J Comp Physiol B (1989) 15:461–472

Journal of Comparative Physiology B Biochemical, Systemic, and Environmental Physiology
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Metabolic correlates to glycerol biosynthesis in a freeze-avoiding insect, *Epiblema scudderiana*

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Accepted May 31, 1989

Summary. The course of glycerol biosynthesis, initiated by exposure to −4 °C, was monitored in larvae of the goldenrod gall moth, *Epiblema scudderiana*, and accompanying changes in the levels of intermediates of glycolysis, adenylates, glycogen, glucose, fructose-2,6-bisphosphate, and fermentative end products were characterized. Production of cryoprotectant was initiated within 6 h after a switch from +16° to −4 °C, with half-maximal levels reached in 30 h and maximal content, 450–500 μmol/g wet weight, achieved after 4 days. Changes in the levels of intermediates of the synthetic pathway within 2 h at −4 °C indicated that the regulatory sites involved glycogen phosphorylase, phosphofructokinase, and glycerol-3-phosphatase. A rapid increase in fructose-2,6-bisphosphate, an activator of phosphofructokinase and inhibitor of fructose-1,6-bisphosphatase, appeared to have a role in maintaining flux in the direction of glycerol biosynthesis. Analysis of metabolite changes as glycerol production slowed suggested that the inhibitory restriction of the regulatory enzymes was slightly out of phase. Inhibition at the glycerol-3-phosphatase locus apparently occurred first and resulted in a build-up of glycolytic intermediates and an overflow accumulation of glucose. Glucose inhibition of phosphorylase, stimulating the conversion of the active *a* to the inactive *b* forms, appears to be the mechanism that shuts off phosphorylase function, counteracting the effects of low temperature that are the basis of the initial enzyme activation. Equivalent experiments carried out under a nitrogen gas atmosphere suggested that the metabolic make-up of the larvae in autumn is one that obligately routes carbohydrate flux through the hexose monophosphate shunt. The consequence of this is that fermentative ATP production during anoxia is linked to the accumulation of large amounts of glycerol as the only means of maintaining redox balance.

Key words: Cryoprotectant synthesis – *Epiblema scudderiana* – Cold hardiness – Regulation of glycolysis – Fructose-2,6-biphosphate

Abbreviations: G6P glucose-6-phosphate; *F6P* fructose-6-phosphate; *F1,6P*$_2$ fructose-1,6-bisphosphate; *F2,6P*$_2$ fructose-2,6-bisphosphate; *G3P* glycerol-3-phosphate; *DHAP* dihydroxyacetonephosphate; *GAP* glyceraldehyde-3-phosphate; *PEP* phosphoenolpyruvate; *PFK* phosphofructokinase; *FBPase* fructose-1,6-bisphosphatase; *PK* pyruvate kinase

Introduction

The last instar larvae of the gall moth, *Epiblema scudderiana* (Clemens) (Lepidoptera, Olethreutidae), overwinter inside spindle-shaped galls on the stems of goldenrod. The larvae utilize the freeze-avoidance strategy of cold hardiness. Autumn cold hardening includes a depression of larval supercooling point to −38 °C, a decrease in body water content, and an accumulation of glycerol to amounts that average 18.7% of fresh weight (over 2000 μmol·g wet weight^{-1}) in mid-winter (Rickards et al. 1987). Larvae may also be shielded from inoculative freezing by environmental ice by a thin cocoon lining the gall cavity.

Aspects of the biosynthesis of cryoprotectant polyols have been investigated by numerous authors. There is general agreement that glycogen is the substrate used, that glycogen mobilization is frequently triggered by cold activation of glycogen phosphorylase, that the hexose monophosphate shunt provides the reducing equivalents needed for synthesis, and that changes in enzyme activities or regulatory properties can channel carbon flux into the appropriate polyol product (for review see Storey and Storey 1988). Questions remain, however, about the control of the process and the energetics of polyol synthesis.

The present experiments were designed to take a close look at the initiation of glycerol synthesis in response to cold exposure. The pattern of changes in glycolytic intermediates and end products as well as adenylates was analyzed to provide information on the pathway of glycerol synthesis, the regulatory enzymes involved, carbon balance, and the energetics of the process. The stress of anoxia was also employed to gain further insights into the patterns of carbon flux and the energetic requirements of polyol synthesis.

Materials and methods

Chemicals and animals. All chemicals and biochemicals were purchased from Sigma Chemical Co., St. Louis, MO or Boehringer Mannheim Co., Montreal, PQ. Galls containing *E. scudderiana* larvae were collected in Ottawa, Canada, in mid-September 1987 and held in the laboratory for 2.5 weeks at 16°±1 °C prior to experimentation. Larvae were removed from their galls and placed in either covered petri dishes or sealed Erlenmeyer flasks for the aerobic versus anoxic experiments, respectively.

Animal experimentation. Experiments were designed to monitor the metabolic events accompanying the activation of glycerol synthesis by low temperature exposure. Larvae, previously acclimated to 16 °C, were moved to a second incubator at −4°±1 °C and were sampled at set time intervals of up to 14 days. At each sampling time, larvae were rapidly removed from the incubator, frozen in liquid nitrogen, and then transferred to −80 °C for storage. Experiments were carried out under either aerobic or anoxic conditions. For the anoxic treatments, Erlenmeyer flasks containing larvae were flushed with a mixture of 95% N_2/5% CO_2 for 30 min and then sealed and transferred to the −4 °C incubator.

Sample preparation and metabolite assay. Samples of 6 frozen larvae were pooled, ground to a powder using a mortar and pestle cooled in liquid nitrogen, and then weighed and extracted 1:5 w/v in 6% perchloric acid (containing 1 m*M* EDTA) (Storey et al. 1981); an aliquot of the well-mixed homogenate was removed for glycogen determination (Keppler and Decker 1974). Precipitated protein was removed by centrifugation and extracts were neutralized by the addition of 3 *N* KOH/0.4 *M* TRIS/0.3 *M* KCl and recentrifuged. Aliquots of neutralized extract were immediately used for assays of pyruvate, ATP, ADP, and AMP assays; the remaining extract was frozen at −80 °C for subsequent use. Metabolites were assayed enzymatically using a Pye Unicam SP8-100 spectrophotometer. Assays used were: glycerol (Eggstein and Kuhlman 1974), sorbitol (Bergmeyer et al. 1974), and all other metabolites (Lowry and Passonneau 1972).

$F2,6P_2$ content was assayed in alkaline extracts of individual larvae (1:20 w/v in 50 *M* NaOH) as described by van Schaftingen (1984).

Extraction and assay of glycogen phosphorylase was as in Storey and Storey (1984) with the exception that enzyme activity was measured in the well-suspended homogenate.

Data are reported as means±SEM for $n=4$ samples with 6 larvae pooled per sample (or 1 larva per sample for $F2,6P_2$). Statistically significant differences, where reported, were performed using the Student's *t*-test with a significance level of $P<0.05$.

Results

Aerobic time course

Upon exposure to −4 °C *E. scudderiana* larvae rapidly initiated glycerol synthesis; after a lag time of 2 h, levels rose over 4 days to 450 µmol/g wet weight (control = 46±8 $\mu mol \cdot g^{-1}$) (Fig. 1). The rate of synthesis was highest over the interval between 2 and 12 h of cold exposure, at 9.6 $\mu mol\ g^{-1} \cdot h^{-1}$, achieving half-maximal concentrations of glycerol within about 30 h. Glycogen content of the larvae was quite variable, but by 36–48 h it had dropped to 50%–60% of the control level, a net decrease sufficient to account for the carbon needs of glycerol synthesis. Glycerol was the only polyol produced; sorbitol did not accumulate (levels less than 0.5 $\mu mol \cdot g^{-1}$; data not shown) even when glucose content peaked at high values.

Changes in the activity state of regulatory enzyme(s) in a pathway alter the distribution of carbon between substrates and products of these loci. For example, enzyme activation typically produces a decrease in substrate content and an increase in product content as flux through the locus is activated; these changes are most pronounced over the transition time while the pathway flux changes from one steady state to another. Such changes in substrate and product contents in a pathway can be used to diagnose enzyme activation or inhibition in response to an external trigger/stress and to identify the rate-controlling enzyme in a pathway (Williamson 1970). When applied to the glycolytic pathway during the active synthesis of glycerol by *E. scudderiana*, this metabolic control theory can help to pinpoint the control points and the sequence of activation/inhibition of these loci over the time course of glycerol biosynthesis. For aerobic larvae exposed to −4 °C, the course of glycerol biosynthesis can be broken into four distinct time periods.

0 to 2 h. Cold exposure resulted in distinct changes in the contents of glycolytic intermediates and related metabolites in *E. scudderiana*. Within the first 2 h the content of G3P, the immediate precursor of glycerol, had decreased significantly ($P<0.05$) by 50% (Fig. 1), suggesting an increased flux through glycerol-3-phosphatase. Over the same time G6P content rose by 4.5-fold. F6P content was assayed, but in all cases levels were below the limits of detection (less than 0.05 $\mu mol \cdot g^{-1}$). However, levels of the product of PFK, $F1,6P_2$, showed a significant rise of 60%, and along with a rise in GAP, this suggests an increase in flux through the PFK locus to provide triose phosphates for

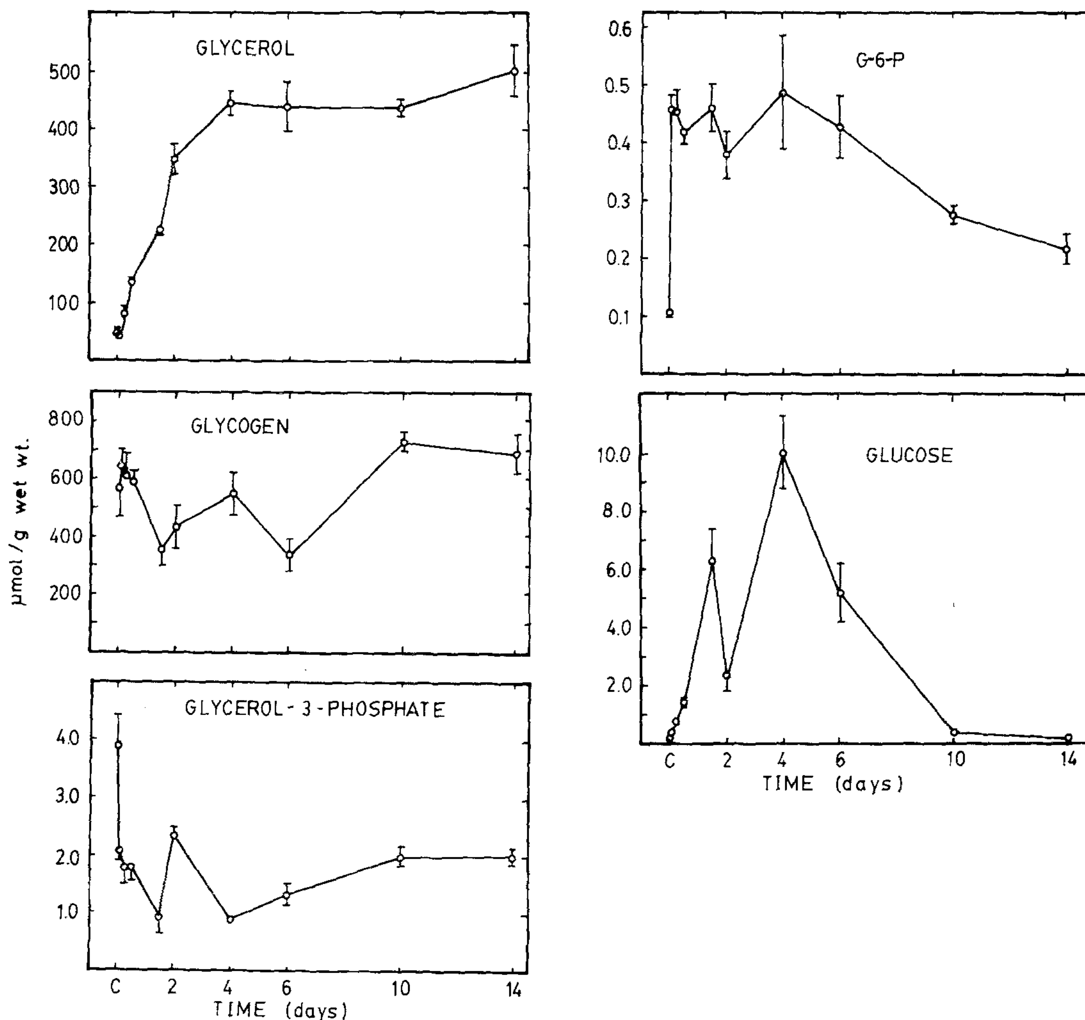

Fig. 1. Glycerol synthesis and associated changes in glycogen, glucose-6-phosphate (*G-6-P*), glucose, and glycerol-3-phosphate in *Epiblema scudderiana* larvae over 14 days after an abrupt temperature change from 16° to −4 °C. Sampling times are 2, 6, and 12 h and 1.5, 2, 4, 6, 10, and 14 days. Data are in μmol·g wet weight^{-1}, means ± SEM, $n=4$ samples each with 6 larvae pooled per sample. *C*, control larvae sampled at 16 °C at time 0. Glycogen is expressed in glucose units

glycerol synthesis (Fig. 2). The content of the PFK activator, $F2,6P_2$, doubled over the first 2 h at −4 °C (Fig. 2).

2 to 12 h. Glycerol accumulated at the highest rate between 2 and 12 h after the transfer to −4 °C. Levels of G3P and G6P did not change over this time, suggesting a relatively constant flux through an activated synthetic pathway. Levels of $F1,6P_2$, DHAP, and GAP showed a transient decrease at 6 h but by 12 h contents were equivalent to or greater than the values at 2 h. $F2,6P_2$ content increased by a further fivefold. Adenylate levels were not immediately perturbed by the cold exposure (a 20 °C decrease in temperature) (Fig. 3), and energy charge ([ATP + 1/2 ADP]/[ATP + ADP + AMP]) remained at 0.92–0.94 over the first 12 h at 4 °C (Table 1). However, lactate content rose significantly by 2.3-fold, suggesting a slight enhancement of glycolytic ATP production by cold exposure (Fig. 4).

1.5 to 6 days. By 36 h larvae showed strong evidence of energy stress; ATP content had dropped by 50%, AMP levels had risen threefold, total adenylates were reduced, and energy charge had dropped to 0.83 (Fig. 3). Pyruvate content had increased significantly twofold (with no change in PEP) (Fig. 2) by 36 h, suggesting an activation of flux through PK. A sharp jump in glucose content (Fig. 1) at the same time suggested that the ATP stress may have limited hexose phosphate conversion to glycerol, with a temporary channeling of G6P into the glucose pool instead.

Adenylate levels and energy charge remained

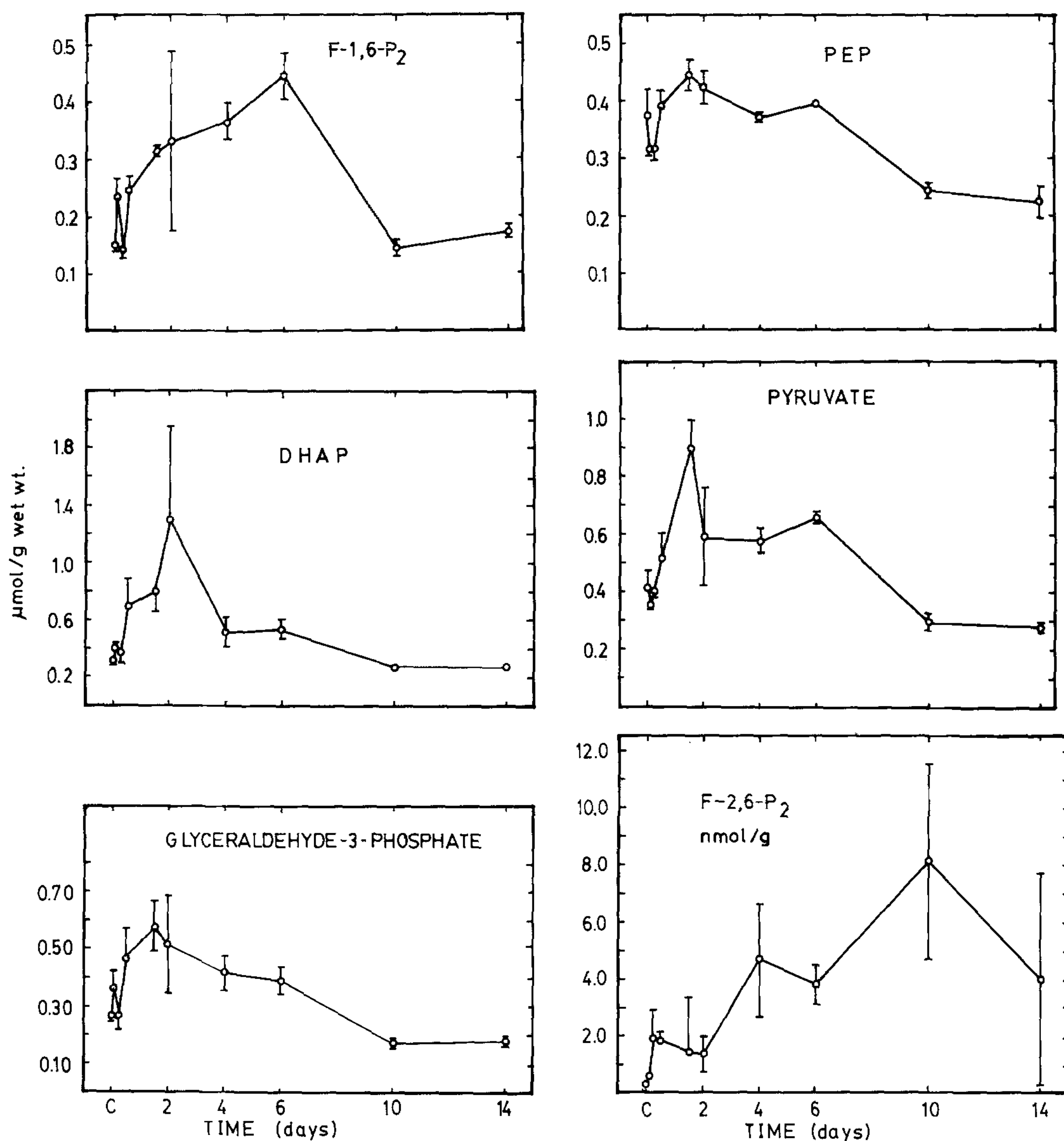

Fig. 2. Changes in the levels of fructose-1,6-bisphosphate (*F1,6-P₂*), dihydroxyacetonephosphate (*DHAP*), glyceraldehyde-3-phosphate, phosphoenolpyruvate (*PEP*), pyruvate, and fructose-2,6-bisphosphate (*F-2,6-P₂*) in *Epiblema scudderiana* larvae over 14 days at −4 °C. Details are as in Fig. 1 except that $F2,6P_2$ content is given in nmol·g wet weight^{-1}

perturbed for several more days but had recovered after 10 days at −4 °C (Fig. 3). During this period L-alanine levels rose by approximately 4 μmol·g^{-1}, suggesting some glycolytic component to ATP production (Fig. 4). Over this same period, glycerol content reached a plateau level; the rate of synthesis declined strongly between 2 and 4 days at −4 °C with no change in glycerol content after 4 days (Fig. 1). Changes in intermediate compounds were consistent with the declining rate of glycerol production. Thus, G3P content jumped sharply at 2 days suggesting the application of inhibitory control at the glycerol-3-phosphatase reaction. DHAP and GAP levels remained high, levels of both fructose bisphosphates increased, and glucose content peaked at 10 μmol·g^{-1}. This build-up of intermediates suggested a slight imbalance in the rates of the controlling enzymes as glycerol production was being turned off. Indeed, glycogen phosphorylase as still in an activated state at 6 days although net glycerol production appeared to have ended; activity was 1.43±0.17 units/g wet weight with 74%±7% *a* compared to control values of 1.04±0.08 U·g^{-1} and 37%±15% *a*.

10 to 14 days. After 10 days glycerol production had clearly ended, and the contents of most inter-

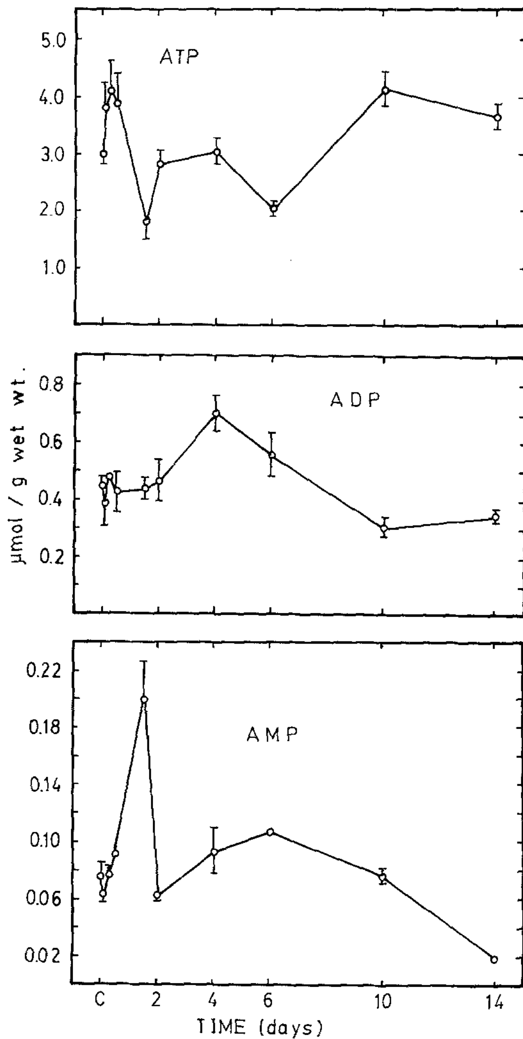

Fig. 3. Changes in the levels of adenosine triphosphate (*ATP*), ADP, and AMP in *Epiblema scudderiana* larvae over 14 days at −4 °C. Details are as in Fig. 1

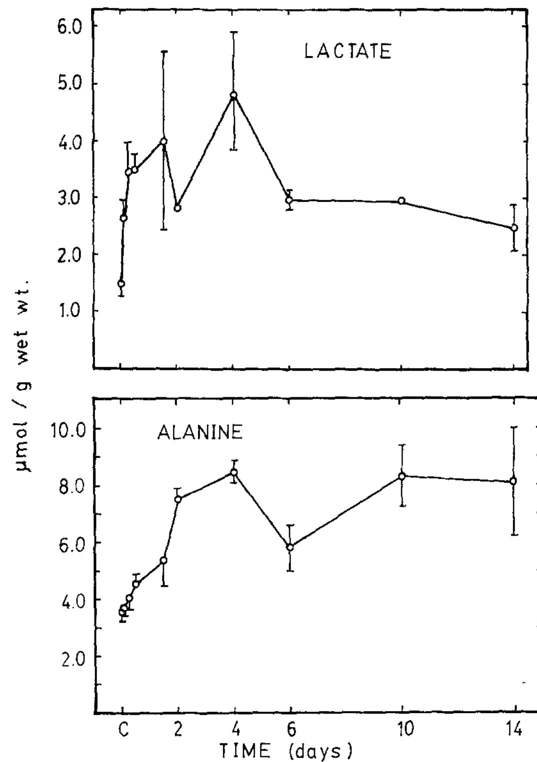

Fig. 4. Changes in the levels of L-lactate and L-alanine in *Epiblema scudderiana* larvae over 14 days at −4 °C. Details are as in Fig. 1

mediates had returned to near control levels by 10 or 14 days. Glucose had been cleared, G6P was falling, $F1,6P_2$ and triose phosphates had returned to control levels. Pyruvate and PEP contents were lower than initial levels in control larvae, but their concentration ratio, 1.1:1.0, was the same. Alanine remained high, but lactate was cleared. Adenylate contents and energy charge had returned to control levels.

Anoxic time course

The equivalent experiments were carried out under nitrogen gas atmosphere to determine whether cryoprotectant production in *E. scudderiana* could be carried out in an energy-restricted state. Figures 5–8 show metabolite levels in control (16 °C, aerobic) larvae, larvae after 30 min of N_2 gas flushing at 16 °C, and then anoxic larvae tranferred to

Table 1. Total adenylate content and energy charge in *Epiblema scudderiana* larvae during exposure to −4 °C under aerobic and anoxic conditions

	Aerobic		Anoxic	
	Total Adenylates	Energy charge	Total Adenylates	Energy charge
Control, 16 °C	3.5	0.94	3.5	0.92
N_2 gassing, 30 min	–	–	3.0	0.94
Time at −4 °C				
2 h	4.3	0.94	3.3	0.77
6 h	4.7	0.93	3.2	0.77
12 h	4.4	0.93	3.0	0.60
36 h	2.5	0.83	3.5	0.59
2 d	3.4	0.91	4.1	0.85
4 d	3.8	0.89	3.0	0.58
6 d	2.7	0.86	3.7	0.81
10 d	4.5	0.95	2.4	0.40
14 d	4.0	0.95	2.3	0.29

Data for adenylates are taken from Figs. 3 and 7. Energy charge is defined as ([ATP] + 1/2 [ADP])/([ATP + ADP + AMP])

−4 °C and held for 2, 6, 12, or 36 h and 2, 4, 6, 10, and 14 days.

Response to 30 min of nitrogen gas exposure. The response to the initial 30 min of anoxia was dra-

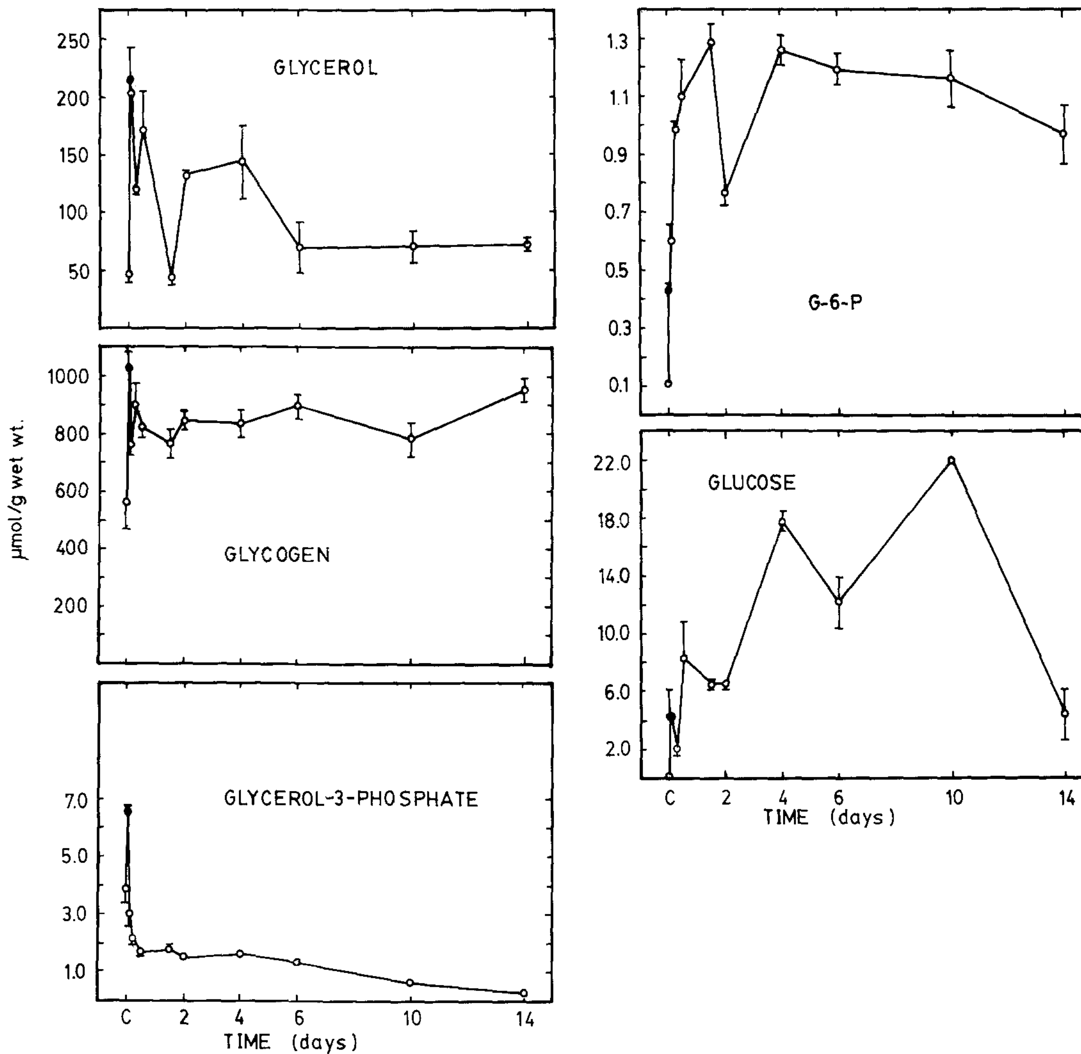

Fig. 5. Changes in glycerol, glycogen, glucose-6-phosphate (*G-6-P*), glucose, and glycerol-3-phosphate contents in *Epiblema scudderiana* larvae held under a nitrogen gas atmosphere in response to an abrupt temperature change from 16° to −4 °C. The *solid dot* represents the response to 30 min gassing with N_2 at 16 °C prior to the transfer to −4 °C. Subsequent sampling times at −4 °C are 2, 6, and 12 h and 1.5, 2, 4, 6, 10, and 14 days. Other details are as in Fig. 1

matic. ATP content fell, and ADP and AMP rose (Fig. 7), although a drop in the total adenylate pool from 3.5 to 3.0 $\mu mol \cdot g^{-1}$ allowed energy charge to remain high (0.94) (Table 1). A strong activation of glycolysis was evident: G6P rose fourfold, glucose content increased about 40-fold, and contents of $F1,6P_2$, GAP, DHAP, and G3P rose significantly by 35%, 35%, 55%, and 72% respectively ($P<0.05$) (Figs. 5, 6). PEP content dropped to 35% of the control value whereas pyruvate content increased by 800%; these changes clearly indicate activation of PK in response to anoxia. Glycerol levels jumped fourfold to 215 ± 28 $\mu mol \cdot g^{-1}$. Sorbitol levels rose about threefold but did not exceed 1.2 $\mu mol \cdot g^{-1}$ at any time (data not shown). Not unexpectedly, levels of glycolytic end products rose sharply with net increases of 3 $\mu mol \cdot g^{-1}$ L-lactate and 9.2 $\mu mol \cdot g^{-1}$ L-alanine over the 30 min. Fructose-2,6-P_2 levels rose 200-fold (Fig. 8).

0 to 12 h at −4 °C. Following the transfer of anoxic larvae to −4 °C, energy stress became more pronounced, this time with energy charge dropping rapidly to 0.60 by 12 h at 4 °C. G6P levels continued a rapid rise to 1.28 $\mu mol \cdot g^{-1}$, a 13-fold increase over control values. However, within 6–12 h at −4 °C contents of $F1,6P_2$, triose phosphates, PEP, and pyruvate had returned to values similar to control levels. $F2,6P_2$ content was also strongly reduced. These changes suggested inhibition at both the PFK and PK loci and a state of reduced flux through glycolysis. Glycerol, alanine, and lac-

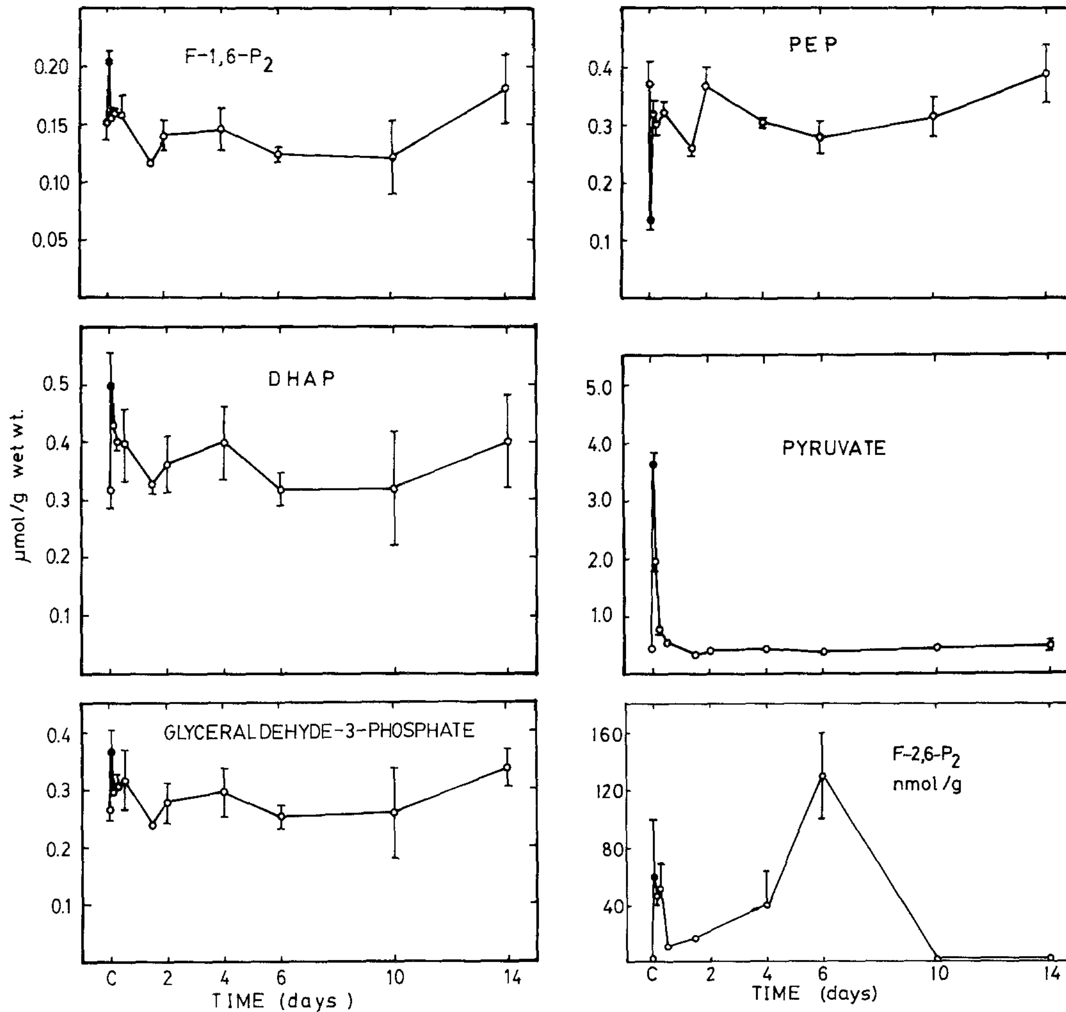

Fig. 6. Changes in the levels of fructose-1,6-bisphosphate (*F-1,6-P*$_2$), dihydroxyacetonephosphate (*DHAP*), glyceraldehyde-3-phosphate, phosphoenolpyruvate (*PEP*), pyruvate, and fructose-2,6-bisphosphate (*F-2,6-P*$_2$) in *Epiblema scudderiana* larvae over 14 days of anoxia exposure at −4 °C. Details are as in Figs. 1 and 5 except that $F2,6P_2$ content is given in $nmol \cdot g^{-1}$

tate levels were essentially constant over this period.

1.5 to 14 days. The subsequent events of anoxic exposure at 4 °C appeared to constitute an effort to sustain cellular energetics as much as possible. No glycerol was synthesized, and indeed, levels of the polyol fell to a constant level of about 75 $\mu mol \cdot g^{-1}$ after 6 days, an amount only 50% greater than control levels. G6P content remained high, and glucose continued to accumulate, reaching 18–22 $\mu mol \cdot g^{-1}$. Alanine and lactate contents further increased. Adenylates followed an oscillating pattern with high ATP and energy charge found after 2 and 6 days of anoxia but depressed levels at 4, 10, and 14 days. Total adenylates remained constant for the first 6 days but dropped by 30% at the last two time points as the fall in energy charge became severe. Adenylate fluctuations at 2, 4, and 6 days correlated with changes in G6P, glucose, and $F2,6P_2$ levels, which may indicate cyclical attempts to re-energize metabolism via increased glycogenolysis and anaerobic fermentations. Overall, high levels of G6P and glucose indicated that carbon provided by glycogenolysis could not be efficiently utilized by the larvae in the anaerobic state.

Water content and survival of experimental larvae

Seasonal changes in water content of *E. scudderiana* altered the apparent stoichiometry between glycogen and glycerol in an earlier study (Rickards et al. 1987). However, the percentage of body

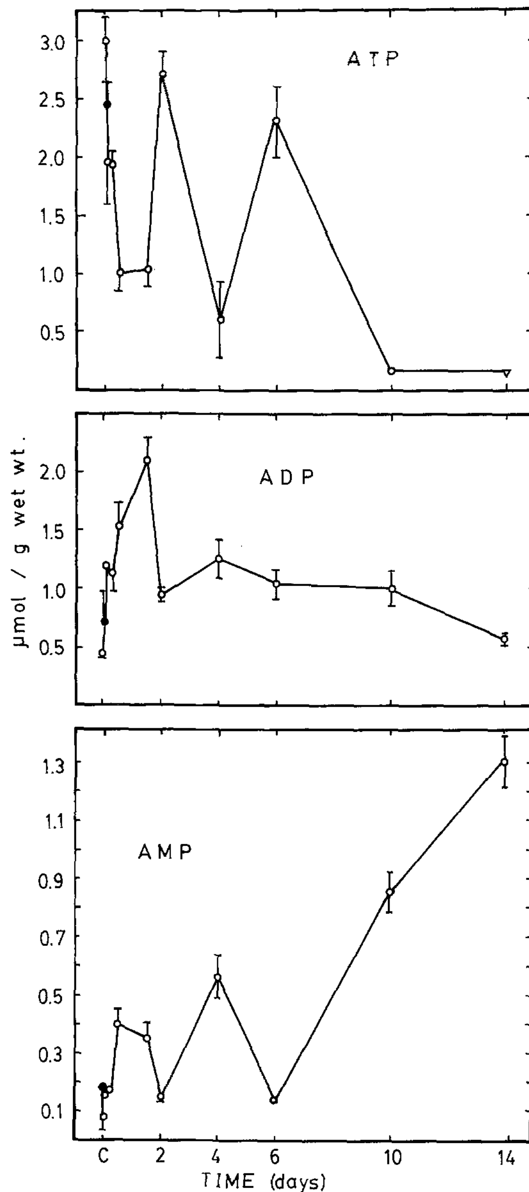

Fig. 7. Changes in the levels of adenosine triphosphate (*ATP*), ADP, and AMP in *Epiblema scudderiana* larvae over 14 days of anoxia exposure at −4 °C. Details are as in Figs. 1 and 5

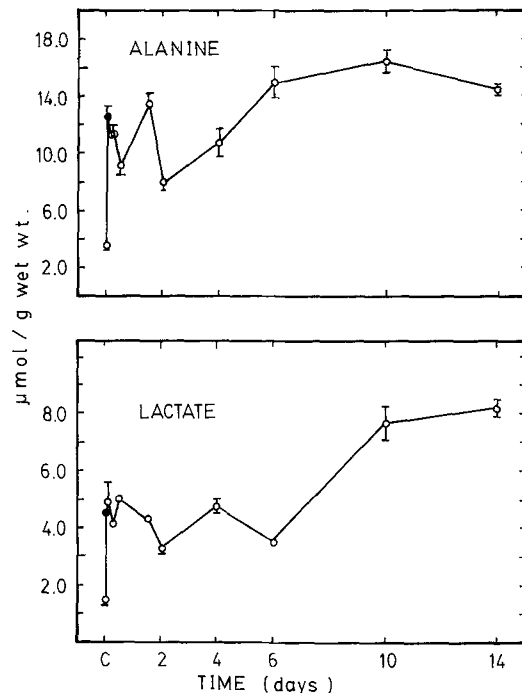

Fig. 8. Changes in the levels of L-lactate and L-alanine in *Epiblema scudderiana* larvae over 14 days of anoxia exposure at −4 °C. Details are as in Figs. 1 and 5

weight as water did not vary over the experimental time course for either aerobic (47% ± 4% H_2O) or anoxic (45% ± 3% H_2O) larvae. Larvae for both the 10 and 14 days of anoxia exposure were tested for survival. All maintained responsiveness to probing with a needle and were still alive 10 days later after being returned to an aerobic atmosphere at 3 °C.

Discussion

The process of cryoprotectant synthesis is characterized in detail by the data for the aerobic time course. As seen previously, glycerol synthesis is rapidly activated by low temperature exposure in *E. scudderiana* (Kelleher et al. 1987). Activation of the synthetic pathway occurred within 2 h at −4 °C with a significant rise in glycerol seen by 6 h. Half-time for maximal synthesis was about 30 h, and content was maximal within 4 days. Changes in G6P (an increase) and G3P (a decrease) by 2 h (Fig. 1) indicate an activation of both the initial (glycogen phosphorylase) and the terminal (glycerol-3-phosphatase) enzymes of the pathway. Furthermore, a sharp rise in $F1,6P_2$ content, the product of PFK, and of the contents of the subsequent glycolytic intermediates (DHAP and GAP) suggest a concomitant activation of PFK upon cold exposure. Sorbitol-producing insects, by contrast, show the opposite changes in PFK substrate and product levels during cold-stimulation of sorbitol synthesis, changes that are consistent with PFK inactivation during production of the hexitol (Storey and Storey 1983). Cold activation of glycogen phosphorylase is well-known among cold hardy insects (Ziegler et al. 1979; Hayakawa and Chino 1982), and direct measurement of enzyme activities here confirm that it also occurs in *E. scudderiana* (see also Churchill and Storey 1989). The mechanism involved is enzyme phosphorylation brought about by differential temperature effects

on the activities of phosphorylase kinase versus phosphorylase phosphatase (Hayakawa 1985). Whether control of glycerol-3-phosphatase and/or PFK also involves covalent modification of the enzyme protein is not known, but such regulation would clearly aid coordination of the biosynthetic response to cold temperature.

Further evidence of the probable importance of the phosphatase reaction to pathway control comes from the analysis of metabolite changes during the cessation of glycerol synthesis. G3P levels jump 2.5-fold between 1.5 and 2 days (Fig. 1) preceding the period of rapidly declining rates of glycerol accumulation. This suggests that inhibitory control at the glycerol-3-phosphatase locus may be involved in shutting down glycerol biosynthesis. The apparent result of such an inhibition is that carbon backs up in the pathway, and the other triose phosphates (GAP and DHAP) as well as $F1,6P_2$ accumulate in parallel (Fig. 2). Carbon also accumulates in the glucose pool, peaking at $10\ \mu mol \cdot g^{-1}$. Parenthetically, it would be interesting to analyze the status of the trehalose pool at this time. Theoretically, the production of the blood sugar should be regulated completely separately from cryoptrotectant biosynthesis, but very high glucose levels might favour increased production of the disaccharide; elevated levels of trehalose are found in many cold hardy insects (Storey and Storey 1988).

Both the evidence of accumulated high levels of glucose and glycolytic intermediates as glycerol production ceases and the direct measurements of phosphorylase activity late in the time course (still 74% *a* at 6 days) suggest that the inactivation of glycogen phosphorylase is somewhat out of phase with inhibition of the glycerol-3-phosphatase locus. However, such a response may indeed be necessary to shut down glycogen phosphorylase activity. The original activation of phosphorylase was due to the stimulating effect of low temperature on the enzyme, triggering the conversion of the inactive *b* form to the active *a* enzyme. High temperature has the reverse effect, inhibiting phosphorylase but activating glycogen synthase (Hayakawa and Chino 1982). However, the cessation of glycerol synthesis under our experimental conditions (constant -4 °C) could not have been caused by a high temperature signal. The same is obviously true in nature; the process of glycerol accumulation during cold hardening occurs as environmental temperatures progressively decrease over the autumn months. What, then, turns off phosphorylase? The answer is glucose.

The regulation of glycogen metabolism has been studied in detail in mammalian liver, and a central regulatory role for glucose has been described (Hers 1976). A primary function of liver is the maintenance of blood sugar levels within narrow limits (a function shared by the fat body in insects), and reciprocal control of glycogen phosphorylase and glycogen synthase is needed. This is provided by glucose in the following way. Glycogen phosphorylase *a* (the active form) is allosterically inhibited by glucose. Phosphorylase phosphatase activity, by contrast, is increased in the presence of glucose and converts phosphorylase *a* to *b*; the effect on this enzyme is not an allosteric one but arises because a glucose-phosphorylase *a* complex is a better substrate for phosphatase action (Hers 1976). Indeed, in the case of insects at low temperature, a glucose phosphorylase *a* complex may be the only effective substrate for phosphorylase phosphatase, the enzyme being otherwise cold-inactivated. Indeed, Hayakawa (1985) found that fat body phosphorylase phosphatase was virtually inactive at 0 °C with rabbit muscle phosphorylase *a* as its substrate. The proposed mechanism, then, would allow phosphorylase to remain active at low temperatures until such time as cryoprotectant levels reached near-maximal amounts. Then, a build-up of intermediates in the synthetic pathway, culminating in an 'overflow' accumulation of glucose, would feed back to inhibit phosphorylase and return phosphorylase *a* content to low levels.

Since its discovery in 1982, $F2,6P_2$ has been shown to be one of the most powerful allosteric signals controlling carbohydrate metabolism (Hue and Rider 1987). The compound is a potent activator of PFK, a strong inhibitor of fructose-1,6-bisphosphatase (FBPase), and focuses the actions of a variety of exogenous signals on these loci for sensitive control over the use of carbohydrate reserves. In mammalian liver, for example, high $F2,6P_2$, via activation of PFK, potentiates the use of carbohydrate reserves for biosynthesis. The sharp rise in $F2,6P_2$ levels (twofold within 2 h, tenfold by 6 h) (Fig. 2) occurring with the initiation of glycerol synthesis is not, therefore, surprising and would serve two purposes: (1) permitting carbon flow through PFK into glycerol biosynthesis and (2) via inhibition of FBPase, preventing the reverse conversion of triose phosphates back to hexose phosphates during the period of active glycerol synthesis when GAP, DHAP and $F1,6P_2$ levels are high. Understandably, then, $F2,6P_2$ levels remained high over the full 14-day time course. $F2,6P_2$ is synthesized from F6P via the enzyme 6-phosphofructo-2-kinase (PFK-2). A potent in-

hibitor of this enzyme is G3P (Hue and Rider 1987). In mammals G3P plus citrate control of PFK-2 is the key to the phenomenon of 'carbohydrate sparing', the inhibitory effect of lipid oxidation on carbohydrate catabolism. G3P regulation of PFK-2 is probably also critical to the regulation of $F2,6P_2$ levels in *E. scudderiana*; the sharp drop in G3P occurring within 2 h at −4 °C would release the inhibition of PFK-2, potentiate $F2,6P_2$ synthesis, and thereby set up the $F2,6P_2$ allosteric controls on PFK and FBPase that ensure unidirectional carbon flux into glycerol synthesis alone.

The experiment in which larvae were exposed to −4 °C in a nitrogen gas atmosphere was initially designed to determine whether glycerol synthesis could be supported in the absence of oxidative phosphorylation as a source of ATP for the PFK reaction. However, the results also provided information on the probable partitioning of carbon flow from G6P to triose phosphates between the ATP-dependent (glycolysis) and ATP-independent (hexose monophosphate shunt) routes in the larvae. The equivalent experiments carried out for freeze-tolerant *E. solidaginis* larvae suggested a substantial ATP dependence of glycerol synthesis, for under anoxic conditions glycerol production was only 57% of the aerobic amount, and yet this required an 18% greater consumption of carbon to fuel anaerobic glycolysis (with lactate and alanine accumulating) (Storey and Storey 1988, 1989). Total hydroxyl equivalents were maintained, however, by substituting sorbitol production (ATP-independent) to boost net polyol content.

The results for anoxia exposure for *E. scudderiana* larvae were unexpected but intriguing. In the end, glycerol levels in anoxic larvae were only 50% higher than in controls, compared to the 1000% increase in aerobic larvae. Clearly, net glycerol accumulation cannot be supported in an anoxic atmosphere in this species nor was the alternative seen in *E. solidaginis* larvae, sorbitol biosynthesis, used. However, two features of the response to anoxia by *E. scudderiana* stood out. One was the dramatic, fivefold rise in glycerol content in response to the 30 min of anoxia exposure alone (Fig. 5). The other was the progressive loss of glycerol over subsequent days of −4 °C exposure. The response to 30 min of nitrogen gassing was an activation of fermentative glycolysis; levels of G6P, $F1,6P_2$, and triose phophates all rose (Fig. 6). PEP content dropped whereas pyruvate content rose, a clear indication of PK activation (Williamson 1970). Metabolite changes were consistent with an activation of glycogen phosphorylase, PFK, and PK to support fermentative ATP synthesis with lactate and alanine accumulating as end products. What is odd, however, is the massive accumulation of glycerol; synthesis of this compound is ATP-consuming, not ATP-producing, and therefore disadvantageous to anoxic survival. However, the accumulation of this product is explainable if there is a fixed routing of carbon flow in autumn larvae. Specifically, the data suggest that most carbon must cycle through the hexose monophosphate shunt whether the fate of that carbon is polyol synthesis, anaerobic fermentation, or oxidation by the tricarboxylic acid cycle. Indeed, we have previously calculated that 86% of carbon must be processed via the hexose monophosphate shunt to support the NAD(P)H needs for glycerol synthesis (Storey and Storey 1988), and several studies have shown that hexose monophosphate shunt activity is high during cold-induced cryoprotectant synthesis (Wood and Nordin 1980; Tsumuki et al. 1987). If such a routing is obligatory, then an anoxia-induced activation of glycogenolysis would cycle carbohydrate through the hexose monophosphate shunt as a means of moving triose phosphates to the ATP-generating reactions of the lower portion of glycolysis. Flux through the hexose monophosphate shunt, however, would generate an enormous output of NADPH reducing equivalents compared with the net lactate and alanine produced. Redox balance could only be maintained, then, by supporting glycerol biosynthesis. Thus, the present data suggest that the routes of carbohydrate catabolism (partitioning between glycolytic versus hexose monophosphate shunt flux) in *E. scudderiana* larvae are fixed in a manner that is of maximal advantage to glycerol biosynthesis during autumn cold hardening but disadvantageous to dealing with stresses (e.g., anoxia) that require fermentative ATP synthesis. However, given that the larvae use the freeze avoidance strategy of winter hardiness and presumably retain oxygen-based metabolism throughout the winter, the need to generate ATP anaerobically should not normally arise. A similar metabolic make-up may be the basis of the glycerol accumulation reported for other species as a result of anoxia stress (Meyer 1978; Sonobe et al. 1979; Gade 1984). The molecular basis of such a fixed routing of carbohydrate may involve the subcellular compartmentation of enzymes, associated with glycogen particles, for the efficient production cryoprotectant during the autumn season. Indeed, in other studies we have found distinct seasonal differences in the relationship between glycogen and glycerol pools in *E. scudderiana* (Churchill and Storey 1989).

Separate from the cold activation of glycerol

synthesis, aerobic larvae showed an energy stress response to the sudden cold exposure. Although the high rates of glycerol biosynthesis may have contributed to the energy stress, the two were temporally shifted. Biosynthesis was well under way before energy stress was first evident at 36 h, and glycerol accumulation was completed by 4 days, whereas energetics recovered between 6 and 10 days at −4 °C (Figs. 1, 3). The molecular basis of the observed energy stress is probably the disruptive effects of the 20 °C drop in ambient temperature on ATP-producing (particularly mitochondrial) versus ATP-utilizing processes in the cell, requiring a reorganization of both membrane and protein components as part of cold acclimation. The initial response at 36 h included a sharp drop in ATP, threefold rise in AMP, and a drop in energy charge. These metabolite changes would lead to allosteric activation of ATP-synthesizing pathways until such time as metabolic reorganization for low temperature function was completed. A corresponding sharp rise in pyruvate at the same time indicated an activation of PK and an increased use of carbohydrate as a fuel at this time; the rise in alanine content beginning at 36 h suggests that fermentative glycolysis may have partially contributed to energy needs. Parenthetically, the preference for alanine versus lactate as the end product of fermentative glycolysis in *E. scudderiana*, seen in both aerobic and anaerobic studies, may be a mechanism of reserving reducing equivalents for polyol synthesis. Alanine results from the amination of pyruvate whereas lactate results from the reduction of pyruvate. Indeed, alanine accumulates in a number of cold hardy insects (Storey and Storey 1988). Interestingly, with the exception of the 36 h time-point, the larvae used a compensation strategy, a drop in the total adenylate pool, to maintain energy charge at high values. This strategy is based on the removal of AMP from the pool, via the action of AMP deaminase, and allows the continued functioning of ATP-consuming processes that are sensitive to the relative ratios of the three adenylates and would otherwise be inhibited by high levels of ADP and AMP. Most biosynthetic pathways in the cell function only when energy charge is high (Atkinson 1977). The preservation of a high energy charge is probably key, therefore, to the maintenance of glycerol biosynthesis by the larvae. This strategy was not, however, used under anoxia; here, total adenylates remained constant whereas energy charge fell. Such an energetic status would prevent biosynthesis and favour energy conservation strategies that would prolong survival under anoxia stress.

Acknowledgements. Thanks to J.M. Storey for assistance in the preparation of the manuscript. Supported by an operating grant from the N.S.E.R.C. Canada to KBS.

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