Division of Comparative Physiology and Biochemistry, Society for Integrative and Comparative Biology

TISSUE-SPECIFIC CONTROLS ON CARBOHYDRATE CATABOLISM DURING ANOXIA IN GOLDFISH[1]

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(Accepted 4/6/87)

Tissue-specific controls on carbohydrate metabolism regulate the response to anoxia in goldfish (*Carassius auratus*) and bring about the overall metabolic rate depression that is a key feature of anoxia tolerance. The effect of anoxia (24 h at 7 C under N_2/CO atmosphere) on glycogen phosphorylase activity and on the content of fructose-2,6-bisphosphate was determined in eight tissues (liver, brain, kidney, gill, spleen, heart, and red and white skeletal muscle) of goldfish. Anoxia resulted in an increase in the percentage of phosphorylase in the active *a* form in brain but significantly reduced phosphorylase-*a* content in liver, kidney, spleen, and heart; total phosphorylase activity (*a* + *b*) was also reduced during anoxia in liver and gill. Levels of the phosphofructokinase activator, fructose-2,6-P_2, dropped 10-fold in liver during anoxia and were also reduced in gill and spleen. Brain and heart showed the opposite response, a 3.5-fold rise in fructose-2,6-P_2 during anoxia. The data support a relative decrease in glycolytic flux in tissues such as liver, spleen, gill, and kidney (in line with a general metabolic rate depression) and a relative increase in glycolysis in brain and heart. In liver, the sharp drop in fructose-2,6-P_2 content is probably also important in directing glycogenolysis toward glucose export into the blood.

INTRODUCTION

Goldfish have a high tolerance for anoxia—survival without oxygen—extending to several days at 4 C in laboratory experiments (Andersen 1975; Walker and Johansen 1977). Adaptive responses allowing this tolerance are twofold. First, metabolic rate is strongly depressed in anoxia. Estimates of anoxic metabolic rate are 20%–30% of normoxic rates measured by calorimetry (Andersen 1975) and one-third normal rates based on the sum of accumulated end products and depletion of endogenous ATP, phosphagen, and O_2 reserves (van den Thillart, Kesbeke, and van Waarde 1976). Second, alternative pathways of fermentative catabolism are used. Metabolic CO_2 is evolved during anoxia; this is derived largely from the conversion of lactate to ethanol (Shoubridge and Hochachka 1981, 1983) but may also include a component from amino acid (protein) catabolism by the tricarboxylic acid cycle (van den Thillart, van Berge-Henegouwen, and Kesbeke 1983). The adaptive advantage of CO_2 plus ethanol production is that the metabolic end products of anoxia can be excreted across the gills, allowing goldfish to avoid the metabolic acidosis associated with lactate accumulation (Shoubridge and Hochachka 1983).

During anoxia, a tissue-specific reorganization of energy metabolism occurs. The quiescent anoxic goldfish depends almost exclusively on carbohydrate catabolism with blood glucose, produced from liver glycogen reserves, the major substrate sustaining long-term anaerobic survival (Shoubridge and Hochachka 1983). Glycogen reserves in heart and brain are rapidly depleted as an immediate response to anoxia (Merrick 1954; McDougal et al. 1968), while reserves in skeletal muscle are retained to fuel swimming (Shoubridge and Hochachka 1983). Relative rates of glycolysis increase in brain and heart but decrease in other tissues in line with a general metabolic rate depression during anoxia (Shoubridge and Hochachka 1983). Lactate is produced as the glycolytic end product in all tissues (rates of production being highest in brain and heart, the most active tissues), but, when anoxia extends beyond a few hours, blood lactate is taken up by skeletal muscle and oxidized to ethanol and CO_2, which are then excreted (Shoubridge and Hochachka 1983; van den Thillart and Verbeek 1982).

[1] I thank Dr. S. Rahman for experimental animals and Mrs. S. Walters for excellent assistance. Supported by an operating grant from NSERC, Canada.

Physiol. Zool. 60(5):601–607. 1987.

 0031-935X/87/6005-8701$02.00

Metabolic depression and metabolic reorganization of tissue fuel utilization patterns for anoxic survival require precise regulatory controls on rate-limiting enzymes. With respect to glycolysis, a tissue-specific balance must be struck that reflects both the general metabolic rate depression (hence, reduced ATP demand in the anoxic state) as well as the requirement that glycolysis alone sustain energy output during anoxia. The present study characterizes two indexes of glycolytic function. Glycogen phosphorylase activity provides a good indication of the relative state of glycogenolysis as a contributor to anaerobic carbohydrate catabolism. Fructose-2,6-P_2 mediates a variety of extracellular signals and, in general, indicates an abundance of glucose and, through its activating effects on phosphofructokinase, potentiates carbohydrate utilization for anabolic purposes in vertebrate tissues (Hue 1983). A strong suppression of fructose-2,6-P_2 content in anabolic tissues is a key feature of facultative anaerobiosis in marine invertebrates (Storey 1985a, 1987). In combination, the two indexes give key insights into tissue-specific responses of carbohydrate metabolism with the transition from aerobic to anoxic function.

MATERIAL AND METHODS

Animals.—Goldfish (*Carassius auratus*) (15–20 cm length) were purchased from Grassy Forks Fishery Co., Indiana. Animals were held in running, aerated water at 7 C and fed ad lib. on goldfish pellets. Fish were fasted overnight prior to use.

Control fish were gently netted from the holding tank and killed by a blow to the head. Tissues were immediately dissected out (within 15 s) and frozen in liquid nitrogen. Neither control nor experimental fish struggled appreciably after removal from the tank, such that effects of stress or muscle work should have been minimal. For anoxia experiments, fish were transferred to sealed containers (two fish per container) with water that had been bubbled with nitrogen gas for 9 h previously. Bubbling with N_2 gas continued for the next 15 h followed by bubbling with carbon monoxide for the next 9 h. Containers were bathed in 7 C water throughout. After 24 h of anoxia, fish were rapidly sampled as described for controls. Frozen tissues were transferred to −80 C for long-term storage.

Chemicals.—Biochemicals and coupling enzymes were purchased from Sigma Chemical Co., St. Louis, Missouri, or Boehringer Mannheim, Montreal.

Fructose-2,6-bisphosphate determinations.—Frozen tissues were ground to a powder under liquid nitrogen. Extraction and assay of fructose-2,6-P_2 followed the method of van Schaftingen (1984), with fructose-2,6-P_2 content determined from the activating effect of extracts on pyrophosphate-linked phosphofructokinase from potato tubers. That enzyme activation was due to fructose-2,6-P_2 content only was confirmed when, after acid treatment, samples no longer activated the enzyme.

Enzyme assay.—Frozen tissue samples (≤200 mg) were weighed and rapidly homogenized (w/v = 1:5 to 1:15) in ice-cold imidazole buffer (50 mM, pH 7.0) containing 15 mM 2-mercaptoethanol, 100 mM NaF, 5 mM EDTA, 5 mM EGTA, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) using a Tekmar Tissuemizer. For glycogen phosphorylase determinations, particulate matter in the homogenate was allowed to settle (without centrifugation), and then enzyme activity was measured in the supernatant. For phosphofructokinase determinations, the homogenate was centrifuged at 27,000 *g* for 20 min at 4 C. The supernatant was removed and dialyzed against homogenizing buffer (minus PMSF) for 2 h at 4 C, with one change of buffer, and then used for assay.

All assays were performed at 23 C using a Pye Unicam SP8-100 recording spectrophotometer.

Assay conditions for glycogen phosphorylase-*a* were 50 mM potassium phosphate buffer, pH 7.0, 2 mg/ml glycogen (previously dialyzed), 0.4 mM NADP, 10 μM glucose-1,6-P_2, 0.25 mM EDTA, 15 mM $MgCl_2$, and excess dialyzed phosphoglucomutase and glucose-6-P dehydrogenase. For measurements of total phosphorylase, 1.6 mM AMP was included in the assay mixture.

Assay conditions for maximal phosphofructokinase activity were 50 mM imidazole buffer, pH 7.0, 10 mM fructose-6-P, 1 mM ATP, 5 mM $MgCl_2$, 50 mM KCl, 0.2 mM NADH, and excess dialyzed aldolase,

triosephosphate isomerase, and glycerol-3-P dehydrogenase. For all measurements, a fructose-6-P/glucose-6-P mixture (M ratio 1:3) was supplied as the substrate. $S_{0.5}$ values were determined from Hill plots using experimentally determined V_{max} measurements. I_{50} and K_a values were determined from plots of v versus [effector] at subsaturating fructose-6-P (0.8 mM for I_{50}, 0.4 mM for K_a).

RESULTS

Table 1 shows the effect of anoxia on the activity of glycogen phosphorylase in goldfish tissues. The percentage of phosphorylase in the active *a* form rose significantly in the anoxic brain, suggesting increased glycogen utilization during anoxia in that tissue. In four other tissues (liver, kidney, spleen, and heart), the percent *a* dropped significantly; the effect was greatest in heart, a sixfold decrease. Gill and skeletal muscle (both red and white) showed no change in phosphorylase-*a* content with anoxia. An alternative mechanism for reducing the amount of phosphorylase-*a* expressed during anoxia was apparent in gill; the total activity ($a + b$) of phosphorylase expressed dropped by one-third, effectively reducing the amount of active enzyme by the same amount. A decrease in total phosphorylase activity during anoxia was also apparent in liver, the combined effect of this plus the drop in percent *a* reducing the amount of phosphorylase-*a* expressed in anoxic liver to 48% that in the aerobic state.

Table 2 shows the effect of anoxia on fructose-2,6-P_2 levels in goldfish tissues. Fructose-2,6-P_2 is a potent activator of phosphofructokinase, and its effects on the enzyme are important in regulating carbohydrate flux through glycolysis in response to a variety of physiological and hormonal signals. Anoxia radically depressed fructose-2,6-P_2 content in liver, levels dropping by 10-fold. A significant reduction in content of the bisphosphate was also seen in gill and spleen. Brain and heart were affected oppositely, each showing an approximately 3.5-fold increase in fructose-2,6-P_2 content during anoxia.

The effect of changes in fructose-2,6-P_2 content on tissue carbohydrate flux during anoxia depends, in part, on the relationship between the K_a for fructose-2,6-P_2 of phosphofructokinase and actual tissue levels of the effector. Therefore, maximal activities and selected kinetic constants (including K_a fructose-2,6-P_2) for phosphofructokinase in goldfish tissues were measured (table 3). Phosphofructokinase from goldfish was a labile enzyme; tissue extracts stored for 3 days lost 100%, 97%, 88%, and 95% of initial activity for liver, skeletal muscle, brain, and heart, respectively. The enzyme was more stable at pH 8.0 than at 7.0, but addition of 3 M sucrose or 50 mM P_i + 40% glycerol could effectively stabilize the enzyme in all tissues except skeletal muscle. Distinct differences in kinetic properties of phosphofructokinase were found between tissues. The enzyme in skeletal muscle

TABLE 1

EFFECT OF ANOXIA ON GLYCOGEN PHOSPHORYLASE IN GOLDFISH TISSUES

	PHOSPHORYLASE-*a* (%)		TOTAL PHOSPHORYLASE (U/g)	
	Aerobic	Anoxic	Aerobic	Anoxic
Liver	80 ± 5	55 ± 5**	3.6 ± .33	2.5 ± .49***
Brain	45 ± 5	68 ± 2*	2.8 ± .58	2.6 ± .35
Kidney	38 ± 5	11 ± 4*	1.7 ± .43	1.4 ± .27
Gill	24 ± 3	28 ± 8	.9 ± .03	.6 ± .10*
Spleen	21 ± 4	7 ± 2*	.5 ± .05	.4 ± .15
Heart	49 ± 4	8 ± 1*	8.6 ± 1.29	8.2 ± 1.24
Red muscle	24 ± 6	27 ± 3	7.4 ± 1.27	7.9 ± 1.41
White muscle	23 ± 5	23 ± 2	6.2 ± 1.98	6.1 ± .37

NOTE.—Data are means ± SEM, n = 4–8 determinations on tissues from separate individuals. Activities of total phosphorylase are μmol glucose-1-P produced min^{-1} g wet $weight^{-1}$ determined in the presence of 1.6 mM AMP.

* Anoxic values are significantly different from aerobic values, by Student's *t*-test, $P < .01$.

** $P < .02$.

*** $P < .10$.

TABLE 2

EFFECT OF ANOXIA ON FRUCTOSE-2,6-P_2 CONCENTRATIONS IN GOLDFISH TISSUES

	nmol/g WET WEIGHT	
	Aerobic	Anoxic
Liver	7.77 ± 1.61	.75 ± .43*
Brain	.20 ± .04	.73 ± .12*
Kidney	1.24 ± .33	.96 ± .14
Gill	.61 ± .09	.35 ± .05**
Spleen	1.43 ± .42	.35 ± .12**
Heart	.13 ± .05	.47 ± .13**
Red muscle	.71 ± .14	.84 ± .13
White muscle	.74 ± .12	.76 ± .07

NOTE.— Data are means ± SEM for n = 4–6 samples from separate individuals.

* Anoxic values are significantly different from aerobic values, by Student's t-test, $P < .01$.

** $P < .05$.

showed a very high affinity for substrate fructose-6-P but was subject to strong ATP inhibition; this probably limits enzyme activity in resting muscle. Activation constants for AMP and fructose-2,6-P_2 of muscle phosphofructokinase were well within the normal ranges of these compounds in vivo. With respect to fructose-2,6-P_2, the skeletal muscle enzyme is probably always saturated. Heart phosphofructokinase showed very different features; affinity for fructose-6-P was very low, but ATP inhibition was weak. Goldfish-heart phosphofructokinase may be highly dependent on the actions of activators for significant activity (and to lower $S_{0.5}$ into the physiological range); similar regulation of mammalian heart phosphofructokinase reflects the normal dependence of heart on lipid oxidation and the activation of carbohydrate catabolism only during times of stress (Narabayashi, Randolph Lawson, and Uyeda 1985). Liver phosphofructokinase also showed a low affinity for fructose-6-P. Adenylate control of the enzyme appears weak (both I_{50} ATP and K_a AMP are high compared to levels of these compounds in vivo). Regulation of the liver enzyme may be largely tied to effects by other modifiers (fructose-2,6-P_2, citrate, etc.) in line with the biosynthetic functions of liver.

DISCUSSION

An overall metabolic depression in the anoxic goldfish to levels of about 30% of resting normoxic metabolic rate results from varying degrees of metabolic depression in individual tissues. Glycolysis and glycogenolysis are altered in each tissue in response to two pressures: (1) the generally reduced energy demands of the depressed state and (2) the dependence, in the anoxic state, on carbohydrate fermentation for energy production. The net result in tissues such as brain and heart is a relative activation of glycolysis during anoxia (as determined by crossover analysis of glycolytic intermediates) and a very active catabolism of blood glucose (Shoubridge and Hochachka 1983). In brain, this results because a much less efficient pathway of energy production (ATP yield from glycolysis = 2–3 per C_6 unit converted to lactate vs. 36–38 for complete oxidation to CO_2 and

TABLE 3

KINETIC CONSTANTS FOR PHOSPHOFRUCTOKINASE FROM GOLDFISH TISSUES

	V_{max}, U/g	$S_{0.5}$ F6P (mM)	I_{50} ATP (mM)	K_a AMP (mM)	K_a F2,6P_2 (μM)
Liver	4.9 ± 1.0	1.9 ± .46	1.8 ± .27	.17 ± .04	.10 ± .04
Brain	7.0 ± .8	2.3 ± .25	1.6 ± .20	.10 ± .02	.22 ± .04
Kidney	7.8 ± .8	.6 ± .03	2.1 ± .65	.04 ± .008	.04 ± .01
Gill	2.1 ± .1	1.0 ± .07	1.8 ± .17	.06 ± .004	.11 ± .04
Spleen	2.3 ± .1	.9 ± .15	1.7 ± .18	.13 ± .02	.09 ± .04
Heart	5.9 ± .7	4.4 ± .58	8.0 ± 1.70	.13 ± .06	.16 ± .06
Red muscle	11.4 ± .9	.12 ± .03	1.9 ± .37	.06 ± .007	.05 ± .03
White muscle	19.0 ± .9	.10 ± .01	1.5 ± .04	.02 ± .01	.03 ± .006

NOTE.—Data are means ± SEM, n = 6 determinations on preparations from individual animals. Maximal activites are μmol fructose-6-P utilized min^{-1} g wet $weight^{-1}$.

H_2O) must meet the energy needs of the anoxic brain (it is not known whether brain sustains a metabolic depression). This is the typical Pasteur effect. In heart, the activation of glycolysis, despite an anoxia-induced bradycardia, occurs as the result of a switch from lipid catabolism in the aerobic state to carbohydrate catabolism in the anoxic state. Other tissues (particulary skeletal muscle) show a relative decrease in glycolytic rate during anoxia (Shoubridge and Hochachka 1983), suggesting a profound metabolic rate depression in the anoxic state. A similar metabolic rate depression occurs during anaerobiosis in intertidal marine invertebrates; not only is no Pasteur effect seen, but glycolytic rate is strongly reduced (de Zwaan and Wijsman 1976; Ebberink and de Zwaan 1980).

The present data begin to explore the molecular mechanisms underlying the tissue-specific response of carbohydrate metabolism to anoxia. Our studies of glycolytic rate control and metabolic depression during anaerobiosis in marine molluscs have revealed three key molecular mechanisms by which glycolysis (and metabolism in general) can be regulated in a coordinated manner during anoxia (Storey 1985*a*, 1987). These are (1) covalent modification via enzyme phosphorylation or dephosphorylation reactions (Storey 1984; Plaxton and Storey 1984*a*, 1984*b*, 1985*a*, 1985*b*); (2) enzyme aggregation/association to form complexes bound to subcellular particles (Plaxton and Storey 1986); and (3) specific to glycolysis, fructose-2,6-P_2 regulation of phosphofructokinase for the control of carbohydrate use in biosynthesis (Storey 1985*b*). The present study begins to explore these regulatory phenomena as they apply to anoxia tolerance in goldfish, focusing on covalent modification of glycogen phosphorylase as an indicator of tissue-specific glycogenolysis during anoxia and fructose-2,6-P_2 levels as an indicator of anoxic glycolytic flux.

Covalent modification of enzymes via phosphorylation or dephosphorylation reactions is an effective mechanism for bringing about both major changes in the kinetic properties of individual enzymes and coordinated changes in the activities of whole pathways. Thus, anaerobiosis in marine molluscs is characterized by widespread phosphorylation of cellular proteins, including specific phosphorylations of pyruvate kinase and phosphofructokinase, that reduce glycolytic flux in concert with the generalized metabolic depression (Storey 1984; Plaxton and Storey 1984*b*, 1985*a*).

Changes in the phosphorylation state of glycogen phosphorylase (a change in the percentage of enzyme in the phosphorylated *a* form) are a response to anoxia in five goldfish tissues. In brain, an increase in the percent *a* allows glycogenolysis to help support a tissue that retains a high metabolic activity during anoxia. A reduction in phosphorylase-*a* activity in four other tissues indicates that the balance between the anoxia-induced metabolic depression and the anoxia-induced reliance on carbohydrate fermentation for energy production favors metabolic depression to the point where the rate of catabolism of endogenous carbohydrate reserves can be reduced during anoxia. The same results are found during facultative anaerobiosis in marine molluscs; phosphorylase-*a* content remains low (6%–8%) and unchanged during anaerobiosis in *Mytilus edulis* (Ebberink and Salimans 1982). The opposite response characterizes anoxia or ischemia in mammalian tissues; phosphorylase in brain, heart, liver, and skeletal muscle is rapidly activated (Dobson and Mayer 1973; Sharma et al. 1980; Siesjo 1978; Harris et al. 1986). This supports an attempt to preserve a constant metabolic rate during an anoxic or ischemic insult by compensating for aerobic rates of energy production with accelerated glycolytic energy production (the Pasteur effect). A reduction in total phosphorylase ($a + b$) content was also evident in gill and liver during anoxia. Although the molecular mechanism behind this is not known, this effect is increasingly identified as a factor in phosphorylase regulation in lower vertebrates (Storey and Storey 1984, 1987).

The effect of anoxia in reducing liver phosphorylase-*a* might be unexpected in light of the role of liver in supplying blood glucose as a major substrate for most or all tissues during anoxia. However, net glucose output owing to glycogenolysis is a function of glucose demand (overall, this is reduced compared to the aerobic state owing to

metabolic depression, although some specific tissues may, individually, have higher demands during anoxia) as well as the relative activities of glycogen phosphorylase and glycogen sythetase in liver. Undoubtedly, synthetase activity in liver is strongly depressed during anoxia.

Although anoxia/ischemia in mammalian systems rapidly stimulates glycolysis, tissue levels of fructose-2,6-P_2 (e.g., liver, skeletal muscle, heart) respond oppositely and are very strongly depressed (Hue 1982). Thus, the activation of phosphofructokinase, which is the key component of the Pasteur effect, is not related to the effects of this allosteric activator but is, instead, due to adenylate control. Fructose-2,6-P_2 appears to control phosphofructokinase with respect to the uses of carbohydrate for biosynthetic purposes; for example, fructose-2,6-P_2 levels in liver increase when glucose availability is high and decrease under the influence of glucagon (Hue 1983). The response of fructose-2,6-P_2 levels to anoxia in liver of goldfish is the same as that seen in mammals (Hue 1982): a very dramatic drop in content. The effects on phosphofructokinase of a 10-fold drop in fructose-2,6-P_2 content in goldfish liver are not only key to the reduction of glycolytic rate during anoxia but also (*a*) facilitate glucose export into the blood as the result of liver glycogenolysis and (*b*) contribute to the depression of various metabolic processes in liver by limiting carbohydrate availability for biosynthesis. A discrepancy between the apparent K_a (0.1 μM) for fructose-2,6-P_2 and levels of the compound in anoxic liver (0.75 nmol/g or about 1 μM in cell water) may suggest that the enzyme in anoxic liver is still fully saturated with respect to fructose-2,6-P_2, but other factors must be considered, including (*a*) the binding of fructose-2,6-P_2 to other cellular proteins; (*b*) the powerful synergistic interactions between fructose-2,6-P_2 and other activators of phosphofructokinase that are reduced as fructose-2,6-P_2 content declines (Storey 1985*c*); and (c) the possible covalent modification of liver phosphofructokinase during anoxia, producing an enzyme that is less sensitive to fructose-2,6-P_2 activation (such occurs during anoxia in marine molluscs [Storey 1984] and is also apparent in several goldfish tissues [S. Rahman and K. Storey, unpublished data]). In other soft tissues (gill, spleen), the reduced fructose-2,6-P_2 content during anoxia probably has the same effect as in liver, facilitating metabolic rate depression by restricting carbohydrate use for anabolic purposes. This is also the typical response to anoxia of soft tissues (e.g., gill, mantle, hepatopancreas) in anoxia-tolerant marine molluscs (Storey 1985*a*, 1985*b*).

Elevated levels of fructose-2,6-P_2 in heart and brain of goldfish during anoxia are probably key to the relative activation of glycolysis during anoxia that is specific to these two tissues (Shoubridge and Hochachka 1983). In both tissues, fructose-2,6-P_2 levels rise from about the K_a level (enzyme half maximally activated with respect to fructose-2,6-P_2) to a saturating level—changes that would stimulate a large increase in phosphofructokinase activity and lower the $S_{0.5}$ for fructose-6-P to a level closer to the physiological range of the substrate. In heart, fructose-2,6-P_2 activation of phosphofructokinase would facilitate the switch from lipid as the primary fuel under aerobic conditions to carbohydrate catabolism during anoxia. In a good anaerobe, changes in adenylate levels would be minimal during the aerobic-anaerobic transition, such that adenylate control of goldfish-heart phosphofructokinase may be inadequate as a means of activating the enzyme during anoxia (although adenylates may be the key factors in adjusting glycolysis to changes in muscle work requirements). Fructose-2,6-P_2, perhaps rising as the consequence of increased glucose uptake by heart cells (owing to increased substrate, fructose-6-P, availability to 6-phosphofructo-2-kinase) may, therefore, be key to the activation of phosphofructokinase during anaerobiosis.

The present data do not provide insights into the regulation of the decreased glycolytic flux (Shoubridge and Hochachka 1983) and metabolic rate depression observed in skeletal muscle during anoxia. Phosphorylase activity was unchanged in anoxic muscle, as was fructose-2,6-P_2 content, amounts of fructose-2,6-P_2 in muscle being manyfold greater than the apparent K_a for fructose-2,6-P_2 of muscle phosphofructokinase. The same findings characterize the response of muscle metabolism to anoxia in marine molluscs (Ebberink and Salimans 1982; Storey 1985*a*). Shoubridge

and Hochachka (1983) found that anoxic skeletal muscle took up relatively little ^{14}C-glucose, so that glycolytic rate in red and white muscle may be regulated by the rate of glucose uptake from blood (substrate availability) in the resting state or by adenylate activation of glycolysis during muscle work.

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