Characterization of mitochondria isolated from the freezing-tolerant larvae of the goldenrod gall fly (*Eurosta solidaginis*): substrate preferences, salt effects, and pH effects on warm- and cold-acclimated animals

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The mitochondria of the freezing-tolerant larvae of the goldenrod gall fly (*Eurosta solidaginis*) have been isolated and characterized. Proline is the preferred substrate of mitochondria from both warm- and cold-acclimated animals based on state 3 rates. Lipid is used as a substrate by warm- and cold-acclimated mitochondria assayed at 20°C, but not by the mitochondria from cold-acclimated animals assayed at 1°C. Cold-acclimated mitochondria assayed at 1°C have a higher and broader optimal range of salt concentration for the oxidation of proline based on the respiratory control ratio (RCR) than those from warm-acclimated animals oxidizing the same substrate at 20°C. The optimal pH for warm-acclimated mitochondria oxidizing proline at 20°C is low (6.2) based on the RCR, but rises to pH 7.0 in cold-acclimated animals at 1°C. It is suggested that the broad optimal salt concentration in the cold-acclimated animals and the very low optimal pH in warm-acclimated animals are adaptations for survival in this freezing-tolerant larva.

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Les mitochondries des larves tolérantes au gel de la mouche gallicole *Eurosta solidaginis* ont été isolées et analysées. D'après les taux au stade 3, la proline est le substrat préféré des mitochondries, à la fois chez les animaux acclimatés à la chaleur et chez les animaux acclimatés au froid. Les lipides servent de substrats aux mitochondries des deux groupes d'insectes à 20°C, mais pas aux mitochondries des animaux acclimatés au froid et analysées à 1°C. Les mitochondries acclimatées au froid et analysées à 1°C ont une étendue optimale de concentrations de sels plus grande pour l'oxydation de la proline (telle que déterminée d'après la valeur du contrôle respiratoire) que les mitochondries des animaux acclimatés à la chaleur qui oxydent le même substrat à 20°C. Le pH optimal pour l'oxydation de la proline à 20°C par les mitochondries des animaux acclimatés à la chaleur est faible (6,2) d'après la valeur du contrôle respiratoire, mais augmente jusqu'à 7,0 pour les mitochondries des animaux acclimatés au froid et analysées à 1°C. Il est possible que la concentration optimale de sel variable chez les animaux adaptés au froid et le pH optimal très faible chez les animaux acclimatés à la chaleur constituent des adaptations qui permettent la survie chez ces larves tolérantes au gel.

[Traduit par le journal]

Introduction

The third-instar larvae of the goldenrod gall fly (*Eurosta solidaginis*) undergo metabolic changes which permit survival of extracellular freezing during the winter months (Morrissey and Baust 1976, Storey 1983; Storey and Storey 1983). Adaptations for overwintering include alterations in the bound water content of cells (Storey, Baust, and Buescher 1981), changes in enzyme activities (Storey and Storey 1981), and accumulation of polyhydric alcohols as cryoprotectants (Morrissey and Baust 1976; Storey, Baust, and Storey 1981).

Extracellular freezing has dramatic effects upon the intracellular environment. As extracellular ice forms, water is drawn out of cells increasing the concentration of solutes within the cell. Subcellular organelles, such as mitochondria, must withstand osmotic stresses during repeated cycles of freeze–thaw over the winter. Indeed, osmotic stress owing to increased salt concentration is thought to be one of the main causes of freezing injury to mitochondria from noncold-hardy organisms (Liu 1979). The cell and its organelles may also face considerable alterations in pH at low temperatures. As temperature decreases the intracellular pH of poikilothermic animals increases (Reeves 1977). The pH of cells at temperatures close to those where freezing occurs (approximately −8°C in *E. solidaginis*) may be crucial to the survival of the animal.

The mitochondria of *E. solidaginis* larvae are potentially faced with these stresses placed upon the intracellular environment during low temperature and freezing exposures. The present study investigates mitochondrial metabolism in the larvae with emphasis on the effects of pH and salt concentration on the oxidation of substrates in mitochondria from both warm- and cold-acclimated animals.

Materials and methods

Animals

Galls of *Solidago* spp. containing third-instar larvae of *Eurosta solidaginis* were collected in the Ottawa area during August and September 1982. The galls were either stored at 5°C for 1 to several months (cold acclimated) or at 20°C for up to 1 month (warm acclimated) prior to use.

Preparation of mitochondria

Immediately before homogenization the larvae (3–5 g) were rinsed 3–5 times with distilled water to remove adhering plant debris. After washing, the larvae were placed in 10 mL of ice-cold isolation medium consisting of 1% bovine serum albumin (BSA) (essentially fatty acid free), 250 m*M* sucrose, 10 m*M* HEPES (pH 7.2 at 20°C), 2 m*M* EDTA, and 1 m*M* EGTA. The larvae were homogenized with three or four passes of a Potter–Elvehjem homogenizer with a loosely fitting Teflon pestle. The homogenate was centrifuged at 100 × *g* for 10 min. The supernatant was decanted and centrifuged at 7670 × *g* for 10 min. The resulting pellet was resuspended and centrifuged again for

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TABLE 1. Mitochondrial oxidation of Krebs cycle intermediates by the warm-acclimated third-instar larvae of *Eurosta solidaginis*

Substrate	State 3	State 4	RCR	ADP/O
Citrate (7.04 m*M*)	21.85±7.40	4.28±1.43	5.08±0.65	2.71±0.10
DL-Isocitrate (1.4 m*M*)	20.64±4.48	7.36±2.33	3.09±0.54	2.77±0.15
α-Ketoglutarate (7.04 m*M*)	24.92±4.99	6.49±1.88	4.03±0.39	2.58±0.18
Succinate (9.86 m*M*) + rotenone (1 μ*M*)	33.48±8.28	11.85±3.69	2.94±0.28	1.56±0.07
Fumarate (7.04 m*M*)	16.75±5.53	5.16±1.64	3.22±0.04	2.35±0.07
L-Malate (7.04 m*M*)	28.70±10.04	9.57±3.82	3.10±0.16	2.40±0.12
Oxaloacetate (1.4 m*M*)	ND	ND	ND	ND

NOTE: Values are means ± SEM (n = 3). State 3 and state 4 rates are expressed as nanomoles O per minute per milligram of mitochondrial protein at 20°C. The pH was adjusted to 7.0 at 20°C. ND, not detected.

TABLE 2. Mitochondrial oxidation of Krebs cycle intermediates in the cold-acclimated third-instar larvae of *Eurosta solidaginis*

Substrate	State 3	State 4	RCR	ADP/O
Citrate (7.04 m*M*)	ND	ND	ND	ND
DL-Isocitrate (1.4 m*M*)	2.42±0.34	0.80±0.09	3.58±0.29	2.76±0.23
α-Ketoglutarate (7.04 m*M*)	3.00±0.32	0.94±0.08	3.43±0.24	2.93±0.07
Succinate (7.04 m*M*)	2.68±0.31	1.17±0.34	1.73±0.05	1.68±0.03
Fumarate (7.04 m*M*)	ND	ND	ND	ND
L-Malate (7.04 m*M*)	2.57±0.26	1.03±0.12	2.52±0.21	2.46±0.42
Oxaloacetate (1.4 m*M*)	ND	ND	ND	ND

NOTE: Values are means ± SEM (n = 3). State 3 and state 4 rates are expressed as nanomoles O per minute per milligram of mitochondrial protein. All assays were conducted at 1°C. The pH was adjusted to 7.0 at 1°C. ND, not detected.

10 min at 7670 × g. The final pellet was resuspended in a small volume of isolation medium.

Oxidative phosphorylation

Oxygen uptake of mitochondrial suspensions was measured polarographically with a Clark-type oxygen electrode. The incubation medium consisted of 100 m*M* KCl (unless KCl concentration was varied), 10 m*M* HEPES (adjusted to pH 7.0 at the temperature of assay unless otherwise stated), 10 m*M* KH_2PO_4, and 1% BSA (essentially fatty acid free). ADP/O and respiratory control ratio (RCR) were calculated as outlined by Estabrook (1967), with respiratory states of mitochondria as defined by Chance and Williams (1956). All substrates were neutralized before addition to the cuvette.

Protein determination

Mitochondrial protein was determined using the procedure of Gornall (1949) with 10% deoxycholate to solubuilize the mitochondria. The protein concentration of the mitochondrial preparation minus 10 mg/mL (1% BSA) was used in subsequent calculations. BSA was used as a standard.

Chemicals

All biochemicals were obtained from the Sigma Chemical Co., St. Louis, MO.

Statistical analysis

The data were analyzed using the Student's t-test.

Results

Mitochondria from warm-acclimated animals assayed at 20°C oxidized all Krebs cycle intermediates to some extent with the exception of oxaloacetate (Table 1). The highest rate in state 3 was observed with succinate as a substrate. Malate was oxidized at nearly the same rate as succinate. α-Ketoglutarate, citrate, and isocitrate were oxidized at similar rates in state 3, at about two-thirds that of succinate and malate. The lowest state 3 rate was obtained with fumarate as substrate. The highest RCR was obtained with citrate as substrate, largely because of the very low state 4 rate. The lowest RCR was obtained with succinate because of the high state 4 rate. The state 4 rate with malate as substrate was also high, resulting in a lower RCR. All substrates except succinate gave ADP/O ratios between 2 and 3. The ADP/O obtained with succinate was 1.56.

Mitochondria from cold-acclimated animals assayed at 1°C oxidized some Krebs cycle intermediates, the exceptions being oxaloacetate, citrate, and fumarate (Table 2). The highest state 3 rates were found with α-ketoglutarate as substrate. ADP/O values between 2 and 3 were obtained for all substrates except succinate. The ADP/O for succinate was 1.68.

Comparison of cold and warm acclimated mitochondria

L-Proline, pyruvate, and palmitoyl-L-carnitine were oxidized at similar rates at 20°C by both warm- and cold-acclimiated mitochondria (Table 3). Lipid was not oxidized at detectable levels by cold-acclimated mitochondria assayed at 1°C (Table 3). Warm-acclimated mitochondria could oxidize lipid at measurable rates. L-Proline was oxidized at higher rates by cold-acclimiated mitochondria at 1°C than by warm-acclimated mitochondria at the same temperature (Table 3) ($p < 0.05$). Pyruvate was oxidized at higher rates by cold-acclimated mitochondria at 1°C than by warm acclimated mitochondria at the same temperature ($p < 0.02$).

pH and salt effects

The optimal range of pH for the oxidation of palmitoyl-L-carnitine in state 3 by warm-acclimated mitochondria assayed at 20°C lies between pH 6.2 and 7.8 (Fig. 1). ADP/O was constant from pH 5.5 to about pH 7.5, after which it declined.

The optimal range of KCl concentration for the oxidation of palmitoyl-L-carnitine by warm-acclimated mitochondria

TABLE 3. Mitochondrial oxidation of substrates by warm- and cold-acclimated third-instar larvae of *Eurosta solidaginis* assayed at 20°C

Substrate	State 3	State 4	RCR	ADP/O
L-Proline (28 m*M*) + pyruvate (0.28 m*M*)				
Cold acclimated	17.78±6.19	6.12±2.00	2.88±0.10	2.65±0.19
Warm acclimated	21.57±0.90	5.12±1.59	3.20±0.23	2.31±0.23
Pyruvate (7.1 m*M*) + L-malate (0.28 m*M*)				
Cold acclimated	17.29±4.79	7.43±1.63	2.29±0.16	2.62±0.02
Warm acclimated	16.80±1.69	5.14±1.14	3.46±0.63	2.25±0.10
Palmitoyl-L-carnitine (113 μ*M*) + L-malate (0.28 m*M*)				
Cold acclimated	13.74±4.25	6.14±1.86	2.23±0.07	2.40±0.18
Warm acclimated	17.25±3.18	7.11±1.35	2.43±0.11	2.77±0.06

NOTE: Values are means ± SEM (n = 3). State 3 and state 4 rates are expressed as nanomoles O per minute per milligram of mitochondrial protein. The pH was adjusted to 7.0 at 20°C.

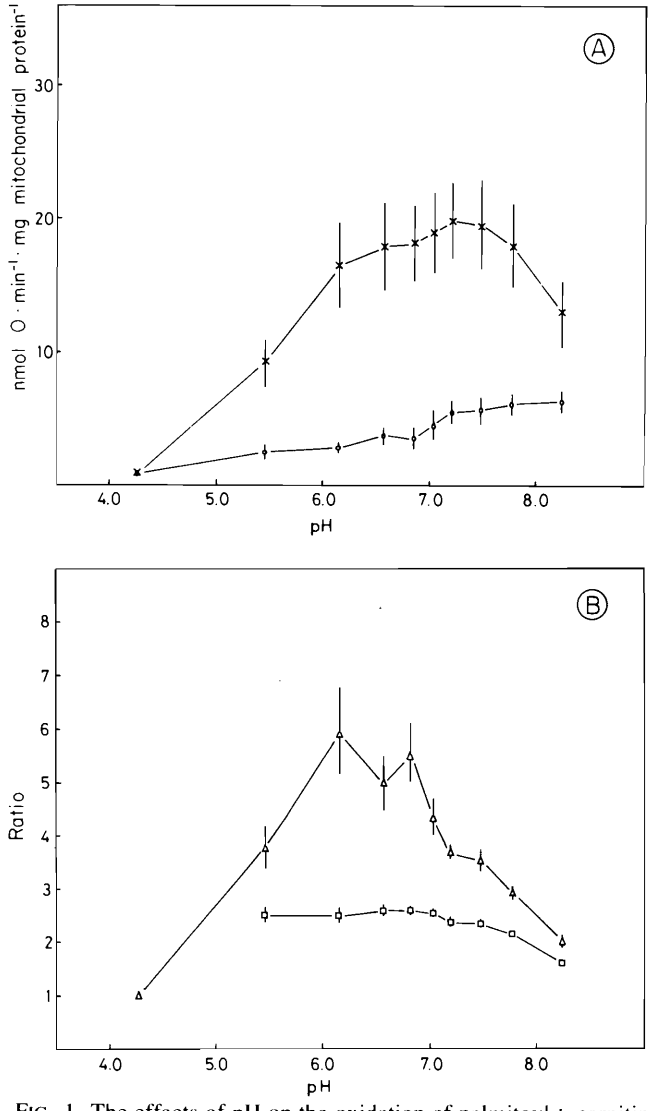

FIG. 1. The effects of pH on the oxidation of palmitoyl-L-carnitine (113 μ*M*) + L-malate (0.28 m*M*) by mitochondria isolated from warm-acclimated third-instar larvae of *Eurosta solidaginis* measured at 20°C. Results are means ± SEM (n = 3). Assay conditions are as described in Materials and methods. (A) Effects of pH on state 3 (×) and state 4 (○) rates of substrate oxidation. (B) Effects of pH on RCR (△) and ADP/O (□).

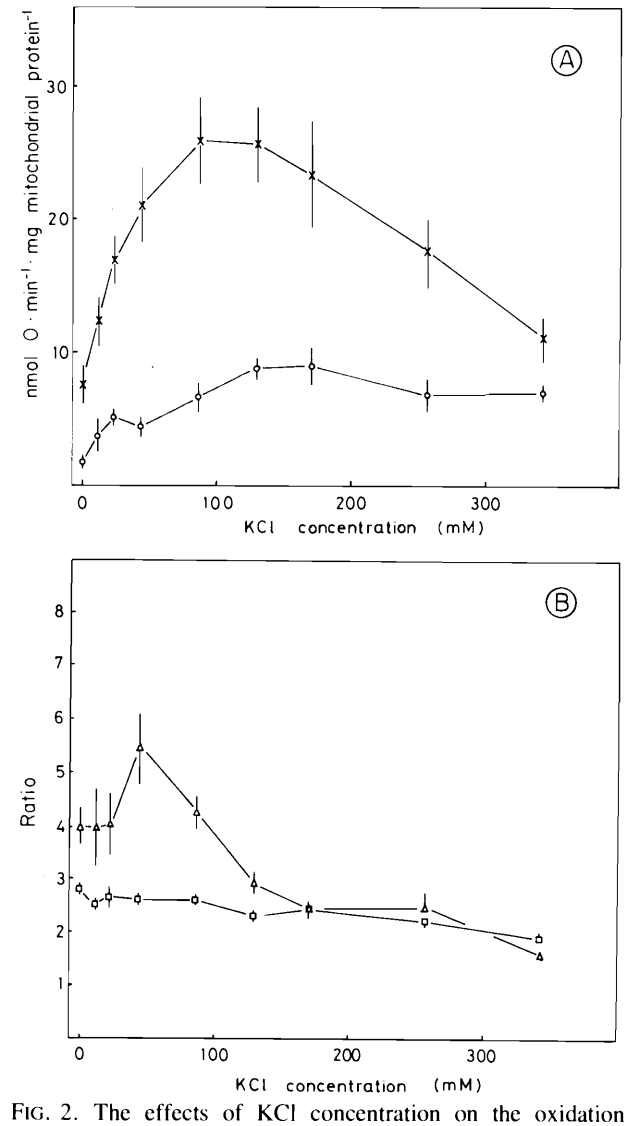

FIG. 2. The effects of KCl concentration on the oxidation of palmitoyl-L-carnitine (113 μ*M*) + L-malate (0.28 m*M*) by mitochondria isolated from warm-acclimated third-instar larvae of *Eurosta solidaginis* measured at 20°C. Results are means ± SEM (n = 3). Assay conditions are described in Materials and methods. (A) Effects of KCl on the state 3 (×) and state 4 (○) rates of oxidation. (B) Effects of KCl on RCR (△) and ADP/O (□).

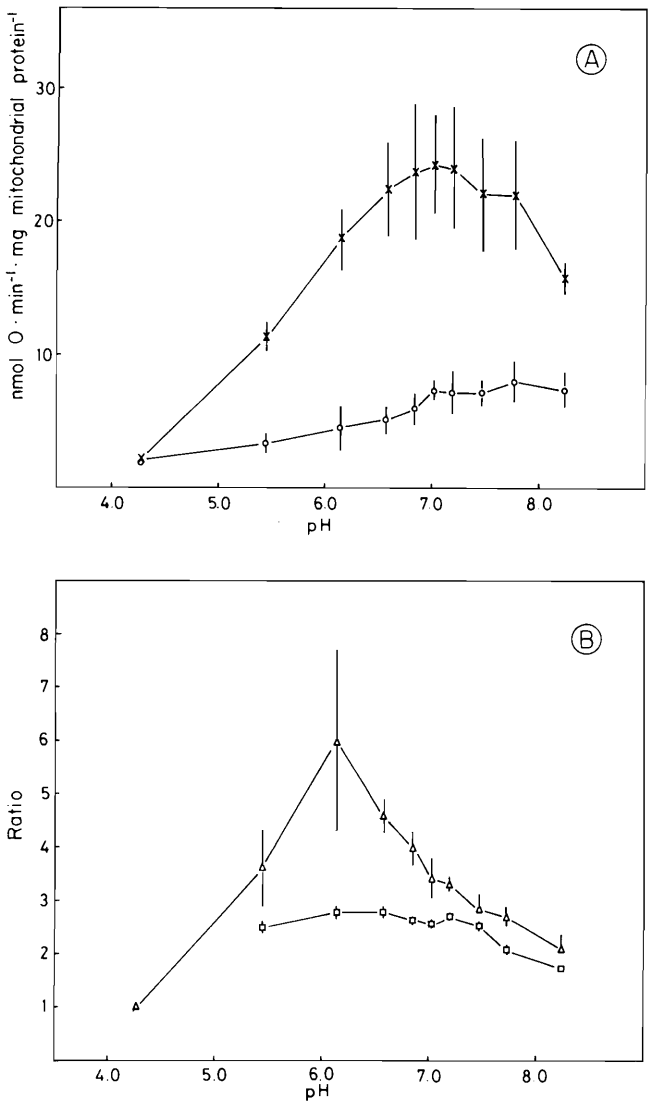

FIG. 3. The effects of pH on the oxidation of L-proline (28 m*M*) + pyruvate (0.28 m*M*) by mitochondria isolated from warm-acclimated third-instar larvae of *Eurosta solidaginis* measured at 20°C. Results are means ± SEM (n = 3). Assay conditions are described in Materials and methods. (A) Effects of pH on state 3 (×) and state 4 (○) rates of oxidation. (B) Effects of pH on RCR (△) and ADP/O (□).

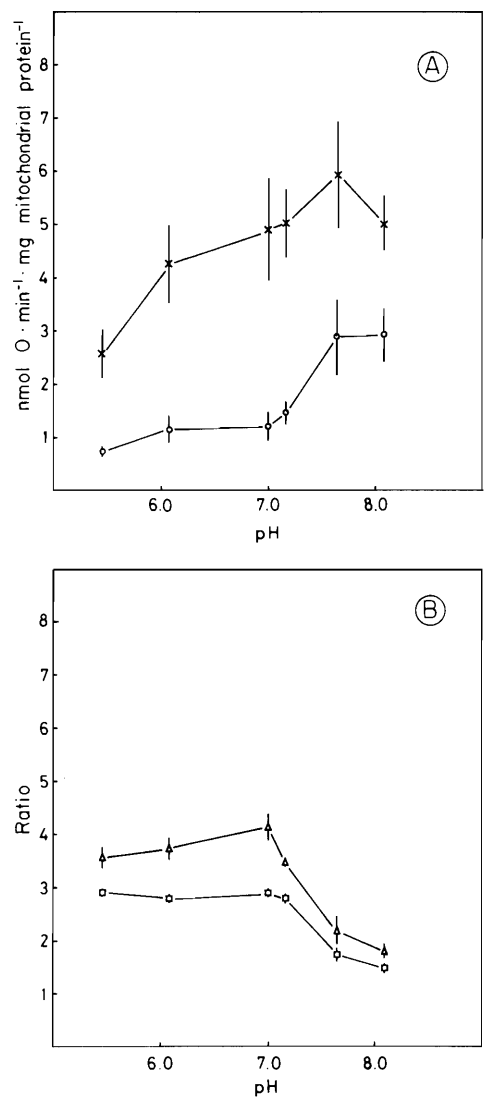

FIG. 4. The effects of pH on the oxidation of L-proline (28 m*M*) + pyruvate (0.28 m*M*) by mitochondria isolated from cold-acclimated third-instar larvae of *Eurosta solidaginis* measured at 1°C. Results are means ± SEM (n = 3). Assay conditions are described in Materials and methods. (A) Effects of pH on the state 3 (×) and state 4 (○) rates of oxidation. (B) Effects of pH on RCR (△) and ADP/O (□).

assayed at 20°C was from 80 to 150 m*M* in state 3 (Fig. 2). The highest RCR value was obtained at 50 m*M* KCl, but relatively high values (approximately 4.0) were obtained between 0 and 25 m*M* KCl. ADP/O was relatively constant over the entire range tested.

The optimal range of pH for the oxidation of proline by mitochondria from warm-acclimated larvae assayed at 20°C was very similar to that for palmitoy-L-carnitine in state 3 (Fig. 3). The state 4 rates also increased over the entire range tested. The highest RCR was obtained at pH 6.15 with lower values at pH's above and below this value. The ADP/O was relatively constant over the range pH 5.5–7.4 and declined above pH 7.4. The highest rate of oxidation of proline by mitochondria from cold-acclimated larvae was pH 7.6 at 1°C (Fig. 4). The state 4 rates increased over the entire range tested. The RCR was high between pH 5.5 and 7.0 and declined sharply at pH values above 7.0. The ADP/O was also constant up to pH 7.0 and declined above this value.

The optimal range of KCl concentration for the oxidation of proline by cold-acclimated mitochondria in state 3 at 1°C was from 100 to 200 mM KCl (Fig. 5). The highest RCRs were obtained over a wide range from 50 to 350 m*M* KCl. ADP/O was constant between 12 and 350 m*M* KCl.

Discussion

Mitochondria from both warm- and cold-acclimated third-instar larvae of *E. solidaginis* oxidize proline at higher state 3 rates than any other substrates under the conditions used. The relative importance of proline as a mitochondrial substrate also increases at low temperatures. Thus, mitochondria from cold-acclimated larvae oxidized proline, pyruvate, and palmitoyl-L-carnitine in a ratio of 100:97:77 (state 3 rates; Table 3) at 20°C. At 1°C, however, the ratio changed to 100:59:0 (Table 4). Proline may, therefore, be the preferred substrate for *E. solidaginis* mitochondria at low temperatures. Supporting

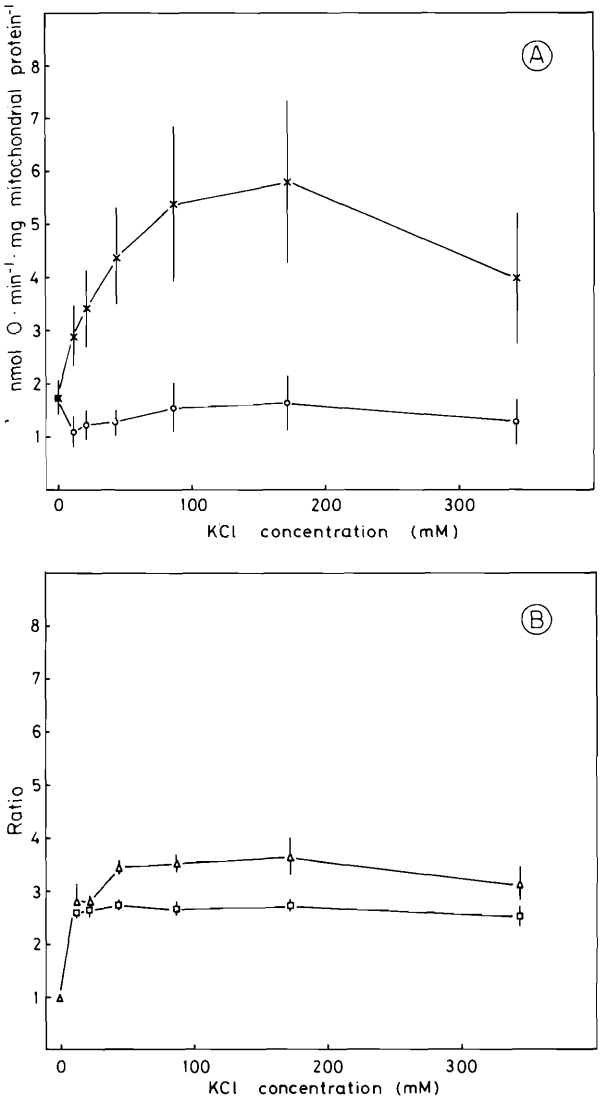

FIG. 5. The effects of KCl on the oxidation of L-proline (28 m*M*) + pyruvate (0.28 m*M*) by mitochondria isolated from cold-acclimated third-instar larvae of *Eurosta solidaginis* measured at 1°C. Results are means ± SEM (n = 3). Assay conditions are described in Materials and methods. (A) Effects of KCl on state 3 (×) and state 4 (○) rates of oxidation. (B) Effects of KCl on RCR (△) and ADP/O (□).

these data, Storey, Baust, and Storey. (1981) and Heady *et al.* (1982) have reported increased concentrations of proline in gall fly larvae acclimated to low temperature.

Oxidation of lipid by *E. solidaginis* mitochondria showed the greatest alteration in comparing warm-acclimated versus cold-acclimated larvae. Thus, mitochondria from warm-acclimated larvae could oxidize lipid at both 20 and 1°C, while those from cold-acclimated larvae oxidized palmitoyl-L-carnitine only at 20°C. The inability of mitochondria from cold-acclimated larvae to oxidize lipid at 1°C must stem from a direct temperature effect at some level of lipid breakdown. Since the oxidation of other substrates is not similarly impaired at low temperature and activities of Krebs cycle enzymes are not altered in low temperature acclimated larvae (Storey and Storey 1981), the inhibition cannot lie at the level of the Krebs cycle, the electron transport system, or oxidative phosphorylation. The site of inhibition must lie between the transfer of palmitoyl-L-carnitine into mitochondria and citrate synthase. The inability of mitochondria from cold-acclimated larvae to oxidize lipid at low temperature suggests that overwintering larvae may make little use of their lipid reserves as fuel, at least at low temperatures. This agrees with an observed constancy of total glyceride reserves in the larvae over a 6-week period of low temperature acclimation (Storey, Baust, and Storey 1981). However, at warmer temperatures, lipid could be a useful fuel; galls exposed above the snow line could experience periods of warming on many winter days.

Cold-acclimated mitochondria oxidized proline and pyruvate at higher rates at 1°C than warm-acclimated mitochondria at the same temperature. No differences were observed between the rates of oxidation of these substrates by warm- and cold-acclimated mitochondria assayed at 20°C. Enhanced rates of oxidation by cold-acclimated animals at lower temperatures would serve to compensate for the apparent inability of these animals to oxidize lipid at cold temperatures.

Based on the rates of pyruvate oxidation, carbohydrate appears to be a substrate of secondary importance for mitochondria of both the warm- and cold-acclimated larvae under low temperature assay conditions. In the overwintering larvae, the large glycogen stores are apparently reserved for functions other than aerobic energy production. Instead, glycogen is converted into the cryoprotectants, glycerol and sorbitol (levels of about 250 and 100 μmol/g wet weight accumulating, respectively), or is preserved to support glycolytic energy production (ending in lactate accumulation) during periods of extracellular freezing (Storey, Baust, and Storey 1981).

The present data on the oxidation of Krebs cycle intermediates indicates that three transporters are present in mitochondria from both warm- and cold-acclimated larvae: the dicarboxylate, tricarboxylate, and α-ketoglutarate−malate transporters. Compared with these mitochondria (from a larval dipteran), mitochondria from the fat body of an adult dipteran (*Phormia regina*) appear to lack the tricarboxylate transporter (Ballantyne and Storey 1983), while mitochondria of *P. regina* flight muscle are impermeable to Krebs cycle intermediates. Mitochondria from warm- and cold-acclimated larvae assayed at 20 and 1°C, respectively, showed some differences with respect to the oxidation of Krebs cycle intermediates, the major difference being the absence of citrate and fumarate oxidation by mitochondria from cold-acclimated animals. This suggests that the membrane transporters for these intermediates may be affected by temperature. This could result from direct temperature effects upon membrane fluidity or adaptational changes in the lipid composition of the membrane in the vicinity of the transporter. The inability of mitochondria from cold-acclimated larvae to oxidize citrate implies that both influx and efflux of citrate into the mitochondria is blocked at low temperature. Since the larvae are fasting, lipid synthesis is likely minimal and citrate efflux would serve no function. The observed constancy of lipid reserves in cold-acclimated larvae (Storey, Baust, and Storey 1981) supports such a cessation of lipid synthesis.

From the present data it appears that warm-acclimated *E. solidaginis* larvae can use lipid, carbohydrate, and at least one amino acid as oxidative substrates for aerobic metabolism. In this respect the mitochondria of these larvae resemble those from the fat body of the adult dipteran *P. regina*, which also oxidizes lipid, carbohydrate, and proline (Ballantyne and Storey 1983). Mitochondria from the adult fat body, however, show a strong preference for lipid (palmitoyl-L-carnitine) as their preferred substrate, while those of the larvae show about

TABLE 4. Mitochondrial oxidation of substrates by warm- and cold-acclimated mitochondria of *Eurosta solidaginis* assayed at 1°C

Substrate	State 3	State 4	RCR	ADP/O
L-proline (0.28 m*M*) + pyruvate (0.28 m*M*)				
Cold acclimated	5.09±0.77[a]	1.40±0.25	2.10±0.36	3.68±0.23
Warm acclimated	2.32±0.56	0.83±0.12	2.09±0.24	2.72±0.32
Pyruvate (7 m*M*) + L-malate (0.28 m*M*)				
Cold acclimated	3.02±0.36[b,c]	1.27±0.04	2.60±0.59	2.35±0.21
Warm acclimated	1.29±0.26	0.65±0.11	2.18±0.34	1.95±0.12
Palmitoyl-L-carnitine (113 μ*M*) + L-malate (0.28 m*M*)				
Cold acclimated	ND	ND	ND	ND
Warm acclimated	2.13±0.40	1.00±0.09	1.95±0.07	2.14±0.37

NOTE: Values are means ± SEM ($n = 3$). State 3 and state 4 rates are expressed as nanomoles O per minute per milligram of mitochondrial protein. The pH was adjusted to 7.0 at 1°C. ND, not detected.
[a] $p < 0.05$, warm acclimated vs. cold acclimated.
[b] $p < 0.02$, warm acclimated vs. cold acclimated.
[c] $p < 0.05$, L-proline vs. pyruvate (cold-acclimated group).

an equal use of lipid and proline when warm acclimated and are unable to oxidize lipid when cold acclimated.

The optimal KCl concentration for the oxidation of palmitoyl-L-carnitine (based on state 3 rates) for mitochondria from warm-acclimated larvae was between 100 and 150 m*M*. This range was also found to be optimal for substrate oxidation by blowfly flight muscle mitochondria (Chappell and Hansford 1969). When RCR (a better indicator of mitochondrial integrity) is considered, the optimal KCl concentration is even lower, at 50 m*M*, for mitochondria from warm-acclimated larvae assayed at 20°C. Mitochondria from cold-acclimated larvae, by contrast, show a somewhat higher optimal salt concentration, between 100 and 200 m*M* KCl, for the oxidation of substrates. High RCRs were obtained over a wide range from 50 to 350 m*M*. Mitochondria from cold-acclimated larvae apparently function optimally at both higher salt concentrations and over a broader range of salt concentration. This may represent an adaptation of the mitochondria for overwintering survival. During cycles of extracellular freezing and thawing, intracellular osmolyte concentration would alternately increase and decrease, intracellular water being drawn out into extracellular ice during freezing and released again during thawing. Thus, mitochondria in the overwintering larvae would have to maintain integrity and function over a wide range of cellular osmolyte concentrations and this appears to be reflected in the ability of mitochondria from cold-acclimated larvae to function over a wide range of salt concentrations.

With respect to pH, mitochondria from warm-acclimated larvae showed a lower pH optimum based on both state 3 rates and RCR than did mitochondria from cold-acclimated larvae. Such pH differences are in line with expected changes in intracellular pH as a function of temperature. The imidazole "alphastat" hypothesis predicts an increase in intracellular pH of 0.018 units per 1°C decrease in temperature and this has been confirmed by measurements of intracellular pH in a number of poikilothermic animals (Reeves 1977) including *E. solidaginis* (Storey *et al.* 1984). Thus, the difference in acclimation temperatures of the larvae (20 vs. 5°C) would predict a 0.27 unit difference in the intracellular pH of the warm- versus cold-acclimated animals with the higher pH in the cold-acclimated larvae. The higher pH optimum for oxidation of substrates by mitochondria from cold-acclimated larvae may reflect adaptations of one or more mitochondrial processes for low temperature functions.

In winter, the daily temperature fluctuations experienced by cold-acclimated *E. solidaginis* larvae cause repeated freezing and thawing. The concentration of intracellular solutes and intracellular pH therefore undergo similar cycles. The results presented here suggest the mitochondria of insects adapted to survive whole body freezing and thawing are different in some properties from other kinds of mitochondria not so adapted. Further investigations are required to examine the mechanisms involved and the effects of the cryoprotectants produced by these animals.

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