J Comp Physiol (1983) 149:495–502

Journal
of Comparative
Physiology · B

Regulation of Cryoprotectant Metabolism in the Overwintering Gall Fly Larva, *Eurosta solidaginis*: Temperature Control of Glycerol and Sorbitol Levels

Janet M. Storey and Kenneth B. Storey
Institute of Biochemistry, Carleton University, Ottawa, Ontario, Canada K1S 5B6

Accepted September 26, 1982

Summary. Abrupt temperature change, from 23 to 13 °C, 13 to 3 °C or vice versa, was used to study the metabolic events associated with cryoprotectant polyol synthesis and the reversibility of polyol accumulations in the overwintering, freezing tolerant larvae of the gall fly, *Eurosta solidaginis*.

Sorbitol synthesis was induced when larvae acclimated to 13 °C were abruptly moved to 3 °C. A precursor-product relationship between glucose-6-P, glucose and sorbitol was apparent with elevated levels of the compounds in the larvae first detected after 1, 2 and 24 h at 3 °C, respectively. A negative cross-over (increase in fructose-6-P, decrease in fructose-1,6-P_2 levels) at phosphofructokinase at 3 °C demonstrated that inhibition at this locus was responsible for the diversion of carbon flow into sorbitol synthesis.

Glycerol synthesis was stimulated when larvae acclimated to 23 °C were chilled to 13 °C, with increased glycerol levels first apparent after 2 days at 13 °C. Synthesis was accomplished via an activation of glycogenolysis coupled with a facilitation of flux through the phosphofructokinase locus and an inhibition (negative cross-over) of flux at the pyruvate kinase reaction resulting in a diversion of triose phosphates into the pathway of glycerol synthesis.

Warming of the larvae resulted in a rapid catabolism of sorbitol, with a restoration of glycogen reserves, when larvae were switched from 3 to 13 °C. Glycerol content of the larvae, however, did not respond to warming and remained constant when larvae were moved from 13 to 23 °C.

The two cryoprotectants appear to have different roles in the overwintering larvae. Glycerol, once synthesized, provides a constant and permanent cryoprotection throughout the winter. Accumulation of this polyol also appears to be anticipatory occurring in response to chilling at relatively high temperatures, well above those at which cryoprotection is needed. Sorbitol, however, is produced only in direct response to cold when freezing temperatures are imminent. Sorbitol provides a variable cryoprotection, levels of the polyol responding to increases or decreases in ambient temperature.

Introduction

The freezing tolerant third instar larva of the goldenrod gall fly, *Eurosta solidaginis*, accumulates high concentrations of two cryoprotective polyhydric alcohols, glycerol and sorbitol, as part of its strategy for overwintering survival. The two polyols show separate and distinct patterns of accumulation. Amongst outdoor populations, glycerol accumulation begins during early autumn; sorbitol synthesis is initiated later and only after the first exposures to frost (Morrissey and Baust 1976). In laboratory acclimation studies, glycerol production was shown to occur at warmer temperatures, with synthesis ceasing when ambient temperature fell below about 5 °C (Storey et al. 1981). Sorbitol production, however, was triggered only when temperature decreased below 5 °C (Storey et al. 1981; Baust and Lee 1982). Synthesis continued down to about −8 °C with further production apparently blocked by the onset of extracellular freezing (Storey et al. 1981). Both polyols are derived from the catabolism of glycogen reserves with two separate activations of glycogenolysis responsible for the separate synthesis of each polyol (Storey 1982a). The kinetic properties of phosphofructokinase from the larvae suggest that a low temperature blockage of glycolytic flux at this

locus is responsible for the cessation of glycerol production at low temperature and for the diversion, instead, of carbon flow into the synthesis of sorbitol (Storey 1982b).

The present study further examines polyol metabolism in *E. solidaginis*. Abrupt temperature change has been used as a trigger to initiate rapid alterations in the metabolic state of the larvae and allow a study of the metabolic events and metabolic control associated with polyol synthesis. The reversibility of polyol accumulations in the overwintering larvae has also been examined.

Materials and Methods

Chemicals and Animals. Biochemicals were purchased from Sigma Chemical Co. or Boehringer Mannheim Corp., FRG.

Galls containing third instar larvae of the gall fly were collected from fields around Ottawa in early October, 1981. Galls were placed at one of three constant temperatures, 3, 13 or 23 °C and held for four weeks. Larvae were then rapidly removed from their galls, placed in plastic cups (in groups of 24) and then returned to their previous temperatures for a further 4 days. Experiments were then initiated by rapidly moving the cups containing larvae from one incubation temperature to another. Larvae acclimated to 23 °C were moved to 13 °C, larvae at 3 °C were moved to 13 °C, and two groups of larvae acclimated to 13 °C were moved, one to 23 °C and one to 3 °C. At timed intervals after switching to the new temperature larvae were sampled and rapidly frozen in liquid nitrogen. Larvae were stored in liquid nitrogen until analysis.

Sample Preparation and Metabolite Assays. Perchloric acid extracts of the larvae were prepared as described by Storey et al. (1981). Four larvae (200–250 mg) were pooled per sample and homogenized in 1 ml 6% perchloric acid. Pyruvate and phosphoenolpyruvate were measured immediately after sample preparation while other metabolites were measured in samples which were stored at −80 °C until use. Glycogen was determined by the method of Keppler and Decker (1974), glycerol by the method of Eggstein and Kuhlman (1974), sorbitol according to Bergmeyer et al. (1974) and glucose, fructose, glucose-6-P, fructose-6-P, fructose-1,6-P_2, phosphoenolpyruvate and pyruvate were determined by the methods of Lowry and Passoneau (1972).

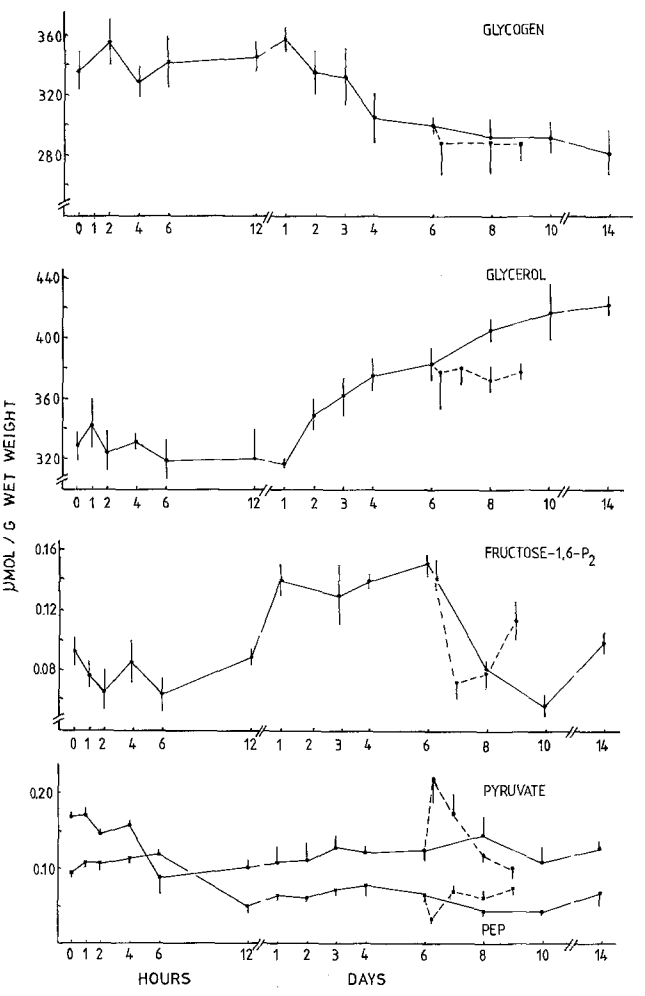

Fig. 1. Effect of temperature change from 23 to 13 °C on the levels of metabolites in *E. solidaginis* larvae. Larvae were acclimated to 23 °C for 4 weeks and then abruptly moved to 13 °C, and sampled at intervals over a 2 week period. Metabolites were measured enzymatically as outlined in Materials and Methods. Glycogen is expressed as µmol glucose after enzymatic hydrolysis. Results are expressed as means ± s.e.m. for $n=3$ samples (4 larvae per sample) at each point. The *dashed line* shows the effect of returning larvae to 23 °C after 6 days at 13 °C

Results

Temperature Switch from 23 to 13 °C

Figure 1 shows the effects of a temperature change from 23 to 13 °C on metabolite levels in *E. solidaginis* larvae over a time course from 1 h to 2 weeks at 13 °C. The effects of returning some larvae to 23 °C after 6 days at 13 °C are also shown (dashed lines). Glycerol levels in the larvae were initially high (330 ± 8 µmol/g wet weight) as larvae had been collected in October when large amounts of glycerol had already been accumulated in natural populations. However, cooling to 13 °C (after 4 weeks acclimation to 23 °C) stimulated a further increase in glycerol levels, a rise of 100 µmol/g being recorded after 2 weeks. A significant elevation of glycerol levels was first apparent 3 days after the switch to the lower temperature. The increase in glycerol content was balanced by a decrease in glycogen reserves of about 60 µmol/g (measured as glucose equivalents), more than accounting for the glycerol synthesized. When larvae were returned to 23 °C after 6 days at 13 °C, glycerol production ceased.

Lowering ambient temperature from 23 to 13 °C did not affect sorbitol levels in the larvae

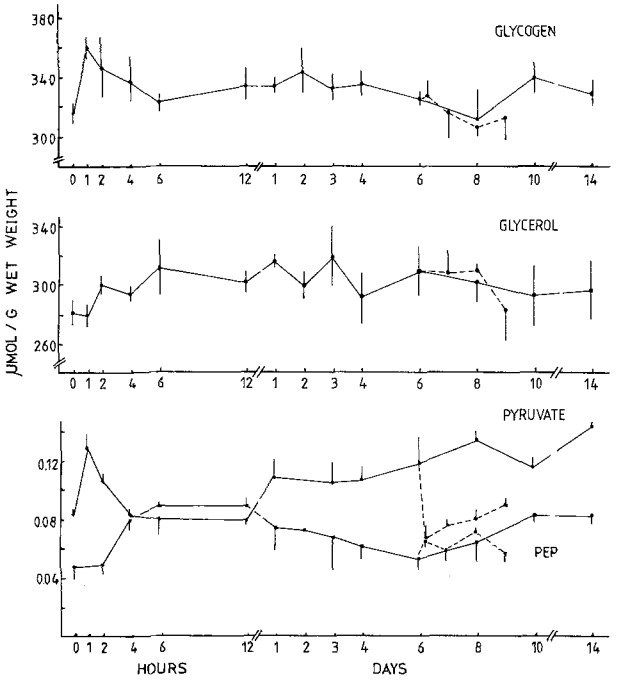

Fig. 2. Effect of temperature change from 13 to 23 °C on the levels of metabolites in *E. solidaginis* larvae. Larvae were acclimated to 13 °C for 4 weeks and then abruptly moved to 23 °C. The *dashed line* shows the effect of returning larvae to 13 °C after 6 days at 23 °C. Results are expressed as means ± s.e.m. for $n = 3$ samples (4 larvae per sample) at each point

which remained very low and constant (averaging 1.00 ± 0.05 μmol/g) throughout. Levels of fructose (0.35 ± 0.036 μmol/g), glucose (0.07 ± 0.024 μmol/g) and glucose-6-P (0.088 ± 0.005 μmol/g) were also unaffected by the experimental temperature change. Accompanying glycerol synthesis, however, was a significant rise in fructose-1,6-P_2 levels, a 75% increase from about 0.08 μmol/g to about 0.14 μmol/g over the period from 1 to 6 days after the switch to 13 °C. This increase in the concentration of the product of the phosphofructokinase reaction suggests an activation of this enzyme during the period of glycerol synthesis. Levels of pyruvate and phosphoenolpyruvate were also affected by temperature change. By 6 h at 13 °C, pyruvate levels had dropped sharply while phosphoenolpyruvate levels had increased. Interpreted via the cross-over theory of Williamson (1970), these results show a negative cross-over at the pyruvate kinase reaction suggesting an inhibition of glycolytic flux through the pyruvate kinase locus. The opposite response, an increase in pyruvate and decrease in phosphoenolpyruvate levels, producing a positive cross-over at the pyruvate kinase locus, was seen when the larvae were returned to 23 °C after 6 days at 13 °C.

Temperature Switch from 13 to 23 °C

Figure 2 shows the effects of a temperature change from 13 to 23 °C on metabolite levels in the larvae. Levels of glycerol in the larvae showed no significant alteration over the two weeks at the higher temperature. Glycogen levels were similarly stable. Larvae which were returned to 13 °C after 6 days at 23 °C also showed no changes in glycogen or glycerol content over the succeeding three days. Sorbitol (0.75 ± 0.042 μmol/g), fructose (0.26 ± 0.127 μmol/g) and glucose (0.084 ± 0.019 μmol/g) again remained low and constant throughout the experiment as did glucose-6-P (0.058 ± 0.005 μmol/g) and fructose-1,6-P_2 (0.077 ± 0.006 μmol/g) levels. Levels of pyruvate in the larvae increased rapidly within 1 h of the switch to the higher temperature while larvae which were moved back to 13 °C after 6 days at 23 °C showed a rapid decrease in pyruvate levels. These responses indicate an activation (positive cross-over) of pyruvate kinase with a change from low to high temperature and a decrease in enzyme activity (negative cross-over) with a change from high to low temperature.

Temperature Switch from 13 to 3 °C

Figure 3 shows the effects of a temperature change from 13 to 3 °C. Previous studies have indicated that sorbitol synthesis by the larvae is initiated only when temperature falls below 5 °C (Storey et al. 1981; Baust and Lee 1982); this experiment was designed, therefore, to examine the induction of sorbitol synthesis in the larvae. Within 1 h of exposure to 3 °C, the larvae showed a significant (2 fold) elevation of glucose-6-P levels which then remained elevated over the first 3 days at 3 °C. By 2 h at 3 °C glucose levels had risen to 1.3 ± 0.37 μmol/g from a level of < 0.1 μmol/g in control animals. Glucose levels continued to rise rapidly, peaking at 11.5 ± 0.94 μmol/g after 3 days before gradually falling. A significant elevation of sorbitol levels was first apparent after 24 h and sorbitol accumulated rapidly over the remainder of the two week period. Sorbitol accumulation was paralleled by a corresponding rise in fructose levels. The larvae also showed an increase in glycerol content of about 30 μmol/g (a 10% increase in pool size) within the first day at 3 °C. Glycerol levels then remained constant over the remainder of the two weeks, however.

Sorbitol production by the larvae was accompanied by a corresponding decrease in glycogen content. After 2 weeks at 3 °C, glycogen content

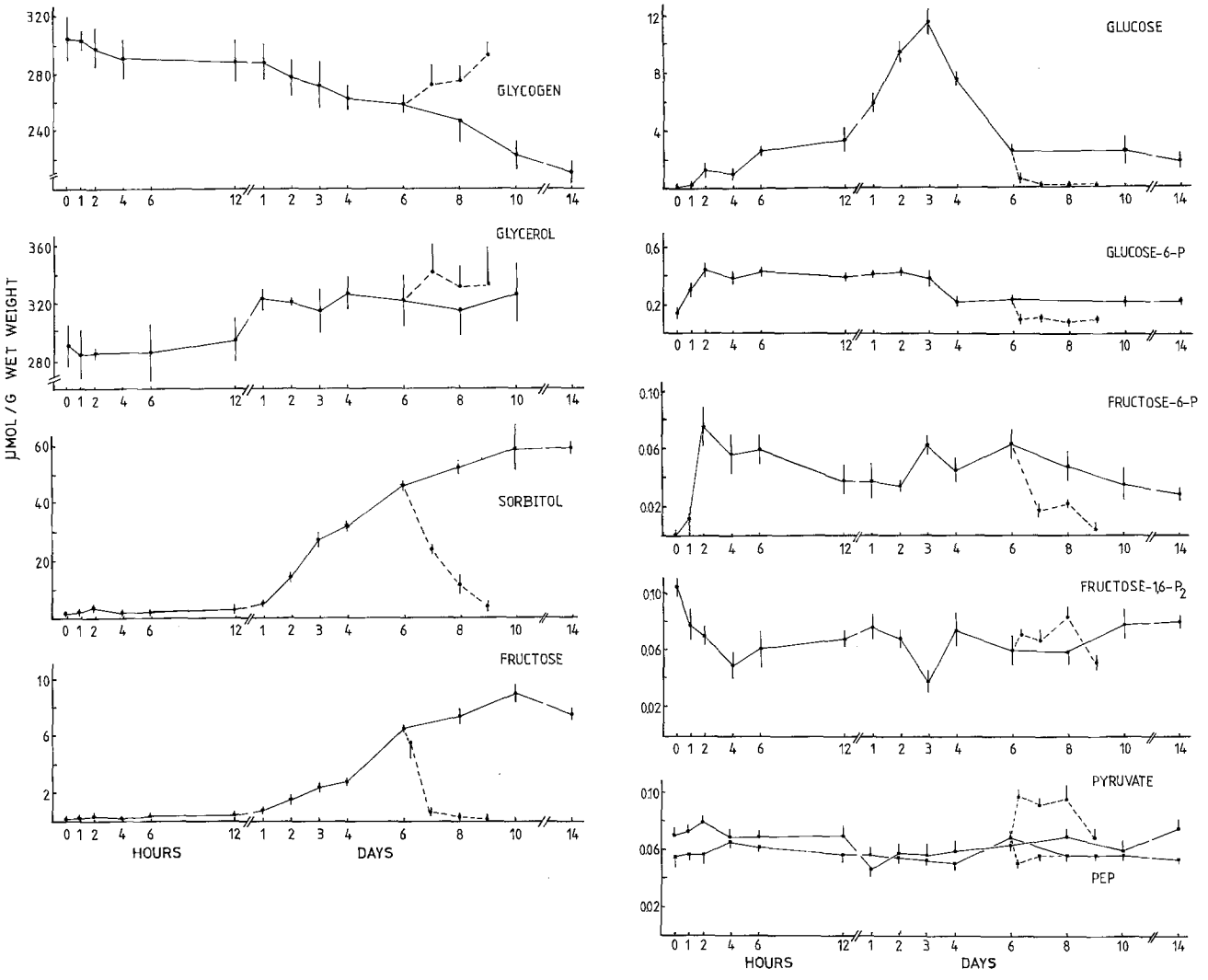

Fig. 3. Effect of temperature change from 13 to 3 °C on the levels of metabolites in *E. solidaginis* larvae. Larvae were acclimated to 13 °C for 4 weeks and then abruptly moved to 3 °C. The *dashed line* shows the effect of returning larvae to 13 °C after 6 days at 3 °C. Results are means ± s.e.m. for $n = 3$ samples (4 larvae per sample) at each point

had decreased by 94 μmol/g, an amount approximately equal to the combined sum of the increases in sorbitol (57 μmol/g), glycerol (30 μmol/g or 15 μmol/g in glucose equivalents) and fructose (8 μmol/g).

Of other glycolytic intermediates, fructose-6-P levels in the larvae paralleled the response of glucose-6-P, a result which is expected due to the equilibrium between these two pools maintained by phosphoglucoisomerase. Fructose-1,6-P_2 levels declined sharply over the first 4 h at 3 °C and together with the alterations in fructose-6-P levels, produced a negative cross-over at the phosphofructokinase reaction. Levels of both of these intermediates returned towards control values after two weeks at 3 °C. Larvae which were returned to 13 °C showed a rapid decrease in fructose-6-P with an accompanying increase in fructose-1,6-P_2 levels. Pyruvate and phosphoenolpyruvate levels in the larvae were not significantly affected by the change to 3 °C. However, when larvae were returned to 13 °C, pyruvate concentration was rapidly elevated while phosphoenolpyruvate showed a small decrease. This suggests an activation of pyruvate kinase (as well as phosphofructokinase) with a transition from cold to warm temperature.

The effects of rewarming the larvae after 6 days at 3 °C were dramatic. The sorbitol pool was rapidly degraded, decreasing by 50% from 45.4 ± 0.27 to 23.3 ± 1.1 μmol/g within 24 h and reaching 4.4 ± 0.3 μmol/g after 3 days at the warmer temperature. Accompanying this was a rapid decrease in the pools of glucose-6-P, fructose-6-P, glucose and fructose. The decrease in sorbitol content was

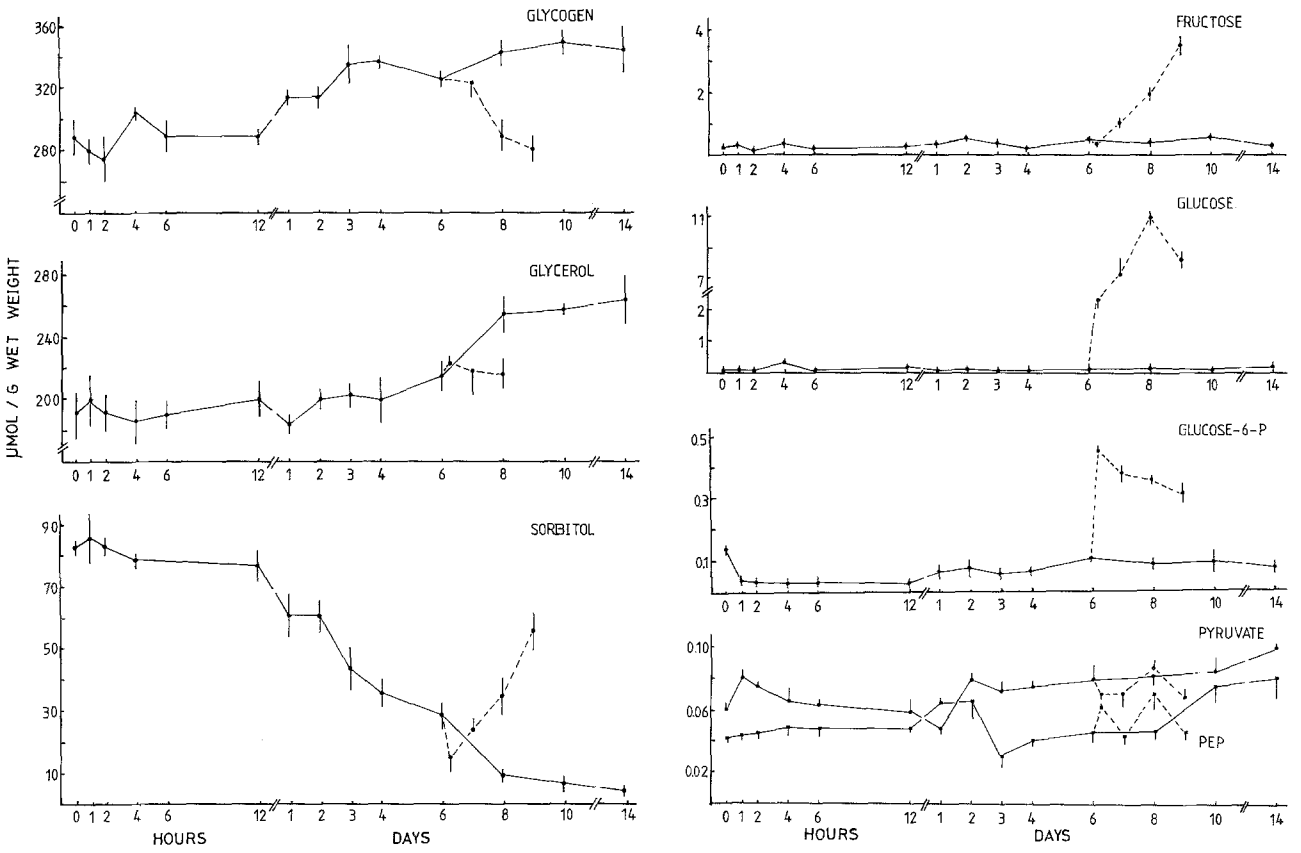

Fig. 4. Effect of temperature change from 3 to 13 °C on the levels of metabolites in *E. solidaginis* larvae. Larvae were acclimated to 3 °C for 4 weeks and then abruptly moved to 13 °C. The *dashed line* shows the effect of returning larvae to 3 °C after 6 days at 13 °C. Results are means ± s.e.m. for $n = 3$ samples (4 larvae per sample) at each point

largely accounted for by an increase in the glycogen content of the larvae.

Temperature Switch from 3 to 13 °C

Figure 4 shows the effects of a temperature change from 3 to 13 °C on metabolite levels in the larvae. The sorbitol pool is rapidly degraded. Sorbitol content decreased by 29% from 85.5 ± 1.6 to 60.7 ± 6.4 μmol/g after 24 h at the warmer temperature and declined rapidly over the remainder of the two weeks. Half-time for the loss of sorbitol was approximately 2.5 days. Much of the carbon derived from the catabolism of sorbitol was restored to glycogen, an increase of 65 μmol/g accounting for 83% of the decrease in sorbitol content in the larvae. After about 6 days at the warmer temperature, however, an elevation of glycerol content began with an increase of about 60 μmol/g (30 μmol/g glucose equivalents) occurring between 6 and 14 days at 13 °C. Carbon for this glycerol synthesis can also be largely accounted for by the decrease in sorbitol content.

The temperature switch from 3 to 13 °C produced an immediate drop in glucose-6-P levels which remained low (average of 0.035 ± 0.001 μmol/g) over the first 12 h at 13 °C but then doubled to an average of 0.079 ± 0.006 μmol/g between 1 and 14 days during the period of sorbitol degradation. No significant alterations in the fructose-6-P (0.014 ± 0.002 μmol/g) or fructose-1,6-P_2 (0.08 ± 0.005 μmol/g) pools were seen during the two weeks at 13 °C nor were alterations in glucose (0.15 ± 0.22 μmol/g) or fructose (0.36 ± 0.033 μmol/g) contents detected. Pyruvate levels showed a small increase when larvae were moved to 13 °C suggesting a warm activation of pyruvate kinase.

When larvae were returned to 3 °C after 6 days at 13 °C, sorbitol catabolism was rapidly reversed. A continued decline in sorbitol content was noted after 6 h but this had reversed after 24 h in the cold. A large increase in glucose-6-P, fructose-6-P and glucose levels in the larvae was noted after 6 h (the first time sampled) with glucose levels peaking at 10.9 ± 0.44 μmol/g after 2 days. An increase in fructose levels again paralleled the rise

in sorbitol content. The carbon source for sorbitol synthesis was, again, glycogen. Glycerol synthesis was stopped at 3 °C. An elevation of fructose-6-P levels, but no detectable change in fructose-1,6-P_2 levels suggested a low temperature block at the phosphofructokinase reaction. Pyruvate and phosphoenolpyruvate levels were not significantly altered when larvae were returned to cold temperature.

Discussion

The data in the present study clearly demonstrate the metabolic pathway of sorbitol synthesis in *E. solidaginis* larvae. Within 1 h of the temperature switch from 13 to 3 °C, glucose-6-P concentration in the larvae was elevated significantly and within two hours glucose levels had increased. Following these changes, a detectable increase in sorbitol content was seen after 24 h at 3 °C. The pattern of accumulation of these three compounds is precisely that of precursor-product relationships. The present and previous (Storey and Storey 1981) data conclusively demonstrate, therefore, that the route of sorbitol synthesis from glycogen in the larvae is via the enzymes glucose-6-phosphatase and polyol dehydrogenase (Fig. 5). The parallel rise in fructose and sorbitol levels in the larvae does not follow a precursor-product relationship but rather is probably the result of an equilibrium between sorbitol and fructose levels necessitated by the presence of sorbitol dehydrogenase in the larvae. An alternative route of sorbitol synthesis, via the enzymes fructose-6-phosphatase and sorbitol dehydrogenase, postulated previously (Storey and Storey 1981), probably does not function in the larvae.

The role of sorbitol dehydrogenase in the larvae appears to be, instead, in sorbitol catabolism. The data show that sorbitol levels in the larvae are flexible with synthesis or degradation of sorbitol pools occurring rapidly in response to decrease or increase in ambient temperature (Figs. 3, 4). Catabolism of accumulated sorbitol was not accompanied by large changes in the levels of compounds intermediate in the pathway between sorbitol and glycogen as was the case during sorbitol synthesis. A small increase in glucose-6-P levels was seen during the period of sorbitol breakdown (Fig. 4) but fructose-6-P and fructose levels were not significantly altered. The kinetic properties of the key enzymes involved in the pathway of sorbitol degradation, sorbitol dehydrogenase and hexokinase (Fig. 5), may account, however, for the absence of large changes in the levels of intermediates of

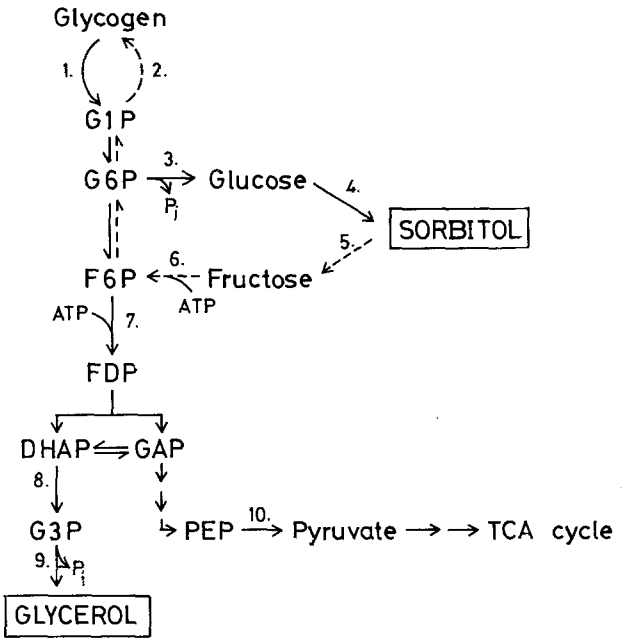

Fig. 5. Metabolic pathways of polyol synthesis and catabolism in *E. solidaginis*. Enzymes involved are: *1.* glycogen phosphorylase; *2.* glycogen synthase; *3.* glucose-6-phosphatase; *4.* polyol dehydrogenase; *5.* sorbitol dehydrogenase; *6.* hexokinase; *7.* phosphofructokinase; *8.* glycerol-3-P dehydrogenase; *9.* glycerol-3-phosphatase; *10.* pyruvate kinase. *Solid lines:* route of synthesis of sorbitol and glycerol; *dashed lines:* sorbitol catabolism

the pathway. Both sorbitol dehydrogenase (K_m (sorbitol) = 0.7 mM in mammalian systems (Gerlach and Hiby 1974)) and hexokinase (K_m (fructose) = 0.21 to 4.3 mM for insect larval isozymes (Yanagawa 1978)) show high affinities for their respective substrates. Reconversion of sorbitol to glycogen via this pathway could be accomplished, therefore, with limited alterations in the concentrations of intermediates. Sorbitol synthesis, however, would require a significant accumulation of glucose to facilitate the action of polyol dehydrogenase (K_m (glucose) = 200 mM for the *E. solidaginis* enzyme (C. O'Grady and K. Storey, unpublished data)).

Previous studies have shown that sorbitol synthesis by *E. solidaginis* larvae is directly stimulated by low temperature, the trigger temperature being 3 °C or 5 °C (Storey et al. 1981; Baust and Lee 1982). The results of the present study are consistent with this view as sorbitol synthesis was rapidly induced when larvae were exposed to 3 °C. The direct influence of temperature on glycerol synthesis has previously been difficult to evaluate. Natural populations of the insect accumulate glycerol in an anticipatory manner, during late summer/early autumn, before the onset of cold weather

(Morrissey and Baust 1976); collecting third instar larvae which have not already begun glycerol synthesis has not been possible to date. Glycerol accumulation by larvae was not stimulated when larvae acclimated to 10 °C were exposed to a 1 °C per day decrease in temperature (Baust and Lee 1982). However, our previous studies suggest that glycerol production in the larvae, which occurred at high rates at temperatures above 10 °C, slowed and stopped over the range 10 to 5 °C (Storey et al. 1981; Storey 1982a). Temperature stimulation of glycerol synthesis was therefore studied at higher temperatures with larvae acclimated to 23 °C and rapidly switched to 13 °C. The results of this experiment indicated that temperature change can directly stimulate glycerol production in the larvae even when very high levels of glycerol (320 μmol/g wet weight) are already present (Fig. 1). A rise in the level of a key intermediate, fructose-1,6-P_2, of the pathway (indicating an activation of phosphofructokinase) was first apparent after one day at the cooler temperature and a significant increase in glycerol levels first appeared after two days at 13 °C. Glycerol synthesis continued for several days, tending to reach a plateau between 10 and 14 days. These data suggest that a temperature stimulation of glycerol synthesis can probably only occur at fairly high temperatures. Cooling of the larvae within the range of about 23 to 10 °C can apparently trigger glycerol synthesis but cooling at temperatures below 10 °C does not produce sustained glycerol synthesis (although a small production of glycerol was found within the first day at 3 °C (Fig. 3)) but stimulates sorbitol synthesis instead. A hormonal or developmental trigger may also be involved in the initiation of glycerol synthesis by the larvae. Second instar larvae failed to synthesize glycerol upon cold exposure (Morrissey and Baust 1976). The second to third instar transition (which occurs in later summer) may be accompanied, therefore, by development of the ability to synthesize glycerol. This might involve a process such as the induction of an enzyme or enzymes specific to the pathway of glycerol synthesis. Primed by a hormonal or developmental trigger, cooling temperatures would then determine the amount and/or rate of glycerol synthesis in the third instar larvae.

The reversibility of polyol accumulations by the overwintering larvae was tested using abrupt temperature increase. Larvae switched from 13 to 23 °C immediately stopped glycerol synthesis (Fig. 1) but did not decrease glycerol levels at the warmer temperature (Figs. 1, 2). Glycerol, once synthesized by the larvae, cannot, therefore, be catabolized in response to increased temperature. Catabolism of accumulated glycerol by the overwintering larvae is probably prevented by the absence of the enzyme glycerol kinase (glycerol + ATP → glycerol-3-P + ADP) (Storey and Storey 1981) which is necessary for the re-introduction of glycerol carbon into the glycolytic pathway. Sorbitol levels, however, are readily reversible. The temperature increase from 3 to 13 °C not only stopped further sorbitol synthesis but resulted in a rapid catabolism of the polyol (and restoration of carbon into glycogen) with a significant decrease in sorbitol levels occurring within 24 h. The presence in the larvae of the enzymes involved in both the synthetic and catabolic pathways of sorbitol metabolism at all times (Storey and Storey 1981) allows the levels of this polyol to be modulated in response to ambient temperature change. The response time for significant alterations (either synthesis or degradation) of sorbitol levels is about 24 h. This time is long enough so that larvae would be unlikely to respond to a transient warming occurring during a warm winter day but would allow the larvae to adjust sorbitol levels to periods of warming or cooling extending over several days.

The two polyols produced by *E. solidaginis*, whose syntheses are separately regulated by the larvae, also appear to perform different functions for the larvae during overwintering. Glycerol production is anticipatory, occurring while ambient temperatures are well above freezing, but providing the larvae with protection against the sudden frosts of autumn. Glycerol also provides the larvae with a *constant* level of cryoprotection during overwintering. Once synthesized, glycerol pools are retained, despite ambient temperature, throughout the winter. Sorbitol production occurs, however, only in direct response to cold temperatures. Cryoprotection by sorbitol is provided only when the threat of subzero temperatures is imminent. Sorbitol also provides a *variable* level of cryoprotection, one which can be adjusted by the larvae in response to ambient temperature change.

The controls operating to direct glycolytic carbon flow into the differential synthesis of glycerol vs sorbitol are illustrated in the present study. Control is probably primarily vested in three enzyme loci: glycogen phosphorylase, phosphofructokinase and pyruvate kinase. The accumulation of glucose-6-P within 1 h when larvae were switched from 13 to 3 °C indicates the rapid activation of glycogenolysis. Cold activation of glycogen phosphorylase (conversion of phosphorylase *b* to *a*) has been well documented for fat body phosphorylase from insects (Ziegler et al. 1979); such an

effect might also be responsible for the rapid activation of glycogenolysis in *E. solidaginis*.

Pyruvate kinase from *E. solidaginis* appears to be cold inactivated. The temperature decrease from 23 to 13 °C resulted in an increase in phosphoenolpyruvate and a decrease in pyruvate concentrations (Fig. 1). Such changes result in a negative cross-over at the pyruvate kinase locus (Williamson 1970) indicative of decreased carbon flow through this enzyme locus at the lower temperature. When larvae were moved from 23 to 13 °C, therefore, the observed activation of glycogen breakdown coupled with an inhibition of glycolytic flux at the level of pyruvate kinase would result in a diversion of carbon flow into the pathway of glycerol synthesis. The switch from cold to warm temperatures (13 to 23 °C or 3 to 13 °C) produced the opposite effect at the pyruvate kinase locus in all cases, a positive cross-over being seen. Warming results in a re-activation of flux through the pyruvate kinase locus, therefore. The absence of significant changes in pyruvate and phosphoenolpyruvate concentrations when larvae were switched from 13 to 3 °C is probably the result, not of a lack of cold inactivation of the enzyme over this temperature range, but of a primary control of glycolytic flux at 3 °C at the phosphofructokinase locus.

When larvae were moved from 23 to 13 °C, elevated levels of fructose-1,6-P_2, the product of phosphofructokinase, indicated a facilitation of this reaction during the period of glycerol synthesis. Carbon flow proceeded past phosphofructokinase to the level of the triose phosphates before being diverted out of the glycolytic pathway and into glycerol synthesis. When larvae were switched from 13 to 3 °C, however, fructose-1,6-P_2 levels decreased sharply while fructose-6-P concentrations increased. A negative cross-over at the phosphofructokinase locus was indicated blocking glycolytic flux at the level of the hexose phosphates. This blockage of carbon flow at the phosphofructokinase reaction, again coupled with an activation of glycogenolysis, resulted in the diversion of carbon into the synthesis of sorbitol. This low temperature inhibition of flux through the phosphofructokinase locus agrees well with kinetic studies of the enzyme from *E. solidaginis* (Storey 1982b). The enzyme is strongly inactivated at low temperatures showing a Q_{10} of 3.64 (between 10 and 0 °C) as well as a decreased affinity for fructose-6-P, a decreased activation by AMP and an increase in inhibitory effects (due to increased levels of these compounds in vivo (Storey et al. 1981)) by glycerol-3-P and sorbitol at low temperature.

Acknowledgement. Supported by an N.S.E.R.C. operating grant to K.B.S.

References

Baust JG, Lee RE (1982) Environmental triggers to cryoprotectant modulation in separate populations of the gall fly, *Eurosta solidaginis* (Fitch). J Insect Physiol 28:431–436

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

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

Gerlach U, Hiby W (1974) Sorbitol dehydrogenase. In: Bergmeyer HU (ed) Methods of enzymatic analysis. Academic Press, New York, pp 569–573

Keppler D, Decker K (1974) Glycogen: determination with amyloglucosidase. In: Bergmeyer HU (ed) Methods of enzymatic analysis. Academic Press, New York, pp 1127–1131

Lowry OH, Passonneau JV (1972) A flexible system of enzymatic analysis. Academic Press, New York, pp 146–218

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

Storey KB (1982a) Metabolism and bound water in overwintering insects. Cryobiology (in press)

Storey KB (1982b) Phosphofructokinase from the overwintering gall fly larva, *Eurosta solidaginis:* Control of cryoprotectant polyol synthesis. Insect Biochem (in press)

Storey KB, Storey JM (1981) Biochemical strategies of overwintering in the gall fly larva, *Eurosta solidaginis:* Effect of low temperature acclimation on the activities of enzymes of intermediary metabolism. J Comp Physiol 144:191–199

Storey KB, Baust JG, Storey JM (1981) Intermediary metabolism during low temperature acclimation in the overwintering gall fly larva, *Eurosta solidaginis*. J Comp Physiol 144:183–190

Williamson JR (1970) General features of metabolic control as applied to the erythrocyte. Adv Biol Med 6:117–136

Yanagawa H-A (1978) Tissue distribution, purifications and properties of multiple forms of hexokinase in the silkworm, *Bombyx mori*. Insect Biochem 8:293–305

Ziegler R, Ashida M, Fallon AM, Wimer LT, Wyatt SS, Wyatt GR (1979) Regulation of glycogen phosphorylase in fat body of *Cecropia* silkmoth pupae. J Comp Physiol 131:321–332