) with adipose tissue and liver mitochondria, we have accomplished this by subtracting the $^{14}CO_2$ produced in the absence of a tissue extract. The muscle extract seemed to contain a factor which prevented spontaneous decarboxylation of [1-^{14}C]pyruvate, however. Consequently the blank obtained by addition of arsenite was slightly lower than the blank obtained by replacing the muscle extract with buffer. In any event, the measured activity of pyruvate dehydrogenase is only slightly affected by the choice of blank and our conclusions do not rest on the method chosen to accomplish the correction.] 'Blank' values (i.e. $^{14}CO_2$ production in the presence of arsenite) were in the range 600–1200d.p.m.; 'experimental' values (i.e. in the absence of arsenite) were at least four times higher. The measured activity of pyruvate dehydrogenase was decreased by approx. 90% when the cofactors NAD^+, CoA and thiamin pyrophosphate were omitted from the assay medium.

Before the assay of (active) pyruvate dehydrogenase, the tissue extract was kept on ice. For determination of total pyruvate dehydrogenase, it was incubated at 30°C for 60min. This produced maximal

conversion into (active) pyruvate dehydrogenase (results not shown).

The activity of pyruvate dehydrogenase is expressed in units; 1 unit is defined as that amount of enzyme that catalyses the production of 1 μmol of $^{14}CO_2$ in 1 min under our assay conditions. Where enzyme activity is expressed per mg of protein, this refers to protein in the resuspended pellet of the 20000*g* centrifugation.

In preliminary experiments, we attempted to assay pyruvate dehydrogenase in unfractionated homogenates of rat skeletal muscle. When carried out in this way, the assay was not linear with respect to protein concentration over the range 5–30 mg/ml. Rather the specific activity of the enzyme appeared to increase with dilution of the extract (results not shown). We considered that this might be due to an inhibitor in the unfractionated homogenate. When the activity of pyruvate dehydrogenase was measured in the supernatant and pellet fractions after centrifugation at 20000*g* (see above), we found that the total activity in the two supernatants was less than 10% of that in the resuspended pellet. Further, the production of $^{14}CO_2$ proceeded at a linear rate for at least 5 min, and the activity of pyruvate dehydrogenase was proportional to protein concentration in the pellet (results not shown). In these studies the supernatants were assayed for pyruvate dehydrogenase activity at high dilution; thus, any inhibitor in the supernatant should not have interfered with the ability to measure enzyme activity which was not precipitated. In addition, centrifugation at a higher speed (e.g. 100000*g* for 30 min) did not increase the yield of pyruvate dehydrogenase in the pellet in preliminary studies. This is in contrast with observations in adipose tissue, where much of the pyruvate dehydrogenase activity in extracts of tissues homogenized in hypo-osmotic buffers cannot be precipitated at 20000*g* within 30 min. (R. L. Jungas & S. I. Taylor, unpublished observations). Although we cannot be certain that all of the enzyme activity was precipitated under these conditions, our evidence makes it seem unlikely that there is a significant amount of enzyme remaining in the supernatant. For these reasons, pyruvate dehydrogenase was assayed as a routine only in the pellet, and we neglected the small amount of activity in the supernatants.

Statistics

Statistical analyses were carried out by using the Student's *t* test (two-tailed). When the effects of exercise were studied, the stimulated limb was compared with the resting limb of the same animal. In all other experiments, statistical analyses were based on group comparisons.

Results

Effects of exercise in vivo

Exercise increases the oxidation of glucose by skeletal muscle very rapidly (see Corsi *et al.*, 1970). To determine whether this is associated with conversion of (inactive) pyruvate dehydrogenase phosphate into (active) pyruvate dehydrogenase, the effect of 2 and 15 min of exercise on the activity of (active) pyruvate dehydrogenase in hindlimb muscles was studied in intact anaesthetized rats. As in the perfusion studies, one limb was electrically stimulated at a rate of 5/s and the contralateral limb served as a control (see the Materials and Methods section). The data shown in Table 1 demonstrate that (active) pyruvate dehydrogenase activity is increased severalfold in the stimulated muscle within 2 min, and that this is associated with significant decreases in ATP and creatine

Table 1. *Effect of isometric exercise in vivo on activity of (active) pyruvate dehydrogenase and tissue metabolite concentrations in hindlimbs of anaesthetized rats*

Results are means ± S.E.M. with numbers of observations in parentheses. Fed, anaesthetized rats were subjected to unilateral sciatic-nerve stimulation, *in vivo*, at a rate of 5/s for the times indicated. Values for resting muscle are from the unstimulated limb. (Active) pyruvate dehydrogenase activity was determined in the 20000*g* pellet of the muscle homogenate.

	Specific activity of (active) pyruvate dehydrogenase (munits/mg of protein)	Content (μmol/g)			
		Lactate	Pyruvate	ATP	Creatine phosphate
2 min					
Rest (5)	0.77 ± 0.12	1.0 ± 0.1	—	5.6 ± 0.2	19.0 ± 0.9
Exercise (5)	2.77 ± 0.53*	11.0 ± 2.1†	—	4.5 ± 0.3*	7.6 ± 1.9†
15 min					
Rest (5)	0.92 ± 0.15	1.0 ± 0.2	0.15 ± 0.004	4.8 ± 0.3	16.9 ± 0.5
Exercise (5)	3.30 ± 0.50	4.2 ± 0.8*	0.22 ± 0.01*	3.6 ± 0.2†	8.3 ± 1.0†

* Value significantly different from that of resting muscle, $P < 0.02$.
† As for *, $P < 0.01$.

phosphate and an increase in lactate. No further increase in (active) pyruvate dehydrogenase activity was noted after 15 min of exercise.

Effects of exercise on the perfused hindquarter

The regulation of pyruvate dehydrogenase was studied in more detail in the isolated perfused rat hindquarter to minimize influences from the rest of the animal and to allow for comparison with previously measured rates of lactate oxidation in the same system (see Berger *et al.*, 1976). The activity of (active) pyruvate dehydrogenase in the resting preparation was similar to that observed in fed rats *in vivo* (cf. Tables 1 and 2). Likewise, exercise increased (active) pyruvate dehydrogenase activity more than twofold in the perfused muscle and caused comparable changes in muscle ATP and creatine phosphate. On the other hand, the increase in (active) pyruvate dehydrogenase activity was not as great as with exercise *in vivo*, and tissue lactate was higher after 15 min of exercise. Exercise did not affect total pyruvate dehydrogenase activity (results not shown).

The total activity of pyruvate dehydrogenase was not altered by starvation or diabetes; however, (active) pyruvate dehydrogenase was diminished in resting muscle in both situations (Table 2). These differences persisted during exercise even though the activity of (active) pyruvate dehydrogenase was increased. Since perfusion with acetoacetate is also associated with a decreased rate of lactate oxidation (Berger *et al.*, 1976), the effect of acetoacetate on (active) pyruvate dehydrogenase activity was studied. As shown in Table 2, addition of 2 mM-acetoacetate to the perfusion medium decreased the activity of (active) pyruvate dehydrogenase in muscle of fed rats at rest, but not during exercise.

Studies on the mechanisms of regulation of pyruvate dehydrogenase

The regulation of pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphate phosphatase has been studied extensively, and effectors of both enzymes have been identified. For example, (active) pyruvate dehydrogenase kinase is inhibited by pyruvate and ADP (Linn *et al.*, 1969*b*; Hucho *et al.*, 1972), and by increases in the ratios acetyl-CoA/CoA and NADH/NAD^+ (Hucho *et al.*, 1972; Pettit *et al.*, 1975; Cooper *et al.*, 1975). Pyruvate dehydrogenase phosphate phosphatase is activated by Mg^{2+} (Linn *et al.*, 1969*a*), Ca^{2+} (Denton *et al.*, 1972; Siess & Wieland, 1972; Hucho *et al.*, 1972; Pettit *et al.*, 1972), and by decreases in the NADH/NAD^+ ratio (Pettit *et al.*, 1975) and may be inhibited by citrate (Taylor & Halperin, 1973). Since altered concentrations of these substances might be responsible for the observed changes in (active) pyruvate dehydrogenase activity, we determined the effects of exercise on the concentrations of some of these metabolites in the same muscle samples used for the studies on (active) pyruvate dehydrogenase activity (Table 3). Exercise caused a slight decrease in the concentration of ATP in all experimental groups. By contrast, it did not affect the concentration of ADP, at least in fed rats. Previous studies have shown that exercise does not alter ADP content in perfused muscle of starved or diabetic rats or in muscle perfused with acetoacetate (Berger *et al.*, 1975; M. Berger, S. A. Hagg & N. B. Ruderman, unpublished work). Despite a marked increase in tissue lactate during exercise, the concentration of pyruvate was not altered. This contrasts with the increase in pyruvate seen with exercise *in vivo* (Table 1). Exercise did not cause significant alterations in tissue citrate (Table 3).

Table 2. *Effect of starvation, diabetes and perfusion with acetoacetate on (active) pyruvate dehydrogenase and total pyruvate dehydrogenase in perfused skeletal muscle at rest and during exercise*

Results are means ± S.E.M. with numbers of observations in parentheses. Hindquarters were perfused with standard medium as described in the Materials and Methods section and were subjected to unilateral sciatic-nerve stimulation (5/s) for 15 min. Enzyme activities in resting muscle were obtained from the unstimulated leg. Values for total pyruvate dehydrogenase are from resting muscle.

	(Active) pyruvate dehydrogenase (munits/mg of protein)		Total pyruvate dehydrogenase (munits/mg of protein)
	Rest	Exercise	
Fed (16)	0.73 ± 0.05	1.60 ± 0.18‖	3.29 ± 0.20
Starved, 48 h (9)	0.44 ± 0.04‡	0.94 ± 0.13*§	3.14 ± 0.10
Diabetic (6)	0.40 ± 0.06†	0.65 ± 0.09*§	2.90 ± 0.18
Fed + acetoacetate, 2 mM (10)	0.34 ± 0.05‡	1.84 ± 0.36§	2.70 ± 0.18*

* Value significantly different from that of the fed group, $P < 0.05$.
† As for *, $P < 0.01$
‡ As for *, $P < 0.001$.
§ Value significantly different from that in resting muscle, $P < 0.01$.
‖ As for §, $P < 0.001$

Table 3. *Effect of starvation and diabetes on tissue metabolite concentrations in perfused skeletal muscle at rest and during exercise*

Results are means ± S.E.M. with numbers of observations in parentheses. See the legend to Table 2 for further details.

	Tissue concentration (μmol/g wet wt.)							
	Fed (16)		Starved, 48h (9)		Diabetic (6)		Fed + 2mM-acetoacetate (10)	
Tissue	Rest	Exercise	Rest	Exercise	Rest	Exercise	Rest	Exercise
Lactate	1.7 ± 0.2	10.8 ± 1.2‡	1.6 ± 0.2	15.2 ± 2.7†	1.0 ± 0.3	7.4 ± 1.2†	1.5 ± 0.2	11.7 ± 1.6‡
Pyruvate	0.17 ± 0.01	0.17 ± 0.01	—	—	—	—	0.14 ± 0.01	0.15 ± 1.6
ATP	4.5 ± 0.2	3.7 ± 0.2*	5.7 ± 0.4	4.1 ± 0.3*	5.5 ± 0.19	3.7 ± 0.3†	5.1 ± 0.3	3.7 ± 0.3†
ADP	0.68 ± 0.04	0.78 ± 0.06	—	—	—	—	—	—
Creatine phosphate	15.4 ± 0.8	9.8 ± 0.9‡	17.8 ± 1.3	7.2 ± 1.8‡	13.8 ± 0.9	5.6 ± 1.6†	16.4 ± 1.3	7.1 ± 1.0†
Citrate	0.19 ± 0.01	0.18 ± 0.01	0.16 ± 0.02	0.15 ± 0.01	—	—	—	—

* Value significantly different from that of hindquarter at rest, $P < 0.05$.
† as for *, $P < 0.01$.
‡ as for *, $P < 0.001$.

To study the effect of Ca^{2+} on the activation of (active) pyruvate dehydrogenase by exercise, Ruthenium Red, an agent which blocks mitochondrial uptake of Ca^{2+} (Moore, 1971; Vasington *et al.*, 1972; Severson *et al.*, 1974), was added to the perfusate. Unfortunately, the results of this experiment are difficult to interpret, because Ruthenium Red appeared to interfere with the ability of the muscle to contract in response to sciatic-nerve stimulation; the contractions of the hindlimb seemed quite feeble. In addition, at high concentrations of Ruthenium Red, the increase in muscle lactate and the decrease in ATP usually associated with exercise did not occur (Table 4). Nevertheless, Ruthenium Red did prevent the increase in (active) pyruvate dehydrogenase activity. Whether this resulted from interference with the ability of the muscle to contract or to a selective inhibition of mitochondrial Ca^{2+} transport remains to be determined. In other experiments (results not shown), procaine (1mM) or $CaCl_2$ (10mM) were added to the medium with which hindquarters from fed rats were perfused. Neither agent altered (active) pyruvate dehydrogenase activity in resting muscle. The effects of procaine and $CaCl_2$ were not investigated during exercise.

Discussion

Methodology

The tissue extract was centrifuged at 20000*g* to obtain a preparation in which pyruvate dehydrogenase activity was proportional to protein content. We think that the non-linearity of the assay in the unfractionated homogenate may be due to an endogenous inhibitor. We did not investigate whether the 'inhibitor' is detectable when pyruvate dehydrogenase activity is assayed by other methods or whether the amount of inhibitory activity varies under different physiological conditions. Studies of pyruvate dehydrogenase in rat adipose tissue, liver and brain have not revealed a similar inhibitor (S. I. Taylor & R. Jungas, unpublished work); however, a similar inhibitor, which alters the kinetic properties of branched-chain 2-oxo acid dehydrogenase, the rate-limiting enzyme of branched chain-amino acid catabolism, has been described in muscle by Odessey (1973).

Under our homogenization conditions of low osmolarity, one would expect the mitochondria to be disrupted. The finding that pyruvate oxidation does not proceed in the absence of CoA, NAD^+ and thiamin pyrophosphate supports this view, since intact mitochondria can oxidize pyruvate without the addition of exogenous cofactors. For this reason, it was somewhat surprising that relatively low centrifugal accelerations were sufficient to precipitate most of the pyruvate dehydrogenase activity. The unusual sub-

Table 4. *Effect of Ruthenium Red on (active) pyruvate dehydrogenase activity in perfused rat skeletal muscle at rest and during exercise*

Results are means ± S.E.M. with numbers of observations in parentheses. Hindquarters of fed rats were perfused with standard media containing Ruthenium Red at the concentrations indicated. See the legend to Table 2 for further details.

Ruthenium Red (mg/litre)	(Active) pyruvate dehydrogenase (munits/mg of protein)		Lactate (μmol/g wet wt.)		ATP (mmol/g wet wt.)	
	Rest	Exercise	Rest	Exercise	Rest	Exercise
0 (5)	0.66 ± 0.09	1.71 ± 0.36*	1.59 ± 0.29	12.06 ± 2.45‡	4.70 ± 0.43	3.70 ± 0.36
5 (4)	0.95 ± 0.05	1.29 ± 0.23				
10 (5)	0.64 ± 0.11	0.88 ± 0.22	1.93 ± 0.51	6.02 ± 2.27	5.09 ± 0.14	4.49 ± 0.15†
20 (6)	0.61 ± 0.13	0.59 ± 0.20	1.82 ± 0.20	4.52 ± 1.46	5.02 ± 0.17	5.10 ± 0.20

* Value significantly different from that of hindquarter at rest, $P < 0.05$.
† As for *, $P < 0.01$.
‡ As for *, $P < 0.001$.

Table 5. *Comparison of rates of lactate oxidation and (active) pyruvate dehydrogenase activity in the perfused rat hindquarter*

Data for lactate oxidation are adapted from Berger *et al.* (1976), and for active pyruvate dehydrogenase activity from values in Table 2. Results are for perfusion carried out in the absence of insulin. To calculate the enzyme activity/g wet wt. it was assumed that the 40% of muscle protein not in the 20000*g* pellet contained negligible (active) pyruvate dehydrogenase activity and that muscle contains 150mg of protein/g wet weight. Results are means of at least six values in each group.

Experimental condition	Lactate oxidation (nmol/min per g wet wt.)	(Active) pyruvate dehydrogenase activity (nmol/min per g wet wt.)
Rest		
Fed	15	65
Starved, 48h	4	40
Diabetic	4	36
Fed + acetoacetate, 1.8–2mM	12	31
Exercise		
Fed	164	144
Starved, 48h	57	84
Diabetic	36	58
Fed + acetoacetate, 1.8–2mM	91	167

cellular fractionation properties of skeletal muscle may relate to the adsorption of mitochondrial fragments to various components of the contractile apparatus as suggested by the studies of Zak *et al.* (1970).

The total activity of pyruvate dehydrogenase in our muscle samples is low compared with results previously published for heart (Whitehouse & Randle, 1973) and for gastrocnemius muscle (Hennig *et al.*, 1975). According to the studies of Baldwin *et al.* (1972) the ability of muscle homogenates to oxidize [2-^{14}C]pyruvate is increased more than threefold in red muscle as compared with superficial white muscle. Thus our results might be explained by a high proportion of white-muscle fibres in the sample of posterior thigh muscle which was used in these studies.

Role of pyruvate dehydrogenase interconversion in the regulation of pyruvate oxidation

In most instances, (active) pyruvate dehydrogenase activity varies in parallel with the rate of lactate oxidation (Table 5); these alterations in its activity are of sufficient magnitude to affect the rate of pyruvate oxidation. However, changes of similar degree are not always found; for example, the activity is doubled during exercise, and lactate oxidation is increased 8–14-fold. Further, (active) pyruvate dehydrogenase activity was measured in the portion of the muscle which contracts with stimulation of the sciatic nerve, i.e. about one-third of the hindquarter. If most of the increment in lactate oxidation occurred in the exer-

cising muscle, the relative disparity may be even greater than the data indicate. The observation that (active) pyruvate dehydrogenase activity in resting muscle is three to ten times greater than the estimated flux through the enzyme suggests that changes in the concentration of substrates, cofactors and inhibitors of (active) pyruvate dehydrogenase are important in determining enzyme activity *in situ*, in addition to the regulation of the enzyme by covalent modification. Taylor & Jungas (1974) have reached a similar conclusion on the basis of studies in adipose tissue.

Activation of pyruvate dehydrogenase during exercise

Exercise increases pyruvate generation by stimulating glucose transport into the cell as well as by enhancing glycogenolysis and glycolysis. This does not by itself account for the large increase in pyruvate oxidation, as demonstrated by Corsi *et al.* (1970). Our data support this conclusion, since we observed a five- to ten-fold increase in lactate oxidation in the exercising perfused hindquarter without a change in muscle pyruvate content (Table 3). Of course, other factors such as transport of pyruvate from cytosol to mitochondrion may also regulate pyruvate oxidation.

Although many substances affect the activities of (active) pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphate phosphatase, our data do not establish which are responsible for the increase in (active) pyruvate dehydrogenase activity during exercise. Tissue concentrations of pyruvate, citrate and ADP were not altered in the contracting muscle (Table 3). We observed a decrease in the ATP/ADP ratio in whole tissue from 6.6 to 4.8; however, studies in liver (Taylor *et al.*, 1975; Siess & Wieland, 1975) suggest that a 27% decrease in the ATP/ADP ratio would increase (active) pyruvate dehydrogenase activity by only 37%. Thus unless changes within the mitochondria are not reflected by measurements in intact tissue, none of these effectors appear to be important physiological modulators of (active) pyruvate dehydrogenase in exercising muscle.

Cytoplasmic nicotinamide nucleotides become increasingly reduced during exercise (Table 5). If, as in liver, mitochondrial redox state moves in parallel (cf. Krebs & Veech, 1969; Greenbaum *et al.*, 1971), changes in nicotinamide nucleotides would not explain the observed increase in (active) pyruvate dehydrogenase activity. There are no data indicating the possible role of CoA derivatives in the regulation of pyruvate dehydrogenase during exercise. Calcium, released from the sarcoplasmic reticulum during muscle contraction, is thought to activate glycogen phosphorylase (Meyer *et al.*, 1964). Uptake of calcium by mitochondria might then activate pyruvate dehydrogenase phosphate phosphatase, favouring the formation of active pyruvate dehydrogenase, and linking activation of glycogenolysis with enhanced pyruvate oxidation. This mechanism has been proposed by Severson *et al.* (1974) to explain the observation that inhibitors of mitochondrial calcium transport, such as Ruthenium Red, block activation of adipose-tissue pyruvate dehydrogenase by insulin. Since Ruthenium Red also interferes with muscle contraction, we were unable to define further the possible regulatory influence of calcium in our system.

Inhibition of pyruvate dehydrogenase in starvation and diabetes

Both starvation and streptozotocin-diabetes are characterized by low concentrations of insulin and high concentrations of fatty acids and ketone bodies in serum. Under these conditions, carbohydrate oxidation contributes a smaller share of the muscle-cell's energy supply than in fed animals (Berger *et al.*, 1976). As noted above, our results suggest that covalent modification of pyruvate dehydrogenase, as occurs in other tissues (Wieland *et al.*, 1971*b*, 1972; Stansbie *et al.*, 1975), as well as changes in the concentration of substrates, cofactors and/or inhibitors of (active) pyruvate dehydrogenase, are responsible for this change.

The conversion of pyruvate dehydrogenase into pyruvate dehydrogenase phosphate *in vivo* by starvation and diabetes has been reproduced *in vitro* by fatty acids and ketone bodies in many organs, including heart (Wieland *et al.*, 1971*a*), liver (Patzelt *et al.*, 1973; Taylor *et al.*, 1975) and adipose tissue (Taylor *et al.*, 1973). Acetoacetate decreases (active) pyruvate dehydrogenase activity in adipose tissue, but it increases it in isolated liver mitochondria (Taylor *et al.*, 1975). Presumably this is because acetoacetate is oxidized in adipose tissue, whereas it is reduced to D-3-hydroxybutyrate in liver at the expense of NAD^+ (Hanson & Ziporin, 1966; Williamson *et al.*, 1971). The present work extends these findings to skeletal muscle. As noted by Berger *et al.* (1976), acetoacetate decreases the oxidation of lactate both at rest and during exercise. We now find that this is associated with a decrease in (active) pyruvate dehydrogenase activity in resting muscle, although not during exercise. We have not observed an effect of exogenous fatty acid on glucose metabolism in the perfused hindquarter; its effect on (active) pyruvate dehydrogenase activity was not studied.

The inhibition of both glucose (lactate) oxidation and the decrease of (active) pyruvate dehydrogenase activity observed in starvation, diabetes and perfusion with acetoacetate may be related to changes in the acetyl-CoA/CoA and $NADH/NAD^+$ ratios in muscle mitochondria. Increases in these ratios have been recognized for more than a decade to inhibit pyruvate dehydrogenase by end-product inhibition (Garland, 1964; Garland & Randle, 1964*b*). Since

then it has been demonstrated that increases in the acetyl-CoA/CoA and NADH/NAD$^+$ ratios favour conversion of pyruvate dehydrogenase into pyruvate dehydrogenase phosphate in heart muscle and liver by activating pyruvate dehydrogenase kinase and/or by inhibiting pyruvate dehydrogenase phosphate phosphatase (Pettit *et al.*, 1975; Cooper *et al.*, 1975). Goodman *et al.* (1974) have demonstrated increases in the acetyl-CoA/CoA ratio in whole muscle from 0.18 in the fed state to 0.37 and 0.54 in starvation and diabetes respectively, and similar changes have been reported in other tissues (Tubbs & Garland, 1964; Garland & Randle, 1964*a*). The intra-mitochondrial NADH/NAD$^+$ ratio increases in liver during starvation and diabetes (Krebs & Veech, 1969; Greenbaum *et al.*, 1971); however, it is not known whether a similar change occurs in skeletal muscle. These considerations do not explain the differential effect of acetoacetate on (active) pyruvate dehydrogenase activity during rest and exercise. Conceivably acetyl-CoA is more effectively oxidized during exercise, so that addition of acetoacetate does not raise its concentration sufficiently to activate the kinase.

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