Effects of seasonal change and prolonged anoxia on metabolic enzymes of *Littorina littorea*

Steven C. Greenway and Kenneth B. Storey

Abstract: The effects of seasonal change (July versus November) and prolonged anoxia (N_2 atmosphere at 5 or 10°C for 6 days) exposure in vivo on the activities of 18 enzymes, as well as the kinetic properties of phosphofructokinase (PFK) and pyruvate kinase (PK), were investigated in foot muscle and digestive gland of the marine periwinkle *Littorina littorea* L. Seasonal differences in enzyme maximal activities were tissue-specific, with generally increased activities during the summer and changes in a greater number of enzymes in digestive gland than in foot muscle. Seasonal differences in the kinetic properties of PFK and PK were observed in both tissues. PK from digestive gland of winter animals showed a much higher $S_{0.5}$ for phosphoenolpyruvate and stronger changes in enzyme kinetic properties in response to anoxia than did the enzyme in summer animals; this may suggest the presence of seasonal isozymes. The effects of anoxia were tissue- and season-specific. Anoxia exposure during the winter induced a greater number of changes in enzyme maximal activities in foot muscle than in digestive gland. Anoxia-induced changes in the kinetic properties of both PFK and PK were also seen in both organs. For PK, these changes were consistent with less active enzyme forms in the anoxic state. Hence, both seasonal and environmental (anoxia) factors influence enzyme maximal activities and kinetic properties in *L. littorea*.

Résumé : Nous avons étudié les effets de changements saisonniers (juillet contre novembre) et de l'anoxie prolongée (atmosphère de N_2 à 5 ou 10°C durant 6 jours) in vivo sur l'activité de 18 enzymes, de même que sur les propriétés cinétiques de la phosphofructokinase (PFK) et de la pyruvate kinase (PK), dans le muscle du pied et la glande digestive du bigorneau marin *Littorina littorea* L. Les différences saisonnières de l'activité enzymatique maximale étaient spécifiques au tissu et, de façon générale, il y avait plus d'activité en été et les changements affectaient un plus grand nombre d'enzymes dans la glande digestive que dans le pied. Nous avons enregistré des différences saisonnières dans les propriétés de la PFK et de la PK dans les deux tissus. La PK de la glande digestive chez les bigorneaux en hiver avait une valeur beaucoup plus élevée de $S_{0,5}$ pour le phosphoénolpyruvate; nous avons constaté aussi des changements plus importants dans les propriétés cinétiques des enzymes en réponse à l'anoxie que chez les bigorneaux en été, ce qui semble indiquer la présence d'isozymes saisonniers. Les effets de l'anoxie se sont également révélés spécifiques au tissu et à la saison. L'exposition à des conditions anoxiques durant l'hiver déclenche un plus grand nombre de changements de l'activité enzymatique maximale dans le muscle du pied que dans la glande digestive. Les modifications des propriétés cinétiques de la PFK et de la PK en réaction à l'anoxie ont été observées dans les deux tissus. Dans le cas de la PK, ces changements correspondent à des formes d'enzymes moins actives durant l'anoxie. Donc, les deux types de facteurs, saisonniers et environnementaux (anoxie), influencent l'activité enzymatique maximale et les propriétés cinétiques des enzymes chez *L. littorea.*

[Traduit par la Rédaction]

Introduction

The demands for ATP and the capacity for its production are altered in many animals for extended periods of time because of seasonal variations in environmental factors (e.g., temperature, oxygen or nutrient availability, salinity), reproductive requirements, and behavioral changes (Newsholme and Crabtree 1986). The marine periwinkle *Littorina littorea* L. (Mollusca, Gastropoda) is one such species that experiences wide variations in environmental parameters over the year and also undergoes metabolic reorganization associated with the reproductive cycle. By synchronizing modifications of their metabolic pathways with the progression of the seasons, organisms can maximize their benefits during favorable conditions and endure when conditions become unfavorable. For example, seasonal changes occur in the metabolism of carbohydrate, lipid, nucleic acids, amino acids, and protein in marine molluscs, as well as in respiration rates and anaerobic metabolism (for a review see Gabbott 1983). Changes in the amounts of metabolic enzymes are important in adaptation to seasonal change or environmental stress. Seasonal or stress-induced alterations in the activities of enzymes can be accomplished by mechanisms that include (*i*) quantitative changes in the amount of enzyme present, owing to changes in gene expression or protein synthesis/degradation rates, (*ii*) differential production of isoforms possessing altered kinetic properties, or (*iii*) post-translational modification (e.g., reversible protein phosphorylation) to alter enzyme function (Livingstone 1981; Storey 1992).

Intertidal molluscs can also experience multiple forms of

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S.C. Greenway[1] and K.B. Storey.[2] Institute of Biochemistry and Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, ON K1S 5B6, Canada.

[1]Present address: Department of Pedriatics, Hospital for Sick Children, 555 University Avenue, Toronto, ON M5G 1X8, Canada.
[2]Corresponding author (e-mail: kenneth_storey@carleton.ca).

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environmental stress within both daily and seasonal time frames, including wide variations in oxygen availability, salinity, and temperature. Oxygen limitation can occur under several circumstances, most commonly because most gill-breathing species have a limited ability to take up oxygen from the air and hence are oxygen-limited during aerial exposure at low tide. Oxygen can also be depleted in tide pools when the temperature is high, and many species also cut themselves off from oxygen supplies when they close their valves or opercula, a strategy that can limit desiccation during aerial exposure, reduce their exposure to poor water conditions (e.g., high levels of silt, pollution), or deter predators. Hence, intertidal species generally have well-developed anoxia tolerance that includes the following biochemical adaptations: (*i*) high reserves of fermentable fuels (glycogen, aspartate) in all tissues, (*ii*) the use of alternative pathways of fermentative metabolism to increase ATP yield (resulting in products such as alanine, succinate, propionate, and acetate), (*iii*) good buffering capacity to minimize metabolic acidosis, and (*iv*) profound anoxia-induced metabolic rate depression (as much as 10- to 20-fold) (Hochachka and Somero 1984; Storey and Storey 1990). Biochemical mechanisms currently known to help regulate anoxia-induced metabolic rate depression in molluscs include (*i*) covalent modification of enzymes by reversible protein phosphorylation, (*ii*) allosteric regulation of key enzymes, and (*iii*) reversible association of enzymes with subcellular structures (Storey and Storey 1990; Storey 1993). Overall, studies to date suggest that transitions to and from the anoxic, hypometabolic state do not seem to involve extensive changes to gene expression and protein synthesis.

In general, then, seasonal changes in metabolic makeup may be attributable, in large part, to "coarse" controls on the amounts of enzymes, whereas shorter term responses to environmental insult (such as anoxia exposure) may be best mediated by modification of existing enzymes (Storey and Storey 1990). This concept has been partially explored in different species; for example, seasonal changes in both aerobic (e.g., supporting gametogenesis) and anaerobic metabolism are known in marine molluscs (Ahmad and Chaplin 1979; Kluytmans et al. 1980; Ho and Zubkoff 1982; Gabbott 1983; Kluytmans and Zandee 1983). The present study presents an analysis of both seasonal and anoxia-induced changes in the maximal activities of 18 enzymes of intermediary metabolism in two tissues of *L. littorea* to see which strategies are used to adjust metabolic potential on both a long-term basis (seasonal) and an acute basis (anoxia stress). In addition, we examine the effects of season and anoxia on the kinetic properties of two regulatory enzymes of glycolysis, phosphofructokinase (PFK) and pyruvate kinase (PK). Both enzymes are well known to be responsive to anoxia stress in molluscs, and typically, anoxia-induced phosphorylation of these proteins strongly suppresses their activities and contributes to both metabolic depression and the redirection of carbon flux into pathways of anaerobic metabolism. However, the seasonality of this response has received little attention.

Materials and methods

Chemicals and animals

All biochemicals and coupling enzymes were obtained from Sigma Chemical Co., St. Louis, Mo., or Boehringer–Mannheim Corp., Montreal, Que. Distilled deionized water was used throughout for the preparation of biochemical solutions. All ATP stock solutions contained added $MgCl_2$ in 1:1 molar amounts. *Littorina littorea* were collected from the New Brunswick seashore; these were shipped via courier to Ottawa with some cold packs in the box to keep the animals cool. "Winter" snails were collected in the first half of November (seawater and air temperatures on the day of collection were 3–5 and 8–10°C, respectively) and "summer" animals were obtained in July (seawater and air temperatures were 12–14 and ~22°C, respectively). Within 1 day of arrival, periwinkles were placed in tanks of aerated artificial seawater (1000 mosmol/L), where they were held for 2 weeks prior to experimentation without feeding; winter animals were held at 5°C and summer animals at 10°C.

Preparation of experimental animals

Control snails (n = 50) were sampled directly from the aerated seawater. The shell was broken open and tissues were quickly excised, immediately frozen in liquid nitrogen, and then transferred to –70°C for storage. For anoxia exposure, snails (n = 50) were placed in jars (10 per jar) with ~1 cm of seawater in the bottom that had been previously bubbled with N_2 gas (100%) for 20 min. The jars were then flushed with N_2 gas for a further 30 min, after which the lid was sealed and coated with several layers of parafilm. Jars were then returned to 5°C (winter) or 10°C (summer) incubators, where they were held for 6 days. After anoxia exposure, jars were opened, quickly assessed for the presence of oxygen using a flame test, and then the N_2 gas bubbler was reintroduced to maintain the anoxic atmosphere while individual snails were removed and rapidly dissected as above. In preliminary trials, 6-day anoxia-exposed snails were returned to aerated seawater and assessed after 1 week; survival was 100%.

Tissue extraction and enzyme assay

Frozen tissue samples were quickly weighed and then homogenized using a Pro200 homogenizer (Diamed Laboratory Supplies Inc., Mississauga, Ont.); a 1:4 w/v ratio was used for PFK and PK kinetic studies and a 1:5 w/v ratio for the enzyme-activity survey. The homogenization buffer contained 50 mM imidazole-HCl buffer (pH 7.2), 100 mM NaF, 5 mM EDTA, 5 mM EGTA, and 15 mM 2-mercaptoethanol. A few crystals of phenylmethylsulphonyl fluoride, a protease inhibitor, were added immediately before homogenization. Homogenates were centrifuged for 20 min at 18 000 × g in a Biofuge 15 centrifuge (Canlab) at 5°C. Supernatants were removed and used directly for the survey of enzyme maximal activities. For PFK and PK kinetic studies, the supernatant was first desalted by centrifugation (benchtop centrifuge at 500 × g for 1 min) through a 5-mL column of fine Sephadex G-25 equilibrated in assay buffer (100 mM imidazole, pH 7.2, 50 mM KCl, 5 mM $MgCl_2$).

All assays were conducted by monitoring changes in the absorbance of NAD(P)H at 340 nm using a MR 5000 microplate reader (Dynatech Laboratories, Chantilly, Va.) connected to a 486 computer running Biolinx version 2.0 software (Dynatech Laboratories). The assays were initiated by adding the enzyme preparation and performed at 21 ± 1°C, with a final microplate well volume of 0.250 mL plus added homogenate. Assay conditions were based on those of Brooks and Lampi (1995, 1996) as designed for the microplate reader, with optimal substrate concentrations determined for the *L. littorea* enzymes. Controls for nonspecific activity were run for all assays, and any blank value (minus the substrate marked with an asterisk) was subtracted to yield final activity values.

Hexokinase (HK; EC 2.7.1.1): 100 mM Tris buffer (pH 8.0), 1 mM EDTA, 2 mM $MgCl_2$, 5 mM glucose*, 1 mM Mg·ATP, 0.2 mM NADP, and 1 unit (U) NADP-dependent glucose-6-phosphate dehydrogenase/mL.

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Phosphofructokinase (PFK; EC 2.7.1.11): 100 mM imidazole-HCl buffer (pH 7.2), 50 mM KCl, 5 mM $MgCl_2$, 10 mM fructose-6-phosphate*, 0.1 mM Mg·ATP, 0.15 mM NADH, 0.1% (v/v) rotenone-saturated ethanol, 0.2 U aldolase/mL, 1.2 U glycerol-3-phosphate dehydrogenase/mL, and 0.16 U triosephosphate isomerase/mL. To remove ammonium sulfate from coupling enzymes, aliquots of stock enzymes were centrifuged for 2 min in a Brinkman 5412 microcentrifuge. The pellet was resuspended in assay buffer and then centrifuged (benchtop centrifuge, top speed, 1 min) through a 5-mL column of fine Sephadex G-25 equilibrated in assay buffer.

Aldolase (EC 4.1.2.13): 50 mM imidazole-HCl buffer (pH 7.0), 2 mM $MgCl_2$, 0.2 mM fructose-1,6-P_2*, 0.15 mM NADH, 1 U triosephosphate isomerase/mL, and 2 U glycerol-3-phosphate dehydrogenase/mL.

Glycerol-3-phosphate dehydrogenase (G3PDH; EC 1.1.1.8): 500 mM triethanolamine buffer (pH 7.9), 5% (w/v) bovine serum albumin, 26 mM glyceraldehyde-3-phosphate*, 0.15 mM NADH, and 1 U triosephosphate isomerase/mL.

Pyruvate kinase (PK; EC 2.7.1.40): 100 mM imidazole-HCl buffer (pH 7.2), 50 mM KCl, 5 mM $MgCl_2$, 10 mM phosphoenolpyruvate* (PEP), 2 mM ADP, 0.15 mM NADH, 0.2% (v/v) rotenone-saturated ethanol, and 1 U lactate dehydrogenase/mL.

Lactate dehydrogenase (LDH; EC 1.1.1.27): 50 mM imidazole-HCl buffer (pH 7.0), 2 mM $MgCl_2$, 4 mM pyruvate*, 0.15 mM NADH.

Phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32): 100 mM imidazole-HCl buffer (pH 6.6), 30 mM 2-mercaptoethanol, 5 mM PEP*, 50 mM $NaHCO_3$, 1.25 mM IDP, 1 mM $MnCl_2$, 0.15 mM NADH, and 2.5 U malate dehydrogenase/mL. Solutions were degassed prior to assaying.

Fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11): 25 mM imidazole-HCl buffer (pH 7.0), 5 mM $MgSO_4$, 1.4 mM fructose-1,6-P_2*, 0.2 mM $NADP^+$, 1 U phosphoglucoisomerase/mL, and 1 U glucose-6-phosphate dehydrogenase/mL.

Glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49): 100 mM Tris-HCl buffer (pH 7.5), 3.3 mM glucose-6-phosphate*, 6 mM $MgCl_2$, and 0.4 mM $NADP^+$.

6-Phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.44): 50 mM Tris-HCl (pH 7.0), 0.5 mM 6-phospho-D-gluconate*, and 0.4 mM $NADP^+$.

Glutamate pyruvate transaminase (GPT; EC 2.6.1.2): 500 mM imidazole-HCl buffer (pH 7.3), 30 mM 2-mercaptoethanol, 500 mM L-alanine*, 0.11 mM pyridoxal phosphate, 15 mM 2-oxoglutarate, 0.15 mM NADH, and 1 U LDH/mL.

Glutamate oxaloacetate transaminase (GOT; EC 2.6.1.1): 500 mM imidazole-HCl buffer (pH 7.8), 30 mM 2-mercaptoethanol, 250 mM L-aspartate*, 0.11 mM pyridoxal phosphate, 15 mM 2-oxoglutarate, 0.15 mM NADH, and 1 U malate dehydrogenase/mL.

Serine dehydratase (SDH; EC 4.2.1.13): 200 mM K_2HPO_4 buffer (pH 8.0), 2 mM EDTA, 100 mM L-serine*, 0.11 mM pyridoxal phosphate, 0.15 mM NADH, 0.2% (v/v) rotenone-saturated ethanol, and 1 U LDH/mL.

Malic enzyme (ME; EC 1.1.1.40): 150 mM triethanolamine buffer (pH 7.4), 5 mM L-malate*, 4 mM $MnCl_2$, and 0.2 mM $NADP^+$.

Malate dehydrogenase (MDH; EC 1.1.1.37): 50 mM imidazole-HCl buffer (pH 7.2), 10 mM $MgCl_2$, 20 mM oxaloacetate*, and 0.15 mM NADH. This assay determines the maximal activity of MDH using its preferred cofactor NADH. The activity of MDH using NADPH as coenzyme was also determined by substituting 0.15 mM NADPH.

Arginine kinase (ArgK; EC 2.7.3.3): 50 mM imidazole-HCl (pH 7.2), 10 mM $MgCl_2$, 15 mM arginine phosphate*, 15 mM glucose, 2 mM ADP, 0.45 mM $NADP^+$, 1 U HK/mL, and 1 U G6PDH/mL.

Adenylate kinase (AK; EC 2.7.4.3): 50 mM potassium phosphate buffer (pH 7.4), 20 mM KCl, 5 mM $MgSO_4$, 2 mM AMP, 2 mM ATP*, 2 mM PEP, 0.15 mM NADH, 2 U PK/mL, and 2 U LDH/mL.

Enzyme kinetics and statistical analysis

Enzyme rate data were processed by the Biolinx 2.0 software of the microplate reader and then imported into the Microplate Analysis Program (Brooks 1994), which processes data from individual cells for different purposes including calculation of enzyme maximal activities (units per gram wet mass of tissues) or assembly of rate versus concentration data (e.g., for substrates, activators, inhibitors) for export into an enzyme-kinetics program. The kinetics program calculated $S_{0.5}$ values (substrate concentration producing half-maximal velocity) by fitting data to the Hill equation using a nonlinear least squares regression program (Brooks 1992). A_{50} values (activator concentration producing half-maximal increase in enzyme velocity) were determined from a modified Hill equation with a V_0 term (rate at zero activator concentration) and I_{50} values (inhibitor concentration that reduces enzyme activity by 50%) from plots of V versus inhibitor concentration. Values for fold activation represent the maximum increase in enzyme activity achieved with saturating concentrations of activator. All data are presented as means ± SEM and statistical testing between control and experimental groups used Student's t test (two-tailed). One unit of enzyme activity was defined as the amount that uses 1 μmol of substrate per minute at 21°C.

Results

Effect of seasonal change on maximal activities of enzymes in foot muscle

Table 1 shows the effect of sampling in different seasons on the maximal activities of 18 metabolic enzymes in foot muscle and digestive gland of periwinkles. In foot muscle there were few seasonal differences in enzyme activities. LDH and MDH (with both NADH and NADPH as substrates) activities in muscle from summer animals were significantly higher (by 38 and 11–16%, respectively) than in muscle from winter animals. By contrast, G6PDH and ArgK activities were lower in summer animals: only 52 and 66% of the corresponding winter values, respectively. Seasonal differences were seen for nine enzymes in digestive gland, and in all cases, activities were higher in the summer. PFK, aldolase, and GPT activities were 34–48% higher, G3PDH, PK, PEPCK, and SDH activities were 85–94% higher, and LDH and ME activities were 120–126% higher in digestive gland of summer versus winter animals. Of note was the fact that in both seasons, a consistent ratio was maintained between PK and PEPCK activities, PK activity being 54- to 61-fold higher than PEPCK in foot and 30-fold higher in digestive gland. These two enzymes gate the aerobic (PK) versus anoxic (PEPCK) use of glycolytic carbon, directing carbon flow into the tricarboxylic acid cycle versus the pathway of succinate formation, respectively.

Effect of anoxia exposure on the maximal activities of enzymes in foot muscle

The effect of anoxia exposure on the maximal activities of all enzymes was also tested in both winter and summer. Snails were held in sealed jars where both the ~1 cm of seawater in the bottom and the atmosphere had been thoroughly flushed with N_2 gas; most snails clung to the walls of the jar. Although hemolymph P_{O_2} was not measured, it would be extremely low or zero after the 6-day exposure, and we have

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Table 1. Maximal activities of enzymes in foot muscle and digestive gland of aerobic control *Littorina littorea* in winter (November) and summer (July).

	Foot muscle		Digestive gland	
Enzyme	Winter	Summer	Winter	Summer
HK	0.64 ± 0.06	0.75 ± 0.04	0.46 ± 0.06	0.68 ± 0.09
PFK	2.11 ± 0.27	1.87 ± 0.17	0.92 ± 0.11	1.36 ± 0.12[a]
Aldolase	8.14 ± 0.88	7.88 ± 0.83	2.94 ± 0.34	4.16 ± 0.37[a]
G3PDH	1.45 ± 0.08	1.15 ± 0.12	1.50 ± 0.18	2.78 ± 0.21[a]
PK	26.7 ± 3.06	21.6 ± 2.38	4.49 ± 0.56	8.69 ± 1.18[a]
PEPCK	0.44 ± 0.04	0.40 ± 0.04	0.15 ± 0.02	0.29 ± 0.03[a]
LDH	14.0 ± 0.57	19.3 ± 1.97[a]	4.99 ± 0.72	11.3 ± 1.57[a]
FBPase	0.04 ± 0.01	0.05 ± 0.01	0.12 ± 0.02	0.16 ± 0.01
G6PDH	1.68 ± 0.25	0.87 ± 0.13[a]	3.86 ± 0.45	3.50 ± 0.46
6PGDH	1.21 ± 0.12	1.16 ± 0.09	2.80 ± 0.18	3.01 ± 0.32
GPT	7.83 ± 0.53	8.61 ± 0.96	7.69 ± 0.73	10.3 ± 0.53[a]
GOT	13.3 ± 0.54	14.3 ± 1.24	11.3 ± 1.07	11.8 ± 0.78
SDH	0.18 ± 0.013	0.19 ± 0.03	0.35 ± 0.06	0.66 ± 0.06[a]
ME	0.37 ± 0.02	0.43 ± 0.04	0.10 ± 0.01	0.22 ± 0.03[a]
MDH (NADH)	52.7 ± 1.29	61.0 ± 1.51[a]	53.0 ± 2.5	50.0 ± 4.90
MDH (NADPH)	5.98 ± 0.12	6.66 ± 0.14[a]	6.10 ± 0.62	5.48 ± 0.27
ArgK	1009 ± 130	662 ± 73[a]	131 ± 9.3	135 ± 3.9
AK	36.6 ± 2.43	30.8 ± 1.86	10.5 ± 2.23	12.6 ± 2.33

Note: Activities are expressed in are units per gram wet mass (mean ± SEM; n = 4–6). For full names of enzymes see Materials and methods.
[a]Significantly different from the corresponding winter value by Student's t test (two-tailed) ($P < 0.05$).

previously shown strong accumulation of anaerobic end-products in littorines held under similar anoxic conditions (Storey et al. 1982; Churchill and Storey 1996). Of the 18 metabolic enzymes assayed, only a few showed significant changes in maximal activities after the 6 days of anoxia exposure, and these are summarized in Fig. 1. Anoxia exposure in the winter (at 5°C) led to significant reductions in the activities of LDH, GPT, and GOT in foot muscle (by 20, 25, and 20%, respectively). Anoxia exposure in the summer (at 10°C) also reduced foot-muscle GPT activity by 33% but, by contrast, HK and 6PGDH activities both rose by ~25%. Anoxia exposure had no effect on the activities of enzymes in the digestive gland of summer snails and affected only two enzymes in winter animals, reducing PK and LDH maximal activities by 55 and 62%, respectively.

Seasonal and anoxia-induced changes in the kinetic properties of PFK

Table 2 summarizes the kinetic properties of PFK from periwinkle foot muscle collected in winter (November) and summer (July). Affinity for the substrate, fructose-6-phosphate (F6P), did not change between the seasons or in response to anoxia, nor did PFK sensitivity to inhibition by high levels of its second substrate, ATP. However, compared with the enzyme from winter snails, PFK in foot of summer snails showed greater sensitivity to two allosteric activators, AMP and fructose-2,6-bisphosphate (F2,6P_2). A_{50} values for the enzyme in summer snails were reduced to 40 and 49% of the corresponding winter values. PFK properties in summer snails also showed an increase in the fold activation by F2,6P_2.

Anoxia exposure elicited significant changes in the properties of foot-muscle PFK in both seasons (Table 2). In winter animals, anoxia caused a significant decrease in the A_{50} value for AMP but an increase in the fold activation by this adenylate. PFK from anoxic animals in summer also showed a significantly higher fold activation by AMP but the fold activation by F2,6P_2 changed oppositely. Anoxia exposure of summer snails also resulted in a 1.6-fold increase in the A_{50} value for F2,6P_2 compared with aerobic controls.

Comparable information for PFK from digestive gland is shown in Table 3. Compared with the enzyme from winter animals, digestive gland PFK from summer snails showed a significantly higher Hill coefficient for F6P and the I_{50} value for ATP was 60% lower. The A_{50} values for both activators were higher in summer animals, by 2.7-fold for A_{50} AMP and by 3.1-fold for A_{50} F2,6P_2. The fold activation of PFK by AMP was also significantly higher in summer digestive gland than in winter digestive gland.

Anoxia exposure caused significant changes to PFK kinetic properties in digestive gland. In winter animals, anoxia exposure resulted in a 34% increase in $S_{0.5}$ F6P and a significant decrease in the Hill coefficient (Table 3). The A_{50} value for AMP also increased 3-fold in anoxic tissue along with an increase in the fold activation by AMP. Oppositely, the fold activation by F2,6P_2 was reduced in anoxic animals. Anoxia-induced changes in the properties of PFK from digestive gland of summer snails included a 20% decrease in the Hill coefficient for F6P, a 50% decrease in A_{50} AMP, a 32% decrease in A_{50} F2,6P_2, and reduced fold activation by both activators.

Seasonal and anoxia-induced changes in the kinetic properties of PK

The effects of season and anoxia exposure on the kinetic properties of PK from foot muscle and digestive gland of periwinkles are shown in Table 4. In foot, small seasonal differences in PK properties were seen in the control situation; the $S_{0.5}$ PEP of PK in summer snails was significantly higher

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Fig. 1. Significant changes in enzyme maximal activities in *Littorina littorea* tissues as a result of anoxia exposure (6 days) in winter or summer. (A) Foot muscle of winter snails. (B) Foot muscle of summer snails. (C) Digestive gland of winter snails. Open bars show control values and solid bars show anoxic values; only enzymes whose activities changed significantly during anoxia exposure are shown. Activities (means ± SEM) are expressed in international units (IU) per gram wet mass (n = 5–6); *, significantly different from the corresponding control using Student's t test (two-tailed) ($P < 0.05$).

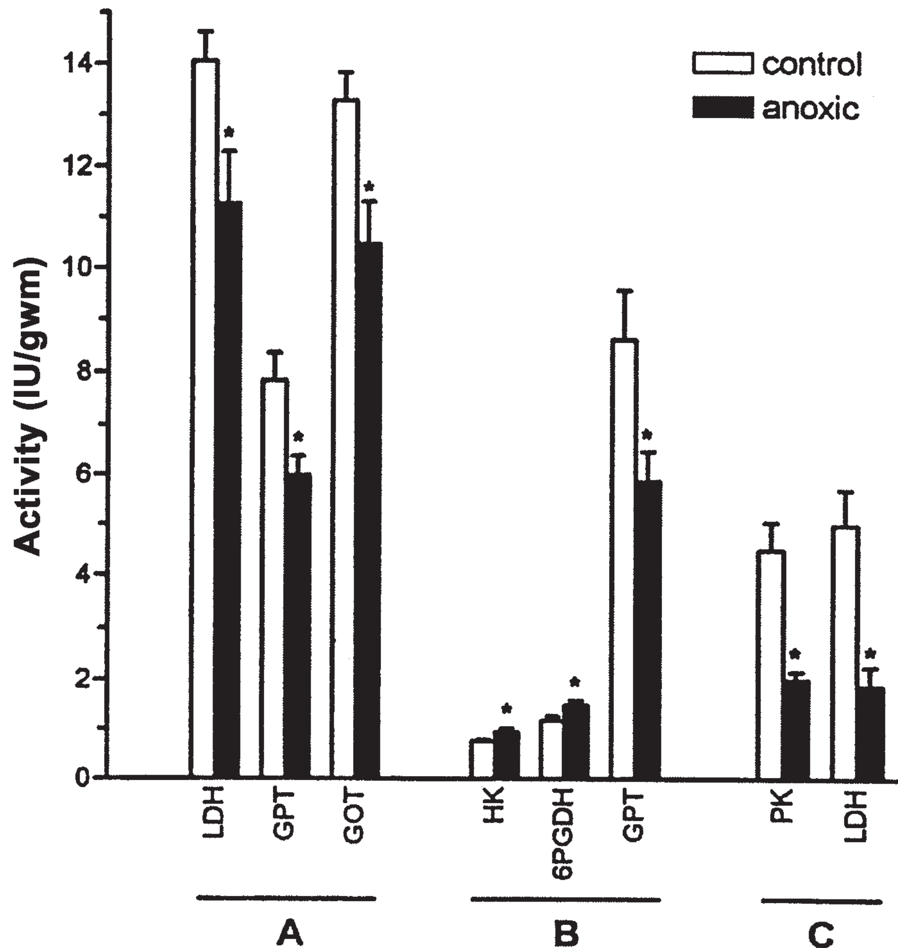

Table 2. Kinetic properties of PFK from foot muscle of aerobic control and 6-day anoxic winter and summer *L. littorea*.

	Winter		Summer	
	Control	Anoxic	Control	Anoxic
$S_{0.5}$ F6P, mM	1.94 ± 0.07	1.70 ± 0.11	2.06 ± 0.06	2.05 ± 0.06
n_H	2.53 ± 0.23	2.37 ± 0.20	2.32 ± 0.07	2.44 ± 0.15
I_{50} Mg.ATP, mM	2.42 ± 0.09	2.74 ± 0.20	2.50 ± 0.16	2.08 ± 0.19
A_{50} AMP, mM	0.05 ± 0.01	0.02 ± 0.001[a]	0.02 ± 0.002[b]	0.03 ± 0.004
Fold activation	2.33 ± 0.22	3.20 ± 0.18[a]	3.48 ± 0.24	4.76 ± 0.33[a]
A_{50} F2,6P$_2$, µM	0.57 ± 0.05	0.80 ± 0.20	0.28 ± 0.01[b]	0.45 ± 0.08[a]
Fold activation	3.78 ± 0.41	4.69 ± 0.40	5.73 ± 0.36[b]	3.81 ± 0.45[a]

Note: The results are given as means ± SEM (n = 5–8). All parameters except I_{50} Mg·ATP were determined at 0.1 mM Mg·ATP. I_{50} Mg·ATP values were determined at 4 mM F6P and A_{50} AMP and A_{50} F2,6P$_2$ values were determined at 1 mM F6P.

[a]Significantly different from the corresponding control value ($P < 0.05$).

[b]Significantly different from the corresponding winter control value ($P < 0.05$).

than that of PK in winter snails, whereas the I_{50} value for L-alanine was lower. PK in summer snails also showed higher fold activation by fructose-1,6-bisphosphate (F1,6P$_2$). Anoxia exposure resulted in major changes in the properties of foot PK that were qualitatively the same in winter and summer animals though differing somewhat in magnitude. Anoxia exposure greatly reduced enzyme affinity for PEP in both seasons, the $S_{0.5}$ PEP rising 6.4- to 7.4-fold during anoxia accompanied by significant increases in the Hill coefficient (indicating a change from a hyperbolic to a sigmoidal

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Table 3. Kinetic properties of PFK from digestive gland of aerobic control and 6-day anoxic winter and summer *L. littorea*.

	Winter		Summer	
	Control	Anoxic	Control	Anoxic
$S_{0.5}$ F6P, mM	2.07 ± 0.09	2.77 ± 0.09[a]	2.29 ± 0.07	2.37 ± 0.10
n_H	2.56 ± 0.05	2.12 ± 0.05[a]	3.04 ± 0.07[b]	2.45 ± 0.18[a]
I_{50} Mg.ATP, mM	3.24 ± 0.25	2.98 ± 0.16	1.28 ± 0.08[b]	1.31 ± 0.08
A_{50} AMP, mM	0.03 ± 0.006	0.10 ± 0.015[a]	0.08 ± 0.01[b]	0.04 ± 0.01[a]
Fold activation	2.01 ± 0.09	3.63 ± 0.40[a]	3.99 ± 0.31[b]	2.67 ± 0.23[a]
A_{50} F2,6P$_2$, µM	0.45 ± 0.06	0.41 ± 0.03	1.38 ± 0.11[b]	0.95 ± 0.07[a]
Fold activation	7.80 ± 0.49	6.17 ± 0.34[a]	7.58 ± 1.02	4.41 ± 0.51[b]

Note: The results are given as means ± SEM (n = 3–8). All parameters except I_{50} Mg·ATP were determined at 0.1 mM Mg·ATP. I_{50} Mg·ATP values were determined at 4 mM F6P and A_{50} AMP and A_{50} F2,6P$_2$ values were determined at 1 mM F6P.
[a]Significantly different from the corresponding control value ($P < 0.05$).
[b]Significantly different from the corresponding winter value ($P < 0.05$).

Table 4. Kinetic properties of PK from foot muscle and digestive gland of aerobic control and 6-day anoxic winter and summer *L. littorea*.

	Winter		Summer	
	Control	Anoxic	Control	Anoxic
Foot muscle				
$S_{0.5}$ PEP, mM	0.22 ± 0.01	1.41 ± 0.10[a]	0.32 ± 0.03[b]	2.37 ± 0.16[a]
n_H	1.06 ± 0.03	1.40 ± 0.09[a]	1.06 ± 0.04	1.67 ± 0.06[a]
I_{50} L-alanine, mM	2.42 ± 0.26	0.12 ± 0.01[a]	1.16 ± 0.14[b]	0.08 ± 0.01[a]
A_{50} F1,6P$_2$, µM	0.04 ± 0.008	2.45 ± 0.45[a]	0.05 ± 0.007	3.69 ± 0.36[a]
Fold activation	2.68 ± 0.17	19.01 ± 0.54[a]	4.52 ± 0.25[b]	11.08 ± 0.45[a]
Digestive gland				
$S_{0.5}$ PEP, mM	1.40 ± 0.11	2.29 ± 0.08[a]	0.18 ± 0.006[b]	0.19 ± 0.02
n_H	1.37 ± 0.09	1.42 ± 0.11	1.09 ± 0.02[b]	1.02 ± 0.008
I_{50} L-alanine, mM	1.18 ± 0.13	0.36 ± 0.03[a]	0.83 ± 0.08[b]	0.68 ± 0.08
A_{50} F1,6P$_2$, µM	3.77 ± 0.47	6.07 ± 0.72[a]	1.53 ± 0.16[b]	2.59 ± 0.29[a]
Fold activation	5.23 ± 0.26	7.14 ± 0.70[b]	2.99 ± 0.21[b]	2.54 ± 0.21

Note: The results are given as means ± SEM (n = 4–11). All parameters were determined at 2 mM ADP. I_{50} L-alanine values were determined at 1 or 4 mM (winter) and 2 or 0.5 mM (summer) PEP for foot or digestive gland, respectively, and A_{50} F1,6P$_2$ values were determined at 0.1 mM PEP for foot and summer digestive gland and at 1 mM PEP for winter digestive gland.
[a]Significantly different from the corresponding control value ($P < 0.05$).
[b]Significantly different from the corresponding winter value ($P < 0.05$).

substrate saturation curve). PK from anoxic foot also showed much greater inhibition by L-alanine, with the I_{50} value reduced to only 5–7% of the corresponding aerobic value. The anoxic enzyme was also much less sensitive to F1,6P$_2$ activation, with 61- to 74-fold increases in A_{50} values, although a greater fold activation by saturating amounts of the activator was seen.

The effects of season and anoxia on digestive gland PK were considerably different. PK from this tissue showed distinct differences in kinetic properties of the control enzymes between the seasons. The $S_{0.5}$ PEP of the enzyme in summer animals was only 13% of that of PK in winter animals and both I_{50} alanine and A_{50} F1,6P$_2$ were reduced in the summer as was the fold activation achieved by F1,6P$_2$. Responses of winter and summer digestive gland PK to anoxia exposure were also very different. The only effect of anoxia on digestive-gland PK from summer animals was a 70% rise in A_{50} F1,6P$_2$. By contrast, the winter enzyme showed responses to anoxia that were similar to those seen in foot muscle: affinity for PEP was reduced ($S_{0.5}$ increased 60%), inhibition by L-alanine increased (I_{50} decreased to 30% of the aerobic value), and sensitivity to F1,6P$_2$ activation was reduced (A_{50} rose by 60%).

Discussion

Seasonal changes in enzyme activities

It has been previously reported that seasonal changes extend to most aspects of metabolism in molluscs, including carbohydrate, lipid, nucleic acid, protein, amino acid, and anaerobic metabolism (Gabbott 1983). These are often closely linked to the reproductive cycle and the periwinkle displays an inverse relationship between growth and gonad development. Following spawning in May, growth occurs, with active feeding and storage of lipid and carbohydrate reserves during the summer and early autumn. Feeding and growth stop in late autumn and maturation of the gonads occurs

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from December until April (Williams 1970). The metabolic reorganization that accompanies these seasonal cycles of growth and reproduction may necessitate changes in the activities of selected enzymes (Livingstone 1981; Gabbott 1983). The two sampling times used in the present study correspond to the period of active growth and feeding (July) and the end of the growth season (November), when metabolic changes have or are being made to support low-temperature winter survival and the initiation of gametogenesis. It should be noted that in both seasons animals were starved while acclimating in the laboratory; although this would not be the natural condition, especially for summer snails, we felt that this would best provide the baseline metabolic status of the tissues at the two seasons and reduce metabolic variability that could arise from variation in feeding status between individuals if we provided food in the laboratory.

Overall, seasonal adjustments in the maximal activities of enzymes were relatively minor in foot muscle. G6PDH and ArgK activities were both lower in foot of summer snails but LDH and MDH activities were higher. Higher LDH activity in summer animals might be due to a higher level of physical activity at this season, although one might then wonder why ArgK did not change in a similar fashion. However, ArgK activities are exceedingly high and undoubtedly far in excess of metabolic flux demands at all times. LDH and the opine dehydrogenases (alanopine dehydrogenase and LDH activities are nearly equal in *L. littorea*; Plaxton and Storey 1982) typically support glycolytic energy production during muscle exercise, whereas glycolysis ending in succinate and alanine production is linked with survival of environmental oxygen deprivation. Under anoxic conditions, MDH functionally replaces LDH in the reoxidation of cytoplasmic NADH and is linked with the succinate-producing pathways; higher levels of both dehydrogenases in summer may support a generally higher glycolytic capacity in summer.

In digestive gland, nine enzymes showed higher maximal activities in July than in November. Activities of four enzymes of glycolysis (PFK, aldolase, PK, LDH) were elevated. PFK is generally considered to be the rate-limiting enzyme of glycolysis and this suggests that overall glycolytic capacity is raised in the summer. This response by glycolytic and other enzymes (Table 1) could support higher rates of biosynthesis during the summer, making use of fuels that are taken in during feeding. For example, G3PDH supplies glycerol-3-phosphate for triglyceride synthesis, whereas GPT and SDH are involved in processing amino acids. Increased levels of these latter enzymes, together with PEPCK and ME, which have gluconeogenic roles, could suggest higher rates of amino acid catabolism from dietary sources in summer animals. GPT, PEPCK, and ME also have significant roles in anaerobic metabolism in marine molluscs. PEPCK converts glycolytic PEP into oxaloacetate, which, after conversion to malate via MDH, moves into the mitochondria. Once it is there, a reversal of MDH releases oxaloacetate for dismutation leading to two fates: the pathway leading to succinate and propionate, which generates ATP and uses NADH, and the pathways leading to alanine and acetate, which use ME to regenerate reducing equivalents and can convert the resulting pyruvate into alanine via GPT (using amino groups transferred during catabolism of aspartate). Hence, seven of the nine enzymes that show higher activities in digestive gland of summer versus winter littorines have defined roles in anaerobic metabolism, and this could suggest that anaerobic capacity may also be enhanced in the summer months. Such an increase in anaerobic capacity of tissues in the summer might be needed for two reasons: the tissue metabolic rate is higher in hot weather, whereas the oxygen concentration is lower in warm seawater than in cold. Hence, situations where internal oxygen reserves of the animal are depleted (e.g., during aerial exposure at low tide) or environmental oxygen availability is highly restricted (e.g., in hot tide pools) would be more common in summer and animals could use a greater anaerobic capacity to deal with potentially more frequent hypoxic or anoxic insults.

Anoxia-induced changes in enzyme activities

A primary response to oxygen deprivation by intertidal invertebrates is metabolic-rate depression, the anoxic metabolic rate typically being only 5–10% of the normal aerobic resting rate at the same temperature (Famme et al. 1981; De Zwaan et al. 1991). Metabolic suppression lowers energy requirements to a level that can be sustained over the long term by the ATP production from fermentative pathways alone. Biochemical controls target and suppress the rates of many ATP-expensive functions including biosynthetic pathways. Protein biosynthesis is one of these targets, hence it is not surprising that few changes to the maximal activities of enzymes occurred during the 6-day anoxia exposure. Furthermore, the majority of changes seen were reductions in enzyme maximal activities during anoxia, which could have been due to slightly higher rates of protein degradation than synthesis under anoxic conditions. This could underlie the reduced activities of several equilibrium enzymes in anoxic tissues: LDH, GPT, and GOT in foot muscle and LDH in digestive gland. The reduction in PK maximal activity may be due to an anoxia-induced change in the phosphorylation state of the enzyme, which is well known to reduce V_{max} and alter the kinetic properties of PK from numerous molluscs, as will be discussed later. Anoxia exposure increased activities of only two enzymes: HK and 6PGDH in summer foot muscle. Increased HK could suggest that the muscle increases its use of exogenous glucose as a metabolic fuel during anoxia.

Seasonal and anoxia-induced changes in PFK kinetic properties

As a regulatory enzyme of glycolysis, PFK has multiple controls on its activity. As discussed above, coarse controls on enzyme maximal activity led to seasonal changes in enzyme activity in digestive gland. PFK also occurs in tissue-specific isoforms that are differentially sensitive to regulation via allosteric modifiers, reversible phosphorylation, polymerization, and enzyme binding to subcellular structural elements (e.g., F-actin). An initial brief analysis of PFK from *L. littorea* foot versus digestive gland suggested the presence of tissue-specific isoforms because the enzyme in the two tissues eluted from an ion-exchange column at very different salt concentrations (Russell and Storey 1995). The present results concur with this by showing differences between the kinetic properties of digestive gland and foot PFK

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in both seasons as well as differential effects of anoxia on the properties of the enzyme in each tissue (Tables 2 and 3). Within each tissue, the properties of PFK also changed seasonally. For foot-muscle PFK, A_{50} values for both AMP and F2,6P_2 were lower for the summer versus winter enzyme, whereas digestive-gland PFK showed a significantly lower I_{50} Mg·ATP in summer but increased A_{50} values for both activators. Such changes might be due to the seasonal expression of a different isoform in each tissue or might also arise from a seasonally dependent difference in the phosphorylation state of the native enzyme.

Anoxia exposure also changed PFK properties in both tissues of winter and summer animals. For foot muscle, anoxia-induced changes were selective and resulted in increased sensitivity to AMP activation (A_{50} decreased) for the enzyme in winter animals (which would make the enzyme more responsive to changes in tissue adenylates during anoxia) and reduced sensitivity to F2,6P_2 (A_{50} increased) for the summer enzyme. Anoxia exposure also reduced sensitivity to F2,6P_2 in muscles of other marine molluscs (*Mytilus edulis*, *Busycon canaliculatum*) among other kinetic changes (Whitwam and Storey 1991; Michaelidis and Storey 1991). For digestive-gland PFK of winter animals, anoxia exposure decreased sensitivity to AMP activation and also slightly decreased affinity for the substrate F6P, whereas the enzyme from summer animals showed increased sensitivity to both activators, AMP and F2,6P_2, during anoxia. Anoxia-induced changes to PFK kinetic properties in digestive gland have also been reported for other species (Michaelidis and Storey 1991; Whitwam and Storey 1991). The molecular basis of stable, anoxia-induced changes in PFK properties in other species is a change in the phosphorylation state of the enzyme (Michaelidis and Storey 1991; Whitwam and Storey 1991). However, whether reversible phosphorylation is also the basis of the changes to *L. littorea* PFK requires further exploration. Some initial results suggested that anoxia-induced changes might not be due to this mechanism. Thus, the presence versus absence of covalently bound phosphate alters the charge on a protein and should therefore change its elution profile on an ion-exchange column. However, anoxia exposure did not alter the elution profile of either foot or digestive-gland PFK in *L. littorea*, nor did prior incubation of tissue extracts in vitro under conditions that would promote the action of endogenous phosphatases or cAMP-dependent protein kinase on PFK (Russell and Storey 1995). More work must be done to resolve the regulation of PFK in *L. littorea*, including an examination of enzyme response to cGMP-dependent protein kinase, which seemed to mediate anoxia-induced changes to PFK in *M. edulis* anterior byssus retractor muscle (Michaelidis and Storey 1990).

Seasonal and anoxia-induced changes in PK kinetic properties

In contrast with the situation for PFK, there seems no doubt that anoxia-induced changes in the properties of PK in *L. littorea* tissues are the result of reversible phosphorylation (Russell and Storey 1995), as is also the case for PK from many other marine molluscs (Plaxton and Storey 1984*a*, 1984*b*; Holwerda et al. 1989; Gaitanaki et al. 1990; Storey 1993). However, in contrast with other species, the data of Russell and Storey (1995) showed that the anoxic enzyme form in both foot and digestive gland of the periwinkle was the low-phosphate form, whereas the aerobic control enzyme was the high-phosphate form. Nonetheless, the current data show that anoxic PK in foot is clearly the less active form (Table 4). In both winter and summer animals, the foot enzyme from anoxic animals (as compared with aerobic controls) showed reduced affinity for PEP substrate (higher $S_{0.5}$), reduced activation by F1,6P_2 (higher A_{50}), and strongly increased inhibition by L-alanine (much lower I_{50}), a product of anaerobic metabolism. These are all common traits of the anoxic form of PK in other molluscan species and serve to greatly reduce PK activity in vivo during anaerobiosis. This helps to channel PEP away from PK and into the PEPCK reaction instead (and, hence, onwards into succinate synthesis) (Storey 1993). The effects of anoxia on digestive-gland PK from winter littorines followed the same pattern but the percentage changes in the values for $S_{0.5}$ PEP, A_{50} F1,6P_2, and I_{50} L-alanine were not as large as were seen in muscle. In summer animals, however, the effect of anoxia on digestive-gland PK kinetics was very different. The only difference between control and anoxic PK was a 70% higher A_{50} F1,6P_2 for the anoxic enzyme form, which is still consistent with a less active enzyme in the anoxic state.

These differences in the response to anoxia by digestive-gland PK between summer and winter animals go along with major seasonal differences in the kinetic properties of the control enzymes. Digestive-gland PK from summer littorines showed a greater affinity for PEP, a hyperbolic n_H value, a lower I_{50} for L-alanine, and a lower A_{50} for F1,6P_2 than did the enzyme from the winter animals. This could suggest a seasonal change in PK isoform in digestive gland. An enzyme with a high substrate affinity ($S_{0.5}$ values are close to in vivo PEP concentrations) and high sensitivity to F1,6P_2 activation could be well suited for function in a tissue that is carrying out high rates of biosynthesis from carbohydrates. In addition, the general lack of anoxia effects on the summer enzyme may suggest that this form is much less susceptible to control by reversible phosphorylation in vivo. PK from the winter animals, however, with its much higher $S_{0.5}$ PEP would be dependent on the actions of activators in order to display substantial activity in vivo, and furthermore, the major changes in kinetic properties induced by anoxia indicate that activity of this enzyme can be readily shut down. Hence, PK from the winter animals may be better suited for the metabolic situation in digestive gland during the nonfeeding season, when rates of biosynthesis would be low and anoxia-induced metabolic-rate depression may be an important feature for winter survival when only endogenous fuel reserves are available.

By contrast with digestive-gland PK, the kinetic differences between summer and winter forms of foot-muscle PK were less pronounced, especially when compared with the very large changes in kinetic properties induced by anoxia. In addition, the changes in kinetic parameters induced by anoxia were quantitatively similar in summer and winter: a 6.4- to 7.4-fold increase in $S_{0.5}$, a 61- to 74-fold increase in A_{50} F1,6P_2, and a reduction in I_{50} L-alanine to 5–7% of the aerobic value. This suggests that the same PK isoform was present in foot muscle in the two seasons, although small

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differences in the basal content of covalently bound phosphate in the control forms may account for the slight seasonal differences in the properties of the control enzymes.

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