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The effect of exposure to low temperature on the metabolism of carbohydrates, lipids and protein in the larvae of *Philosamia ricini*

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Abstract. Exposure of the silkworm (*Philosamia ricini*) larvae to cold temperature (2°C) and subsequent exposure to room temperature (29° C) resulted in the mortality of the larvae.

Cold exposure brought about significant decrease in enzymic activity of proteases, aminotransferases, diacylglycerol lipase and in the amounts of some haemolymph sugars and polyols. However, glycerol increased sharply in response to severe cold exposure. There was also a marked increase in the levels of protein, pyruvate, total free amino acids, total lipid, phospholipid and triacylglycerols.

In the colder environment, carbohydrates served as the energy source. Glycerol appears to have conferred cryoprotection to the cold-stressed *Philosamia ricini* larvae.

Keywords. Cryoprotection; cold exposure; *Philosamia ricini;* carbohydrates; protein; lipids; triacylglycerols; lipases.

Introduction

Although the adaptive differences shown by insects in response to temperature are well known, there have been relatively fewer studies on the rate of acclimation in insects (Anderson and Mutchmor, 1971).

Studies on cold acclimation of insects have shown that carbohydrates undergo profound metabolic changes following cold stress (Lenartowicz and Niemierko, 1968). Many insect species which are exposed to temperatures below the freezing point accumulate glycerol, to which several protective functions have been ascribed (see review by Baust, 1973). It is also known that glycerol in combination with a mono- or disaccharide-based cryoprotectant increases low-temperature survival of organisms (Redway and Lapage, 1974). Interestingly, there was an accumulation of glycerol in the mature fifth instar larva of the non-diapausing lepidopteran, *Philosamia ricini* (Pant and Gupta, 1977). Hence, studies on cold stress were carried out with a view to provide an insight into the physiological strategy of survival adopted by this silkworm species at winter temperature (4–12°C). Since there were a few studies which dealt with rates of acclimation of enzymes in insects (Anderson and Mutchmor, 1971), in general, it was also considered pertinent to investigate the effect of low temperature on some key metabolites and enzymes involved in protein and lipid metabolism.

Materials and methods

The Eri silkworm *P. ricini* larvae were reared in the laboratory on tender fresh leaves of castor (*Ricinus communis*) at 29 ± 2° C and humidity 90% as described earlier (Pant and Agarwal, 1965).

Two-day old fifth instar larvae (110 in number) were reared in a wooden tray. at 2°C and the rate of mortality was recorded. From the colony, five sets of larvae each consisting of two or three insects were homogenised (20%, w/v) as described earlier (Pant and Morris, 1972) and assayed for enzymes as well for carbohydrates glycerides, lipids, amino acids and amino sugars as described below. Collection of haemolymph was made according to Pant and Gupta (1977).

Proteolytic activity was determined according to Matsushita and Iwami (1967), while the aminotransferases (glutamate : oxaloacetate and glutamate : pyruvate) were assayed by the method of Reitman and Frankel (1957). Tri- and diacylglycerol lipases were determined by the method of Cherry and Crandall (1931) with slight modifications (Pant *et al.,* 1978) and employing trioleylglycerol and dipalmitylglycerol as their respective substrates. Cholesteryl esterase was assayed by the method of Deykin and Goodman (1962). Free fatty acids liberated were determined according to Anderson and McCarty (1972). Protein was estimated according to Lowry *et al.* (1951).

Total lipids were extracted by the method of Folch *et al.* (1957), fractionated into free fatty acids, neutral lipids and phospholipids and determined as described by Pant *et al.* (1973). Triacylglycerols were determined according to Fletcher (1968), while sterols were quantitated by the method of Harrison (1959).

For analysis of total carbohydrates, trehalose, aminosugars and total free amino acids, alcoholic extract of the haemolymph was employed. Total periodate oxidisable substances, glycerol, sorbitol-6-phosphate and sorbitol were assayed in the trichloroacetic acid extract.

Soluble carbohydrates were determined by the method of Trevelyan and Harrison (1952) while trehalose was determined by that of Wyatt and Kalf (1957). Total amino acids were determined as described by Rosen (1957) while aminosugars were quantitated by the procedure of Elson and Morgan (1933). Total periodate-oxidisable substances were assayed by the procedure of Korn (1955). Glycerol, sorbitol and sorbitol-6-phosphate were separated by descending paper chromatographic technique employing butanol-acetic acid-water (5:1:2, v/v/v) as the solvent system. Appropriate places corresponding to their Rf' s were marked on the developed chromatograms, cut and eluted in 5% acetic acid in ethanol. They were quantitated according to Korn (1955).

Results

Effect of cold exposure on survival

At 2°C, 75% of the experimental insects survived for 7 days. Maintaining them for a further period (15 days), resulted in the death of another 30%. LT_{50} which is defined as the time taken to cause 50% mortality in insects at low temperature, was found to be 15 days for *P. ricini* fifth instar larvae. When transferred to the ambient temperature (29 ± 2° C) after a 15-day exposure to 2° C, all insects died in three days.

Influence of low temperature on some key enzymes and the metabolites in insect homogenate

The changes observed in protein and lipid metabolism have been represented in tables 1 and 2, respectively. Following 7 days' exposure to cold, the total protein

Table 1. The effect of low temperature (2°C) on some enzymes and metabolites of protein metabolism.

	Control (Mean ± SEM(N))	After 7 days at 2° C (Mean ± SEM(N))	Levels after cooling (% of control)
Protein (mg/g fresh wt)	22·53±1·68 (5)	31·32± 1·41 (5)	140
Proteolytic enzyme	23·32±7·46 (4)	14·21± 4·34 (4)	60
GOT*	58·41±4·05 (5)	30·10± 2·82 (5)	51
GPT*	144·52±9·00 (5)	94·24±14·16 (5)	66
Total amino acids (mg/g fresh wt)	3·95±0·71 (5)	4·14± 1·64 (5)	105
Pyruvate (μg/g fresh wt)	124·52±3·78 (5)	116·00±16·53 (5)	86

N = No. of experiments performed
* μg pyruvate formed/mg/protein/h.

content increases by 40% and proteolytic activity decreases by 40%. Significant decrease in the activity of the two important amino-transferases, glutamate-oxaloacetate (GOT) and glutamate-pyruvate (GPT) is also observed (table 1). Pyruvate and total free amino acids also register a small, but a significant increase by day 7.

Triacylglycerol lipase activity increased by over 45% by the 7th day when compared to the control group larvae (table 2).

Diacylglycerol lipase surprisingly lost more than 88% of its activity. Cholesteryl esterase activity also underwent a decline by over 55% (table 2).

Following exposure of the insects to 2°C for 7 days, the phospholipid and triacylglycerol concentration attained a significantly higher level (40% and 50%, respectively) over that in control insects. Marginal changes were also observed in the content of total lipids and total sterols.

Effect of cold exposure on haemolymph carbohydrates

As presented in table 3, the haemolymph metabolites show significant changes in response to low temperature stress. Total soluble carbohydrates particularly trehalose and sorbitol are greatly influenced by cold exposure.

Total periodate-oxidisable substances of haemolymph origin experience drastic increase recording 260% of the content of control insects. The concentration of glycerol, which is believed to confer cryoprotection to insects, also increases following 7 days' cooling to 2° C.

Table 2. The effect of exposure to low temperature on lipases and lipid content of silkworm.

	Control (Mean ± SEM(N))	After exposure (Mean ± SEM(N))	Levels after cooling (% of control)
Triacylglycerol lipase (n mol fatty acids/mg protein	8·7	12·8 ± 2·86 (5)	146
Diacylglycerol lipase (n mol fatty acids/mg protein)	223·8 ±50·24 (5)	25·2 ± 5·03 (5)	11
Cholesteryl esterase (n mol fatty acids/mg protein)	75·75±26·12 (4)	33·25± 4·37 (4)	44
Total lipids[a]	5·0 ± 0·19 (5)	5·5 ± 0·62 (5)	110
Free fatty acids[b]	31·20± 1·67 (5)	152·13±29·54 (5)	487
Neutral lipids[a]	4·15± 0·62 (5)	3·98± 0·40 (5)	97
Phospholipids[a]	0·27± 0·10 (5)	1·02± 0·24 (5)	140
Acylglycerol[b]	5·53± 0·60 (5)	5·45± 0·77 (5)	99
Triacylglycerols[c]	0·67± 0·67 (5)	1·01± 0·12 (5)	150
Sterols[a]	1·11± 0·19 (5)	1·37± 0·15 (5)	123
Monoacylglycerols[a]/diacylglycerols	2·37	1·50	

[a] mg/g fresh wt.

[b] μg/g fresh wt.

[c] Values obtained by subtracting the sum total of acylglycerol, triacylglycerols and sterols from neutral lipids.

N =No. of experiments performed

Table 3. The effect of low temperature on carbohydrates of haemolymph.

	Control	After exposure (mg/ml haemolymph)	Levels after cooling (% of control)
Total soluble carbohydrates	4·17	2·70	65
Trehalose	0·497	0·247	50
Total periodate-oxidisable substances	0·568	1·4806	260
Sorbitol	0·63	0·26	41
Sorbitol-6-PO_4	0·545	0·230	42
Aminosugars	0·161	0·121	75
Glycerol	0·99	1·22	123
Total amino acids	3·28	3·65	111

The silkworms were exposed to 2°C for seven days.

Discussion

The mortality of *P. ricini* on cold exposure is time-dependent. When reverted to ambient temperature (29 ± 2° C) the larvae became flaccid and lost the capacity to spin normal cocoons. Since the control 5th instar larvae spun cocoons on the fifth day while the duration of fifth larval instar was prolonged for 15 days, it is assumed that following severe cold stress, *Corpora allata* are stimulated to increase the haemolymph juvenile hormone titre which consequently inhibits the activity of prothoracic glands and arrests larval-pupal ecdysis. Cymborowski and Bogus (1976) also noticed serious disturbances following cooling in *Galleria mellonella* during late fifth instar larval development.

A great deal of evidence has been forwarded implicating the importance of enzymes as a factor involved in temperature acclimatisation and heat death (see Davidson, 1969). In particular, there are three major strategies of adjusting rates of enzymic activity in response to low temperature, *viz.,* qualitative, quantitative and modulation (see Hochachka and Somero, 1973). One currently available example of qualitative strategy is that of Rainbow trout acetylcholinesterases (Baldwin and Hochachka, 1970).

Pant and Morris (1969) observed that a high proteolytic activity in second instar larva of *P. ricini* declined steadily till late fifth instar development. The decrease was attributed to the composition of food ingested. The low proteolytic activity was due to the host plants which are rich sources of amino acids and which are consumed in large quantities by this insect. The observed 40% decrease in proteolytic activity and 40% increase in the protein content in the coldstressed larvae reflect on the reduced amount of food intake at low temperature. It is clear that proteins are not being used for deriving energy in the colder environment.

Presence of marked aminotransferase activity has been demonstrated in several tissues of *P. ricini* (Pant and Morris, 1972). Fat body and haemolymph both exhibit higher GOT activity than the GPT all through development. However the haemolymph aminotransferases are present in less significant levels (Pant and Morris, 1972). In contradistinction, the intestinal enzymes exhibit elevated GPT activity over that of GOT. The elevation in the activities of the two transaminases appears to parallel the rate of protein synthesis in *P. ricini* fat body (Pant and Morris, 1972) as well as in the haemolymph during larval development. However, the intestinal tissues participate in protein biosynthesis as well as in incorporating amino acids into carbohydrates. Increased glycogenesis concurrent with decrease in free amino acids was observed in *P. ricini* adults.

It seems likely that following cold stress, the activity of these enzymes appears to be greatly reduced. Results with *P. ricini* larvae suggest that the transaminases (GOT and GPT), which are implicated to have a role in protein synthesis, are seriously affected by cold stress. Increase in free amino acid content further confirms this.

The data obtained for the haemolymph carbohydrates (table 3) definitely suggest that during a week's exposure to cold temperature, carbohydrates are the main source of energy in the exposed insects. Except glycerol which shows substantial increase, all the metabolites (table 3) undergo significant depletion in the new environment for energy purposes. Sorbitol is not synthesised above zero degrees. It is possible that this lepidopteran insect survives severe cold exposure for one week mainly by a cryoprotectant, glycerol, which protects the insects from damage due

to freezing. The observed decrease in the activity of the enzymes, *viz.*, proteases GOT and GPT, diacylglycerol lipase and cholesteryl esterase is suggestive of their subdued functions at low temperature.

The increase in the contents of total lipids, free fatty acids and phospholipids occurs with the increase in the food consumption and it seems likely that these parameters do not provide energy to the cold stressed larvae. The increase in free fatty acids may provide the lipoprotein enzymes, with an environment to modulate the latter's activity at low temperature (see Hochachka and Somero, 1973). Triacylglycerols do not function as energy supplier as seen by a near two-fold increase in their content.

The observed increase in the phospholipid content in *P. ricini* larvae points to the greatly increased activity of those mitochondrial enzymes which are membrane-associated, and thus the former retains their reticular structures intact during cold-exposure. Therefore, *P. ricini* larvae are seen to employ both the quantitative and modulation strategies in response to low temperature.

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