J. Insect Physiol. Vol. 32, No. 10, pp. 897-902, 1986
Printed in Great Britain

0022-1910/86 $3.00 + 0.00
Pergamon Journals Ltd

EFFECT OF TEMPERATURE ACCLIMATION ON HAEMOLYMPH COMPOSITION IN THE FREEZE-TOLERANT LARVAE OF *EUROSTA SOLIDAGINIS*

KENNETH B. STOREY*, D. GORDON MCDONALD† and CHARLES E. BOOTH†‡
Institute of Biochemistry and Department of Biology, Carleton University, Ottawa, Ontario and †Department of Biology, McMaster University, Hamilton, Ontario K1S 5B6, Canada

(*Received* 13 *December* 1985; *revised* 24 *February* 1986)

Abstract—The physiological and biochemical composition of haemolymph from warm (23°C) and cold (3°C) acclimated *E. solidaginis* larvae was assessed in a search for haemolymph adaptations for freeze tolerance. Haemolymph pH and total carbon dioxide content did not vary between the two groups although the effect of temperature on pH ($\Delta pH/\Delta T$) was higher for warm acclimated (−0.0142 U/°C) than for cold acclimated (−0.0116 U/°C) larvae. Haemolymph from cold-acclimated larvae had a 2-fold higher Mg^{2+} content and was also higher in Na^+ and Ca^{2+} but lower in K^+ than haemolymph from warm-acclimated larvae. Both groups accumulated cryoprotectants over the course of acclimation, glycerol in warm acclimated and glycerol + sorbitol in cold-acclimated larvae, with equal hydroxyl equivalents achieved in the two groups but a higher osmolarity in the haemolymph of the warm-acclimated larvae. Citrate content of the haemolymph was 48% higher in cold-acclimated larvae. Contents of polyols, sugars, organic acids, amino acids and protein were also measured in whole larvae and comparisons with haemolymph levels revealed that polyols, organic acids and all amino acids except glutamate were probably in equilibrium between intra- and extracellular fluids while sugars (glucose, fructose) were preferentially concentrated in the haemolymph and protein content was higher in cells. Amino acid composition varied between warm and cold-acclimated larvae with the larger pool, due to a higher proline content, in the haemolymph of warm-acclimated animals. Concentrations of several neutral amino acids (threonine, serine, glycine, valine) were higher in the haemolymph of cold-acclimated larvae while levels of charged amino acids (asparagine, arginine) were lower.

Key Word Index: *Eurosta solidaginis*, freeze tolerance, cold hardiness, haemolymph composition, acid-base balance, haemolymph ions, amino acid composition

INTRODUCTION

The larvae of the goldenrod gall fly, *Eurosta solidaginis*, are freeze tolerant in the 3rd instar, overwintering form (Morrissey and Baust, 1976). These animals have been extensively used as a model system for studies of the biochemical and physiological adaptations required for freeze tolerance. Adaptations include: (a) Accumulation of high concentrations of glycerol and sorbitol as cryoprotectants with different roles for the two polyols (Morrissey and Baust, 1976; Storey *et al.*, 1981a; Storey and Storey, 1983a; Rojas *et al.*, 1983), (b) the use of nucleating agents in the haemolymph to control extracellular freezing (Zachariassen *et al.*, 1982), (c) alterations to cellular composition to increase the content of bound (versus free) water (Storey *et al.*, 1981b), (d) some modifications to cellular enzyme activities and to mitochondrial oxidative metabolism (Storey and Storey, 1981; Ballantyne and Storey, 1985), and (e) a good capacity for surviving anaerobic conditions in the frozen state (Storey and Storey, 1984). Freezing is limited to extracellular compartments. The presence of proteinaceous nucleating agents in the haemolymph allows the controlled formation of ice at relatively high subzero temperatures (about −8°C). Cryoprotectants in the haemolymph, although excluded from growing ice crystals (leading to a higher concentration inside cells) may act to reduce the amount of ice on a colligative basis, blunt the growing crystals, and reduce potential physical damage to cell membranes.

Although some physical properties (freezing, melting and supercooling points) of *E. solidaginis* haemolymph have been characterized (Morrissey and Baust, 1976; Baust, 1981) and the presence of nucleating agents and cryoprotectants has been established (Zachariassen *et al.*, 1982; Morrissey and Baust, 1976), the majority of studies to date have focussed on cellular metabolic adaptations for freeze tolerance (for reviews see Storey, 1983, 1984; Storey and Storey, 1983b). The present study concentrates on the physiological and biochemical properties of *E. solidaginis* haemolymph to determine whether cold acclimation results in other types of changes in the haemolymph composition which would be adaptive for freeze tolerance.

MATERIALS AND METHODS

Animals

Galls containing *E. solidaginis* larvae were collected from fields around Ottawa in late September

*To whom correspondence should be addressed.
‡Present address: Department of Biology, Eastern Connecticut State University, Willimantic, CT 06226, U.S.A.

1983. Galls were placed at one of two temperatures, 23°C (room temperature) or 3°C (in a refrigerator), and were acclimated to these temperatures for 1 month.

Chemicals

Biochemicals and coupling enzymes were from Sigma Chemical Co. or Boehringer Mannheim Corp.

Sampling of haemolymph

A small hole was clipped in the cuticle of larvae with scissors and larvae were then placed in 1.5 ml conical plastic centrifuge tubes. Animals were centrifuged at low speed for 2 min using a bench top centrifuge. Haemolymph collected in the bottom of the tube was sampled with a Pasteur pipette or with a Hamilton 500 μl gas-tight syringe.

Metabolite analysis

Haemolymph. Haemolymph from about 25 larvae was pooled per sample and mixed with 4 vol 6% perchloric acid containing 1 mM EDTA. This was centrifuged at 6000 *g* for 15 min to pellet protein. The supernatant was removed and neutralized by the addition of 3 N KOH–0.3 M imidazole–0.4 M KCl. After a second centrifugation, the neutralized supernatant was stored at −80°C until analysis.

Whole larvae. Whole larvae were weighed and homogenized 1:4, w/v, in perchloric acid and extracts were processed as above. Four larvae were pooled per sample.

Metabolite measurements. Glycerol, sorbitol, glucose, fructose, citrate and malate were determined enzymatically as described by Storey *et al.* (1981). Precipitated protein was dissolved in 0.2 N NaOH and then measured using the Coomassie blue dye-binding method with prepared reagent from Bio-Rad Laboratories and bovine gamma globulin as the standard. Amino acids were analyzed on a Beckman amino acid analyzer.

Analysis of haemolymph ion and acid-base status

Haemolymph samples of 200–400 μl volume (obtained as above but on 6–10 animals pooled) were collected anaerobically from the centrifuge tube into a gas-tight syringe and analyzed immediately for pH and total carbon dioxide. The remaining volume was stored at 3–4°C for later analysis of osmolarity and ion concentrations. For pH measurements, 30–50 μl samples were injected into a Radiometer G297 capillary pH electrode calibrated with standard phosphate buffers (Analytical Products Inc., Nos. 14-191 and 14-209) and thermostated with an Endocal RTE-9 chilling recirculator to either 5 or 18°C. Total carbon dioxide measurements (C_{CO_2} in Mol/ml) were made on 15–20 μl samples injected into a Capnicon carbon dioxide analyzer (Cameron Instruments Inc.). To increase the precision of this measurement each unknown was bracketed by injections of 20 μl of 4.0 μMol/ml $NaHCO_3$ standard. Haemolymph cations (Na^+, K^+, Ca^{2+} and Mg^{2+}) were determined on appropriately diluted 8 μl aliquots using a Varian AA 1275 atomic absorption spectrophotometer. Chemical interferences with Ca^{2+} and Mg^{2+} measurements were eliminated by addition to the diluted sample of $LaCl_2$ to a final concentration of 0.02%. Chloride measurements were made without dilution on 8 μl samples injected into a Radiometer CMT-10 chloridometer. Osmolarity (in millosmoles/litre) was determined on 8 μl samples injected into a Wescor vapour pressure osmometer. Measurement precision was estimated to be within ± 0.5 μMol/ml for C_{CO_2} and all ions and within ± 5 μOsm/ml for osmolarity.

RESULTS

Average animal weights were 36.9 ± 2.5 mg for warm (23°C)-acclimated larvae and 55.1 ± 1.4 mg for cold (3°C)-acclimated larvae, the lower weight of warm-acclimated animals reflecting the utilization of endogenous fuels during the acclimation period. However, water content of both groups was identical, $58.6 \pm 0.9\%$ of total weight for warm-acclimated larvae and $58.5 \pm 0.3\%$ for cold-acclimated larvae. This agrees with the 60–64% water content reported by Baust (1983).

Physiological parameters of the haemolymph are given in Table 1. Haemolymph pH was not significantly different between the two groups when measured at either 5 or 18°C. However, both warm- and cold-acclimated larvae showed increasing haemolymph pH as the assay temperature decreased; the change, $\Delta pH/\Delta T$, being −0.0142 units/°C for warm-acclimated larvae and −0.0116 unit/°C for cold-acclimated larvae. Total haemolymph carbon dioxide content was the same in both groups but haemolymph osmolarity was 18% higher in warm-acclimated larvae. The osmolarity difference was largely due to differences in haemolymph cryoprotectant content (see below). Total concentration of measured ions was slightly higher in the haemolymph of cold-acclimated larvae, 155.3 ± 3.0 vs 143.3 ± 4.5 μEquiv/ml. Individual ions varied somewhat between the two groups, the largest difference being a 2-fold higher Mg^{2+} content in the haemolymph of cold-acclimated animals. Na^+ and Ca^{2+} contents were also higher in the haemolymph of cold-adapted larvae while K^+ content was higher in warm-acclimated larvae.

Table 1. Haemolymph pH, osmolarity and ion composition for warm (23°C)- and cold (3°C)-acclimated *E. solidaginis* larvae

	Warm (23°C)-acclimated	Cold (3°C)-acclimated
pH: at 5°C	6.978 ± 0.048	6.917 ± 0.041
at 18°C	6.794 ± 0.025*	6.766 ± 0.014*
Osmolarity	947 ± 16.5	804 ± 14.7
Ions: Na^+	8.4 ± 0.2	11.9 ± 0.7
K^+	59.5 ± 2.1	50.8 ± 1.5
Ca^{2+}	13.7 ± 0.2	17.6 ± 0.4
Mg^{2+}	14.9 ± 2.6	31.5 ± 1.6
Cl^-	40.1 ± 1.0	37.7 ± 0.5
HCO_3^-	6.7 ± 0.5	5.7 ± 0.8
"Unidentified anions"	68.5 ± 2.7	49.8 ± 4.0

Data are means ±1 SEM, $n = 5$ for pH, $n = 7$ for ion and osmolarity measurements with each data point determined on haemolymph pooled from 6–10 animals. Units for osmolarity and ion concentrations are μOsm/ml and μEquiv/ml respectively. $[HCO_3^-]$ was calculated from pH and C_{CO_2} measurements using the Henderson–Hasselbalch equation. The "unidentified anions" or "anion gap" was calculated as $(Na^+ + K^+ + Ca^{2+} + Mg^{2+}) - (Cl^- + HCO_3^-)$. *Significantly different from pH at 5°C, $P < 0.01$, Student's t-test.

Table 2. Concentrations of selected metabolites and protein in the haemolymph and whole larvae of warm (23°C)- and cold (3°C)-acclimated *E. solidaginis*

	Haemolymph		Whole larvae			
	(μmol/ml)		(μmol/g wet weight)		(μmol/ml larval water)	
	Warm	Cold	Warm	Cold	Warm	Cold
Glycerol	445 ± 17.9	204 ± 6.2	306 ± 28.2	157 ± 8.3	510	261
Sorbitol	1.58 ± 0.63	97.3 ± 1.43	1.38 ± 0.31	61.2 ± 0.69	2.3	102
Glucose	5.48 ± 0.73	2.69 ± 0.67	0.45 ± 0.12	0.16 ± 0.07	0.75	0.27
Fructose	1.42 ± 0.68	2.61 ± 0.47	0.05 ± 0.05	0.66 ± 0.15	0.08	1.10
Citrate	5.49 ± 0.07	8.15 ± 0.09	3.26 ± 0.49	4.48 ± 0.40	5.43	7.47
Malate	1.07 ± 0.08	1.02 ± 0.08	0.50 ± 0.05	0.37 ± 0.06	0.83	0.61
	mg/ml		mg/g		mg/ml	
Total protein	161 ± 18.3	131 ± 9.4	121 ± 3.6	94 ± 5.8	200	157

Data are means ± SEM for $n = 4$ samples with pooled haemolymph from about 25 larvae in each haemolymph sample and 4 larvae pooled for each whole larvae sample. Assuming that 60% of larval weight is water, data for whole larvae (in μmol/g wet wt) were recalculated and expressed as mol/ml total larval water to facilitate determinations of whether or not metabolites were in equilibrium between the haemolymph and intracellular water.

Table 2 shows the contents of selected metabolites in the haemolymph of warm- and cold-acclimated larvae. For comparison, levels of these compounds in whole larvae are presented. These values have also been converted to μmol/ml total larval water based on a 60% water content of the larvae and an assumed even distribution of metabolites in all water compartments of the larvae. The cryoprotectants, glycerol and sorbitol, were both present in cold-acclimated larvae while only glycerol was found in the warm-acclimated group. A comparison of haemolymph concentration with the concentration in whole larvae expressed as μmol/ml total larval water reveals that cryoprotectants are apparently in equilibrium between intra- and extracellular water. The same is not true of the sugars, glucose and fructose, whose concentrations are much higher in the haemolymph. Citrate concentration was significantly higher in cold-acclimated larvae than in warm-acclimated animals but levels of both citrate and malate appeared to be in equilibrium between the haemolymph and other aqueous compartments. Total protein content was significantly higher in warm-acclimated whole larvae than in cold-acclimated larvae but no significant difference was found in the haemolymph protein concentration between the two groups. Expressed per ml larval water, protein content was higher in whole larvae than in the haemolymph, no doubt reflecting a high protein content of cells.

Amino acid concentrations in the haemolymph and whole larvae are given in Table 3. Total amino acid content of warm-acclimated larvae was 22% higher than that of cold-acclimated animals, the result of the much higher proline content of these animals. In addition to proline, asparagine and arginine contents were also higher in the haemolymph of warm-acclimated animals and these along with alanine were also higher in warm-acclimated whole larvae. Other amino acids were in higher concentration in cold-acclimated larvae. These included threonine, serine, glycine, valine and ornithine in the haemolymph and these five as well as glutamine when whole larvae were considered. When concentrations in whole larvae, expressed in μmol/ml larval water, were com-

Table 3. Levels of amino acids in the haemolymph and whole larvae of warm (23°C)- and cold (3°C)-acclimated *E. solidaginis*

	Haemolymph		Whole larvae			
	(μmol/ml)		(μmol/g wet weight)		(μmol/ml larval water)	
	Warm	Cold	Warm	Cold	Warm	Cold
Aspartate	0.32 ± 0.03	0.21 ± 0.01	0.44 ± 0.17	0.53 ± 0.03	0.73	0.88
Threonine	3.76 ± 1.37	7.94 ± 0.41	2.07 ± 0.33	4.66 ± 1.24	3.45	7.77
Serine	2.78 ± 0.30	6.18 ± 0.34	2.15 ± 0.32	3.53 ± 0.01	3.58	5.88
Asparagine	9.78 ± 0.61	1.67 ± 0.31	5.40	0.32	9.0	0.53
Glutamate	0.78 ± 0.07	0.46 ± 0.04	3.33	3.70 ± 0.75	5.5	6.17
Glutamine	26.7 ± 10.6	21.2 ± 0.15	10.5 ± 0.17	14.5 ± 0.51	17.6	24.2
Proline	58.4 ± 3.30	41.1 ± 2.85	36.5 ± 4.39	21.9 ± 0.41	60.8	36.4
Glycine	1.66 ± 0.10	3.66 ± 0.29	1.38 ± 0.04	2.68 ± 0.55	2.3	4.47
Alanine	4.36 ± 1.06	4.14 ± 0.60	3.24 ± 0.24	0.89 ± 0.12	5.4	1.48
Valine	1.05 ± 0.15	2.51 ± 0.20	0.66 ± 0.14	1.29 ± 0.24	1.1	2.15
Cysteine	0.53 ± 0.01	0.59 ± 0.10	0.36 ± 0.05	0.41 ± 0.09	0.6	0.68
Methionine	0.48 ± 0.08	0.60 ± 0.18	0.19 ± 0.06	0.23 ± 0.01	0.32	0.38
Isoleucine	0.33 ± 0.05	0.59 ± 0.03	0.20 ± 0.03	0.31 ± 0.04	0.33	0.52
Leucine	0.57 ± 0.09	0.36 ± 0.02	0.35 ± 0.04	0.08 ± 0.02	0.58	0.13
Tryosine	1.57 ± 0.30	0.89 ± 0.05	1.09 ± 0.04	0.47 ± 0.03	1.81	0.78
NH_4^+	13.5 ± 0.34	14.8 ± 0.95	11.3 ± 0.33	13.1	18.9	10.5
Ornithine	1.20 ± 0.12	3.86 ± 0.35	0.62 ± 0.01	1.36 ± 0.23	1.04	2.26
Lysine	5.75 ± 0.63	7.39 ± 0.53	2.24 ± 0.11	2.41 ± 0.03	3.73	4.02
Histidine	2.13 ± 0.31	1.45 ± 0.20	1.12 ± 0.15	0.61 ± 0.26	1.87	1.02
Arginine	6.10 ± 0.83	1.56 ± 0.58	3.76 ± 0.58	1.93	6.30	3.22
Total amino acids	128.8	106.4	75.8	61.8	126	103
Amino acids + NH_4^+	142.3	121.2	87.2	74.8	145	125

Data are means ± SEM of $n = 3$ samples. All other details are as in Table 2.

pared with haemolymph concentrations it was apparent that amino acids are, in general, evenly distributed throughout the aqueous compartments of the larvae. The only exception to this was glutamate whose concentration was much higher in whole larvae suggesting a preferential sequestering of glutamate in cells.

DISCUSSION

The biochemical composition of haemolymph from warm- and cold-acclimated larvae differed markedly with respect to cryoprotectants although based on previous studies the composition was not unexpected. Sorbitol synthesis in this species is triggered only at temperatures below 5°C while glycerol production occurs only at higher temperatures (Storey *et al.*, 1981a; Storey and Storey, 1983a; Rojas *et al.*, 1983). When larvae were collected from outdoors, they had a glycerol content of approx 120 μmol/g, substantially less than midwinter values (Morrissey and Baust, 1976). Laboratory acclimation at 3°C for 1 month, maintained glycerol at about this level but stimulated sorbitol synthesis. Laboratory acclimation to 23°C resulted in continued glycerol synthesis but not sorbitol production. Thus we arrive at the anomaly of higher total cryoprotectant levels in warm- vs cold-acclimated animals in this experiment. Total available cryoprotection, in terms of hydroxyl equivalents (glycerol has 3, sorbitol has 6 hydroxyl groups), however, was virtually the same in both groups (1337 vs 1199 μ equivalents per gram for warm- versus cold-acclimated haemolymph) although osmolarity of the haemolymph from warm-acclimated larvae was much higher due to the high glycerol content. That glycerol continues to be synthesized at a constant 23°C indicates that glycerol production in this species follows an endogenous rhythm over the autumn months despite environmental temperature (although temperature may modulate the rate of synthesis); we have explored this phenomenon further in experiments yet to be published. Less pronounced but significant differences in haemolymph composition were found for citrate (higher in 3°C acclimated larvae) and for several amino acids. Chief among these was proline, the major free amino acid of the larvae. High levels of proline have been noted previously in both *E. solidaginis* and other cold-tolerant insects leading to the suggestion that proline could have a cryoprotective function (Storey *et al.*, 1981a). Alternatively, proline accumulation in the larvae may serve the needs for flight fuel in the non-feeding adult dipteran (Sacktor, 1970). The higher levels of proline in warm-acclimated larvae may suggest that, like glycerol synthesis, proline production is potentiated by warmer temperatures; of note in this regard is the higher content of ornithine, a proline precursor, in cold-acclimated larvae. Although the total amino acid pool size (when proline is discounted) is virtually identical in warm- and cold-acclimated larvae several differences in composition were noted between the groups in both the haemolymph and whole larvae contents. Thus, levels of four neutral amino acids (threonine, serine, glycine, valine) were higher in cold-acclimated larvae while the contents of two of the highly charged amino acids, asparagine and arginine, were lower in cold-acclimated larvae. Perhaps a more neutral amino acid pool would be adaptive for cold tolerance.

The ion levels measured in the haemolymph of both warm- and cold-acclimated larvae are very characteristic of a pattern found in insects feeding on higher plants (Type IV of Sutcliffe, 1963), where K^+ is greater in the haemolymph than Na^+ and where the total inorganic cations contribute a relatively small amount (20% or less) to the total osmolar concentration of the haemolymph. The only deviation from this pattern is the relatively higher osmolarity in *E. solidaginis* (800–900 μOsm/ml) compared to most other insects (300–600 μOsm/ml; Sutcliffe, 1963); this relative dehydration of both warm- and cold-acclimated animals can reasonably be viewed as an effective pre-adaptation to cold exposure. Furthermore, low-temperature acclimation led to alterations in the ion composition of the haemolymph, the overall result being an increased content of divalent (particularly Mg^{2+}) versus monovalent cations in cold-acclimated larvae. However, another recent study (Duman *et al.*, 1985) found no change in the haemolymph ion composition in the freeze-tolerant cranefly larvae measured over a winter season.

Biochemical composition of the haemolymph was compared with that of the whole larvae to determine whether or not concentrations of metabolities in the haemolymph were in equilibrium with cellular contents. The results show that polyols, organic acids, and all amino acids except glutamate (which appears to be preferentially found in cells) are apparently evenly distributed throughout the larval fluid compartments. For polyols this is an expected result indicating no barrier to movement between intra- and extracellular compartments; during freezing, then, polyols in the haemolymph, when excluded by ice formation, could freely move into the intracellular space to protect this environment. Sugars (glucose, fructose), however, were found preferentially in the haemolymph perhaps reflecting their role as tissue energy substrates. The less-efficient transport properties of the open circulatory system of insects apparently necessitates rather high haemolymph concentrations of substrates; use of sugar substrates by cells is regulated by membrane transporters and the activity of hexokinase so that intracellular sugar concentrations are low. Not unexpectedly the results showed a higher protein content in whole larvae than in the haemolymph, the result, undoubtedly, of the high protein content of cells.

Physiological acid-base parameters of the haemolymph were not affected by temperature acclimation. Thus, haemolymph pH (at either 5 to 18°C) and [HCO_3^-] were not significantly different between the two groups (Table 1). However, the haemolymph pH in both groups increased when measured at the lower temperature. This is common for both blood and intracellular pH in many poikilothermic animals and is due to the effect of temperature on the pK_a of physiological buffers (White and Somero, 1982). The major physiological buffering is based on imidazole containing compounds; these maintain a constant fractional dissociation state (α_{imid}), and thereby maintain a constant ionization state on cellular proteins

(thus stabilizing protein function), with a $\Delta pH/\Delta T$ of -0.017 unit/°C, a coefficient virtually identical to the effect of temperature on the neutral pH of water (Hochachka and Somero, 1984). Studies of *E. solidaginis* larvae *in vivo* using [^{31}P]nuclear magnetic resonance have indicated that intracellular pH of the larvae follows alphastat regulation with a $\Delta pH/\Delta T$ of -0.0185 unit/°C (Storey *et al.*, 1984). Results for the haemolymph pH given here show lower coefficients for change, warm-acclimated larvae having a value (-0.0142 unit/°C) similar to that reported for blood of many other species (about -0.015 unit/°C) and due to the combined actions of histidine-containing compounds and bicarbonate ($\Delta pH/\Delta T$ for bicarbonate buffer $= -0.006$ unit/°C) in determining blood pH (White and Somero, 1982). Haemolymph from cold-acclimated larvae showed an even lower coefficient (-0.0116 unit/°C). This may be a consequence of changes in the haemolymph composition occurring with low temperature acclimation or may perhaps be adaptive in helping to limit the range of pH fluctuations in animals which can face massive ambient temperature changes throughout a winter. Our previous study using [^{31}P]NMR failed to find a change in the pH of extracellular compartments with temperature (over the range $+15$ to -18°C) and also measured a significantly lower extracellular pH (6.1–6.3) than did the present study (Storey *et al.*, 1984). The difference in results is hard to explain but may be due to one of the following reasons: (a) a longer low temperature acclimation of larvae used in the NMR study (b) measurement *in vivo* (NMR) versus *in vitro* (pH electrode) and (c) differences in the parameters measured, the chemical shift of inorganic phosphate (NMR) versus the hydrogen ion content of the haemolymph.

Haemolymph composition should balance with respect to two parameters, osmolarity and cations vs anions. The sum of polyols, sugars, organic acids, amino acids and measured ions comes to 731 μMol/ml for haemolymph of warm-acclimated animals and to 567 μMol/ml for that of cold-acclimated animals, representing 77 and 68% respectively of the measured osmolarity (c.f. Table 1). The remaining percentages are probably due to compounds which were not measured (e.g. inorganic phosphate) and to proteins. The difference in measured osmolarity between the haemolymph from warm- and cold-acclimated larvae (143 μOsm/ml, Table 1) is totally accounted for by the difference in total polyol content of the two groups (445 μmol/ml in the haemolymph of warm-acclimated larvae versus 300 μmol/ml) for cold-acclimated animals. The charge balance of cations and anions in the haemolymph can also be considered from the present measurements. The difference between the measured inorganic cations and inorganic anions indicates missing anion(s) amounting to 68 ± 2.7 and 49.8 ± 4.0 μEquiv/ml respectively for warm- and cold-acclimated animals (Table 1). If the measured organic ions (Tables 2 and 3) are also considered (i.e. cations: $NH_4^+ + Arg + Lys$; anions: $Cit \times 3 + mal \times 2 + Asp + Glu$) then the difference remains nearly the same; missing anion(s) of 65.8 and 43.1 μEquiv/ml respectively for warm- and cold-acclimated animals. Relative to vertebrate plasma this is a large concentration of unmeasured anion. In cally only about 12 μEquiv/ml and is largely attributed to the net negative charge on serum albumin (Oh and Carroll, 1977). Measured protein in the haemolymph (Table 2) is about double that for human plasma (60–80 mg/ml) so this would account for perhaps half the gap. Possible candidates for the remainder include inorganic phosphate, nitrate, sulphate, keto- and fatty acids. In any case, the relatively small difference in the gap between warm- and cold-acclimated animals suggests that the unidentified anions are not important in the acquisition of cold tolerance.

Acknowledgements—KBS acknowledges the technical assistance of Mrs C. Shay in running the amino acid analyses and the support of an N.S.E.R.C. operating grant. Work at McMaster University was funded by an N.S.E.R.C. operating grant to DGM.

REFERENCES

Ballantyne J. S. and Storey K. B. (1985) Characterization of mitochondria isolated from the freezing tolerant larvae of the goldenrod gall fly, *Eurosta solidaginis*: Substrate preferences, salt and pH effects on warm and cold acclimated animals. *Can. J. Zool.* **63,** 373–379.

Baust J. G. (1981) Biochemical correlates to cold hardening in insects. *Cryobiology* **18,** 186–198.

Baust J. G. (1983) Protective agents: Regulation of synthesis. *Cryobiology* **20,** 357–364.

Duman J. G., Neven L. G., Beals J. M., Olson K. R. and Castellino F. J. (1985) Freeze-tolerance adaptations including haemolymph protein and lipoprotein nucleators in the larvae of the cranefly *Tipula trivittata*. *J. Insect Physiol.* **31,** 1–8.

Hochachka P. W. and Somero G. N. (1984) *Biochemical Adaptation*. pp. 392–396. Princeton University Press.

Morrissey R. E. and Baust J. G. (1976) The ontogeny of cold tolerance in the gall fly *Eurosta solidaginis*. *J. Insect Physiol.* **22,** 431–437.

Oh M. S. and Carroll H. J. (1977) The anion gap. *New Engl. J. Med.* **297,** 814–817.

Rojas R. R., Lee R. E., Luu T.-A. and Baust J. G. (1983) Temperature dependence-independence of antifreeze turnover in *Eurosta solidaginis* (Fitch). *J. Insect Physiol.* **29,** 865–869.

Sacktor B. (1970) Regulation of intermediary metabolism with special reference to control mechanisms in insect flight muscle. *Adv. Insect Physiol.* **7,** 267–348.

Storey K. B. (1983) Metabolism and bound water in overwintering insects. *Cryobiology* **20,** 365–379.

Storey K. B. (1984) A metabolic approach to cold hardiness. *Cryo. Lett.* **5,** 147–161.

Storey K. B. and Storey J. M. (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 J. M. and Storey K. B. (1983a) Regulation of cryoprotectant metabolism in the overwintering gall fly larva. *Eurosta solidaginis*: Temperature control of glycerol and sorbital levels. *J. comp. Physiol.* **149,** 495–502.

Storey K. B. and Storey J. M. (1983b) Biochemistry of freeze tolerance in terrestrial insects. *Trends Biochem. Sci.* **8,** 242–245.

Storey J. M. and Storey K. B. (1984) Freezing and cellular metabolism in the gall fly larva, *Eurosta solidaginis J. comp. Physiol.* **155,** 333–337.

Storey K. B., Baust J. G. and Storey J. M. (1981a) Inter-

mediary metabolism during low temperature acclimation in the overwintering gall fly larva, *Eurosta solidaginis*. *J. comp. Physiol.* **144,** 183–190.

Storey K. B., Baust J. G. and Buescher P. (1981b) Determination of water bound by soluble subcellular components during low temperature acclimation in the gall fly larva, *Eurosta solidaginis*. *Cryobiology* **18,** 315–321.

Storey K. B., Micelli M., Butler K. W., Smith I. C. P. and Deslauriers R. (1984) ^{31}P NMR studies of metabolism in the freeze tolerant larvae of the gall fly, *Eurosta solidaginis*. *Eur. J. Biochem.* **142,** 591–595.

Sutcliffe D. W. (1963) The chemical composition of haemolymph in insects and some other arthropods, in relation to their phylogeny. *Comp. Biochem. Physiol.* **9,** 121–135.

White F. N. and Somero G. N. (1982) Acid-base regulation and phospholipid adaptations to temperature: Time courses and physiological significance of modifying the milieu for protein function. *Physiol. Rev.* **62,** 40–90.

Zachariassen K. E., Baust J. G. and Lee R. E. (1982) A method for quantitative determination of ice nucleating agents in insect hemolymph. *Cryobiology* **19,** 180–184.