J Com Physiol B (1990) 160:77–84

Journal of Comparative Physiology B Biochemical, Systemic, and Environmental Physiology

Carbon balance and energetics of cryoprotectant synthesis in a freeze-tolerant insect: responses to perturbation by anoxia

Janet M. Storey and Kenneth B. Storey

Institute of Biochemistry and Department of Biology, Carleton University, Ottawa, Ontario, Canada K1S 5B6

Accepted December 8, 1989

Summary. The capacity for polyol synthesis by larvae of *Eurosta solidaginis* was evaluated under aerobic versus anoxic (N_2 gas atmosphere) conditions. Glycerol production occurred readily in aerobic larvae at 13 °C. Under anoxic conditions, however, net glycerol accumulation was only 57% of the aerobic value after 18 d, but the total hydroxyl equivalents available for cryoprotection were balanced by the additional synthesis of sorbitol. The efficiency of carbon conversion to polyols was much lower in anaerobic larvae. The ATP requirement of glycerol biosynthesis necessitated a 22% greater consumption of carbohydrate, when anaerobic and resulted in the accumulation of equimolar amounts of L-lactate and L-alanine as fermentative end products. The ratio of polyols produced to glycolytic end products formed was consistent with the use of the hexose monophosphate shunt to generate the reducing equivalents needed for cryoprotectant synthesis. A comparable experiment analyzed sorbitol synthesis at 3 °C under aerobic versus anoxic conditions. Sorbitol synthesis was initiated more rapidly in anaerobic larvae, and the final sorbitol levels attained after 18 d were 60% higher than in aerobic larvae. The enhanced sorbitol output under anoxia may be due to an obligate channeling of a high percentage of total carbon flow through the hexose monophosphate shunt at 3 °C. Carbon processed in this way generates NADPH which, along with the NADH output of glycolysis, must be reoxidized if anaerobic ATP synthesis is to continue. Redox balance within the hexose monophosphate shunt is maintained through NADPH consumption in the synthesis of sorbitol.

Key words: *Eurosta solidaginis* – Hexose monophosphate shunt – Anaerobic metabolism – Cryoprotectant synthesis – Freeze-tolerant insects

Introduction

Larvae of the goldenrod gall fly *Eurosta solidaginis* (Fitch) have been widely used as a model system to investigate natural freeze-tolerance in insects (reviews: Baust 1983; Baust et al. 1985; Storey 1983; Storey and Storey 1988, 1990). A key feature of cold-hardening in this species is the accumulation of two polyhydric alcohols, glycerol and sorbitol, as cryoprotectants. Although both polyols are derived from the glycogen reserves of the larvae, production of each is triggered and regulated separately. Glycerol accumulates first, building up over the autumn months and persisting well into the spring, even into the pupal stage in northern populations (Morrissey and Baust 1976; Storey and Storey 1986). The capacity for glycerol synthesis begins with the molt to the overwintering form, the 3rd instar, and the rate of glycerol synthesis is modulated by cooling over the range between 15 and 5 °C, and in response to dehydration of the surrounding gall tissue (Storey and Storey 1983; Rojas et al. 1986). Sorbitol production is triggered only by direct exposure to low temperature (0–5 °C) with maximal rates of synthesis between about 0 and −5 °C (Storey 1983; Storey and Storey 1983; Rojas et al. 1983). The independent syntheses of the two polyols has been linked to control of the glycogen phosphorylase and phosphofructokinase reactions and the differential effects of temperature on the function of these enzymes (Storey 1982; Storey and Storey 1988).

A large number of insect species utilize dual cryoprotectants, most often glycerol plus sorbitol (Miller and Smith 1975; Duman 1980; Sømme 1982; Furusawa et al. 1982). The reasons for using dual protectants are not fully understood although there have been a variety of suggestions (Storey and Storey 1988, 1990). Certainly, the production of the 3-carbon glycerol maximizes the yield of osmolytes produced from the glycogen pool as compared to the synthesis of the 6-carbon sorbitol. Sorbitol and glycerol might also act differently in the cryoprotection of different subcellular components or, alternatively, synergistic interactions between the two polyols might offer improved cryopreservation compared to that of a single protectant. Indeed, recent studies have shown that mixtures of the two polyols vitrify at about −25 °C, suggesting that intracellular water in frozen larvae may undergo a transition to the glassy state at temperatures

approaching the lower lethal limit (Wasylyk et al. 1988). Differences in the metabolic regulation of the two polyol pools may also be the determining factor in the choice of a dual versus a single polyol system. Thus, sorbitol is reconverted to glycogen during spring dehardening in *E. solidaginis* but glycerol is not (Storey and Storey 1986). Therefore, the size of the winter sorbitol pool determines the size of the subsequent carbohydrate reserve available for the non-feeding pupal and adult stages. Furthermore, glycerol snythesis requires ATP input whereas sorbitol production does not. Therefore under energy-limited conditions sorbitol production can go forward but glycerol synthesis may be limited.

The present study examines this latter concept. Using temperature conditions known to differentially stimulate glycerol versus sorbitol biosynthesis, we examined the capacity for polyol synthesis when ATP production was limited by a lack of oxygen. The results confirm the ATP-dependence of glycerol synthesis and offer new insights into the carbon stoichiometry and energy balance of polyol synthesis.

Materials and methods

Animals and experimental treatments. Galls containing third instar larvae of *E. solidaginis* were collected from fields around Ottawa in mid-September 1984. Larvae were removed from the galls and placed in plastic Petri dishes, 50 per dish. Animals were acclimated to constant temperature, either 23 °C or 13 °C, for 2 weeks before use. Larvae to be used in anoxia experiments were transferred to 25-ml Erlenmeyer flasks two days before experiments began.

To impose anoxia, flasks containing larvae were sealed with serum stoppers and flushed with nitrogen gas, introduced via a syringe needle and with a second needle to vent the gas. After 10 min of constant flushing, the needles were pulled out and the stoppered flasks were transferred to the appropriate experimental temperature.

An abrupt temperature change from the acclimation temperature to a lower temperature was used to stimulate polyol synthesis by the larvae, as described previously (Storey and Storey 1983). For the 23 °C-acclimated larvae, the experimental treatment was an abrupt transfer to 13 °C, whereas larvae acclimated to 13 °C were transferred to 3 °C. Groups of larvae exposed to aerobic versus anoxic conditions were treated identically. Larvae were sampled over a time course of up to 18 d at the new temperature. Animals were immediately frozen in liquid nitrogen and then transferred to −80 °C until analysis. Control larvae were maintained at the acclimation temperatures, 23 °C or 13 °C, and were sampled from these temperatures at both the beginning (0 h) and the end (18 d) of the time course.

Chemicals. All biochemicals were obtained from Sigma Chemical Co., St. Louis MO, or Boehringer Mannheim, Montreal PQ.

Metabolite analysis. For the measurement of metabolites other than fructose-2,6-bisphosphate, frozen larvae (2 pooled per sample, approximately 100–120 mg) were weighed and then immediately homogenized in 1 ml ice-cold 6% perchloric acid (containing 1 m*M* EDTA). Homogenates were centrifuged at $6000 \times g$ for 15 min at 4 °C. The supernatants were removed, neutralized by addition of ice-cold 3 *M* KOH/0.3 *M* imidazole/0.4 *M* KCl and recentrifuged as above. Neutralized extracts were stored at −80 °C until analysis. Metabolites were measured spectrophotometrically using the assays of Eggstein and Kuhlmann (1974) for glycerol, Bergmeyer et al. (1974) for sorbitol, and Lowry and Passonneau (1972) for all others.

For fructose-2,6-P_2 determination, alkaline extracts (1:20 w/v) of larvae (2 larvae per sample) were prepared and assayed by the method of van Schaftingen (1984).

Data are presented as means ± SEM for n = 3 samples with 2 larvae pooled per sample. Statistical significance was tested using the Student's *t*-test, with a significance level of $P < 0.05$.

Results

Abrupt temperature changes from 23 °C to 13 °C and 13 °C to 3 °C were used to stimulate the synthesis of glycerol and sorbitol, respectively, by *E. solidaginis* larvae. Experiments were carried out under air or nitrogen gas atmospheres to test the capacity of the larvae for polyol synthesis in the absence of oxygen, and to analyze the energetic requirements of polyol synthesis.

Temperature change from 23 °C to 13 °C

Figure 1 shows changes in metabolite levels in *E. solidaginis* larvae over 18 d at 13 °C under aerobic versus anoxic atmospheres. Larvae under both conditions showed a statistically significant increase ($P < 0.05$) of about 30 μmol·g wet weight^{-1} in glycerol content within the first 6 h at 13 °C. Aerobic larvae showed little subsequent change in glycerol over the next four days, but by 10 d their glycerol content had risen again and the final level of glycerol was 219 ± 9 μmol·g^{-1} after 18 d. This was double the initial content of 106 ± 9 μmol·g^{-1}. The net accumulation of glycerol in anaerobic larvae after 18 d was, by contrast, only 64 μmol·g^{-1} or 57% of that in aerobic larvae.

However, anaerobic larvae also accumulated a second cryoprotectant, sorbitol. Sorbitol levels increased significantly from 0.17 ± 0.01 μmol·g^{-1} in controls to 1.8 ± 0.5 μmol·g^{-1} after 6 h, and continued a linear increase over the entire 18 d to a final content of 22.0 ± 1.1 μmol·g^{-1}. Underlying sorbitol synthesis in anoxic larvae was a rise in glucose content peaking at 14.8 μmol·g^{-1} after 4 d, but reduced to 4.2 μmol·g^{-1} at 18 d. Aerobic larvae at 13 °C showed no accumulation of either sorbitol or glucose. Control larvae were also held at a constant 23 °C over the entire 18-d period (labelled CT in Fig. 1) and were assessed for seasonal rhythms in cryoprotectant production that are separate from the temperature-stimulated cryoprotectant production. These larvae also accumulated glycerol with net increases of 130 μmol·g^{-1} for aerobic, and 53 μmol·g^{-1} for anaerobic larvae. Sorbitol accumulation by anoxic larvae at 23 °C was low, being only 3.5 μmol·g^{-1}.

Figure 1 also shows levels of glycolytic end products, L-lactate and L-alanine, as well as the precursor of glycerol, glycerol-3-phosphate. As expected for aerobic larvae there was only a minor accumulation of lactate (1.4 μmol·g^{-1}) that was significantly greater than the control value only at the 18-d time point. Alanine levels did not change over the time course. However, levels of both products rose significantly within 6 h in anaerobic larvae and accumulation was progressive over the

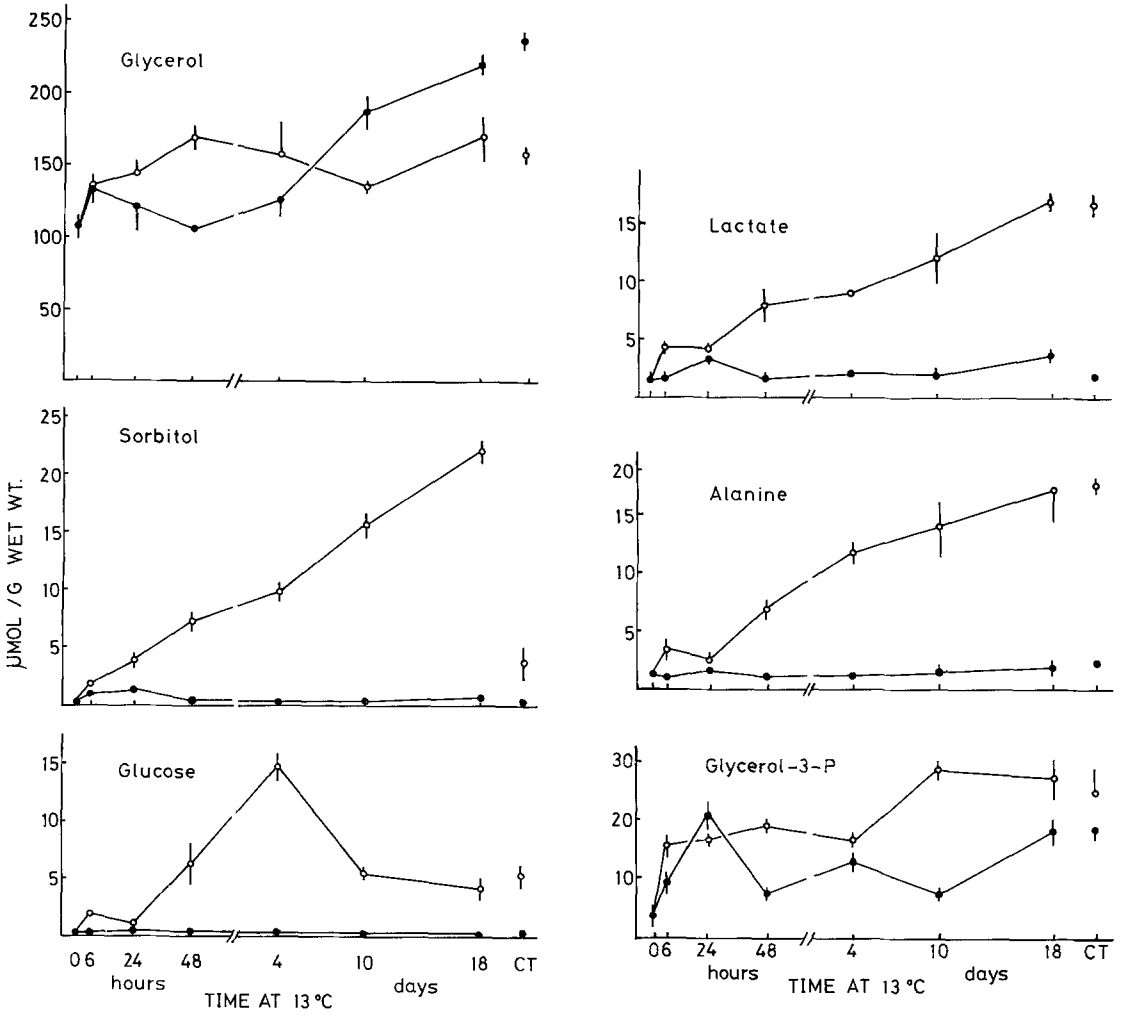

Fig. 1. Metabolite levels in *Eurosta solidaginis* larvae over a time course of 18 d at 13 °C under aerobic versus anoxic (nitrogen gas atmosphere) conditions. Larvae were previously acclimated to 23 °C and immediately switched to 13 °C at time zero. Controls at 23 °C were sampled at both 0 h and 18 d. Filled symbols are values for aerobic larvae; open symbols are for anoxic larvae. Data are μmol·g wet weight $^{-1}$, means±SEM, $n=3$ samples with 2 larve pooled per sample. Where error bars are not shown these are enclosed within the symbol used

remainder of the experimental period with a net increase of 15 μmol·g^{-1} lactate and 15.8 μmol·g^{-1} alanine. Glycerol-3-P levels rose significantly from a control level of 3.4±0.6 μmol·g^{-1} to 9.0±1.4 μmol·g^{-1} in aerobic, and 15.6±2.1 μmol·g^{-1} in anaerobic larvae over the first 6 h of 13 °C-exposure. Glycerol-3-P content in aerobic larvae subsequently fell and remained constant throughout the period of glycerol accumulation. In anaerobic larvae, however, glycerol-3-P continued to build up with a final net accumulation of 24 μmol·g^{-1}.

Life without oxygen involves the use of alternative pathways of fermentative energy production for many invertebrate species; commonly, the catabolism of glycogen and aspartate as substrates is coupled to the accumulation of alanine and succinate as end products (Hochachka and Somero 1984). We assessed the possible involvement of such a pathway in supporting anaerobic survival in *E. solidaginis* by measuring levels of aspartate and succinate in selected groups of larvae: control (time zero at 23 °C), 18 d aerobic at 13 °C, and 2 d and 18 d of anoxia at 13 °C. The levels of both compounds were the same in all groups with overall average levels of 1.73±0.27 μmol·g^{-1} for aspartate and 1.32±0.18 μmol·g^{-1} for succinate.

Fructose-2,6-P_2 is a potent activator of phosphofructokinase and, in multicellular organisms, generally facilitates the use of carbohydrate reserves for anabolic purposes (Hue and Rider 1987). Aerobic larvae showed a significant 2-fold increase in fructose-2,6-P_2 content after 6 h at 13 °C, but subsequent levels were reduced and little different from control values over the remaining days (Fig. 2). Fructose-2,6-P_2 also rose significantly over the first hours of 13 °C-exposure in anaerobic larvae, with a peak content at 48 h that was 2.6-fold higher than control values at time zero. With longer exposure at 13 °C, fructose-2,6-P_2 content fell and after 18 d was reduced to only 30% of the control level.

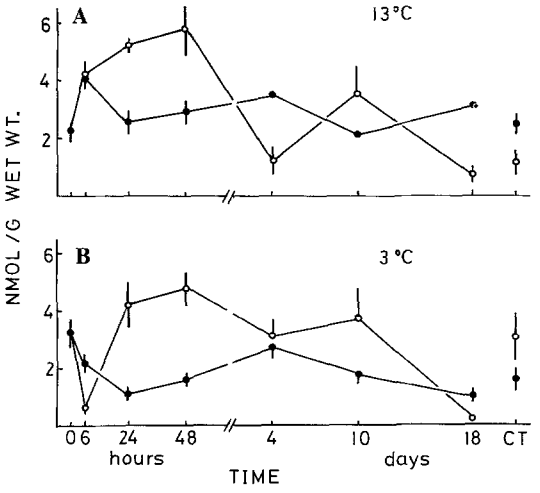

Fig. 2A, B. Content of fructose-2,6-bisphosphate in *Eurosta solidaginis* larvae over a time course of aerobic versus anoxic exposure. Data are nmol·g wet weight^{-1}, means±SEM, $n=3$ samples with 2 larvae pooled per sample. **A.** Time course of exposure at 13 °C, as described in Fig. 1. **B.** Time course of exposure at 3 °C, as described in Fig. 3

Temperature change from 13 °C to 3 °C

Larvae acclimated to 13 °C and then switched to 3 °C showed distinctly different metabolic responses to temperature change than were described for the 23 °C to 13 °C switch (Fig. 3). Neither aerobic nor anaerobic larvae showed a significant accumulation of glycerol, but both produced sorbitol. A signifiant rise in sorbitol content occurred by 24 h and continued to a final net sorbitol accumulation of 50 μmol·g^{-1} for the aerobic larvae and significantly higher, 79 μmol·g^{-1}, for the anaerobic animals. High rates of sorbitol production were also initiated earlier in the anaerobic larvae. Glucose levels were elevated to 10–12 μmol·g^{-1} (from control=0.4± 0.03 μmol·g^{-1}) during the period of rapid sorbitol synthesis but thereafter declined in aerobic larvae. In anaerobic larvae glucose content continued to increase to a final 27±3 μmol·g^{-1} after 18 d at 3 °C. Larvae held at a constant 13 °C throughout accumulated glycerol in the aerobic situation (a significant net rise of 70 μmol·g^{-1} over 18 d) and sorbitol in anoxia (a significant net increase of 39 μmol·g^{-1}).

Aerobic larvae at 3 °C showed no significant production of L-lactate or L-alanine, a confirmation of their aerobic status (Fig. 3). The final glycerol-3-P content of the larvae was also not significantly different from the control value. By contrast, anaerobic larvae accumulated both lactate and alanine but not glycerol-3-P. Both products increased significantly over the first 6 h of anoxia but subsequently remained nearly constant for the next 10 d. Amounts of both rose sharply, however, between 10 and 18 d, with final net increases of 8.3 μmol·g^{-1} lactate and 10.4 μmol·g^{-1} alanine. Levels of aspartate and succinate in the larvae were assessed but again were not affected by the experimental protocol; average

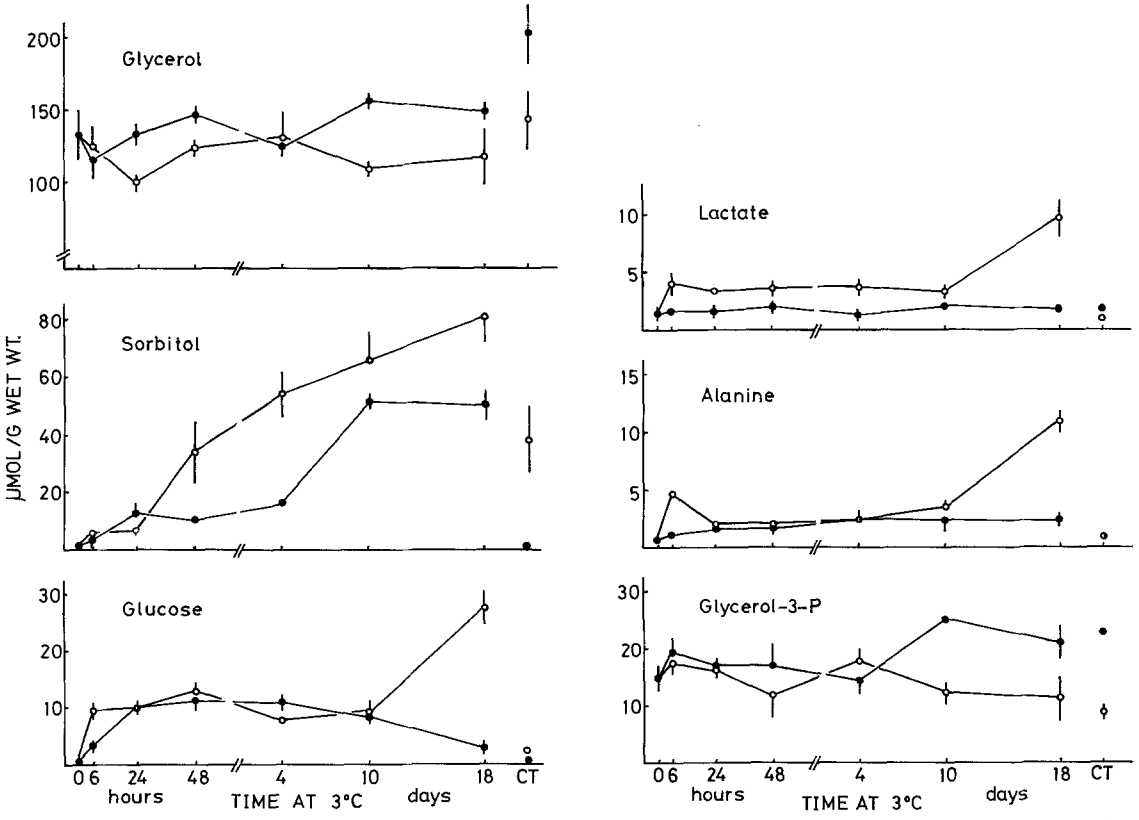

Fig. 3. Metabolite levels in *Eurosta solidaginis* larvae over a time course of 18 d at 3 °C under aerobic versus anoxic conditions. Larvae were previously acclimated to 13 °C and immediately switched to 3 °C at time zero. Controls at 13 °C were sampled at both 0 h and 18 d. Other information is as in Fig. 1

levels were 1.19 ± 0.08 μmol·g^{-1} for aspartate, and 0.93 ± 0.15 μmol·g^{-1} for succinate.

Fructose-2,6-P_2 levels dropped quickly when larvae were transferred to 3 °C (Fig. 2). Subsequently, levels in aerobic larvae remained below the control values over the 18-d experimental course. Content in anaerobic larvae followed a different pattern with levels greater than or equal to control amounts between 1 and 10 d followed by a sharp decrease in fructose-2,6-P_2 content at day 18.

Discussion

Polyol synthesis is an important metabolic event for the cold-hardy insect. It involves mobilization of virtually the entire glycogen reserve and results in a cryoprotectant content that can be as high as 20% of the fresh weight of the animal (Ring and Tesar 1981; Sømme 1982; Furusawa et al. 1982; Storey and Storey 1986; Rickards et al. 1987). Obviously such a massive undertaking requires close regulation to achieve optimal conversion efficiency and supply the required amounts of NAD(P)H and ATP. The present study examines the capacity of *E. solidaginis* larvae to carry out glycerol or sorbitol synthesis in the absence of ATP availability from oxidative phosphorylation. The results provide insights into the metabolic organization required for optimal polyol synthesis.

Energetics and carbon balance for glycerol synthesis

Synthesis of glycerol requires ATP input at the phosphofructokinase reaction. Although most (86% in theory) of the glucose-6-P generated by glycogenolysis must cycle through the hexose monophosphate shunt to generate the reducing equivalents required for glycerol biosynthesis, less than 20% of the carbon skeletons needed for glycerol production are retrieved from the shunt as a direct output of triose (i.e., glyceraldehyde-3-P; Storey and Storey 1988, 1990). The remainder of the carbon exits the shunt as fructose-6-P and must be converted to fructose-1,6-P_2, via phosphofructokinase, before being processed to glycerol. For this reason glycerol synthesis benefits from an aerobic metabolism. The required ATP can be generated via oxidative phosphorylation with only a minor cost (theoretically 1.1% of the total glycogen mobilized; Storey and Storey 1988, 1990).

The present study demonstrates, however, that glycerol production can still take place in the absence of oxygen but the metabolic cost to the larvae is much greater. Net glycerol production in the anaerobic larvae at 13 °C was only 57% of the amount in aerobic larvae and had to be supported by a large accumulation of glycolytic end products L-lactate and L-alanine (Table 1). This is necessitated by the much lower ATP output of anaerobic glycolysis compared to oxidative phosphorylation; one mol of hexose phosphate (derived from glycogen) fermented to lactate or alanine yields a net of only 3 mol of ATP by substrate-level phosphorylations compared to 38 mol by mitochondrial oxidative phosphorylation. Every mol of fructose-6-P converted to lactate or alanine generates 4 mol ATP, or a net of 3 mol ATP when the ATP input at phosphofructokinase is taken into account. These 3 mol of ATP can then support the conversion of a further 3 mol of fructose-6-P to glycerol. Thus, to maintain ATP balance for anaerobic glycerol synthesis, the expected partitioning of fructose-6-P must be 3:1 channeled into glycerol versus lactate/alanine pools. The observed ratio was very close to this, 2.85:1 for glycerol (plus its precursor glycerol-3-P) versus lactate/alanine (Table 1).

Because of the lactate + alanine accumulation associated with anaerobic ATP production the calculated efficiency of glycogen conversion to polyols was much lower for the anaerobic compared to aerobic larvae. Table 1 shows that the net accumulation of glycerol under anoxic conditions represented only 57% of the total hexose used. The remainder of the hexose was used in supporting biosynthesis (and/or basal metabolism). This percentage rises to 70% if we assume that all the accumulated glycerol-3-P was destined for conversion to glycerol, but this is still much less efficient than the theoretical balance for the aerobic synthesis of glycerol which is 84% of hexose converted to glycerol versus 16% used to support biosynthesis (Storey and Storey 1988). The actual observed values for aerobic larvae at 13 °C were 73% conversion to glycerol or 82.5% conversion to glycerol + glycerol-3-P (Table 1).

In addition to the low conversion efficiency of anaerobic glycerol synthesis, polyol production under such conditions has other disadvantages. The enormous carbon loss into anaerobic end products is wasteful, and the acidification that results from the accumulation of lactic acid is metabolically disruptive. It is obvious, therefore, that efficient glycerol synthesis is best served by aerobic metabolism. Not surprisingly, then, glycerol accumulates naturally in *E. solidaginis* during an autumn cold-hardening period that comes well in advance of the first exposures of the larvae to freezing temperatures. Freeze-avoiding insects also depend upon aerobic metabolism for optimal glycerol production. *Epiblema scudderiana* larvae produced 10 times more glycerol when aerobic than when anaerobic (Churchill and Storey 1989).

Glycerol production and the hexose monophosphate shunt

The present results also support the view, established via radiotracer studies (Wood and Nordin 1980; Tsumuki et al. 1987), that the hexose monophosphate shunt is the primary source of reducing equivalents for polyol synthesis. Without the provision of reducing equivalents by this route, anoxic glycerol synthesis at 13 °C could proceed only by using the NADH generated at the glyceraldehyde-3-P dehydrogenase step of glycolysis. Lactate could not accumulate as an end product of glycoly-

Table 1. Effect of anoxia on the net carbon, hydroxyl equivalent, and ATP balance over 18 days of polyol synthesis in *Eurosta solidaginis* larvae held at 13 °C or 3 °C

	13 °C-exposure		3 °C-exposure	
	Aerobic	*Anoxic*	*Aerobic*	*Anoxic*
	(μmol·g wet weight^{-1})			
Net synthesis of carbon compounds				
C_6: Sorbitol	0.2	21.3	49.7	79.3
Glucose	0.2	4.0	2.2	27.0
C_3: Glycerol	112.0	64.0	15.0	–
Glycerol-3-P	14.4	23.7	6.8	–
Lactate	1.4	15.0	0.5	8.3
Alanine	0.5	15.8	1.9	10.4
Total products converted to hexose equivalents	64.6	84.5	64.0	125.0
Aerobic hexose oxidized to produce ATP for glycerol synthesis	1.4	0	0.2	0
Hexose consumed in pentose phosphate cycle (1 C_6 lost for 12 NAD(P)H)	10.5	9.1	6.0	6.6
Total hexose consumption	76.5	93.6	70.2	131.6
Percent of total hexose converted to polyols	73%	57%	81.5%	60%
Hydroxyl equivalents (glycerol × 3, sorbitol × 6)	336	320	343	476
Anaerobic ATP balance				
Net production, (lact + ala) × 1.5	–	46.2	–	28.1
Use in polyol synthesis (glycerol + G3P)/2	–	43.9[a]	–	–

[a] This represents the maximum possible use of ATP if all carbon is processed through phosphofructokinase; since up to 20% of the carbon could be derived from glyceraldehyde-3-P outputs of the hexose monophosphate shunt, total ATP use for glycerol synthesis probably ranges between 35.1 and 43.9 μmol·g^{-1}, leaving a substantial anaerobic ATP output to support basal metabolism

sis since lactate dehydrogenase utilizes this NADH. Redox balance would be maintained only with a 1:1 glycerol:alanine product ratio. In other words, triose phosphates would have to be split evenly between NADH-utilizing and NADH-generating pathways. Since this was not the observed result, and since another NAD(P)H-utilizing product (sorbitol) was also produced during anaerobiosis, the data strongly support the use of the hexose monophosphate shunt for the production of reducing equivalents.

Sorbitol production at 13 °C

The accumulation of sorbitol under anoxic conditions at 13 °C was an unexpected result. In nature or under aerobic conditions in the laboratry *E. solidaginis* never produces sorbitol at such a high temperature. Natural sorbitol synthesis is triggered solely by low temperature exposure below 5 °C (Storey and Storey 1983; Rojas et al. 1983), and indeed, sorbitol is rapidly reconverted to glycogen when temperatures rise above 5 °C in nature (Storey and Storey 1983, 1986). Unlike glycerol synthesis, however, sorbitol production is not ATP-dependent and so sorbitol synthesis can proceed when ATP availability is limiting. By accumulating the alternative polyol, sorbitol, during anaerobiosis at 13 °C the larvae can achieve nearly the same cryoprotective capacity as the aerobic larvae. When assessed as total hydroxyl equivalents (glycerol has 3, sorbitol has 6 hydroxyl groups; Baust and Morrissey 1977) accumulated over 18 d at 13 °C, the value for aerobic larvae was 336 μmol·g^{-1}, and 320 μmol·g^{-1} for anaerobic larvae (Table 1). Indeed, the data suggest that hexose phosphate output (determined by glycogen phosphorylase activity) and the flux through the hexose monophosphate shunt were little affected in the anaerobic larvae. However, because ATP production was compromised under anoxic conditions, carbon and reducing equivalents that would have normally been used to build the glycerol pool were redirected into the production of the alternative polyol, sorbitol.

Carbon balance and partitioning for sorbitol synthesis

At 3 °C aerobic polyol synthesis was directed into sorbitol production (a 15 μmol·g^{-1} rise in glycerol content was not significantly different from control values), as has been described previously (Storey and Storey 1983; Rojas et al. 1983). In the anaerobic larvae, sorbitol was also produced (with no glycerol) and amounts were 58% higher than in the aerobic state. However, although the

total amount of sorbitol produced was higher in anaerobic than aerobic larvae, the conversion efficiency of hexoses to polyols was substantially lower: 60% in anaerobic compared to 81.5% in aerobic larvae. This was due to the additional accumulation during anaerobiosis of lactate, alanine and glucose (Table 1). These results indicate therefore, that an aerobic metabolic state is also the favoured situation for optimal conversion efficiency in the production of sorbitol. Indeed, in nature most sorbitol production probably occurs under aerobic conditions. Sorbitol synthesis by *E. solidaginis* larvae is triggered at 3–5 °C and optimal rates of synthesis occur between 0 and −5 °C (Storey and Storey 1983; Rojas et al. 1983). Accumulation of the sorbitol pool is largely complete before environmental temperatures of about −10 °C initiate the extracellular freezing that imposes a natural anaerobiosis on the larvae.

However, although the efficiency of carbon conversion is lower, sorbitol synthesis certainly proceeds in anaerobic *E. solidaginis* larvae, confirming that production is not ATP-limited. Indeed, net sorbitol accumulation was higher in anaerobic larvae than in aerobic animals. A possible reason for this may be the occurrence of obligate routings of carbon flow in polyol-producing insects. A strict percentage of carbon must pass through the hexose monophosphate shunt in order to generate the NADPH needed for polyol synthesis. The theoretical value is 46% for sorbitol synthesis, reflecting the fact that one mol of glucose-6-P directed into the shunt generates 2 mol of NADPH, sufficient reducing power for the production of 2 mol of sorbitol (the value is not 50% because some carbon is lost as CO_2 in the shunt; Storey and Storey 1988).

To optimize the conversion of glycogen to polyols in cold-hardy insects, the glycolytic and hexose monophosphate shunt enzymes involved in polyol synthesis may be physically associated with glycogen particles in enzyme complexes. When glycogenolysis is activated, carbon flow through the two pathways is correctly apportioned to create the proper balance of NADPH and glucose outputs needed for sorbitol synthesis. During anaerobiosis, however, glycogenolysis is activated for a different reason, i.e., to support glycolytic ATP production. If the routing of carbon is fixed within enzyme complexes in fat body, then carbon would still be directed through both glycolysis and the hexose monophosphate shunt in anoxia. The portion of carbon that is routed through the hexose monophosphate shunt necessarily generates NADPH. Just as the NADH generated by the glyceraldehyde-3-P dehydrogenase reaction of glycolysis is reoxidized in the synthesis of lactate in anaerobic larvae, so must the NADPH generated by carbon flow through the hexose monophosphate shunt be reoxidized. Sorbitol synthesis serves this function for the anaerobic larvae. The same reasoning would also explain the accumulation of sorbitol in the larvae during anaerobiosis at 13 °C (Fig. 1).

Several other studies have reported the accumulation of both lactate and sorbitol during anoxia in cold-hardy insect species (Sømme 1967; Meyer 1978; Gäde 1984). In addition, Meyer (1978) compared the ^{14}C-labelling pattern of lactate from ^{14}C-1-glucose versus ^{14}C-6-glucose as precursors and concluded that carbon routing through the hexose monophosphate shunt increased during anoxia. These results can also be explained by the scheme outlined above, and they also suggest that the fixed routing of a high proportion of carbon flow through the hexose monophosphate shunt may be a common feature of fat body metabolism in cold-hardy insects. In *E. solidaginis*, this routing may also be necessary to channel carbon to the lowr portion of glycolysis during anaerobiosis at low temperature since phosphofructokinase in this species is known to be cold-inactivated (Storey 1982).

In one sense, then, sorbitol is an end product of anaerobic metabolism in *E. solidaginis*, but this is only because of the obligate links between glycolysis and the hexose monophosphate shunt that are required in the polyol-producing insect. As a general scheme of anaerobic metabolism, however, the routing of carbon through the hexose monophosphate shunt during anoxia is wasteful since hexose phosphates cannot be quantitatively converted to triose phosphates. Instead, carbon is lost as CO_2 in passing through the hexose monophosphate shunt and, in addition, high levels of sorbitol must accumulate to maintain redox balance. Indeed, the theoretical ratio would be 6:1 sorbitol:lactate to accommodate the relative amounts of NADPH versus NADH produced in converting hexose phosphates to lactate when carbon flow passes through the hexose monophosphate shunt.

Anaerobic end products

Lactate and alanine are accumulated as glycolytic end products in *E. solidaginis* during both anaerobiosis (this study) and freezing exposure (Storey and Storey 1985). In both situations they accumulate in parallel and in roughly equal amounts. The total end product accumulation was higher at 13 °C, compared to 3 °C, but this is to be expected because of the higher body temperature and the active glycerol synthesis (requiring ATP) at 13 °C. Several patterns of anaerobic metabolism have been identified among the Insecta, involving various types of end products and obviously adapted to the particular environmental and physiological circumstances that accompany oxygen lack in each instance (Gäde 1984). Lactate and alanine accumulate in near-equal amounts during anoxia in several other cold-hardy species (Sømme 1967; Conradi-Larsen and Sømme 1973; Churchill and Storey 1989) suggesting that this combination of end products may be optimal when limiting oxygen availability and low environmental temperature occur together. Glycerol-3-P also frequently builds up during anaerobiosis in insects, particularly in species that power flight by the aerobic oxidation of carbohydrate and use the glycerol-3-phosphate cycle for transporting reducing equivalents into the mitochondria (Gäde 1984; Storey 1985). In *E. solidaginis*, however, glycerol-3-P appears to be primarily an intermediate of glycerol biosyn-

thesis and the present study shows that glycerol-3-P levels were modulated during active glycerol production at 13 °C but did not change over time at 3 °C.

Fructose-2,6-bisphosphate

Fructose-2,6-P_2 is a powerful activator of phosphofructokinase. In mammals, high levels of fructose-2,6-P_2 typically potentiate the use of carbohydrate for anabolic purposes, whereas the content of bisphosphate is depressed under conditions where carbohydrate must be conserved (e.g., starvation, anoxia; Hue and Rider 1987). Fructose-2,6-P_2 rose rapidly (by 10-fold in 6 h) during active glycerol synthesis in *E. scudderiana* (Churchill and Storey 1989). In *E. solidaginis*, less dramatic changes were seen but the initial response in aerobic larvae was a significant rise in fructose-2,6-P_2 content with the 23 °C to 13 °C transfer and a significant fall in fructose-2,6-P_2 with the 13 °C to 3 °C transfer, responses consistent with the stimulation of glycerol versus sorbitol synthesis, respectively, and the requirement for an active versus inactive phosphofructokinase (Storey 1982) under these conditions.

Acknowledgements. We are very grateful for the technical assistance of Mrs. S. Walters and Mrs. M. Brown. This study was supported by an operating grant from the N.S.E.R.C. Canada to KBS.

References

Baust JG (1983) Protective agents: regulation of synthesis. Cryobiology 20:357–364

Baust JG, Morrissey RE (1977) Strategies of low temperature adaptation. Proc Int Cong Entomol XV:173–184

Baust JG, Rojas RR, Hamilton MD (1985) Life at low temperatures: representative insect adaptations. Cryo Lett 6:199–210

Bergmeyer HU, Gruber W, Gutman I (1974) D-Sorbitol. In: Bergmeyer HU (ed) Methods of enzymatic analysis. Academic Press, New York, pp 1323–1326

Churchill TA, Storey KB (1989) Metabolic correlates to glycerol biosynthesis in a freeze-avoiding insect, *Epiblema scudderiana*. J Comp Physiol B 159:461–472

Conradi-Larsen EM, Sømme L (1973) The overwintering of *Pelophila borealis* Payk. II. Aerobic and anaerobic metabolism. Nor Entomol Tidsskr 20:325–332

Duman JG (1980) Factors involved in the overwintering survival of the freeze-tolerant beetle, *Dendroides canadensis*. J Comp Physiol 136:53–59

Eggstein M, Kuhlmann E (1974) Triglycerides and glycerol: determination after alkaline hydrolysis. In: Bergymeyer HU (ed) Methods of enzymatic analysis. Academic Press, New York, pp 1825–1831

Furusawa T, Shikata M, Yamashita O (1982) Temperature dependent sorbitol utilization in diapause eggs of the silkworm, *Bombyx mori*. J Comp Physiol 147:21–26

Gäde G (1984) Anaerobic energy metabolism. In: Hoffmann KH (ed) Environmental physiology and biochemistry of insects. Springer, Berlin Heidelberg New York, pp 119–136

Hochachka PW, Somero GN (1984) Biochemical adaptation. Princeton University Press, Princeton

Hue L, Rider MH (1987) Role of fructose-2,6-bisphosphate in the control of glycolysis in mammalian tissues. Biochem J 245:313–324

Lowry OH, Passonneau JV (1972) A flexible system of enzymatic analysis. Academic Press, New York

Meyer SGE (1978) Effects of heat, cold, anaerobiosis and inhibitors on metabolite concentrations in larvae of *Callitroga macellaria*. Insect Biochem 6:471–477

Miller LK, Smith JS (1975) Production of threitol and sorbitol by an adult insect: association with freezing tolerance. Nature 258:519–520

Morrissey RE, Baust JG (1976) The ontogeny of cold tolerance in the gall fly, *Eurosta solidaginis*. J Insect Physiol 22:431–437

Rickards J, Kelleher MJ, Storey KB (1987) Strategies of freeze avoidance in larvae of the goldenrod gall moth, *Epiblema scudderiana*: winter profiles of a natural population. J Insect Physiol 33:443–450

Ring RA, Tesar D (1981) Adaptations to cold in Canadian arctic insects. Cryobiology 18:199–211

Rojas RR, Lee RE, Luu TA, Baust JG (1983) Temperature dependence-independence of antifreeze turnover in *Eurosta solidaginis* (Fitch). J Insect Physiol 29:865–869

Rojas RR, Lee RE, Baust JG (1986) Relationship of environmental water content to glycerol accumulation in the freezing tolerant larvae of *Eurosta solidaginis* (Fitch). Cryo Lett 7:234–245

Schaftingen E van (1984) D-Fructose-2,6-bisphosphate. In: Bermeyer HU (ed) Methods of enzymatic analysis. Verlag Chemie, Weinheim, pp 335–341

Sømme L (1967) The effect of temperature and anoxia on hemolymph composition and supercooling in three overwintering insects. J Insect Physiol 13:805–814

Sømme L (1982) Supercooling and winter survival in terrestrial arthropods. Comp Biochem Physiol A 73:519–543

Storey KB (1982) Phosphofructokinase from the overwintering gall fly larva, *Eurosta solidaginis*: control of cryoprotectant polyol synthesis. Insect Biochem 12:501–505

Storey KB (1983) Metabolism and bound water in overwintering insects. Cryobiology 20:365–379

Storey KB (1985) Metabolic biochemistry of insect flight. In: Gilles R (ed) Circulation, respiration and metabolism. Springer, Berlin Heidelberg New York, pp 193–207

Storey JM, Storey KB (1983) Regulation of cryoprotectant metabolism in the overwintering gall fly larva, *Eurosta solidaginis*: temperature control of glycerol and sorbitol levels. J Comp Physiol 149:495–502

Storey JM, Storey KB (1985) Freezing and cellular metabolism in the gall fly larva, *Eurosta solidaginis*. J Comp Physiol B 155:333–337

Storey JM, Storey KB (1986) Winter survival of the gall fly larva, *Eurosta solidaginis*: profiles of fuel reserves and cryoprotectants in a natural population. J Insect Physiol 32:549–556

Storey KB, Storey JM (1988) Freeze-tolerance in animals. Physiol Rev 68:27–84

Storey KB, Storey JM (1990) Biochemistry of cryoprotectants. In: Denlinger D, Lee RE (eds) Insects at low temperatures. John Wiley & Sons, New York, in press

Tsumuki H, Rojas RR, Storey KB, Baust JG (1987) The fate of [^{14}C]glucose during cold-hardening in *Eurosta solidaginis* (Fitch). Insect Biochem 17:347–352

Wasylyk JM, Tice AR, Baust JG (1988) Partial glass formation: a novel mechanism of insect cryoprotection. Cryobiology 25:451–458

Wood FE, Nordin JH (1980) Activation of the hexose monophosphate shunt during cold-induced glycerol accumulation by *Protophormia terranovae*. Insect Biochem 10:87–93