



Title	Freezing Injury in Fat-Body Cells of the Poplar Sawfly
Author(s)	TANNO, Kouzou
Citation	Cellular Injury and Resistance in Freezing Organisms : proceedings, 2, 245-257
Issue Date	1967
Doc URL	<a href="http://hdl.handle.net/2115/20420">http://hdl.handle.net/2115/20420</a>
Type	bulletin (article)
Note	International Conference on Low Temperature Science. I. Conference on Physics of Snow and Ice, II. Conference on Cryobiology. (August, 14-19, 1966, Sapporo, Japan)
File Information	1_p245-257.pdf



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# Freezing Injury in Fat-Body Cells of the Poplar Sawfly\*

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## Abstract

The mechanisms of freezing injury in poplar sawfly, *Trichiocampus populi* Okamoto, was studied in relation to the types of cell freezing in their fat-body cells. The prepupa of this insect is remarkably frost resistant; it can survive a slow freezing down to a temperature of liquid nitrogen. After rewarming from the super-low temperature, some of them are able to resume development to normal pupae. Upon emergence, however, they cannot shed their pupal skins and fail to appear on the wing. Such freezing injury can be abolished by cooling them very slowly down to a temperature of  $-30^{\circ}\text{C}$  when they are subjected to freezing.

The prepupa of this insect has two fat-body layers; a parietal and a visceral layer. The occurrence of freezing injury in fat-body cells at temperatures from  $-30^{\circ}\text{C}$  to the liquid nitrogen temperature are much easier in the visceral layer than in parietal layer. During metamorphosis in the insect, the fat-body cells of the visceral layer are consumed mainly for the formation of the imago, while that of the parietal layer are utilized mainly for the formation of the pupa. It seems, therefore, that failure to emerge into adults in this insect may perhaps be explained to be a result of the freezing injury in fat-body cells of the visceral layer.

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## Introduction

It has been known that after freezing and thawing, some larval or pupal insects are observed to be apparently intact, but their further development is subnormal and they fail to emerge on the wing (Asahina, 1959; Asahina and Takehara, 1964; Tanno, 1963). A remarkable example of such freezing injury was demonstrated in the prepupa of poplar sawfly; after thawing from liquid nitrogen temperature, some of the prepupae were able to resume development up to the formation of imago, but most of them could not shed their pupal skins (Tanno, 1964). This type of injury may result from freezing damage in some tissues of frozen insects which does not directly cause the death of the entire insect. Judging from their role in the metamorphosis in insects, fat-bodies may reasonably be one of the assumable tissues responsible for such an indirect mode of freezing injury. To solve the mechanism of such freezing injury, the mode of freezing of fat-body cells in the prepupa and the behavior of these cells during metamorphosis is described in the present paper, in relation to frost injury in the entire insect.

## I. Materials and Methods

*Material.* The full-grown larvae of the poplar sawfly, *Trichiocampus populi* Okamoto,

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\* Contribution No. 795 from the Institute of Low Temperature Science.

were collected in Sapporo, during autumn in 1963, 1964 and 1965. In this season, the full-grown larvae are moving on dead trees or grasses under the food plant, a poplar tree, to find a suitable place for wintering. The dry stems of a herbaceous plant, *Rudbeckia laciniata* L., cut in 10 cm lengths, were placed in a glass vessel in which the collected sawfly larvae were reared. The insects soon entered into the pith of the stems. The larvae began to spin cocoons within the stems, and transformed into prepupae after three days. The prepupae in the stems were then placed in a room in which the room temperature was kept nearly the same as outdoor temperature throughout winter. There were some differences in seasonal changes of frost-resistance between male and female in the insects, although the general pattern was almost the same (Tanno, 1964 c). Therefore, female prepupae were only used for experiments.

*Observation of frost-resistance in an entire insect.* More than ten individual prepupae were generally used in one experiment for determining the degree of frost-resistance. The insects were placed in a petri dish, and were cooled in a cold box. The cooling rate in the insects was about 2°C per minute (the standard rate). When an insect was exposed to a temperature below -10°C without ice seeding, body freezing always occurred in the insect within ten minutes. The supercooling point in the prepupae was  $-8.6 \pm 0.4^\circ\text{C}$ . Insects were subjected to freezing at -10, -15, -20, -25 and -30°C respectively. After body freezing for 24 hours, insects were thawed at room temperature and were transferred to the rearing box at 20°C. The degree of frost-resistance in the insects was determined by examining the lowest tolerable temperature. When over 60 per cent of frozen insects could survive for more than 30 days after thawing, the freezing temperatures concerned was regarded as a tolerable temperature. A very high degree of frost-resistance in the insects below -30°C was determined by testing the effective prefreezing temperature to survive in liquid nitrogen (Asahina, 1959; Sakai, 1956). As the prefreezing temperatures, -15, -20, -25 and -30°C were employed. The cooling rate during prefreezing was about 2°C per minute. After a prefreezing for 9 hours, insects in a small cage made of wire gauze were immersed directly in liquid nitrogen. After overnight freezing at the super-low temperature, the insects were thawed slowly at room temperature.

Since the above described prefreezing method failed to produce an entirely normal insect, the following two freezing methods were tried. First method; the insects were kept at dry ice temperature for 18 hours after prefreezing to -30°C with a cooling rate of about 2°C per minute and were then immersed directly in liquid nitrogen. Second method; after very slow prefreezing to -30°C with a cooling rate of about 0.5°C per minute, the insects were immersed directly in liquid nitrogen.

*Observation of freezing fat-body cells.* The prepupae were frozen at a standard and very rapid rate of cooling. The cooling rate in the standard freezing was about 2°C per minute and the final temperature was about -30°C. Very rapid freezing was achieved by directly immersing them into liquid nitrogen from room temperature. In a cold room at -15°C, the frozen sections were made from the frozen insect body which was adhered to a wood block with a small amount of aniline. The frozen section was 25  $\mu$  in thickness, and was observed under a polarizing microscope (Tanno, 1964 b).

After thawing of a frozen insect, the fat-body cells were removed from the insect

body and were transferred into Ringer's solution for a silkworm, and were observed under a microscope.

*Utilization of the fat-body cells during metamorphosis of the insect.* The utilization of the fat-body cells during the metamorphosis in the insect was observed in both normal unfrozen prepupae and frozen-thawed ones by measuring the volume of the fat-body cells. The volume was estimated from the diameter of these fat-body cells. Five insects were used respectively for each measurement. The average volume was calculated using more than 30 fat-body cells from one insect.

## II. Results

### *Developmental stages and frost-resistance*

A remarkable seasonal change in frost-resistance and sugar content in the insect was observed (see Fig. 1). In the middle of August, no larvae could survive freezing even at a temperature of  $-10^{\circ}\text{C}$  for 30 minutes. After spinning cocoons they transformed into prepupae. During such transformation, their frost-resistance was rapidly enhanced in accordance with the increase of sugar content. Prepupae at the 40th day after spinning the cocoon were able to withstand liquid nitrogen temperature, provided they had been previously frozen at  $-30^{\circ}\text{C}$ . At this time their sugar content reached a maximum level of  $4.27 \pm 0.56$  per cent, based on their fresh body weight. In January, prepupae were able to survive liquid nitrogen temperature after previous freezing at

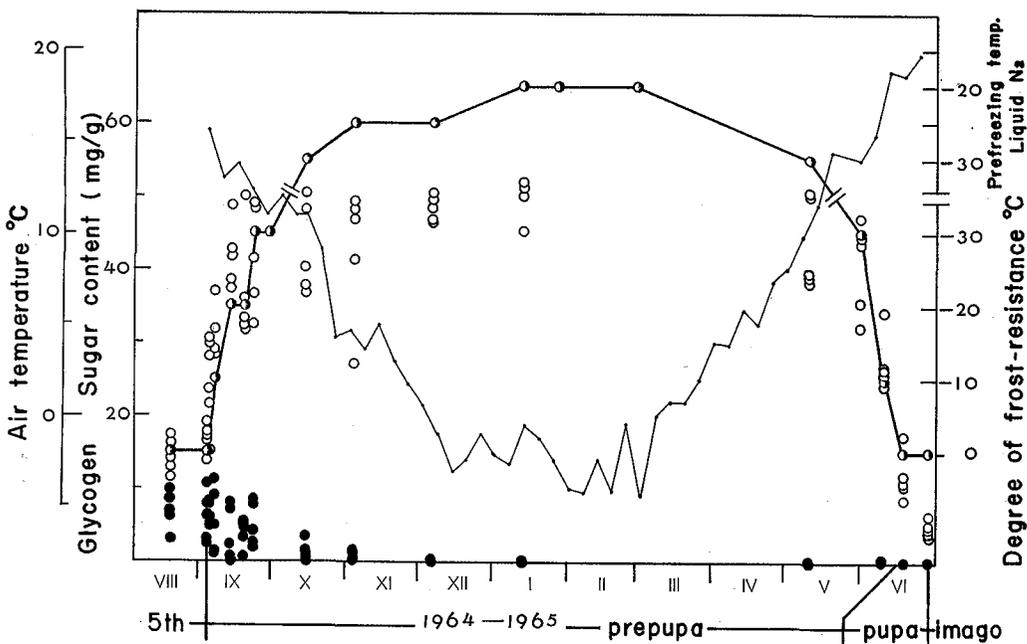


Fig. 1. Seasonal changes in the frost-resistance, sugar content and glycogen content in the sawfly

- : Degree of frost-resistance
- : Sugar content
- : Environmental temperature
- : Glycogen content

-20°C. Such high levels of frost-resistance and of sugar content persisted throughout the five month cold season. After rewarming from the liquid nitrogen temperature, some of them were able to resume development normally up to pupae, but all of them showed some injuries during the following development to imago, and failed to appear on the wing. Compared with normal imago, the adult insects developed from the prepupae frozen at and thawed from the liquid nitrogen temperature had tender bodies with a somewhat swollen abdomen. They were weak in movement. They could not shed their transparent pupal skins, and died within several days after transformation into imagoes.

With the rising temperature in spring the metamorphosis to pupa began to occur in prepupa at the middle of June in the following year, the frost-resistance decreased to a temperature of -10°C in accordance with the decrease of sugar content. A few pupae could survive freezing at -10°C for one day. All adults were invariably killed by freezing at a temperature of -10°C for several minutes.

*Survival at liquid nitrogen temperature without any injury*

The two-step freezing methods already described were employed on the prepupa in the most frost-resistant stage. Results are shown in Table 1. Both slow and standard

**Table 1.** Survival at liquid nitrogen temperature in prepupae of the sawfly

Prefreezing at -30°C**	Freezing at		No. of insect used	Survival after thawing*			
	-70°C***	-195°C****		at 10th day	pupae	imagoes normal    abnormal	
Standard	-	-	10	9	9	9	0
Standard	-	+	30	28	22	0	19
Standard	+	-	10	10	7	3	3
Standard	+	+	10	10	8	1	3
Slow	-	-	53	48	48	48	0
Slow	-	+	46	42	40	9	19

\* After thawing, the development of prepupae at 25°C were observed

\*\* Cooling rate of prefreezing down to -30°C: Standard, about 2°C/min; Slow, about 0.5°C/min. They were kept at -30°C for 9 hrs

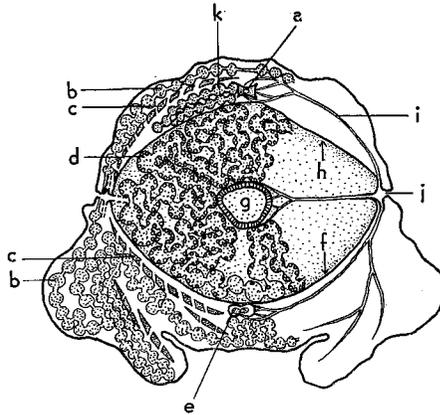
\*\*\* After prefreezing at -30°C, they were kept at dry ice temperature for 18 hrs

\*\*\*\* After prefreezing, they were immersed in liquid nitrogen for 3 to 24 hrs

freezing to a temperature of -30°C was harmless in prepupae. Most of the prepupae transferred to -70°C and then into liquid nitrogen after standard prefreezing at -30°C suffered considerable injury. On the other hand, many could survive liquid nitrogen temperature without any injury after very slow prefreezing. These results suggest that very slow prefreezing to a temperature of -30°C is very useful to protect the insect against freezing injury occurring at temperatures between -30°C and the liquid nitrogen temperature.

*Freezing states in fat-body cells*

The prepupa, as known in other insects, has two kinds of fat-bodies. One is the



**Fig. 2.** Cross-section of the prepupa of the sawfly

- |  |                      |
|--|----------------------|
| a : dorsal vessel                        | g : gut              |
| b : fat-body cells in the parietal layer | h : dorsal diaphragm |
| c : muscle                               | i : trachea          |
| d : fat-body cells in the visceral layer | j : spiracle         |
| e : ventral nerve cord                   | k : pericardial cell |
| f : ventral diaphragm                    |                      |

parietal layer, and the other is the visceral layer. The arrangement of these layers in the body cavity is shown in Fig. 2. Fat-body cells are connected by fine canals with each other. They are arranged continuously from the head part to the tail in the body cavity as a longitudinally folded thin sheet. Both parietal and visceral layers consisted of many fat-body cells. Each layer has nearly the same number of cells and are estimated to be about 2,700. There are some differences between these two kinds of fat-bodies, especially in the deposit of uric acid in the cells. The fat-body cells in the visceral layer are yellow colored spheres which are about  $230\ \mu$  in diameter (Fig. 5-M). They have many pouches, filled with uric acid, about 22 in number at the surface of each fat-body cell (Tanno, 1965 a). The fat-body cells in the parietal layer are orange colored spheroids which are about  $170\ \mu$  in diameter (Fig. 5-N). These cells have no pouches filled with uric acid. Each cell in both layers is tightly covered with a thin membrane. When a fat-body cell is slightly flattened between a slide glass and a cover glass, the thin membrane separated from the protoplasm surface (Fig. 5-O).

When the prepupae were cooled to  $-30^{\circ}\text{C}$  at a cooling rate of about  $2^{\circ}\text{C}/\text{min}$  (standard rate), extracellular freezing occurred in most of the fat-body cells except for a few of them in the visceral layer which froze intracellularly. The extracellularly frozen cells contracted irregularly, and the oil droplets within them coalesced into larger drops (Fig. 5-P, Q). After thawing, the cell recovered their normal size and shape except for the size of oil drops (Fig. 5-P, Q). The number of intracellularly frozen cells was observed to be less than 1 per cent of the total fat-body cells in the visceral layer. These cells contracted very slightly, and there were many large ice crystals within them (Fig. 4-G, H). Prepupae frozen in this way could normally develop up to the imago stage after thawing.

In the insect at the end of the prepupal stage, intracellular freezing readily occurred under the same freezing conditions; sometimes over 70 per cent of the total cells in the visceral layer froze intracellularly. In the parietal layer, on the other hand, extracellular freezing only occurred in fat-body cells. Those prepupae frozen in this way could survive only for a few days after thawing.

When the prepupae were immersed directly into liquid nitrogen, intracellular freezing invariably occurred in all fat-body cells. These cells did not contract, and numerous tiny ice crystals were found within them (Fig. 4-I, J, K, L). Immediately after thawing, however, these cells appeared nearly normal, because they were tightly covered with the thin membrane. Within the following few minutes, the thin membrane was separated from protoplasm in all cells of both fat-body layers showing a destroyed structure of protoplasm (Fig. 5-R). The rapidly frozen prepupae were invariably found to be killed just after thawing.

When the prepupae were immersed in liquid nitrogen after a prefreezing at  $-30^{\circ}\text{C}$ , the appearance of freezing fat-body cells was almost the same as that frozen at  $-30^{\circ}\text{C}$ . Extracellular freezing occurred in most of the fat-body cells. The number of intracellularly frozen cells was as small as in the case of freezing at  $-30^{\circ}\text{C}$ , and was estimated to be less than 1 per cent of the total fat-body cells in the visceral layer. All of the fat-body cells in the parietal layer froze extracellularly. When the fat-body cells in the visceral layer were removed from the thawed insect and put into Ringer's solution, the thin membranes of some fat-body cells separated from the protoplasm within a few minutes and the cells appeared to be destroyed. However, this was not the case in the cells in parietal layer. When the prepupae were immersed into liquid nitrogen after very slow prefreezing to a temperature of  $-30^{\circ}\text{C}$ , most of the fat-body cells in both layers appeared, after thawing, as intact as that frozen at  $-30^{\circ}\text{C}$ . These results show that intracellular freezing occurred easier in the fat-body cells of the visceral layer than that of the parietal layer, and that very slow prefreezing to  $-30^{\circ}\text{C}$  was very effective to prevent the injury which may occur during the process of freezing in liquid nitrogen.

The cooling rate of the freezing insect affected the grain size of ice crystals formed within the insect. The grain size of ice crystals formed within an insect frozen very slowly to  $-30^{\circ}\text{C}$  was as about twice large in diameter as that in the insect frozen rapidly to the same final temperature.

#### *Utilization of fat-bodies during metamorphosis in the insect*

The nature of the utilization on fat-bodies during metamorphosis in the insect from

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**Fig. 3.** Extracellular freezing in fat-body cells, photographed at  $-15^{\circ}\text{C}$ .  $\times 100$

A. Fat-body cells in the visceral layer frozen extracellularly. The prepupa was frozen at the cooling rate of about  $2^{\circ}\text{C}/\text{min}$  and was kept at  $-30^{\circ}\text{C}$  for 24 hours before it was sectioned. The freezing section was  $25\ \mu$  in thickness

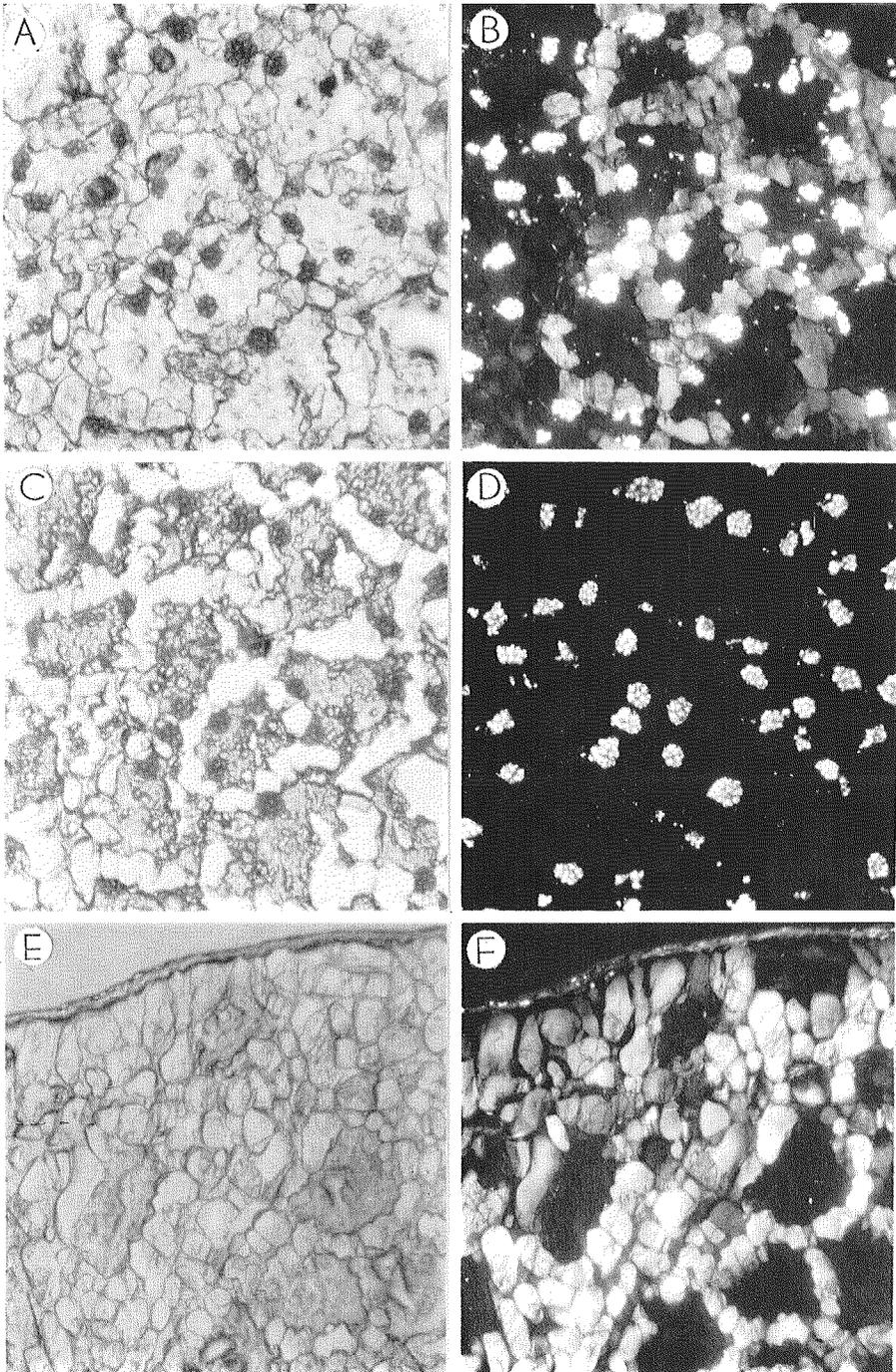
B. The same as A under a polarizing microscope. Extracellular ice and spherulites of uric acid in these cells are clear

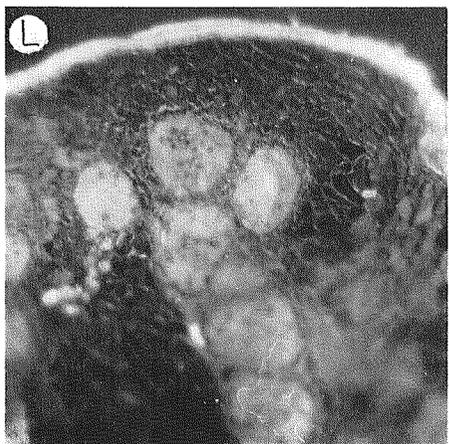
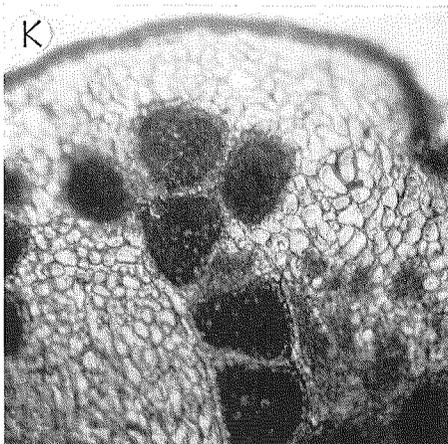
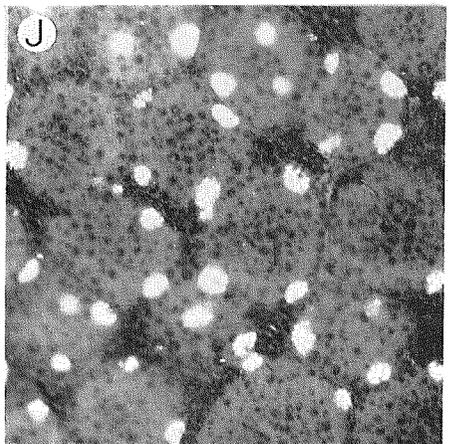
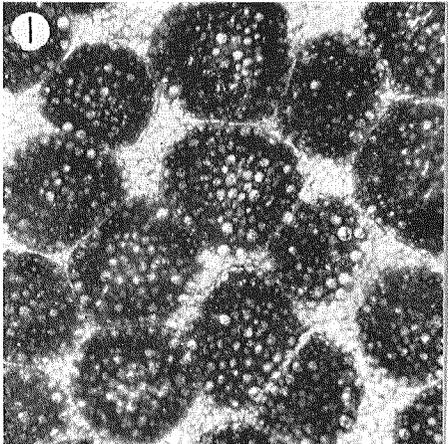
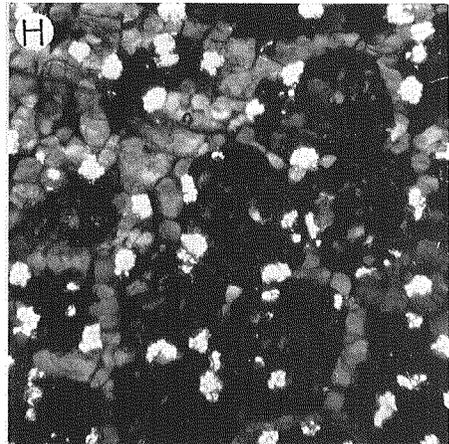
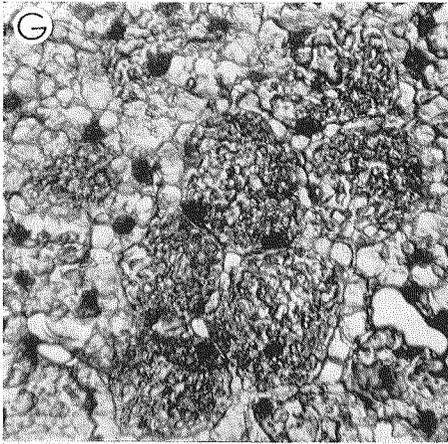
C. The same as A after sublimation of ice at  $-15^{\circ}\text{C}$

D. The same as C under a polarizing microscope. Only spherulites of uric acid can be seen

E. Fat-body cells in the parietal layer frozen extracellularly. The prepupa was frozen in the same way

F. The same as E under a polarizing microscope





prepupa to imago is shown in Fig. 6. It is clear that the fat-body cells in the parietal layer are mainly utilized for the formation of pupa and those in the visceral layer are mainly consumed for the formation of imago. In this connection, it is of interest to note that the adult sawflies developed from the prepupae frozen in liquid nitrogen after a standard rate of prefreezing  $-30^{\circ}\text{C}$  has much more fat-body cells remaining in the visceral layer in their abdomen than normal adult insects.

### III. Discussion

All the observations described above seem to suggest that when the prepupae are immersed into liquid nitrogen after prefreezing at  $-30^{\circ}\text{C}$ , some fat-body cells in the visceral layer suffer injury with or without apparent intracellular ice formation, but the cells in the parietal layer do not. After thawing, they can consume the fat-body cells in the parietal layer to transform to pupa. When they transform from pupa to imago, however, they cannot resume normal development, because they have an insufficient amount of uninjured fat-body in the visceral layer to be consumed for the adult formation. They are, therefore, too weak in movement to shed their thin pupal skins by themselves, which results in failing to appear on the wing.

A similar freezing injury was also observed in other insects. When the overwintering prepupae of the slug caterpillar, *Monema flavescens* Walker, were immersed into liquid nitrogen after prefreezing at  $-30^{\circ}\text{C}$ , and then were thawed, some of them were able to transform to normal pupae, and even to almost normal imagoes, but all of them failed to shed their pupal skins at the time of the emergence (Asahina, 1966). The cause of the freezing injury in the slug caterpillar may be the same as that in the case of the sawfly mentioned above, though the role of the fat-body cells during the metamorphosis has not been determined yet in the slug caterpillar.

It was reported that overwintering pupae of the swallow tail, *Papilio machaon hipocrates* Felder et Felder, could survive at a liquid nitrogen temperature for 2 days after prefreezing at  $-30^{\circ}\text{C}$ . About half the number of them were able to resume their development at  $20^{\circ}\text{C}$  after thawing. In these insects, however, the transformation from the pupa to the imago was restricted to the tissue of the anterior half in the pupal bodies. The abdomen behind the third or fourth segment remains in the pupal state, and had much fat-bodies within itself. They survived for some ten days with an active heart beat even after the anterior halves had died (Asahina, 1959). Such a half-imago

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**Fig. 4.** Intracellular freezing in fat-body cells, photographed at  $-15^{\circ}\text{C}$ .  $\times 100$

G. Fat-body cells in the visceral layer frozen intracellularly. The prepupa was frozen in the same way mentioned in Fig. 3. Ice crystals are found within these cells

H. The same as G under a polarizing microscope

I. Fat-body cells in the visceral layer frozen intracellularly. The prepupa was immersed directly in liquid nitrogen. Ice crystals within these cells are so small that the cells appear dark except for the space occupied by oil drops

J. The same as I under a polarizing microscope

K. Fat-body cells in the parietal layer frozen intracellularly. The prepupa was frozen in the same way mentioned above

L. The same as K under a polarizing microscope

formation may be assumed to be the result of freezing injury in the prothoracic gland which supplies moulting hormone at the time of transformation to imago in the insect. However, it is to be noted that the abnormal adult insect from the frozen-thawed pupa was imperfect not only in the moulting but also in the formation of normal internal tissues of imago. In the swallow tail, it is now possible to interpret the half-imago formation to be the result of the freezing injury in some fraction of fat-bodies. In a freezing susceptible pupae of a butterfly, *Papilio xuthus* Linne, the half-imagoes were invariably produced by a previous body freezing, even if they were rapidly thawed within a few minutes immediately after the initiation of body freezing (Tanno, 1963). Thus the half-imago formation is not a special injury produced by a previous freezing in the insect at a super-low temperature.

By freezing at super-low temperatures, almost all kinds of insects, so far as our experiments are concerned, suffered the above mentioned mode of injury except for the poplar sawfly, and failed to appear normally on the wing. One of the reasons why only the poplar sawfly can completely escape the freezing injury at a super-low temperature may be the application of the special freezing method, that is, very slow prefreezing to a temperature of  $-30^{\circ}\text{C}$  before immersing them in liquid nitrogen.

The freezing injury does not occur in the prepupa of the poplar sawfly by both standard ( $2^{\circ}\text{C}/\text{min}$ ) and very slow rate of freezing ( $0.5^{\circ}\text{C}/\text{min}$ ) at temperatures above  $-30^{\circ}\text{C}$ . In the following process of freezing to the liquid nitrogen temperature in the prepupa, why is the very slow prefreezing procedure to  $-30^{\circ}\text{C}$  more effective to protect fat-bodies from freezing injury than the standard rate of prefreezing? The appearance of extracellularly frozen fat-body cells was almost the same in both cases of prefreezing at the same final temperature of  $-30^{\circ}\text{C}$ . The occurrence of intracellular freezing in a few cells in the prepupae frozen with standard rate of cooling, cannot be the main factor to cause such a serious injury following immersion in liquid nitrogen, since the number of intracellularly frozen cells is too small. The grain size of ice crystals formed within the prepupal body by very slow freezing was about twice as large in diameter than that in the frozen insect by the standard rate of freezing. Estimated from the grain size of the ice crystals, the layer of concentrated hemolymph between ice grains and fat-body cells seems to be about twice thicker in very slowly frozen insects than in the frozen

**Fig. 5.** Fat-body cells before and after freeze-thawing

M. Fat-body cells in the visceral layer. There are many spherulites of uric acid in each cell.  $\times 100$

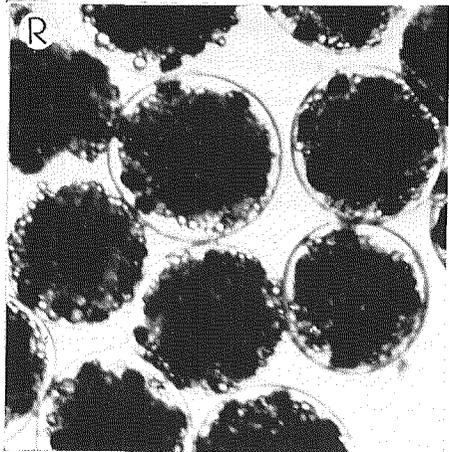
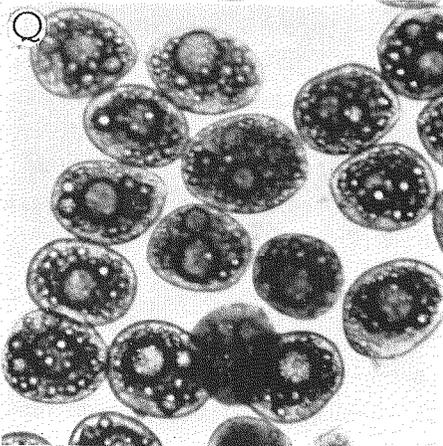
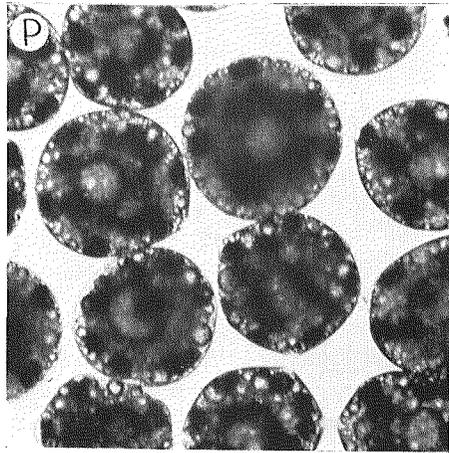
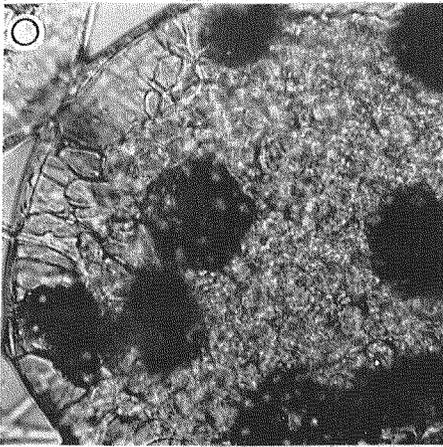
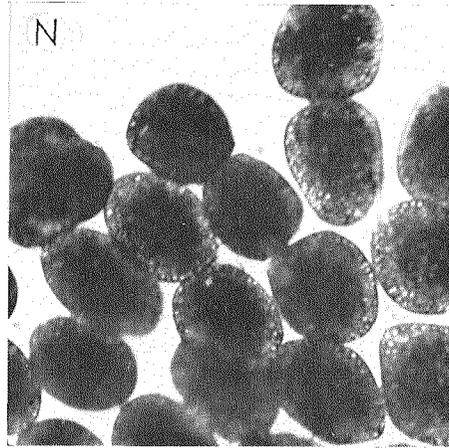
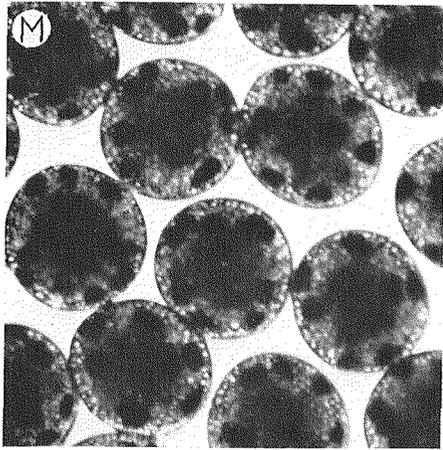
N. Fat-body cells in the parietal layer. There are no spherulites of uric acid in these cells.  $\times 100$

O. A part of a fat-body cell in the visceral layer showing a thin surface membrane. When the fat-body cells are slightly pressed between two plates, the thin membrane of fat-body cells is separated from the protoplasm.  $\times 380$

P. Fat-body cells in the visceral layer after thawing from extracellular freezing. The oil droplets within the cell coalesced into large drops.  $\times 100$

Q. Fat-body cells in the parietal layer after thawing from extracellular freezing.  $\times 100$

R. Fat-body cells in the visceral layer after thawing from intracellular freezing. The prepupa was immersed directly in liquid nitrogen, and was thawed at a room temperature. The thin membrane is separated from the protoplasm of fat-body cells and the disorganized cell structure is apparent.  $\times 100$



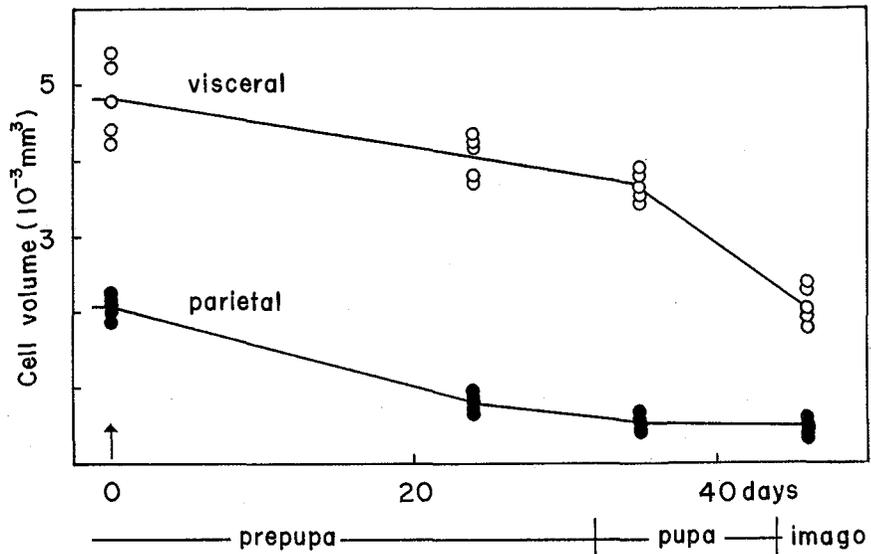


Fig. 6. Changes of volume in fat-body cells in accordance with the transformation of the sawfly

- : Fat-body cells in the visceral layer
- : Fat-body cells in the parietal layer

The arrow shows the time when prepupae were removed to a temperature of 25°C from an outdoor temperature

insects cooled by the standard rate. When a prepupa is rapidly immersed in liquid nitrogen after prefreezing, fat-body cells may suffer drastic mechanical stress brought about by the rapid thermal contraction of the insect body. In fact, a faint but sharp sound was sometimes heard at the time of immersing them in liquid nitrogen, and some of them were found to be broken. The mechanical stress may be somewhat lowered by the layer of concentrated hemolymph which surround fat-body cells. The thick layer of concentrated hemolymph may be effective to decrease the stress by which some fat-body cells may suffer mechanical injury. A natural occurrence of glycerol or other polyol has been found in a variety of frost-resistant insects (Asahina, 1966; Asahina and Takehara, 1964; Takehara and Asahina, 1960; Salt, 1965; Sømme, 1964, 65). Prepupae of the poplar sawfly contain much trehalose but no glycerol in their body (Tanno, 1964, 1965 c; Asahina and Tanno, 1964). The trehalose in the prepupae may be useful in increasing the thickness of the layer of the concentrated hemolymph when they are frozen.

The injury produced in the step of freezing from  $-30^{\circ}\text{C}$  to the liquid nitrogen temperature occurred more readily in the fat-body cells of the visceral layer than those of the parietal layer. This may be explained by the difference in size between these two kinds of fat-body cells. Under the same mechanical stress, the larger fat-body cells of the visceral layer will reasonably suffer larger strain than the much smaller fat-body cells of the parietal layer.

### Acknowledgments

The author wishes to express his gratitude to Prof. E. Asahina for offering some experimental results and valuable advice in the preparation of the manuscript.

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