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Intermediary Metabolism During Low Temperature Acclimation in the Overwintering Gall Fly Larva, *Eurosta solidaginis*

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Summary. 1. The levels of glycogen, lipid, protein, polyols (glycerol and sorbitol), sugars, amino acids, adenylates, and other intermediary metabolites were measured in the overwintering, third instar larvae of the gall fly, *Eurosta solidaginis*, sampled at specified temperatures during a controlled (1° C per day decrease) low temperature acclimation of the larvae from 15° to −30° C.

2. Glycogen reserves were depleted as temperature was decreased, the decrease in glycogen fully accounting for the observed increases in glycerol, sorbitol, glucose, and trehalose in the larvae at low temperatures. Protein and total glyceride reserves of the larvae, however, were not altered during low temperature acclimation.

3. Temperature specific patterns of glycerol and sorbitol accumulation were found. Glycerol concentrations, which were 65% of maximum at 15° C, reached a plateau in concentration of 235 μmol/g wet wt. between 5 and 0° C. Sorbitol first appeared in larvae at 5° C and then increased in concentration rapidly as temperature decreased further to reach a plateau level of 145 μmol/g wet wt. by −10 °C.

4. The free amino acid pool increased in size by 50% during acclimation from 15 to −5° C, this increase due largely to a 24 μmol/g wet wt. increase in proline concentration and a smaller 4.4 μmol/g wet wt. increase in alanine.

5. Arginine phosphate and ATP levels, as well as energy charge and the ratio $[ATP]/[ADP]\cdot[P_i]$, remained high and constant in larvae acclimated to temperatures as low as −5 °C but in larvae acclimated to −30 °C phosphagen and ATP levels had declined by 54 and 29% respectively and energy charge had decreased from 0.92 to 0.82.

6. The data suggest that aerobic metabolism with continued polyol synthesis is fully active in these larvae at temperatures as low as −10 °C. However, below −10 °C, the temperature at which hemolymph freezing takes place, mitochondria appear to be metabolically inactive. Evidence for this includes the cessation of polyol, sugar, and amino acid accumulation by this temperature and the drop in arginine phosphate, ATP, and energy charge and build-up of lactate at −30 °C.

7. The regulation of metabolism in *E. solidaginis* larvae during low temperature acclimation is discussed with particular emphasis on the possible metabolic 'switches' regulating the flow of carbon to glycerol versus sorbitol synthesis.

Introduction

The third instar larvae of the goldenrod gall fly, *Eurosta solidaginis*, overwinter inside stem galls on goldenrod plants and display a cold tolerance which allows survival of temperatures as low as −40 °C (Morrissey and Baust 1976); indeed, exposed above the snowline, the larvae face and survive the rigors of a full Canadian winter. Several physiological and biochemical factors have been identified which contribute to overwintering survival. Hemolymph of the third instar has a supercooling point of −10 °C with freezing taking place only below this temperature (Morrissey and Baust 1976). Low temperature acclimation also results in an increase in the amount of water 'bound' by both low and high molecular weight subcellular components. This serves to limit intracellular dehydration during freezing and to protect proteins from denaturation (Storey et al. 1981). The larvae also build up high concentrations of two cryoprotectant polyhydric alcohols, glycerol and sorbitol, in their tissues. Concentrations as high as 0.6 M glycerol and 0.3 M sorbitol have been recorded in the hemolymph of naturally overwintering populations (Morrissey and Baust 1976). In laboratory acclimation ex-

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periments it was found that only glycerol was present when larvae were held at temperatures above 0 °C while both sorbitol and glycerol were present in larvae held at subzero temperatures; sorbitol production appeared to be 'triggered' by exposure to 0 °C temperature (Morrissey and Baust 1976).

In the present study we have investigated intermediary metabolism in the gall fly larvae during low temperature acclimation. Measurements of polyol production are correlated with parallel measurements of stored fuel reserves (glycogen, lipid, protein), glycolytic and Krebs cycle intermediates, amino acids and energy status (arginine phosphate, adenylates). The data are utilized to provide an assessment of the biochemical adaptations to overwintering survival and to provide insights into the regulation of metabolism, in particular the control of polyol synthesis, during cold acclimation in these larvae.

Materials and Methods

Chemicals and Animals

All biochemicals and enzymes were purchased from Sigma Chemical Co., St. Louis, MO.

Round galls containing the third instar larvae of the gall fly, *Eurosta solidaginis*, were collected from goldenrod bushes in fields around Ottawa during early October. Average outdoor temperature at this time was approximately 15 °C. Acclimation was begun by holding the galls for 1 week at 15 °C in an incubator. The temperature of the incubator was then lowered 1 °C per day until −30 °C was reached, an acclimation regimen developed by Baust et al. (1979). At specified temperatures, samples of galls were removed from the incubator, quickly dissected open, and the larvae removed and killed by freezing on dry ice. Larvae were then transferred to a freezer at −80 °C for long term storage.

Measurement of Metabolite Levels

Frozen larvae were rapidly weighed and transferred to tubes in a dry ice/methanol bath at −8 °C. Ten volumes of −8 °C 6% perchloric acid containing 1 mM EDTA were quickly added and the larvae homogenized using a Tekmar tissuemizer. Precipitated protein was removed by centrifugation at 6,000 × *g* for 10 min. The acid supernatant was neutralized by the addition of 1.5 N KOH/0.4 M imidazole/0.3 M KCl and precipitated $KClO_4$ removed by centrifugation. Neutralized extracts were stored at −80 °C until analysis except for pyruvate determinations which were made immediately after completion of the extraction.

Metabolite concentrations were measured in coupled enzyme assays at 340 nm using a Guilford spectrophotometer. Glucose, sn-glycerol-3-phosphate, pyruvate, lactate, alanine, citrate, malate, ATP, ADP, AMP, and inorganic phosphate were determined as described by Lowry and Passonneau (1972). Arginine phosphate and arginine were measured by the methods of Storey and Storey (1978). Glycerol was determined by the method of Eggstein and Kuhlman (1974) and sorbitol was measured according to Bergmeyer et al. (1974). Glycogen was quantitated as glucose after amyloglucosidase digestion (Keppler and Decker 1974).

Trehalose and fructose were determined by HPLC (Waters Associates) using a Bio-Rad HPX-87 column as outlined by Baust and Edwards (1979). Glycerol and a combined sorbitol + glucose peak were also determined by HPLC; the results, while not reported here, were directly comparable to those determined by the enzymatic methods.

Inorganic phosphate was quantitated in two ways. 'Total' P_i was measured in neutralized perchloric acid extracts. For 'free' P_i, larvae were homogenized in 50 mM Tris-HCl buffer, pH 7.4, centrifuged to precipitate phosphate-containing spherites, the resultant supernatant treated with perchloric acid, and P_i measured in the neutralized extracts.

For total glyceride determination, larvae were homogenized in 20 vol 2:1 chloroform:methanol and glycerides extracted in the chloroform layer (Folch et al. 1957). After saponification, glycerides were determined as glycerol released using the enzymatic assay.

Protein was determined by the Lowry method. Total protein was measured in the perchloric acid-precipitated pellets produced in the isolation of intracellular metabolites. Soluble protein was measured in the supernatant after homogenization of gall fly larvae in Tris-HCl buffer, pH 7.4 and centrifugation at 27,000 × *g*.

For amino acid analysis, frozen larvae were homogenized in 10 volumes of ice-cold sulphosalicic acid (0.5% w/v). After centrifugation to remove precipitated protein, the supernatant was clarified by passage through a 0.05 μm Swinnex filter. Amino acids were then measured using a Beckman 119 BL amino acid analyzer.

Results

Figure 1 shows the changes in glycogen, glycerol, sorbitol, glucose, and trehalose levels in *E. solidaginis*

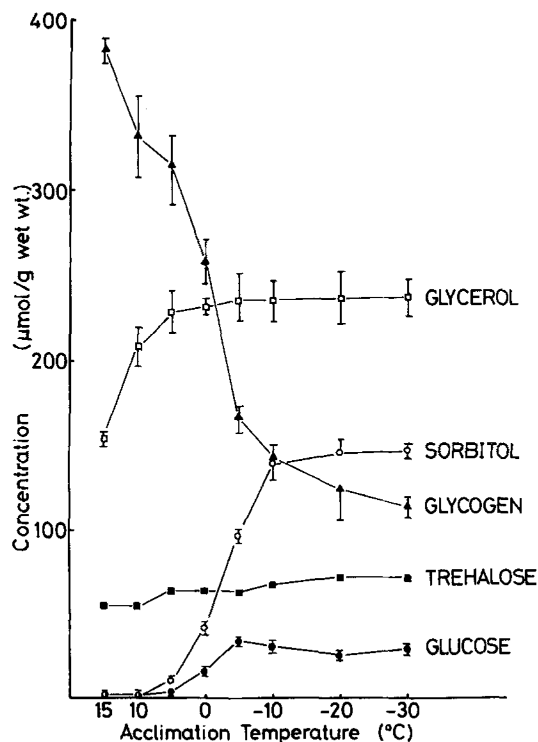

Fig. 1. Levels of glycogen, polyols, and sugars in *E. solidaginis* larvae acclimated to low temperatures. Metabolites were measured as described in Materials and Methods. Results are means ± S.E.M. indicated by the error bars for $n = 11$ samples (3 larvae per sample) for 15, 0, −5, and −30 °C, and $n = 3$ for other temperatures. Symbols are: ▲ glycogen; □ glycerol; ○ sorbitol; ■ trehalose; ● glucose

Table 1. Levels of some metabolites in *Eurosta solidaginis* larvae acclimated to low temperatures

Metabolite	Concentration (μmol/g wet weight)[a]			
	15 °C	0 °C	−5 °C	−30 °C
Glycogen[b]	381.4 ±6.9	258.1 ±12.8*	165.5 ±8.0*	113.3 ±6.2*
Total glycerides[b]	167.1 ±5.3	170.2 ±3.8	167.8 ±10.0	158.7 ±9.7
Protein[c]: total	80.9 ±1.1	81.8 ±2.6	80.4 ±2.2	81.9 ±1.9
soluble	46.6 ±1.6	50.7 ±1.7	48.8 ±0.5	48.9 ±1.3
Glycerol	153.5 ±4.3	232.0 ±2.9*	236.9 ±14.2*	237.6 ±10.7*
Sorbitol	0.8 ±0.4	42.3 ±3.5*	97.4 ±3.7*	146.6 ±4.0*
Glucose	0.1 ±0.03	16.2 ±0.6*	33.8 ±1.6*	28.7 ±1.5*
Fructose[d]	6.1	–	8.9	8.3
Trehalose[d]	55.3	63.5	63.2	70.8
Glycerol-3-P	0.19±0.03	0.93±0.12*	1.59±0.15*	1.04±0.04*
Pyruvate	0.16±0.02	0.11±0.02	0.14±0.01	0.16±0.02
Lactate	0.16±0.04	0.11±0.03	0.14±0.04	0.53±0.09*
Alanine	1.16±0.23	2.81±0.70**	3.87±0.24*	5.45±0.41*
Citrate	4.09±0.18	5.27±0.15*	5.22±0.15*	4.46±0.28
Malate	0.22±0.02	0.34±0.04**	0.47±0.05*	0.27±0.03

[a] Values are means ± S.E.M., $n=7$ samples, 3 larvae per sample
[b] Glycogen and glyceride levels are expressed as μmol glucose and glycerol, respectively
[c] Protein concentration is given as mg/g wet wt
[d] Trehalose and fructose were determined by HPLC, single samples combining 8 larvae per sample
* Significantly different (Student's *t*-test) from 15 °C, $P<0.01$; ** $P<0.05$

larvae during low temperature acclimation. The data for 15, 0, −5, and −30 °C are also tabulated in Table 1 along with the levels of other intermediary metabolites. An inverse relationship can be seen between the levels of glycogen and the concentrations of polyols and sugars indicating that glycogen is the probable carbon source for the synthesis of these compounds. As Table 1 shows, there is no significant change in the levels of total glycerides (tri-, di- or monoglyceride) or of total or soluble protein during the low temperature acclimation suggesting that these reserves do not serve as a carbon source for polyol/sugar synthesis. Indeed, as Table 2 demonstrates, there is an excellent correlation between the decrease in glycogen content and the total increase in the amounts of polyols and sugars produced (all determined in C_6 units) in the larvae from 0, −5, and −30 °C as compared to 15 °C. This same relationship can be seen to hold for the larvae from other temperatures (Fig. 1).

Table 2. Changes in sugar, polyol and glycogen levels with acclimation to low temperatures in comparison to metabolite concentrations in larvae at 15° C

Metabolite	Change from 15 °C (μmol C_6/g wet weight)		
	0 °C	−5 °C	−30 °C
Glycerol/2	+ 39.3	+ 41.7	+ 42.1
Sorbitol	+ 41.5	+ 96.6	+145.8
Glucose	+ 16.1	+ 33.7	+ 28.6
Trehalose × 2	+ 18.0	+ 16.0	+ 32.0
Sum	+114.9	+188.0	+248.1
Glycogen	−123.3	−215.9	−268.1

Distinct patterns of glycerol versus sorbitol accumulation in the larvae were seen with respect to temperature. Glycerol concentration was 65% of its maximum in the larvae at 15 °C and increased rapidly to reach a plateau of about 235 μmol/g wet wt. at temperatures between 5 and 0 °C. Sorbitol, however, was present in only minute amounts (< 1 μmol/g wet wt.) at higher temperatures. Sorbitol began to increase in concentration at 5 °C and thereafter increased rapidly in concentration over the 5 to −10 °C range reaching a plateau of approximately 145 μmol/g wet wt. by −10 °C. Glucose levels paralleled those of sorbitol although never exceeding 34 μmol/g wet wt. Trehalose concentrations in the larvae increased by approximately 15 μmol/g wet wt. between acclimation temperatures of 15 and −30 °C. Fructose was present in low concentrations in all groups of larvae and appeared to remain unchanged in concentration with decreasing temperature.

The concentrations of some glycolytic and Krebs cycle intermediates in the larvae sampled from different low temperatures are also shown in Table 1. Glycerol-3-P, the probable immediate precursor of glycerol, was low in concentration in the larvae at 15 °C

Table 3. Levels of free amino acids in *Eurosta solidaginis* larvae acclimated to low temperatures. Values are means ± S.E.M. for $n=3$ samples, 2 larvae per sample

Amino acid	Concentration (μmol/g wet weight)			
	15 °C	0 °C	−5 °C	−30 °C
Aspartic acid	0.6 ± 0.18	0.6 ± 0.06	0.8 ± 0.03	0.4 ± 0.09
Threonine	2.7 ± 1.16	3.2 ± 0.18	3.0 ± 0.99	1.4 ± 0.11
Serine	2.6 ± 0.31	2.9 ± 0.10	3.0 ± 1.06	2.9 ± 0.03
Asparagine	0.6 ± 0.18	0.7 ± 0.10	0.4 ± 0.07	–
Glutamate	3.5 ± 0.23	5.8 ± 0.20*	4.7 ± 0.26**	3.9 ± 0.30
Glutamine	5.4 ± 0.40	6.0 ± 1.14	6.3 ± 0.33	4.8 ± 0.32
Proline	32.2 ± 2.65	41.8 ± 2.09**	56.5 ± 2.38*	55.8 ± 4.05*
Glycine	0.9 ± 0.21	1.4 ± 0.17	1.4 ± 0.20	1.3 ± 0.08
Alanine	0.4 ± 0.02	2.0 ± 0.43**	2.7 ± 0.40*	4.8 ± 0.70*
Cysteine	1.0 ± 0.21	1.0 ± 0.02	1.0 ± 0.10	0.9 ± 0.04
Methionine	0.3 ± 0.04	0.5 ± 0.01	0.4 ± 0.06	0.4 ± 0.02
Isoleucine	0.4 ± 0.20	0.4 ± 0.21	0.4 ± 0.19	0.3 ± 0.05
Leucine	0.6 ± 0.23	0.6 ± 0.21	0.6 ± 0.22	0.4 ± 0.01
Tyrosine	0.7 ± 0.21	0.7 ± 0.13	0.6 ± 0.04	0.5 ± 0.04
Phenylalanine	0.2 ± 0.07	0.1 ± 0.01	0.3 ± 0.01	0.2 ± 0.02
Ornithine	0.6 ± 0.02	0.5 ± 0.08	0.5 ± 0.11	0.6 ± 0.03
Lysine	2.7 ± 0.51	3.8 ± 0.55	4.5 ± 0.65	4.2 ± 0.10
Histidine	0.4 ± 0.02	0.3 ± 0.05	0.4 ± 0.15	0.6 ± 0.10
Arginine	2.2 ± 0.57	2.8 ± 0.39	2.6 ± 0.07	2.6 ± 0.08
Total amino acids	58.0	75.1	87.1	86.0

* Significantly different from the value for 15 °C using Student's t-test, $P<0.01$; ** $P<0.05$

but increased by 5-fold in larvae acclimated to 0 °C. The build-up of glycerol-3-P correlates well with the region of the plateau in glycerol level and the cessation of glycerol accumulation. Glycerol-3-P is also a known anaerobic end product in insect tissues and accumulates in a 1:1 ratio with pyruvate under anoxic conditions in flight muscle (Sacktor 1970). Pyruvate levels, however, were low and constant at all acclimation temperatures in the larvae. Lactate, another possible anaerobic end product in *E. solidaginis* (larval lactate dehydrogenase activity is 6.5 μmol/min·g wet wt. or 60% of the activity of glycerol-3-P dehydrogenase; K.B. Storey, unpublished data) was not altered in larvae acclimated to temperatures as low as −5 °C but increased significantly in the larvae by −30 °C. Alanine is also known to accumulate as an anaerobic end product in insects (Sacktor 1970) and a progressive increase in alanine levels in the larvae was noted with decreases in acclimation temperature. However, the total increase in these four compounds which could be called 'anaerobic' end products was not more than 6 μmol/g wet wt. Citrate and malate concentrations were found temporarily elevated in the larvae at 0 and −5 °C but by −30 °C had decreased to the same levels as those in the larvae at 15 °C.

Table 3 shows the levels of free amino acids in *E. solidaginis* larvae acclimated to decreasing low temperatures. Total free amino acids increased by 28 μmol/g wet wt. during the acclimation from 15 to −30 °C, a 50% increase in the pool size. The bulk of this increase is accounted for by a large scale increase in proline concentration from 32 to 56 μmol/g wet wt. between 15 and −5 °C (and remaining at 56 μmol/g wet wt. at −30 °C). A transient increase in glutamate (the precursor of de novo proline synthesis) concentrations was seen at 0 and −5 °C. The only other amino acid which was altered in concentration during the low temperature acclimation was alanine. The preferential increase in proline levels during low temperature acclimation argues strongly for a de novo synthesis of this amino acid rather than for a breakdown of protein at low temperature. Indeed, the carbon needed for proline synthesis can be accounted for from the decrease in glycogen reserves while protein content was unaltered by low temperature acclimation.

The energy status of *E. solidaginis* larvae acclimated to low temperature is assessed in Table 4. Arginine phosphate reserves in these larvae are not high in agreement with measures of arginine phosphate in other insect larvae (Mandel et al. 1980). Levels of the phosphagen remained constant in larvae from temperatures as low as −5 °C but decreased by 50% in the larvae at −30 °C. Arginine concentrations followed the inverse pattern and doubled in concentration in the larvae at −30 °C as compared to 15 °C. The discrepancy between arginine concentrations given in Tables 3 and 4 likely reflects the hydrolysis

Table 4. Levels of arginine phosphate, arginine, adenylates, and inorganic phosphate in *Eurosta solidaginis* larvae acclimated to low temperatures. Values are means ± S.E.M. for $n=7$ samples, 3 larvae per sample. Energy charge = $[ATP]+{}^1/_2[ADP]/[ATP]+[ADP]+[AMP]$.

Metabolite	Concentration (μmol/g wet weight)			
	15 °C	0 °C	−5 SC	−30 °C
Arginine phosphate	2.18 ± 0.26	2.12 ± 0.04	2.02 ± 0.09	1.01 ± 0.19*
Arginine	0.87 ± 0.08	0.84 ± 0.06	0.84 ± 0.03	2.10 ± 0.08*
P_i free	8.15 ± 0.46	6.62 ± 0.28**	6.22 ± 0.12*	4.87 ± 0.69*
total	46.7 ± 2.8	48.7 ± 2.5	49.9 ± 3.1	53.4 ± 2.7
ATP	2.23 ± 0.04	2.21 ± 0.04	2.21 ± 0.04	1.58 ± 0.08*
ADP	0.28 ± 0.03	0.30 ± 0.03	0.35 ± 0.02	0.66 ± 0.04*
AMP	0.05 ± 0.01	0.04 ± 0.003	0.04 ± 0.01	0.10 ± 0.02**
Total adenylates	2.56	2.55	2.60	2.34
Energy charge	0.93	0.93	0.92	0.82
$[ATP]/[ADP]\cdot[P_i]$ (M^{-1}) [a]	977	1,113	1,015	492

[a] Calculated using the concentrations of free P_i

* Significantly different (Student's *t*-test) from the value for 15 °C, $P<0.01$; ** $P<0.05$

of arginine phosphate in the samples used for amino acid analysis as these samples were stored under acid conditions. Total adenylate levels in the 15 °C group of larvae were similar to those measured in other insect larvae (Mandel et al. 1980). ATP levels and energy charge remained high and constant in larvae from acclimation temperatures as low as −5 °C but both had dropped in larvae acclimated to −30 °C (ATP decreased by 29% and energy charge by 12%). Total P_i assayed in perchloric acid extracts of *E. solidaginis* larvae was nearly 50 μmol/g wet wt., a very high concentration. However, Turbeck (1974) has reported the existence of spherites of magnesium or calcium phosphate occurring in larval insect tissues. The function of these crystalline structures is thought to be excretory and they are highly acid labile such that measurement of P_i in perchloric acid extracts overestimates the actual content of free intracellular P_i. Using techniques of neutral (see Materials and Methods) or basic (Mandel et al. 1980) extraction of tissues, the spherites can be pelleted intact and free P_i can then be measured. The content of free P_i in the larvae was much lower, ranging between 5 and 8 μmol/g wet wt. While the levels of total P_i were not altered with acclimation temperature in the larvae, free P_i levels, those which would be important in the control of metabolic reactions, decreased significantly with decreasing acclimation temperature. The term $[ATP]/[ADP]\cdot[P_i]$ was calculated using the measured concentrations of free P_i, the results showing a high and constant ratio in the larvae from 15, 0, and −5 °C with a 50% decrease in $[ATP]/[ADP]\cdot[P_i]$ in the larvae acclimated to −30 °C. This ratio is now believed to be the most influential factor controlling respiratory rate (Erecinska et al. 1977).

Discussion

Previous studies have indicated that a major factor in overwintering survival of the gall fly larvae is the accumulation in tissues of high concentrations of two polyhydric alcohols, glycerol and sorbitol (Morrissey and Baust 1976). The cryoprotectant system of *E. solidaginis* is of particular interest not only because of the involvement of two different polyols but also because of the distinct temperature dependent sequence with which first glycerol, and secondly sorbitol, are accumulated in larval tissues. In outdoor, northern U.S.A. populations of *E. solidaginis* Morrissey and Baust (1976) showed that glycerol accumulation began during early autumn when daily temperatures were still well above 0 °C. Sorbitol accumulation, however, was not initiated until the first exposures of the larvae to 0 °C temperature, much later in autumn.

In the present study, we have used laboratory low temperature acclimations to examine various aspects of intermediary metabolism, including polyol synthesis, in *E. solidaginis* larvae. The use of laboratory acclimations allows an effective separation to be made between the biochemical events associated strictly with adaptation to low temperature and those developmental changes which could occur over time in the naturally overwintering populations studied previously. The study revealed that glycerol was present in the larvae even at the highest (15 °C) acclimation temperatures (Fig. 1), well above temperatures at which cryoprotection would be needed. The initiation of glycerol synthesis in *E. solidaginis* may, therefore, result from a cue other than temperature (ex. photoperiod or a developmental cue), the larvae 'anticipat-

ing' the need for cryoprotection against sudden unseasonal frosts. The initiation of sorbitol synthesis, however, is more likely the result of a temperature cue, sorbitol accumulation beginning quite suddenly when acclimation temperature decreases to approximately 5 °C. The initiation of sorbitol synthesis correlated well with the final cessation of glycerol accumulation under our experimental conditions, suggesting the operation of a temperature dependent metabolic switch which acts to divert carbon flow from the glycerol pathway into the sorbitol pathway. Sorbitol synthesis continued as temperature decreased from 5 to −10 °C but synthesis ceased at −10 °C, the temperature at which freezing of the larval fluids takes place (Morrissey and Baust 1976).

The carbon source for polyol synthesis in *E. solidaginis* is glycogen, a strong inverse relationship occurring between glycogen levels and the accumulated increase in polyol levels as temperature is decreased (Fig. 1). Indeed, glycogen depletion can quantitatively account for the total increase seen in glycerol + sorbitol + glucose + trehalose in the larvae at all temperatures (Table 2) and can even provide for the carbon necessary for proline synthesis. By contrast, total protein and glyceride reserves of the larvae were not significantly altered during low temperature acclimation; these reserves are likely retained for use during pupation and the non-feeding adult stages.

The metabolic pathways utilized in polyol synthesis in *E. solidaginis* are indicated by the data in this study. Polyol synthesis results from aerobic glycogen catabolism. The pathway of glycerol production utilizes the reactions of glycolysis down to the level of the triose phosphates. Reduction of dihydroxy-acetone-P followed by dephosphorylation of glycerol-3-P are the probable final reactions in glycerol production. Support for this pathway comes from the observed accumulation of glycerol-3-P in the larvae concomitant with the cessation of glycerol synthesis. An alternate pathway is the dephosphorylation of glyceraldehyde-3-P and reduction of glyceraldehyde. The enzymes catalyzing these latter reactions occur in high activities in diapausing silkworm eggs which also accumulate high levels of glycerol (Chino 1960; Kageyama at al. 1973; Takahashi et al. 1974). Glycerol synthesis requires the provision of reducing power (NAD(P)H) and ATP (at the phosphofructokinase reaction). The production of this polyol could be considered more 'efficient' than that of sorbitol in terms of net production of polyol because 2 mol of glycerol, but only 1 mol of sorbitol, is produced per mol of glucose-1-P derived from glycogen. The synthesis of sorbitol, however, utilizes a simpler metabolic pathway (3–5 enzymatic steps versus 7 for glycerol) and is not ATP dependent. The probable route of sorbitol production would involve dephosphorylation of either glucose-1-P, glucose-6-P or fructose-6-P followed by a reduction of the sugar produced. In the present study, the parallel increases in glucose and sorbitol levels points to a precursor role for glucose in sorbitol synthesis.

Several types of controls could be operative in regulating polyol synthesis in *E. solidaginis* and in operating the metabolic 'switch' to divert carbon flow from glycerol synthesis into sorbitol synthesis. These could include: a) temperature effects upon enzyme catalysis, b) end product effects by glycerol in regulating its own synthesis and/or in activating sorbitol synthesis, c) synthesis/degradation of enzymes involved in glycerol or sorbitol synthesis, and d) activation/inhibition of enzymes by changes in the levels of metabolite effectors. The switching off of glycerol synthesis may involve actions at several enzymatic sites. The temperature dependence of glycerol synthesis suggests that there may be inhibitory effects of low temperature on one or more enzymes involved in glycerol, but not sorbitol, synthesis. One probable site for this regulation is phosphofructokinase, a complex, multimeric regulatory enzyme which is the key rate-limiting enzyme in the upper portion of glycolysis (Ramaiah 1974). Low temperature affects upon the catalysis of this enzyme could severely limit carbon flux past the midpoint of glycolysis. Other enzymatic sites at which glycerol production might be regulated include glycerol-3-P dehydrogenase and glycerol-3-P phosphatase. The build-up of glycerol-3-P accompanying the cessation of glycerol synthesis may suggest an inactivation of the phosphatase by temperature or modulator effects at low acclimation temperatures. In addition to controls shutting off glycerol synthesis, sorbitol synthesis could be positively enhanced by mechanisms such as the induction of key enzymes (ex. polyol dehydrogenase, sorbitol dehydrogenase) and activating effects of modulators (perhaps even glycerol) upon enzymes of sorbitol synthesis.

Morrissey and Baust (1976) suggested that trehalose may also have a cryoprotectant role in *E. solidaginis* larvae after demonstrating an accumulation of this disaccharide in larval hemolymph during late winter, well after both glycerol and sorbitol levels had stabilized. In their study, haemolymph trehalose levels rose by 6–7 mM and remained elevated until sharply decreasing along with sorbitol levels just before pupation. In the present work we found a 16 μmol/g wet wt. increase in trehalose during low temperature acclimation as well as a 30 μmol/g wet wt. increase in glucose levels. Perhaps both of these sugars could be considered to have roles as cryopro-

tectants in the gall fly larvae. Sorbitol, glucose and trehalose synthesis all appeared to be initiated at about 5 °C, when glycerol production had ceased. Apparently, then, when carbon flow through glycolysis to glycerol is restricted, the glucose-1-P derived from glycogenolysis is redistributed into the formation of C_6 sorbitol and glucose and C_{12} trehalose.

A cryoprotectant role for amino acids in overwintering insects has never been directly investigated. However, proline appears to be specifically accumulated in *E. solidaginis* during low temperature acclimation. Proline accounted for 86% of the total increase in the free amino acid pool of the larvae during low temperature acclimation with alanine making up most of the rest of the observed increase. Similarly, Mansingh (1967) noted an accumulation of proline and alanine (a 25 and 20 mM increase over larval or non-diapausing pupal haemolymph concentrations, respectively) in the hemolymph of diapausing, overwintering pupae of *Antheraea pernyi* held at 6 °C. Thus the accumulation of proline in tissues may be characteristic of overwintering tolerance in insects although the cryoprotective function of proline is as yet unknown. However, it is well known that proline typically accumulates during cold hardening in plants (Yelenosky 1979). The increase in proline levels in the larvae probably results from de novo synthesis. This is suggesed by a) the selective increase in this single amino acid and b) the transient elevation of glutamate levels, the precursor of proline, during the period of proline accumulation. The synthesis of proline, which requires mitochondrial function, argues strongly for the maintenance of mitochondrial activity at temperatures at least as low as −5 °C in these larvae.

Metabolism can be divided into two distinct phases in these larvae with respect to temperature. During the first phase, which extends from high temperatures down to approximately −10 °C, normal, aerobic, oxidative metabolism, based upon carbohydrate catabolism, is maintained. Energy levels (ATP, arginine-P) remain high and synthesis and accumulation of polyols, sugars, and proline continues. Within this phase we find temperature (or other) effects on synthetic pathways resulting in a shifting of metabolism from glycerol to sorbitol production.

The larvae enter a second metabolic phase when temperatures decrease below about −10 °C, the point at which hemolymph freezing takes place. While single or even repeated freezing does not kill the larvae or impair adult development (Salt 1957), the phase transition does appear to have a strong depressing effect upon oxidative metabolism. Below −10 °C, we find a cessation of synthetic metabolism with no further significant production of polyols, sugars or amino acids. Energy status cannot be maintained and arginine-P and ATP levels drop with a concomitant fall in energy charge and in the ratio $[ATP]/[ADP] \cdot [P_i]$. Thus oxidative metabolism appears to be blocked at these low temperatures. The blockage may perhaps result from a limitation of O_2 delivery to the tissues or an impairment of cell membrane function brought about by the freezing of extracellular water (Raison 1973). However, a low rate of basal metabolism does continue in the larvae as demonstrated by the elevated levels of lactate found in the larvae at temperatures below −10 °C. But lactate production results from glycolytic function only and it is produced at a very low rate, less than 0.4 µmol/g wet wt. of lactate accumulating in the larvae during the three weeks necessary to lower temperatures from −10 to −30 °C. Metabolism in the frozen state in *E. solidaginis* is, apparently, essentially dormant.

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