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Biochemical Adaptation for Cold Hardiness in Insects [and Discussion]

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Biochemical adaptation for cold hardiness in insects

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Specific biochemical adaptations permit winter survival at subzero temperatures by both freeze-tolerant and freeze-avoiding insects. Common to both survival strategies is the accumulation of high concentrations of polyols, providing deep supercooling point depression for freeze-avoiding forms and regulating cell volume reduction during extracellular freezing in freeze-tolerant insects. Studies in my laboratory have elucidated the molecular mechanisms (temperature effects on enzyme properties, allosteric regulation, reversible protein phosphorylation) that control the massive conversion of glycogen to polyols and, in some species, regulate the differential synthesis of dual polyols. New studies have highlighted the importance of aerobic ATP production for glycerol biosynthesis, suggested the importance of micro-compartmentation for optimal conversion efficiency, documented seasonal changes in the capacity for polyol synthesis versus reconversion to glycogen and analysed the role of protein phosphorylation in enzyme regulation during polyol synthesis.

1. Introduction

Most terrestrial ectotherms living at high latitudes must have a survival strategy for dealing with the cold temperatures of winter. For many species this includes adaptations that provide seasonal tolerance of temperatures below the equilibrium melting point of body fluids. Insect species may use one of two strategies: freeze tolerance or freeze avoidance. Freeze-tolerant insects initiate and control ice formation in extracellular fluid spaces while employing adaptations that regulate the concomitant reduction of cell volume and stabilize macromolecular structure (Storey & Storey 1988). Freeze-avoiding insects maintain a liquid state throughout the winter by a colligative depression of freezing and supercooling points and an inhibition of ice nucleation via the action of thermal hysteresis proteins (Zachariassen 1985; Storey & Storey 1989*a*). Common to both strategies is the use of low molecular mass polyhydric alcohols as cryoprotectants. For freeze-avoiding insects a high content of polyols (often up to 2 M or about 20% of the fresh mass of the animal) permits the supercooling point to be pushed to −40 °C or lower (compared to a limit of about −12 °C without cryoprotectants) (Zachariassen 1985). For freeze-tolerant insects, the colligative action of cryoprotectants limits the reduction of cell volume during extracellular ice formation to prevent the freeze concentration of cells from reaching an injurious level. The choice of polyols as cryoprotectants is well suited to these functions. Polyhydric alcohols are highly soluble in aqueous solution, they are non-toxic and compatible solutes that have few effects on enzymatic or metabolic processes even at very high concentrations. They are freely penetrating across membranes (Storey & Storey 1988). Furthermore, polyols stabilize the native state of proteins to prevent denaturation as a consequence of low temperature or freezing.

Many of the fundamental aspects of cryoprotectant metabolism and function in insects have been studied. It is well known, for example, that polyols are synthesized from fat-body

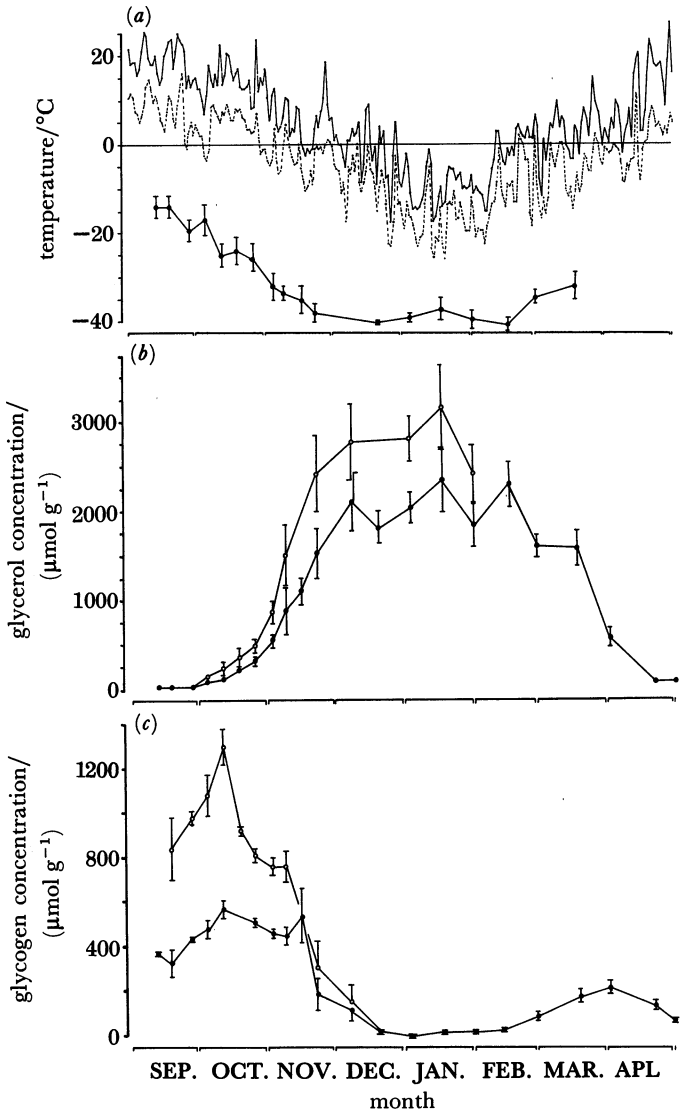

FIGURE 1. The winter profile of (*b*) glycerol and (*c*) glycogen contents in the freeze-avoiding larvae of the goldenrod gall moth *Epiblema scudderiana*. Also plotted (*a*) are daily temperature minima and maxima and whole larvae supercooling points. Metabolite contents are expressed relative to both wet mass (●) and dry mass (○) of the animal. Modified from Rickards *et al.* (1987).

glycogen reserves (accumulated for this purpose during late summer feeding) and that the initiation of polyol synthesis in most species results from a cold stimulation of the activity of glycogen phosphorylase (Ziegler *et al.* 1979; Hayakawa 1985). Typically, polyols accumulate over the autumn months, with the rate of synthesis often highest when average environmental temperatures are about 0 to −5 °C. Cryoprotectant pools remain stable over the mid-winter months and then are gradually cleared during spring warming.

This article focuses on recent studies in my laboratory aimed at resolving specific problems in the metabolic regulation of cryoprotectant metabolism in cold-hardy insects. The two model insects that we use are the larvae of goldenrod gall formers, the freeze-avoiding gall moth *Epiblema scudderiana* (Clemens) (Lepidoptera, Olethreutidae) and the freeze-tolerant gall fly

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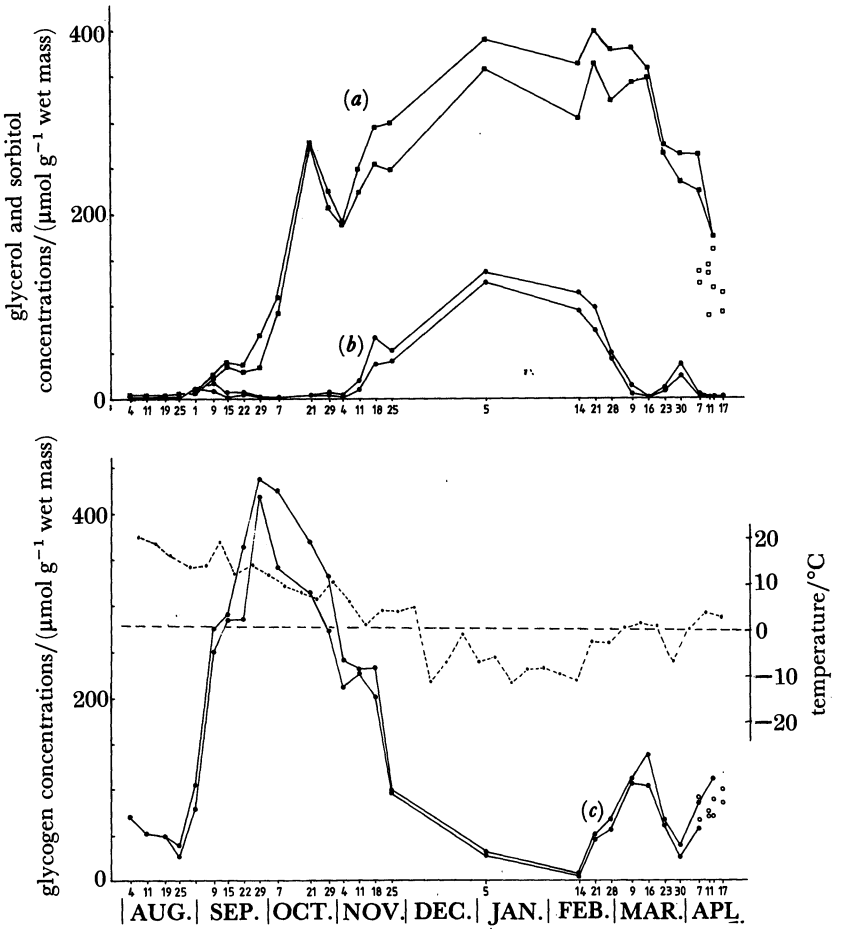

FIGURE 2. The winter profiles of glycerol, sorbitol, and glycogen in the freeze tolerant larvae of the goldenrod gall fly, *Eurosta solidaginis*. Also plotted are mean weekly environmental temperatures (●---●). Open symbols in April represent pupae. Data from Storey & Storey (1986). (*a*) glycerol; (*b*) sorbitol; (*c*) glycogen.

Eurosta solidaginis (Fitch) (Diptera, Tephritidae). Autumn cold hardening for *E. scudderiana* includes a depression of supercooling point to −38 °C, a decrease in body water content, and an accumulation of glycerol to an average amount of 18.7% of fresh body mass (over 2000 μmol g^{-1} wet mass) (figure 1) (Rickards *et al.* 1987). Larvae may also be shielded from inoculative freezing by environmental ice via a thin cocoon lining the gall cavity. Cold hardening of *E. solidaginis* larvae, by contrast, includes an increase in supercooling point to about −10 °C (so that controlled freezing is induced at a relatively mild temperature), tolerance of up to 65% of total body water as ice, and the independent accumulation of two cryoprotectants, glycerol and sorbitol (figure 2) (Morrissey & Baust 1976; Storey *et al.* 1981; Storey & Storey 1986; Lee & Lewis 1985).

2. METABOLIC ORGANIZATION FOR OPTIMAL CRYOPROTECTANT SYNTHESIS

The production of cryoprotectants is a major metabolic event in the life cycle of cold-hardy insects. Synthesis involves the conversion of virtually the entire glycogen reserve of the animal into polyol products (figures 1 and 2). Such a huge undertaking needs to be closely regulated

for maximal efficiency, and mechanisms should be in place that ensure that the conversion of glycogen to polyols is unidirectional over the autumn months and that the pool is maintained throughout the winter. Such efficiency is necessary as about 16% of the total carbohydrate reserve must be consumed during the synthesis of glycerol to provide the required reducing equivalents and ATP; the corresponding value for sorbitol is about 8% (Storey & Storey 1988). Recent data suggest that such efficiency of glycogen to polyol conversion is achieved, at least in part, by altering the subcellular organization of metabolism in a seasonally dependent manner to promote carbon flow through specific routings.

Seasonal dependence of glycogen–glycerol interconversions

In recent studies with *E. scudderiana* larvae we have found distinct seasonal differences in the relation between glycogen and glycerol pools (Churchill & Storey 1989*a*). Alternating exposures (of 10 or 12 days each) to cold (−4 °C) versus warm (16 °C) temperatures were used to probe the interconvertibility of these pools in autumn, mid-winter, and late winter.

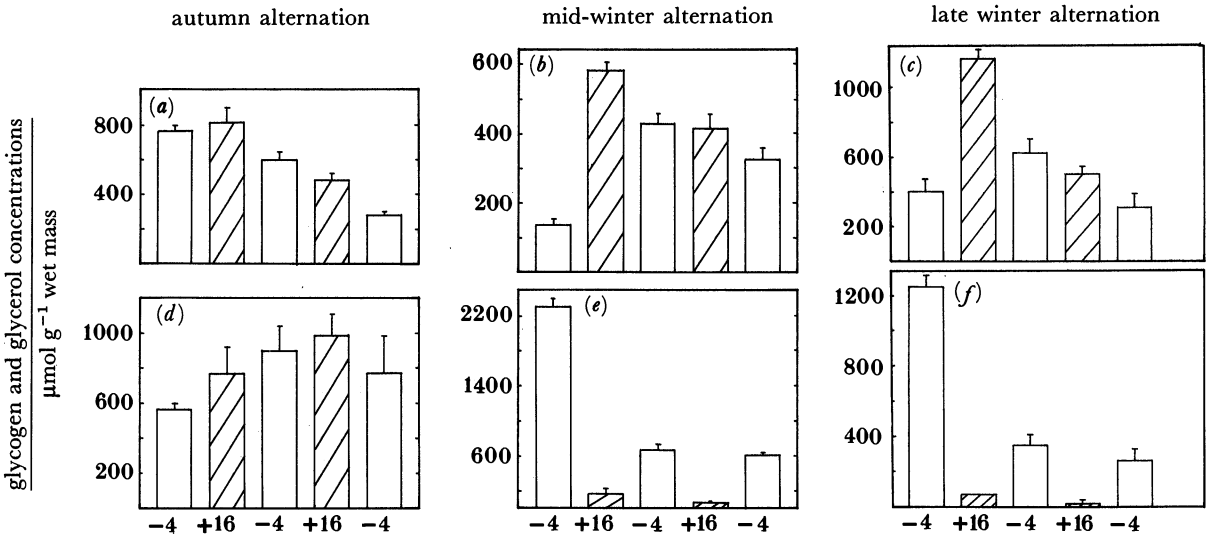

FIGURE 3. The responses of glycogen and glycerol pools in *Epiblema scudderiana* to cycles of low versus high temperature exposure in autumn, mid-winter, and late winter. Before beginning, autumn larvae were acclimated to 16 °C for 2.5 weeks and had a glycerol content of 48 ± 6 μmol g^{-1}. From Churchill & Storey (1989*a*); ((*a*)–(*c*), glycogen; (*d*)–(*f*), glycerol).

As figure 3 shows, cold exposure in the autumn readily stimulated the production of glycerol with the inverse depletion of glycogen. However, subsequent warm exposure did not halt synthesis. In the autumn then, the metabolism of the larvae is poised for the unidirectional conversion of glycogen into glycerol and, once initiated, cryoprotectant synthesis proceeds until glycogen reserves fall to low levels. A similar response has been reported for the autumn accumulation of glycerol in *E. solidaginis* (Pio & Baust 1988). By mid-winter, however, the metabolism of *E. scudderiana* larvae had changed and glycerol levels fell in response to warming with a concomitant increase in glycogen content. Subsequent cold exposure none the less restimulated polyol synthesis. The same pattern held true in the late winter, but by then the rate at which the resynthesis of glycerol could take place in response to cold stimulation had been reduced by at least 50%.

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Seasonal changes in the capacity for activating polyol synthesis were also apparent from the response of glycogen phosphorylase to cold stimulation in *E. scudderiana*. When larvae were switched from 16 to −4 °C, the content of active phosphorylase *a* jumped from 0.38 to 6.5 U g^{-1} wet mass in autumn larvae, the change made up of an eightfold increase in the total activity of phosphorylase (*a*+*b*) plus a twofold increase in the percentage of the active *a* form (Churchill & Storey 1989*a*). The same low temperature stimulation given in mid-winter raised phosphorylase *a* activity from 0.26 to 4.8 U g^{-1} whereas by late winter the response to cold was much reduced, 0.13 rising to 1.4 U g^{-1}, and due solely to a change in the percent phosphorylase *a* upon cold exposure. These data further demonstrate that both the metabolic make-up of the larvae and responsiveness to low temperature stimulation change over the winter season.

Subcellular organization of polyol production

The bulk conversion of glycogen to polyols during autumn cold hardening could be greatly aided by a subcellular organization to metabolism that compartmentalized the requisite enzymatic pathways. Evidence for the microcompartmentation of individual metabolic functions within cells has increased enormously in recent years (Masters *et al.* 1987; Srere 1987). Even so-called soluble enzymes may form binding associations with each other or with the cytoskeleton matrix to form functional pathway complexes that increase the efficiency of carbon flow caused by both the physical proximity of consecutive enzymes and binding-induced changes to the kinetic properties of individual enzymes. Such associations could prove key to polyol synthesis. For example, the very high K_m values for sugars displayed by insect polyol dehydrogenases, often greater than 50 mM and much above sugar concentrations *in vivo*, have made it difficult to visualise how these enzymes can function in the cell. However, microcompartmentation or binding associations, or both, of polyol dehydrogenase with the other enzymes of the reaction pathway may effectively channel substrate through this locus. Yamashita *et al.* (1975) have provided some evidence that such compartmentation probably occurs in cold-hardy insects by noting that the site of glycogen deposition was markedly different for polyol-producing diapause eggs of *Bombyx mori* (intergranular matrices) compared to non-diapause eggs (within the yolk granules). A 'glycogen particle' composed of the requisite glycolytic and hexose monophosphate shunt enzymes in association with glycogen granules may provide the microcompartmentation that ensures efficient conversion of glycogen to polyols.

Metabolic design for glycerol synthesis in E. scudderiana

A recent study of the metabolic events associated with glycerol biosynthesis in *E. scudderiana* provided us with additional evidence that the metabolic make-up of the larvae in the autumn is tailored to facilitate polyol synthesis. Experiments were designed to test the energetics of glycerol synthesis and the partitioning of carbon between ATP-dependent, via phosphofructokinase (PFK), and ATP-independent, via the hexose monophosphate shunt, routes for triose phosphate production by testing the ability of the autumn larvae to synthesize glycerol under anoxic conditions (Churchill & Storey 1989*b*). We predicted that efficient glycerol biosynthesis would require an aerobic metabolism, particularly in a species that converts about 18% of its total body mass into glycerol. Indeed, this was the finding; final glycerol levels in anoxic larvae after 14 days at −4 °C were only 50% higher than control values compared to a 1000% increase in glycerol in aerobic larvae. What was surprising, however, was that the first response to the N_2 gassing alone (30 min of gassing at 16 °C before

−4 °C exposure) was a fivefold increase in glycerol content of the larvae, a faster and greater response than was triggered by the subsequent cold exposure of the anoxic larvae. The activation of this pathway that is a net consumer of ATP should be detrimental to anoxia survival. However, glycerol production is explainable if there is a fixed routing of carbon flow in autumn larvae. Specifically, these data suggest that a high percentage of total carbon flow must cycle through the hexose monophosphate shunt whether the fate of that carbon is polyol synthesis, anaerobic fermentation, or oxidation by the tricarboxylic acid cycle. Indeed, it can be calculated that for the biosynthesis of glycerol from glycogen, 86% of the total carbon must pass through the shunt (before exiting as fructose-6-P or glyceraldehyde-3-P) to generate the required NAD(P)H (Storey & Storey 1988). If such a routing is obligatory during the period of autumn cold hardening, then an anoxia-induced activation of glycogenolysis as a means of stimulating fermentative ATP synthesis would generate an enormous output of NADPH reducing equivalents in the process of routing carbon to the ATP-producing reactions of glycolysis. Redox balance could only then be maintained by supporting glycerol biosynthesis. The result was a net increase in anoxia of 165 μmol g^{-1} wet mass for glycerol versus 12 μmol g^{-1} net accumulation of lactate plus alanine (Churchill & Storey 1989*b*). The data suggest that the routes of carbohydrate catabolism in *E. scudderiana* are fixed in a manner that is of maximal advantage to the efficient biosynthesis of glycerol during autumn cold hardening but are disadvantageous for dealing with stresses (e.g. anoxia) that require fermentative ATP synthesis. For a freeze-avoiding insect, however, circumstances of natural anoxia exposure should rarely occur. A similar metabolic make-up may be the basis of reports of glycerol accumulation during anoxia stress in other insect species (Meyer 1978; Sonobe *et al.* 1979; Gade 1984).

Metabolic design for polyol synthesis in E. solidaginis

Studies of the effects of anoxia stress on sorbitol and glycerol biosynthesis in the freeze tolerant larvae of *E. solidaginis* further confirm that a specialized organization to metabolism probably underlies polyol synthesis in cold-hardy insects (Storey & Storey 1989*b*). In this species, sorbitol production is linked to the low temperature trigger and a temperature drop from 13 to 3 °C stimulates sorbitol synthesis. When 3 °C exposure was given under a nitrogen gas atmosphere, net sorbitol accumulation was 58% higher than in the corresponding aerobic larvae (table 1).

As discussed above for *E. scudderiana*, polyol synthesis in anoxia has no energetic advantage for the larvae, but the accumulation of sorbitol in anoxia appears to reflect a strict partitioning of carbon flow between glycolytic versus hexose monophosphate shunt routes during the autumn cold-hardening period. Theoretically, optimal sorbitol synthesis requires that 46% of total hexose phosphates pass through the hexose monophosphate shunt to generate the required NADPH (Storey & Storey 1988). If this routing remains fixed in anoxia, then redox balance during fermentative ATP production must provide the means of reoxidizing both the NADH generated by the glyceraldehyde-3-P dehydrogenase reaction (via lactate synthesis) and the NADPH generated by carbon routed through the hexose monophosphate shunt (via sorbitol synthesis). Other authors have also noted sorbitol accumulation during anoxia in insects (Somme 1967; Meyer 1978; Gade 1984), Meyer (1978) concluded from the [^{14}C]-labelling pattern of lactate that hexose monophosphate shunt activity was increased in anoxia in *Callitroga macellaria*. In one sense sorbitol is an anaerobic end product of anoxia, along with lactate and alanine (table 1), as its production is a consequence of the required routes for the

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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 OR 3 °C

	13 °C exposure		3 °C exposure	
	aerobic	anoxic	aerobic	anoxic
	(μmol g^{-1} wet weight)			
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 in C_6 equivalents	64.6	84.5	64.0	125.0
aerobic C_6 oxidized to produce ATP for glycerol synthesis	1.4	0.0	0.2	0.0
C_6 consumed in pentose phosphate cycle (1 C_5 lost for 12 NAD(P)H)	10.5	9.1	6.0	6.6
total C_6 consumption	76.5	93.6	70.2	131.6
percentage of total C_6 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; as up to 20% of the carbon could be derived from glyceraldehyde-3-P outputs of the hexose monophosphate shunt, total ATP use for synthesis probably ranges between 35.1 and 43.9 μmol g^{-1}, leaving a substantial anaerobic ATP output to support basal metabolism. (Data are from Storey & Storey (1989).)

anaerobic fermentation of glycogen in this species. However, the production of sorbitol does nothing to enhance anoxic ATP output and, indeed, because CO_2 is lost whenever carbon cycles through the hexose monophosphate shunt, the net conversion of hexose phosphates to triose phosphates cannot be quantitative and this lowers the net efficiency of ATP output from the anoxic fermentation of glycogen.

Table 1 also shows the consequences of anoxia on the metabolism of *E. solidaginis* larvae under temperture conditions (a switch from 23 to 13 °C) designed to stimulate the synthesis of glycerol in this species (Storey & Storey 1983, 1989*b*). Under anoxic conditions, net glycerol synthesis was only 57% of the corresponding aerobic production indicating, again, that ATP availability limits glycerol output. Indeed, to balance ATP use versus ATP production when only fermentative metabolism is available, the theoretical partitioning of fructose-6-P into glycerol versus glycolytic end products (lactate plus alanine) would have to be 3:1. The observed ratio was 2.85:1 (glycerol–glycerol-3-P:lactate–alanine) (table 1). Furthermore, the overall carbon balance under anoxia was 57% converted to polyols versus 43% consumed to support biosynthesis. This is much less efficient than the theoretical balance for the aerobic production of glycerol (84% to glycerol versus 16% for support) (Storey & Storey 1988) or the observed value of 73% conversion to glycerol seen for the aerobic situation at 13 °C (table 1). The low efficiency of polyol production in anoxia, coupled with the accompanying high accumulation of glycolytic end products, further confirms that optimal efficiency in the synthesis of glycerol by cold-hardy insects requires aerobic metabolism. This is at odds with a recent suggestion by Kukal *et al.* (1988) that glycerol builds up as an 'anaerobic' product when oxidative metabolism is blocked by a degradation of mitochondria as larvae prepare for winter.

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Our present analysis clearly shows that although the two events (the decrease in mitochondria numbers and glycerol biosynthesis) may be correlated, they cannot be cause and effect. Furthermore, cryoprotectant synthesis is too major an event in the life cycle of an organism to be caused by default because of blocked oxidative metabolism.

Under anoxia at 13 °C, *E. solidaginis* larvae produced two polyols; glycerol was expected but larvae also accumulated substantial amounts of sorbitol. Under normal, aerobic conditions sorbitol never accumulates in *E. solidaginis* at this temperature but is triggered only by exposures below 5 °C (Storey *et al.* 1981). The production of sorbitol in anoxia at 13 °C may be for two reasons. Firstly, by the addition of sorbitol, larvae could arrive at virtually the same total cryoprotectant capacity (assessed as total hydroxyl groups) under either aerobic or anoxic conditions (table 1). Secondly, as was also seen at 3 °C, the accumulation of sorbitol in anoxia suggests an obligate routing of a high proportion of carbon flow through the hexose monophosphate shunt with the consequent need to reoxidize NADPH produced via polyol synthesis. Indeed, without hexose monophosphate shunt participation, net glycerol synthesis in anoxia could only proceed by using the NADH generated at the glyceraldehyde-3-P dehydrogenase reaction of glycolysis. Lactate could not accumulate as an end product of glycolysis (as lactate dehydrogenase utilizes NADH) and the ratio of glycerol:alanine produced would have to be 1:1; i.e., carbon would have to be split evenly between NADH-producing and NADH-utilizing pathways. This was obviously not the observed result and indeed, the total NAD(P)H use associated with product formation in anoxia (124 μmol g^{-1} for glycerol + glycerol-3-P + lactate + sorbitol) far exceeded the NADH availability associated with alanine formation (16 μmol g^{-1}). Again, it is obvious that a high proportion of carbon flow must by routed through the hexose monophosphate shunt in the cold-hardy insect.

3. New insights into the enzymatic control of cryoprotectant synthesis

Recent studies of the metabolic events associated with glycerol synthesis in *E. scudderiana* have added new information about the probable enzymatic controls regulating glycogen conversion to polyols. Figure 4 shows the timecourse of glycerol biosynthesis at −4 °C along with changes in the levels of other associated compounds (Churchill & Storey 1989*b*). Upon exposure to −4 °C, glycerol production was initiated after a lag time of 2 h and levels rose tenfold over 4 days to 450 μmol g^{-1} wet mass. The rate of synthesis was highest between 2 and 12 h, at 9.6 μmol g^{-1} h^{-1}, and half-maximal glycerol concentrations were reached within 30 h.

The first metabolic events seen upon exposure to −4 °C were a 4.5-fold increase in glucose-6-P content and a 50% decrease in glycerol-3-P content after 2 h. The sharp rise in glucose-6-P indicates a rapid activation of glycogenolysis, initiated by the cold-stimulation of glycogen phosphorylase that has been described for this and other species. The drop in glycerol-3-P, the substrate of glycerol-3-phosphatase suggests the concomitant activation of this final enzyme of the biosynthetic pathway. A 60% increase in fructose-1,6-bisphosphate concentration, the product of phosphofructokinase (PFK), also occurred after 2 h and further indicated an activation of the rate-limiting enzyme of glycolysis. The content of fructose-2,6-P_2, a potent activator of PFK, also rose twofold within the initial 2 h at −4 °C and continued to increase (by as much as 40-fold) over the period of active glycerol biosynthesis. The initial activation of glycerol biosynthesis in *E. scudderiana* therefore appears to involve regulation of the glycogen

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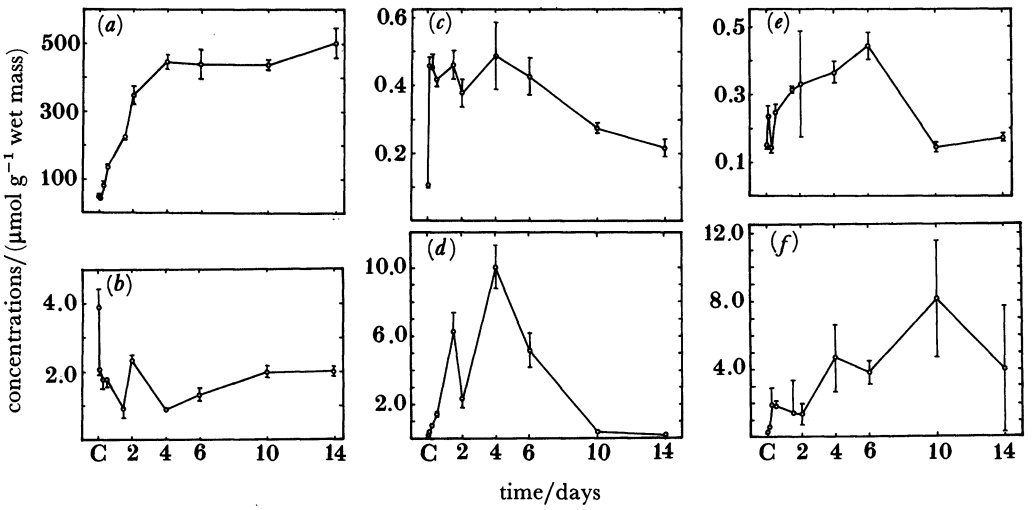

FIGURE 4. Contents of glycerol (*a*), glycerol-3-P (*b*), glucose-6-P (*c*), glucose (*d*), fructose-1,6-P_2 (*e*), and fructose-2,6-P_2 (*f*) in *Epiblema scudderiana* larvae over a timecourse of exposure to −4 °C. Modified from Churchill & Storey (1989*b*).

phosphorylase, PFK, and glycerol-3-phosphatase reactions. The mechanism of glycogen phosphorylase activation is well-known to be protein phosphorylation, brought about by differential temperature effects on the activities of phosphorylase kinase versus phosphorylase phosphatase (Hayakawa 1985). Whether the activations of glycerol-3-phosphatase or PFK, or both, are similarly initiated by covalent modification mechanisms is not yet known but such a system would clearly aid in coordinating enzyme activation to initiate polyol synthesis.

Fructose-2,6-bisphosphate

The role of fructose-2,6-P_2 in the control of glycolysis during glycerol biosynthesis deserves discussion. Since its discovery in 1982, fructose-2,6-P_2 has proven to be one of the most powerful allosteric effectors of PFK and one of the most important regulatory metabolites controlling carbohydrate metabolism in the cell (Hue & Rider 1987). The compound is both a powerful activator of PFK and a strong inhibitor of fructose-1,6-bisphosphatase. Fructose-2,6-P_2 control over these enzymes focuses the actions of a variety of exogenous signals (that alter fructose-2,6-P_2 levels) on these key loci of carbohydrate metabolism. High fructose-2,6-P_2 content typically potentiates the use of carbohydrate reserves for biosynthetic purposes (by activating PFK). However, fructose-2,6-P_2 content falls under conditions when carbohydrate reserves must be conserved (for example, in anoxia) or when gluconeogenic flux is required. The sharp rise in fructose-2,6-P_2 content during the initiation of glycerol biosynthesis in *E. scudderiana* would serve two important purposes. Firstly, by activating PFK, glycolytic flux and the rate of glycerol synthesis can be increased. Secondly, by inhibiting fructose-1,6-bisphosphatase, the potential reconversion of triose phosphates back to hexose phosphates is prevented during the period of active glycerol production when levels of glyceraldehyde-3-P, dihydroxyacetone-P, and fructose-1,6-P_2 are high (Churchill & Storey 1989*b*). Fructose-2,6-P_2 is synthesized by the enzyme 6-phosphofructo-2-kinase (PFK-2). A potent inhibitor of this enzyme is glycerol-3-P (Hue & Rider 1987) and in mammals glycerol-3-P control over PFK-2 is an important component of 'carbohydrate sparing', the inhibitory effect of lipid oxidation

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over carbohydrate catabolism. Glycerol-3-P regulation of the enzyme may also be key to controlling fructose-2,6-P_2 levels in *E. scudderiana*. The sharp drop in glycerol-3-P content that occurred within 2 h at −4 °C (figure 4) would release the inhibition of PFK-2, potentiate fructose-2,6-P_2 synthesis, and thereby set up the allosteric controls on PFK and fructose-1,6-bisphosphatase that ensure unidirectional carbon flow into the glycerol pool.

Inhibitory controls leading to the cessation of glycerol synthesis

Metabolic events associated with the cessation of glycerol synthesis in *E. scudderiana* also provided insights into the enzymatic regulation of the process. Rates of glycerol synthesis decline rapidly over the period from 2 to 4 days at −4 °C (figure 4). Metabolite changes at the 2 day time point included a sharp increase in glycerol-3-P content as well as elevated levels of glyceraldehyde-3-P, dihydroxyacetone-P and fructose-1,6-P_2 (Churchill & Storey 1989*b*). It appears that the first event in slowing glycerol synthesis is probably an inhibition of the glycerol-3-phosphatase reaction that then leads to an accumulation of intermediates in the glycolytic pathway. Excess carbon begins to pour into the glucose pool and glucose levels rise as high as 10 μmol g^{-1} after 4 days. Both the accumulation of glucose and glycolytic intermediates and direct measurements of glycogen phosphorylase activity (still 74 % of *a* after 6 days at −4 °C) suggest that the inactivation of glycogen phosphorylase is somewhat out of phase with the inhibition of the glycerol-3-phosphatase locus. However, this apparent lack of coordination between the initial and terminal enzymes of the synthetic pathway may, in fact, be necessary to shut off the phosphorylase. The initial activation of phosphorylase was in response to the stimulating effect of low temperature on the enzyme (figure 5). High temperature is known to have the reverse effect in insects, inhibiting phosphorylase and activating glycogen synthase (Hayakawa & Chino 1982) and the effects of high temperature on these enzymes are undoubtedly key to the clearance of cryoprotectant in the spring. However, the cessation of glycerol synthesis under the conditions of the present experiment, exposure to constant −4 °C, could not be caused by a high temperature signal. The same would be true in nature as glycerol accumulation during autumn cold hardening occurs against a background of constantly decreasing environmental temperatures. What then turns off phosphorylase at low temperatures? The answer is glucose.

The regulation of glycogen metabolism has been extensively studied in mammalian liver and a central role for homeostatic regulation by glucose has been described for the reciprocal control of glycogen phosphorylase versus glycogen synthase activities (Hers 1976). The mechanism involved is as follows. Glycogen phosphorylase *a* (the active form) is allosterically inhibited by glucose. Phosphorylase phosphatase activity is increased in the presence of glucose and converts phosphorylase *a* back to *b* (figure 5). This is not an allosteric effect of glucose on the phosphatase but arises because a glucose–phosphorylase *a* complex is the preferred substrate for phosphatase action (Hers 1976). Indeed, in the case of insects at low temperature, a glucose–phosphorylase *a* complex may be the only effective substrate for phosphorylase phosphatase, the enzyme being otherwise cold-inactivated. Hayakawa (1985) found that fat-body phosphorylase phosphatase was virtually inactive at 0 °C with rabbit muscle phosphorylase *a* as its substrate. This mechanism would provide the means of maintaining high phosphorylase activity until such time as cryoprotectant levels reached a maximum. Then, as synthesis slowed and intermediates of the pathway began to be accumulated, an 'overflow' accumulation of glucose would occur. Glucose inhibition of phosphorylase and activation of

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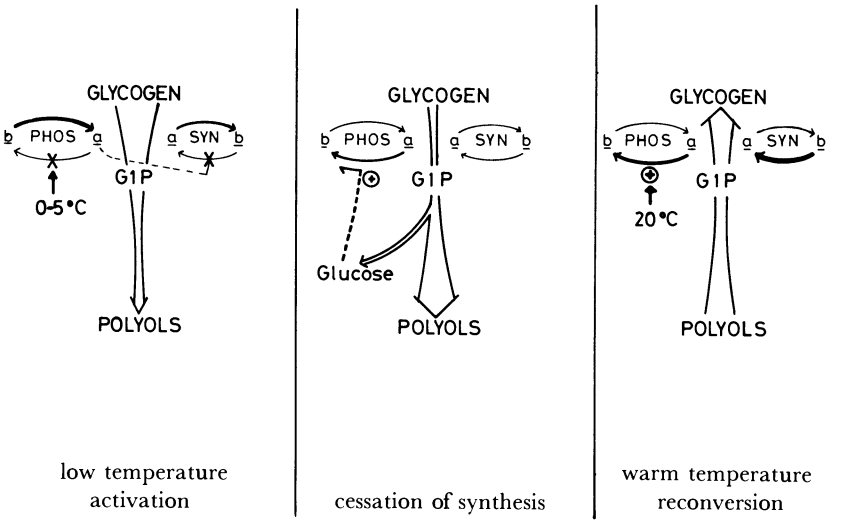

FIGURE 5. Control of glycogen metabolism in cold-hardy insects showing the reciprocal regulation of glycogen phosphorylase and glycogen synthetase by metabolite effectors and reversible protein phosphorylations and the interactions of temperature with these processes.

phosphorylase phosphatase would provide the means of returning glycogen phosphorylase activity to low levels.

4. MECHANISMS OF REGULATION OF GLYCOLYTIC ENZYMES IN A FREEZE-TOLERANT INSECT

E. solidaginis produces both glycerol and sorbitol as cryoprotectants. The capacity for glycerol synthesis is put in place with the molt to the third instar, the over-wintering form; synthesis occurs over the early autumn with the rate of production responding to environmental stimuli: cooling below about 15 °C or the dehydration/death, or both, of the goldenrod plant (figure 2) (Storey & Storey 1983; Rojas *et al.* 1986). Sorbitol synthesis, in contrast, occurs in the late autumn and is directly stimulated by low temperature, the trigger lying between 0 and 5 °C (Storey & Storey 1983; Rojas *et al.* 1983). To accomplish the differential synthesis of the two polyols requires specific regulation of the synthetic pathways involved, in particular, control over key regulatory enzymes. The activation of glycogen phosphorylase provides the substrate for polyol synthesis but cannot determine the resulting polyol product. That requires regulation at other sites in the synthetic pathway. During glycerol production, for example, inhibition at pyruvate kinase helps to divert triose phosphates into the pathway of glycerol synthesis (Storey & Storey 1983). For the differential production of sorbitol versus glycerol, control at PFK is very important. An active PFK is required to permit glycerol synthesis whereas inhibition of PFK is the key to diverting hexose phosphates out of the glycolytic pathway for sorbitol production.

PFK is a complex regulatory enzyme that is subject to many types of controls including allosteric regulation by a wide variety of inhibitors and activators, reversible protein phosphorylation, polymerization of the enzyme tetramers, and reversible binding associations with subcellular particles. We have previously analysed the allosteric regulation and temperature dependence of *E. solidaginis* PFK. Total enzyme activity increases slightly (about

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20%) during cold acclimation of the larvae (Storey & Storey 1981) but the properties of the enzyme are such that PFK activity would be strongly reduced at low temperature, providing the means for diverting carbohydrate flux into sorbitol synthesis (Storey 1982). Included in the mechanism of PFK control are: (i) strong temperature effects on enzyme activity, a Q_{10} of 3.6 compared to 2 for beef-heart PFK; (ii) a decrease in enzyme affinity for fructose-6-P at low temperature; (iii) reduced effects by activators (AMP, fructose-2,6-P_2) at low temperature; (iv) elevated levels of enzyme inhibitors (glycerol-3-P, sorbitol) and reduced levels of enzyme activators (fructose-2,6-P_2) *in vivo* at low temperature (Storey 1982; Storey & Storey 1989*b*). Interestingly, our recent studies of *E. solidaginis* metabolism during active polyol synthesis have shown that although glycerol-3-P builds up rapidly (from 3 to about 20 μmol g^{-1}) when glycerol synthesis is initiated, the compound is not cleared when glycerol production ends (Storey & Storey 1989*b*). Instead levels of 15–20 μmol g^{-1} remain in cold acclimated larvae producing sorbitol and this high inhibitor content may be important for limiting PFK function during sorbitol synthesis. Furthermore, as discussed previously, glycerol-3-P is also a powerful inhibitor of PFK-2 and this inhibition may be the means of depressing fructose-2,6-P_2 levels (content of the PFK activator drops by 50%) during the initiation of sorbitol synthesis (Storey & Storey 1989*b*) despite the increased availability of hexose phosphates as potential substrates for PFK and PFK-2 at this time.

Another key mechanism of PFK control in animal systems is post-translational modification of the enzyme via the incorporation of covalently bound phosphate. PFK in both vertebrate and invertebrate systems can be regulated in this way; organ-specific effects of phosphorylation can modify affinities for substrates or allosteric effectors as well as enzyme binding to F-actin to increase or decrease activity *in vivo* (Pilkis *et al.* 1987; Storey 1988). Typically, changes in the phosphorylation state of PFK are coordinated with the covalent modification of glycogen phosphorylase, pyruvate kinase, and PFK-2 to create an integrated response by glycolysis to a metabolic stimulus. Because it is well known that the activation of glycogen phosphorylase during polyol synthesis in cold-hardy insects is caused by enzyme phosphorylation (Hayakawa 1985), we wondered whether reversible phosphorylation also played a role in the control of PFK and pyruvate kinase during the activation of glycerol or sorbitol synthesis in *E. solidaginis*. Changes to the phosphorylation state of these enzymes typically alter the measured kinetic constants of the enzymes, often by several orders of magnitude, and as such, distinct changes in enzyme kinetic constants as a reponse to metabolic stimulus are frequently used to diagnose enzyme phosphorylation or dephosphorylation (Pilkis *et al.* 1987; Engstrom *et al.* 1987; Storey 1988).

Abrupt temperature switches from 23 to 13 °C or from 13 to 3 °C were used to stimulate the production of glycerol or sorbitol, respectively, by *E. solidaginis* larvae. The metabolic events accompanying polyol synthesis were analysed over a timecourse of up to 18 days at the new temperature (Storey & Storey 1989*b*). After 10 days at the new temperature, synthesis of either polyol was well underway, supported by increased activities of the phosphorylated, active form of glycogen phosphorylase (table 2). Table 2 also shows the kinetic constants of PFK and pyruvate kinase assayed from larvae in four situations: controls at 23 °C, 10 days after switching from 23 to 13 °C, controls at 13 °C, and 10 days at 3 °C. There were, however, no significant changes in the kinetic constants of either enzyme between the four experimental conditions. It appears that alterations to the phosphorylation state of PFK and pyruvate kinase are not part of the mechanism, therefore, for the control of polyol synthesis in *E. solidaginis*.

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TABLE 2. KINETIC PROPERTIES OF PHOSPHOFRUCTOKINASE AND PYRUVATE KINASE IN *EUROSTA SOLIDAGINIS* LARVAE UNDER CONDITIONS OF LOW TEMPERATURE EXPOSURE THAT STIMULATE THE SYNTHESIS OF GLYCEROL (23 TO 13 °C) VERSUS SORBITOL (13 TO 3 °C)

(Acclimation and experimental manipulation of larvae and data for polyol concentrations are taken from Storey & Storey (1989*b*). The preparation of enzyme extracts and enzyme assay were essentially as described by Storey (1987), including the use of EDTA, EGTA, and NaF in homogenates to inhibit the action of endogenous protein kinases and protein phosphatases and assay at 23 °C. Modifications to assay conditions included: for PFK, optimal ATP substrate concentration was 2 mM; I_{50} and K_a values were determined at 0.8 and 0.4 mM fructose-6-P, respectively; for PK, I_{50} values were determined at 0.1 mM P-enolpyruvate. Data are means ± s.e.m., $n = 3$–5 determinations in separate samples.)

	23 °C → 13 °C		13 °C → 3 °C	
	control	10 days at 13 °C	control	10 days at 13 °C
polyol content				
glycerol	106 ± 9	187 ± 12	133 ± 25	156 ± 3
sorbitol	0.17 ± 0.01	0.49 ± 0.10	1.6 ± 0.09	52.3 ± 3.3
glycogen phosphorylase				
total activity $(a+b)$/U g^{-1} wet mass	0.59 ± 0.06	1.14 ± 0.50	0.77 ± 0.13	2.04 ± 0.10
percentage a	45 ± 5.5	71 ± 3.7	46 ± 5.8	62 ± 3.3
phosphofructokinase				
V_{max}/U g^{-1} wet mass	1.74 ± 0.15	2.07 ± 0.09	1.84 ± 0.13	1.89 ± 0.10
K_m fructose-6-P/mM	1.62 ± 0.12	1.68 ± 0.11	1.72 ± 0.14	1.58 ± 0.43
Hill coefficient	2.18 ± 0.33	2.00 ± 0.14	1.72 ± 0.12	1.88 ± 0.58
I_{50} Mg.ATP/mM	4.53 ± 0.84	3.03 ± 0.32	4.00 ± 0.46	4.80 ± 0.99
K_a fructose-2,6-P_2/μM	0.43 ± 0.06	0.37 ± 0.04	0.43 ± 0.07	0.25 ± 0.05
pyruvate kinase				
V_{max}/U g^{-1} wet mass	75.4 ± 6.7	82.9 ± 3.6	85.9 ± 1.7	71.9 ± 1.9
K_m P-enolpyruvate/mM	0.21 ± 0.05	0.18 ± 0.04	0.21 ± 0.04	0.16 ± 0.01
Hill coefficient	1.30 ± 0.23	1.46 ± 0.27	1.27 ± 0.19	1.37 ± 0.15
K_m in presence of 200 μM fructose-1,6-P_2/mM	0.06 ± 0.005	0.06 ± 0.002	0.08 ± 0.01	0.07 ± 0.004
I_{50} L-alanine/mM	16.9 ± 3.6	13.0 ± 2.3	10.4 ± 1.7	9.0 ± 0.76

Covalent modification regulation may be limited to the control of glycogen phosphorylase activity although other potential enzymes that might be regulated in this manner could include PFK-2 and glycerol-3-phosphatase.

An alternative role for phosphorylation control of the activity state of enzymes in freeze-tolerant insects could be in regulating the transition to the frozen state. The presence of extracellular ice imposes upon cells an ischemic state in which oxygen is rapidly depleted; cells must rely, for long term survival, upon the fermentation of endogenous fuel reserves to supply the ATP needs of metabolism (Storey & Storey 1985). Frequently, the response by animals to an environmental insult is a withdrawal into a hypometabolic or dormant state; anoxia tolerance, hibernation, estivation, and diapause all incorporate metabolic-rate depression (lowering metabolic rate to 1–20% of normal, resting, rate) as a key component of the survival strategy (Storey 1988). One of the universal molecular mechanisms of metabolic rate depression has proven to be the covalent modification of proteins. The mechanism is widely applicable to many proteins in the cell (including many regulatory enzymes, membrane ion channel proteins, and ATP-linked ion pumps) and via coordinated covalent modification of key cellular reactions, a balanced reduction in the rates of all cellular processes can be achieved. We wondered whether covalent modification controls would be applied to regulatory enzymes in *E. solidaginis* as a part of the transition to the frozen state. As the control of glycolysis is key to fermentative ATP production while frozen (lactate and alanine accumulate as glycolytic end

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TABLE 3. EFFECT OF NATURAL FREEZING AT −16 °C ON THE PROPERTIES OF REGULATORY ENZYMES OF FERMENTATIVE ATP PRODUCTION IN *EUROSTA SOLIDAGINIS* LARVAE

(Enzyme preparation and assay were as in table 2 but data are $n = 1$ determination.)

	control	time frozen at −16 °C		
	3 °C	4 h	2 days	14 days
glycogen phosphorylase				
total activity $(a+b)$/U g^{-1} wet mass	1.63	1.92	1.88	2.51
percentage a	51	78	100	80
phosphofructokinase				
V_{max}, U g^{-1} wet mass	1.63	1.72	1.88	2.34
K_m fructose-6-P/mM	2.49	1.62	1.78	1.82
Hill coefficient	1.94	1.74	2.08	2.07
I_{50} Mg.ATP/mM	7.4	4.6	6.6	10.6
K_a fructose-2,6-P$_2$/μM	0.6	0.55	0.45	0.55
pyruvate kinase				
V_{max}/U g^{-1} wet mass	80.2	70.4	79.5	77.8
K_m P-enolpyruvate/mM	0.14	0.15	0.18	0.13
Hill coefficient	1.54	1.28	1.04	0.97
K_m in presence of 200 μM fructose-1,6-P$_2$ mM	0.05	0.06	0.07	—
I_{50} L-alanine/mM	11.5	9.0	11.3	10.8

products) (Storey & Storey 1985), we focused on the properties of glycogen phosphorylase, PFK, and pyruvate kinase over a timecourse of freezing exposure at −16 °C (table 3).

Freezing resulted in an activation of glycogen phosphorylase, the percentage of phosphorylase *a* doubling after 2 days at −16 °C. This probably represents the cold-shock activation that is typical of insect phosphorylases but as phosphorylase activity remained high (80 % *a*) even after 14 days of freezing, enzyme activation may also contribute to the supply of hexose phosphates for fermentative ATP production in the frozen state. Again, however, no indication of covalent modification of PFK or pyruvate kinase was found; neither enzyme showed a modification of kinetic properties over time in the frozen state. It may be that the transition to the frozen state requires few specific metabolic adjustments. The reduction in metabolic rates imposed by the low ambient temperature may be sufficient to sustain long-term survival, based on anaerobic metabolism alone, during periods of freezing without the need for additional and specific metabolic rate depression. Alternatively, the physical alterations of the intracellular environment (reduced water content, high solute and polyol content) that are the result of extracellular freezing may influence cellular metabolism in a manner that enhances long-term survival in the ischemic frozen state. However, whatever mechanisms are involved, the capacity for freezing survival appears to greatly exceed the actual need. Thus *E. solidaginis* larvae readily survived 12 weeks of constant freezing at −16 °C (Storey & Storey 1985) although assessment of the ambient temperature profile faced by the same animals in the natural environment suggested that 9 days was probably the maximal time that constant freezing would be experienced (Storey & Storey 1986).

5. CONCLUDING REMARKS

The picture that is emerging of polyol synthesis in cold-hardy insects is one of a seasonally-dependent reorganization of carbohydrate metabolism that appears to bring together the requisite glycogenolytic, glycolytic, and hexose monophosphate shunt enzymes into an efficient

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association that can channel glycogen into polyol products and is regulated in response to specific temperature and metabolite controls. A variety of problems remain to be assessed. Our studies have, for example, implied the occurrence of regulatory controls over glycerol-3-phosphatase activity and the probable role for glucose in the enzymatic regulation of glycogen phosphorylase but neither of these proposals has yet been assessed experimentally. A cold-hardy insect model may also prove to be an excellent system in which to study enzyme-complex formation as the proposed association between glycogen, glycogen-metabolizing enzymes, and polyol-producing enzymes should be a prominent feature of the fat-body cells of these animals. The metabolic 'reasons' for the production of alternative cryoprotectants such as ribitol, threitol, erythritol, and ethylene glycol also need to be explored in appropriate species. Because of CO_2 loss in the conversion of glycogen to these C5, C4, and C2 polyols, their synthesis appears to be inefficient in terms of carbon conservation but there may be energetic or redox balance reasons for the production of these compounds.

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Discussion

D. R. Wilkie, F.R.S. (*Physiology Department, University College London, U.K.*). Two ways of expressing the temperature-dependence of chemical and biological processes. My question is directed not only to Professor Storey but also to the meeting as a whole. I noticed that Professor ap Rees expressed the variation with temperature of the rates of various biochemical reactions in the empirical way usually favoured by biologists, that is, as a Q_{10} (the ratio by which the rate increases for every 10 °C or K rise in temperature).

$$k_2/k_1 = Q_{10}^{(T_2-T_1/10)}, \tag{1}$$

where T_2 and T_1 are the two temperatures and k_2 and k_1 are the corresponding rates.

On the other hand, Professor Franks and Professor Storey have preferred to relate k_2 and k_1 by using the Arrhenius equation:

$$k = A \times \exp(-E_{act}/RT), \tag{2}$$

where A is the pre-exponential constant and E_{act} is the activation energy.

Although the two equations are fundamentally different from the physical point of view, they often seem to fit experimental results equally well. A few years ago I began to wonder whether there might be at least a mathematical relation between the two equations; I concluded that there was one:

$$E_{act} = R \times \ln Q_{10} \times T_1\, T_2/10. \tag{3}$$

As the temperature-interval being considered is reduced, this equation approaches:

$$E_{act} = R \times \ln Q_{10} \times T^2/10. \quad (4)$$

These equations unite the empirical and the physico–chemical approaches to temperature dependence, and make it simple to convert from one to the other. From the practical point of view, if T is set at 20 °C, and a value of E_{act} or Q_{10} obtained, by using equation (4), it will be found that equations (1) and (2) fit experimental results almost equally well over a temperature range of at least 0 °C–40 °C. There is not space here to explain the merits and drawbacks of the two equations.

I doubted that I could be the first to discover these simple and fundamental relations, but an extensive search through books on physical chemistry and general biology failed to reveal either equation (3) or (4), so they are certainly not readily accessible. Accordingly, I asked everybody at the meeting whether they had come across either equation.

The only response was from Dr Kruger of the Rothamsted Experimental Station (Harpenden, U.K.) who said that he knew of a treatment of this problem, and an equation somewhat similar to my equation (4), in the Encyclopedia of plant physiology, for which he kindly sent me a reference. Dr Kruger and I have entered into a fruitful correspondence on the subject; I hope that this will lead to a fuller publication.

Reference

Berry, J. A. & Raison, J. K. 1981 Responses of macrophages to temperature. In Physiological plant ecology I (ed. O. L. Lange, P. S. Nobel, C. B. Osmond & H. Ziegler), pp. 292–294. Berlin, Heidelberg, New York: Springer-Verlag.

A. Cairns (*Welsh Plant Breeding Station, Plas Gogerddan, Aberystwyth, U.K.*). Professor Storey presented convincing evidence showing the accumulation of glycerol and sorbitol in the tissues of *Eurosta* larvae at the expense of glycogen breakdown, in response to low temperature. The accumulation of these compounds is implicated in the cryotolerance of the organism, although Professor Storey did not attempt to correlate the presence or absence of these compounds with the cold tolerance of larvae.

The biochemical explanation for this phenomenon is based upon the measured differential cold-sensitivity of enzymes of glycolysis. Low temperature causes a block of specific steps in the pathway and a resultant accumulation of glycolytic intermediates. The consequence of this accumulation is an 'overspill' of carbon compounds into the pathways leading to glycerol and sorbitol formation. Measurements of the inhibition of specific glycolytic enzyme activities by low temperature were presented in support of this mechanism.

Professor Storey did not present data concerning the activities of enzyme(s) of sorbitol synthesis, particularly, whether these activities changed before accumulation, implicating the activation of latent activity or synthesis of new enzyme protein, or whether these enzymes have specific low temperature adaptation, for example, a low temperature activity optimum.

My interest in the system was to decide between the following possibilities: (i) is the increase in sorbitol synthesis a specific adaptive mechanism resulting in the accumulation of a functional cryoprotectant? or (ii) is the accumulation of sorbitol an accidental consequence of altered metabolism at low temperature?

If the former is true, then we might expect to observe an increase in the activity of sorbitol-synthesising enzymes before the accumulation of sorbitol; if the latter is true we may expect no increase in extractable amounts of enzyme.

Dr Storey said that the enzymes showed a slight increase in activity (roughly twofold) before sorbitol accumulation. When asked if he felt that sorbitol had a specific function in cryotolerance, he replied that as a biochemist, he observed only the changes in metabolism and he was not prepared to speculate on a specific crytoprotective role for sorbitol.

I would be interested to know if larvae which have had the opportunity to accumulate glycerol and sorbitol have improved cryotolerance, relative to larvae in which no accumulation has taken place.

K. B. Storey. Details of the control of cryoprotectant production and the levels of enzymes present in freeze tolerant and freeze intolerant insects can be found in Storey *et al.* (1981); Storey & Storey (1988).

As to the final point, larvae which have not built up glycerol have less cryotolerance than larvae that have high levels of glycerol. However, warming larvae causes dramatic drops in sorbitol levels and these warmed larvae are still cryotolerant despite little or no sorbitol. Glycerol levels do not decrease upon warming, however, so no real conclusions can be drawn.

Additional reference

Storey, K. *et al.* 1981 *J. comp. Physiol.* **144**, 183–190.

H. Thomas (*Institute of Grassland and Animal Production, Aberystwyth, U.K.*). Malonate and arsemite are known to induce the synthesis of certain heat proteins and to improve thermotolerance. It has been suggested that flux through respiratory pathways is the factor responsible for induction. Is there evidence of protein induction under the conditions of low-temperature restriction of respiratory flux that Professor Storey describes?

K. B. Storey. There is no available evidence to support the production of 'shock' proteins at this point, in either the freeze tolerant or freeze intolerant insects that I have presented data on. We have looked for new isozymic forms of enzymes, newly synthesized proteins and new glycoproteins in previous studies (Storey *et al.*) and found no new proteins formed. We have not yet tried the use of radioactive label (S^{35}-Met, for example) incorporation during cold/freeze stress. This type of experiment may show new synthesis.

Reference

Storey, K. *et al.* 1981 *Cryoletters* **2**, 279–284.

D. A. Cowan (*Department of Biochemistry, University College London, U.K.*). Is there any evidence that concentration of solutes in freeze-tolerant organisms can result in changes in enzyme activity or specificity via a reduction in water activity?

K. B. Storey. High concentrations of the solutes produced as 'cryoprotectants' in freeze tolerant organisms can cause changes in enzyme activity, enzyme polymeric state and in

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enzyme specificity (for one example, see Yamamoto & Storey (1988)). It is not yet clear whether these changes in the enzyme are a result of reduced water activity *per se*.

Reference

Yamamoto, S. & Storey, K. B. 1988 *Int. J. Biochem.* **20**, 1267–1271.

J. Baust (*Centre for Cryobiological Research, State University of New York, U.S.A.*). Should not the freeze concentration that occurs in the cells of *Eurosta* following their crystallization cause an apparent increase in adenylates, as with other solutes? If the adenylates are so concentrated, the cells may then be considered to be 'supercharged' when compared to the unfrozen cell at the same temperature?

K. B. Storey. Freeze concentration does occur for the cells of *Eurosta* during freezing, with solute levels rising as a result of over 60% of total body water freezing as ice. Interestingly, although the adenylates should be concentrated in this situation, the amount of *free* ATP, ADP and AMP actually drop in *Eurosta* even before freezing occur. This curious phenomena is shown by N.M.R. studies (see Storey, K. B. *et al. Eur. J. Biochem.* **142**, 591–595): as temperature decreases even though total adenylates stay constant, the free adenylate pool – the pool that is metabolically active – decreases. It approaches zero and remains very low even though after freezing starts cell contents are being concentrated. Adenylates are being bound up at low temperatures by protein components and so are not concentrated even though the cell loses water.

The low temperature and/or freeze-concentrated *Eurosta* cell has almost no metabolically active ATP available for metabolic function and this may be one of the reasons that many cell processes cease under these conditions.

A. S. Pullin (*Department of Pure and Applied Biology, Agricultural Sciences Building, University of Leeds, U.K.*). Would Professor Storey comment on the possible mechanisms controlling cryoprotectant synthesis in those insect species which are known to accumulate cryoprotectants, in the absence of any low temperature exposure, but in response to the induction of diapause?

K. B. Storey. The critical biochemical 'switch' that turns on the production of sugar and polyol cryoprotectants appears to be the activation of glycogen phosphorylase. This enzyme is normally mainly in a sub-active (b) form and is stimulated by enzymatic phosphorylation to an active (a) form (see Storey & Storey (1988) for further details). This stimulation of glycogen phosphorylase can be triggered by other factors than temperature and so insects that do not see low temperature, but which are stimulated by another signal to activate phosphorylase will build up cryoprotectants via the breakdown of stored glycogen. The signals that turn on glycogen phosphorylase can be varied: increased cell calcium levels, hormones that act via cAMP, even physical wounding causes conversion of the enzyme to an active form. Some factor in diapausal transition, acting at high temperatures, is responsible for the activation of glycogen phosphorylase, the breakdown of glycogen and the eventual production of sugar and polyols.

N. J. Russell (*Department of Biochemistry, University of Wales College of Cardiff, U.K.*). If there is

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quantitative conversion of glycogen to sugar polyols following a decrease in temperature, what does the insect use as a source of energy?

The conversion of glycogen into 'cryoprotectants' – sorbitol plus glycerol in *Eurosta*, glycerol in *Epiblema*, even glucose in the freeze tolerant frog *Rana sylvatica* is nearly quantitative. Some carbon (between 1% and 10% of the total, escapes down glycolysis and into the TCA cycle and is utilized for energy (see Storey & Storey (1988) for details). In addition, at high temperatures at least, the insects are capable of oxidizing fat as a substrate via mitochondrial oxidations (see Ballantyne & Storey (1985)). Oxidation of fatty acids would yield ATP and serve to spare glycogen stores that would then be diverted to cryoprotectant production.

Additional Reference

Ballantyne, J. & Storey, K. B. 1985 *Can. J. Zool.* **63**, 373–379.

R. Jaenicke (*Institüt für Biophysik und Physikalische Biochemie, Universität Regensburg, Regensburg, F.R.G.*). One of Professor Storey's slides combined the effect of polyols and high salt on the enzymes involved in glycerol and sorbitol synthesis. Do the enzymes exhibit halophilic characteristics?

K. B. Storey. Our work on the effects of salts and cryoprotectants on the enzymes of *Eurosta* (freeze tolerant insect) and *Epiblema* (freeze intolerant insect) have concentrated on enzymes of energy metabolism only and do not let us generalize to other enzymes or to structural proteins found in these insects. Within these limitations, however, we have found that in general the enzymes of these insects are not specially constructed and show no greatly increased tolerance to high levels of salt compared with the enzymes of other insects.

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