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Frost Injury and Resistance in the Poplar Sawfly, *Trichiocampus populi* OKAMOTO*

Kouzou TANNO

丹 野 皓 三

*Biological Section, Institute of Low Temperature Science
Hokkaido University*

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Abstract

The Japanese poplar sawfly, *Trichiocampus populi* OKAMOTO, was investigated with special regard to frost resistance.

Overwintering prepupae of this insect were able to survive liquid nitrogen temperature following prefreezing treatment at temperatures below -20°C . Such high levels of frost resistance in this insect persisted throughout the five month cold season. A remarkably large amount of sugar, estimated to be about 5 percent of the fresh body weight, was found in the prepupae. The main part of this sugar was trehalose. A clear correlation was observed in this insect between frost resistance and sugar levels. The insect did not have glycerol or any other polyhydric alcohols. It would seem, therefore, that the high sugar content of the sawfly may contribute to its survival during freezing.

The prepupae readily suffers frost injury by artificial rapid freezing even at -20°C . A clear correlation was observed in frozen-thawed insects between the number of intracellularly frozen fat-body cells and the mode of injury in the insect as a whole which appears upon metamorphosis after thawing. It was observed that more than half the volume of the fat-body cells in the prepupa was utilized during metamorphosis to the imago. It would seem, therefore, that an occurrence of fatal intracellular freezing in some of the fat-body cells may result in sublethal injury in the insect as a whole after thawing. Sublethal injury was also observed in prepupae frozen down to -190°C and thawed. Even after rewarming from the super-low temperatures, transformation into pupae is possible. Upon emergence, however, they cannot shed their pupal skins. Such frost injury could be prevented by keeping prepupae at -5°C for several hours in the step of pre-freezing to -30°C before immersion in liquid nitrogen. Judging from the micro-

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scopical observation of frozen sections of prepupae, the following mechanism of such frost injury was considered. When a prepupa is rapidly immersed in liquid nitrogen after prefreezing, fat-body cells show a distinct tendency to suffer drastic mechanical stress brought about by the rapid thermal contraction of the insect body, which may even lead to death. It was surmised that the prefreezing procedure at 5°C may provide a favorable arrangement of both fat-body cells and ice particles in the insect to decrease such mechanical stress which may arise between them.

Immediate termination of diapause occurred in prepupae of the sawfly by freezing the insect down to -15°C or more for 30 minutes. No prepupae, however, terminated their diapause after body freezing at -10°C, even when the duration of body freezing was prolonged for 21 days. It seems probable that the immediate termination of diapause by body freezing may be explained to be the result of the inactivation of the corpora allata by freezing.

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1. Introduction

There are two common natural means by which insects may avoid frost injury, namely supercooling and frost resistance. The former is the avoidance of freezing, the latter the tolerance to freezing. It has been known in several species of insects that the accumulation of glycerol or sorbitol in insects

enhances the supercooling ability¹⁻⁹⁾. The majority of hibernating insects can pass the winter in a supercooled state. When the environmental temperature sinks below the supercooling point in an insect, ice forms in the insect body. In many of these insects ice formation in the insect body is fatal, while others can survive such freezing. The former group is called freezing-susceptible and the latter freezing-resistant or frost resistant¹⁰⁾. In several insects, a clear correlation was found between the seasonal changes of the frost resistance ability and of the amount of glycerol accumulated in the insect body^{1,4,9,11-14)}. It seems, therefore, that the accumulation of glycerol in overwintering insects may contribute to the avoidance of frost injury by enhancing not only the supercooling ability but also the frost resistance. In fact, with a few exceptions⁷⁾, the majority of remarkably frost resistant insects have been shown to accumulate glycerol or sorbitol in their bodies⁹⁾.

The overwintering prepupae of the Japanese poplar sawfly, *Trichiocampus populi* OKAMOTO, however, were found to survive freezing down to liquid nitrogen temperatures without the accumulation of any polyhydric alcohol, but with an extraordinarily large amount of trehalose^{15,16)}. It has been shown that very frost-hardy plants, as a rule, contain a large amount of sugar¹⁷⁾. In insects, however, accumulation of a quantity of sugar which may be useful in the avoidance of frost injury has not been reported except in the case of this sawfly. The present paper deals with various properties concerning frost resistance in the poplar sawfly.

Some insects can survive freezing at an extremely low temperature far below the climatic winter temperatures^{9,18)}. Some larval or pupal insects were observed to be apparently intact after warming from an extremely low temperature, but their further development was subnormal and they failed to emerge on the wing. A remarkable example of such sublethal frost injury was demonstrated in the prepupae of this sawfly: after thawing from liquid nitrogen temperature, while some of the prepupae were able to resume development up to the formation of imago, in the majority of the cases they could not shed their pupal skins^{19,20)}. To clarify the mechanism of such frost injury, the mode of freezing of fat-body cells was observed in relation to the resulting injury to the entire insect.

Since fat-body cells in insects are generally far larger in size than other tissue cells²¹⁾, intracellular freezing has a high tendency to occur in fat-body cells by rapid freezing²²⁻²⁴⁾. It has been known that an clear formation of intracellular ice crystals is fatal in almost all living cells²⁵⁻²⁷⁾. Salt, however, reported that an intracellular freezing is not necessarily fatal in fat body cells in the frost resistant larvae of *Eurosta solidaginis*²⁸⁾. On the other hand,

in fat-body cells of the prepupae of the poplar sawfly intracellular freezing seems to be fatal to the cells. The effect of the intracellular freezing in fat-body cells upon the metamorphosis after thawing of the frozen insect is described in the present paper. As to the effect of freezing on the termination of diapause of this sawfly, a remarkable experimental result was also briefly referred to.

II. Materials and Methods

Materials

The Japanese poplar sawfly, *Trichiocampus populi* OKAMOTO, is one of the common pests of poplar trees. The distribution of the insect is limited to a few regions in Japan, including the Hokkaido island. The insect is mono-voltine in this island and appears on the wing during a period from the end of June to the middle of July. In early autumn many of the 5th stage larvae are observed gathering and feeding on leaves of poplar trees (Pl. I-1, 2). After completing the feeding stage, the full-grown larvae move to dead trees or grass under the poplar trees to find suitable places for overwintering (Pl. I-3). They are frequently found in small holes or slits in the surface of dead trees or in the empty pith of dead grass throughout the cold season (Pl. I-4).

The full-grown larvae of this sawfly were collected in Sapporo, during the autumn season from 1963 to 1968. 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 the pith of the stems. The larvae began to spin cocoons and two stoppers at the both ends of each cocoon within the stem (Pl. I-1, 5). After three days in the cocoon, the larva transformed into prepupa. When the prepupae were removed from the cocoon, they assumed a U-shape position, and were not able to crawl (Pl. I-6). The prepupae in the stems were then placed in a room in which the room temperature was maintained as close to the outdoor temperature as possible throughout the winter. There were some differences in seasonal changes of frost resistance between the male and female in the insects, although the general pattern was almost the same²⁹. Thus, only female prepupa were used for the present 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 of the insects

was about $2^{\circ}\text{C}/\text{min}$. When an insect was exposed to a temperature below -10°C without ice seeding, body freezing invariably occurred in the insect within ten minutes. The supercooling point in the prepupae was $-8.6 \pm 0.4^{\circ}\text{C}$ ¹⁶⁾. 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 insect was determined by examining the lowest tolerable temperature. When over 60 per cent of the frozen insects could survive for more than 30 days after thawing, the freezing temperature 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 enabling them to survive in liquid nitrogen^{18,30)}. As the prefreezing temperatures, -15 , -20 , -25 and -30°C were employed. The cooling rate during prefreezing was about $2^{\circ}\text{C}/\text{min}$. After 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.

Freezing with various cooling rates

Treatments A to E in Table 3 indicate the different modes of freezing with various cooling rates. A. Twenty prepupae in a small cage made of wire gauze were immersed directly in liquid nitrogen from room temperature. After freezing in liquid nitrogen for one minute, they were transferred to a cold box at -20°C , and were kept there for 2 hours. B. Prepupae on a piece of wire gauze were exposed to cold vapour ($-60 \sim -70^{\circ}\text{C}$) from liquid nitrogen. After 25 seconds, they were transferred to a cold box at -20°C , and were kept at the same temperature for 2 hours. C. In a cold room at -30°C , prepupae on wire gauze were exposed to cold winds from an electric fan for 5 minutes. Then, they were transferred to a cold box held at -20°C and were kept there for 2 hours. D. In a cold room at -20°C , prepupae on wire gauze were exposed to cold winds from an electric fan for 5 minutes. Then, they were transferred to a cold box at -20°C , and were kept there for 2 hours. E. Twenty prepupae were placed in a petri dish, and were cooled in a cold box at -20°C for 2 hours. In each treatment, the cooling rate of the insects were estimated by determining the body temperature with a fine thermocouple as will be described later.

Methods of cooling to avoid frost injury in liquid nitrogen

The following methods were attempted (Tables 4, 5). Experiment No. 1. The prepupae were frozen down to -30°C with a cooling rate of about

2°C/min. They were kept at -30°C for 9 hours. Experiment No. 2. After the treatment No. 1, prepupae were directly immersed in liquid nitrogen. They were kept in liquid nitrogen for 3 to 24 hours. Experiment No. 3. After the No. 1 treatment, the prepupae were kept at -70°C using dry ice for 18 hours. Experiment No. 4. After the No. 3 treatment, the prepupae were immersed in liquid nitrogen from a temperature of -70°C . They were kept there for 3 hours. Experiment No. 5. The prepupae were frozen down to -30°C with a cooling rate of about $0.5^{\circ}\text{C}/\text{min}$. They were kept at -30°C for 9 hours. Experiment No. 6. After the same treatment as in No. 5, the prepupae were directly immersed in liquid nitrogen. They were kept there for 3 to 24 hours. Experiment No. 7. Prepupae were frozen down to -30°C with a cooling rate of about $1^{\circ}\text{C}/\text{min}$ and kept at the same temperature for 20 hours. Experiment No. 8. Prepupae, previously frozen to -20°C with a cooling rate of about $1^{\circ}\text{C}/\text{min}$, were warmed to and kept at -5°C for 18 hours. They were then cooled down to -30°C with a cooling rate of $1^{\circ}\text{C}/\text{min}$ and kept there for 24 hours. Experiment No. 9. After the No. 7 treatment, the prepupae were immersed in liquid nitrogen for 24 hours. Experiment No. 10. After the No. 8 treatment, the prepupae were immersed in liquid nitrogen for 24 hours.

Recording of the freezing curves

Since prepupal bodies were soft and flexible, they were doubled up and tied lightly with a cotton thread to hold a U-shape form. A fine copper constantan thermocouple was set in the fold of the doubled up prepupa, the temperature of the prepupa was recorded by an electronic recorder. Freezing curves in five individual prepupae were obtained in this manner in each experiment. Cooling rates shown in Tables 2 and 3 indicate the average cooling rates from the freezing point of prepupa to -20°C which were determined from the freezing curves. Cooling rates except for that indicated in Tables 2 and 3 were expressed by tangents at -15°C along the freezing curves. In this expression, cooling rates of $2^{\circ}\text{C}/\text{min}$ and $1^{\circ}\text{C}/\text{min}$ in Tables 4 and 5 are almost the same as $0.8^{\circ}\text{C}/\text{min}$ and $0.4^{\circ}\text{C}/\text{min}$ in Tables 2 and 3 respectively.

Estimation of total sugar content

A single insect was ground in 5 or 10 ml of 80% ethanol and an extract was obtained by centrifuging. The extract of the volume from 0.05 ml to 0.5 ml was heated at 100°C for 10 minutes with 5 ml of anthrone reagent³¹⁾. The amount of total sugar was determined colorimetrically and was expressed as milligrams per gram of fresh body weight.

Paper chromatographic analysis of sugar and polyhydric alcohol

Ten to twenty insects (527–1450 mg) were ground in 80% ethanol and an extract was obtained by centrifuging. The remaining residue was washed twice with 80% ethanol. The ethanol extracts obtained in this manner were combined and dried in a warm air stream. The resulting residue was suspended in a small amount of anhydrous pyridine and insoluble matter was removed by centrifuging. The supernatant obtained was used for paper chromatographic analysis. Aliquots of the supernatant were applied to a strip of Whatman No. 1 paper and developed by the ascending method with *n*-butanol-acetic acid-water (4 : 1 : 2) as the solvent³²⁾. Permanganate periodate reagent was sprayed on this strip to detect the spot of trehalose³³⁾. For detecting the spots of other sugars and polyhydric alcohol, Trevelyan's alkaline silver nitrate method³⁴⁾ was used. The appropriate area was cut from the unsprayed paper chromatogram strip and the sugars and polyhydric alcohol were eluted with 20 ml of deionized water respectively. The sugar content was determined colorimetrically with anthrone reagent. The polyhydric alcohol content was also determined colorimetrically with chromotropic acid reagent³⁵⁾.

Chemical method of trehalose estimation

With the chemical character of trehalose in mind which has a high stability against both acid and alkaline, Wyatt and Kalf (1957) reported a method of trehalose estimation in sugar mixtures in insects³⁶⁾. Because of the simplicity of the procedure, this method was mainly used for estimating trehalose. Ethanol extract was obtained by the same method described above in sugar estimation. The extract of 0.05 to 0.5 ml was dried at 100°C. The residue was dissolved in 0.3 ml of 1 N H₂SO₄, and was heated at 100°C for 10 minutes. After this treatment, 0.2 ml of 6 N NaOH was added to this solution. This was heated again at 100°C for 10 minutes. After cooling, the trehalose content in the solution was determined with anthrone reagent.

Estimation of glycogen

To the resulting residue after the ethanol extraction of sugar, 0.5 ml of 30% KOH was added, and the mixture was heated at 100°C for 20 minutes³⁷⁾. After cooling, 4.5 ml of 80% ethanol and active carbon were added to the mixture. A supernatant was obtained by centrifuging. Aliquots of the supernatant were used to determine the glycogen content with anthrone reagent.

Estimation of water content

The insect was dried at 105°C for 8 hours. The water content of the

insect was determined from the difference in weight between fresh and dried insects. This was expressed in percentage of the initial fresh weight.

Observation of freezing fat-body cells

The prepupae were separately frozen using various cooling rates as described previously. In a cold room at -15°C or -20°C , these frozen insects were fixed to wood blocks with a small amount of aniline, and the frozen sections were made by using a microtome. The frozen section was $20\ \mu$ in thickness for ordinary light microscopy, and was $25\ \mu$ for polarizing microscopy.

Since the frozen sections tended to curl, they were unrolled with a fine needle on a slide glass. By keeping the sections on the slide glass for about one hour without a cover glass in cold room, ice crystals within the section sublimated. In this manner, the 'freeze-dried tissue section' was obtained on the slide glass²³⁾. By this method, the remaining tissue could be clearly observed under a microscope, although it was difficult to obtain smooth sections. In addition the following method³⁸⁾ was developed. A small amount of liquid paraffine saturated with ice was placed on a slide glass at -15°C or -20°C . When the frozen sections unrolled with a fine needle were placed on the liquid paraffine layer on the slide glass, the sections became smooth. The ice crystals within the sections were then sublimated. In this method, however, the 'freeze-dried tissue section' could not be clearly observed under an ordinary microscope because of the very small difference in light refraction between the tissues and the surrounding liquid paraffine. The 'freeze-dried tissues' prepared in this manner were, therefore, observed under a phase-contrast microscope.

After thawing of frozen insects, the fat-body cells were removed from the insect body and were transferred into Ringer's solution to be observed under a microscope.

Morphological observations of fat-body cells

The insects were fixed for 2 days in Gender's fixative. The fixed insects were embedded in paraffine wax after dehydration through a ethanol-xytol series. Transverse sections of $5\ \mu$ in thickness were made from the fixed insects. The sections obtained were dyed with eosin and hematoxiline.

Since almost all oil droplets and spherulites of uric acid are lost from the fat-body cells by the above described treatment, frozen sections were used to observe these materials. The insects were immersed directly in liquid nitrogen from room temperature. In a cold room at -15°C , the frozen sections were made from the insect frozen in liquid nitrogen. After sub-

limiting ice crystals in the sections, the remaining tissue was observed under an ordinary microscope or a polarizing microscope in a cold room. The cut surface of the remaining insect body from which the frozen sections were obtained, was also observed in a cold room after slight sublimation of the ice crystals on the surface.

Determination of the prepupal diapause

The progress of metamorphosis in prepupae was observed in the rearing box at 20°C. When the prepupae resumed development to pupae within 40 days at 20°C, they were regarded as non-diapausing prepupae. Diapausing prepupae could not transform into pupae and were alive for over 30 days at 20°C. The body weight showed a gradual drops (Pl. I-11) and finally died without developing beyond the prepupal stage. The percentage of the diapause is indicated by the number of the diapausing prepupae in the total number of prepupae used in an experiment.

III. Results

Seasonal change in frost resistance and sugar content in the insect

A remarkable seasonal change in frost resistance and sugar content in the sawfly was observed (Fig. 1). In the middle of August, no larvae could survive freezing even at a temperature of -10°C for 30 minutes. After spinning cocoons, they transformed into prepupae. During such transformation, their frost resistance was rapidly enhanced in proportion to the increase of the sugar content. Prepupae only three days after the cocoons were spun, could survive freezing at -10°C for 24 hours. On the 20th day after cocooning, the prepupae could withstand freezing at -30°C, even though the number is limited a few survived liquid nitrogen temperature. In this season, the outdoor air temperature gradually falls, however, such thermal conditions do not seem to be the direct cause of the remarkably rapid enhancement of their frost resistance, because the velocity of the increase in frost resistance after the cocoons are spun is exactly the same in the prepupae both in the middle of August and the middle of September.

Almost all prepupae on the 40th day after the cocoons were spun could withstand liquid nitrogen temperature, provided that they were previously frozen at -30°C. At about this time, the sugar content reached a maximum level of 4.27 ± 0.56 per cent, based on their fresh body weight. In January, prepupae survived liquid nitrogen temperature after prefreezing at -20°C. Such high levels of frost resistance and sugar content persisted throughout the five month cold season. With the rising temperature in spring metamor-

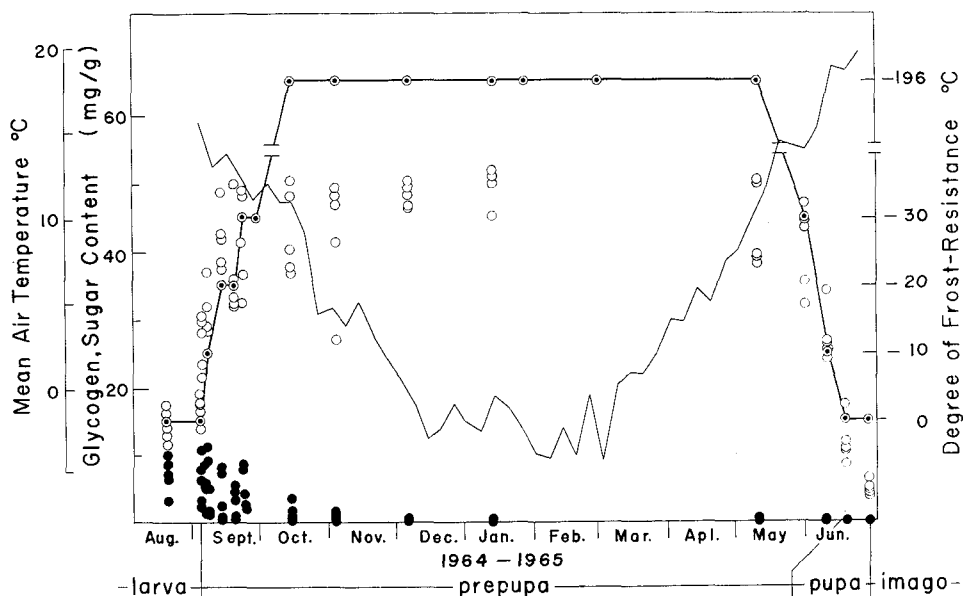


Fig. 1. Seasonal changes in the frost resistance, sugar and glycogen content in the sawfly

○: Degree of frost resistance ○: Sugar content
—: Environmental temperature ●: Glycogen content

phosis to pupae commenced in prepupae (Pl. I-7) in the middle of June of the following year. The degree of frost resistance decreased to -10°C in accordance with the decrease of sugar content. Almost all pupae could not survive freezing at -10°C for one day. All adults were invariably killed by freezing at -10°C for several minutes.

The full grown larvae initially contained glycogen of $6.0 \pm 3.4 \text{ mg/g}$, based on their fresh body weight. After cocooning, the glycogen content decreased as compared against the increase of sugar content, and almost all of the glycogen disappeared on the 40th day in the prepupae. Glycogen contained in the insect, therefore, may convert to sugar, however, the amount of increased sugar seems to be much larger than that of the decreased glycogen (Fig. 1). As shown in Fig. 3, a remarkably rapid decrease of water content occurs in the insect upon transformation from larva to prepupa. Since the amount of sugar is expressed as the weight percentage based on fresh body weight, the sugar content in prepupae is apparently larger than that in larvae. It is clear from Figs. 1 and 2 that the absolute sum of sugar and glycogen in the insect hardly changes during the developmental period from the full grown larva to the end of the prepupal stage.

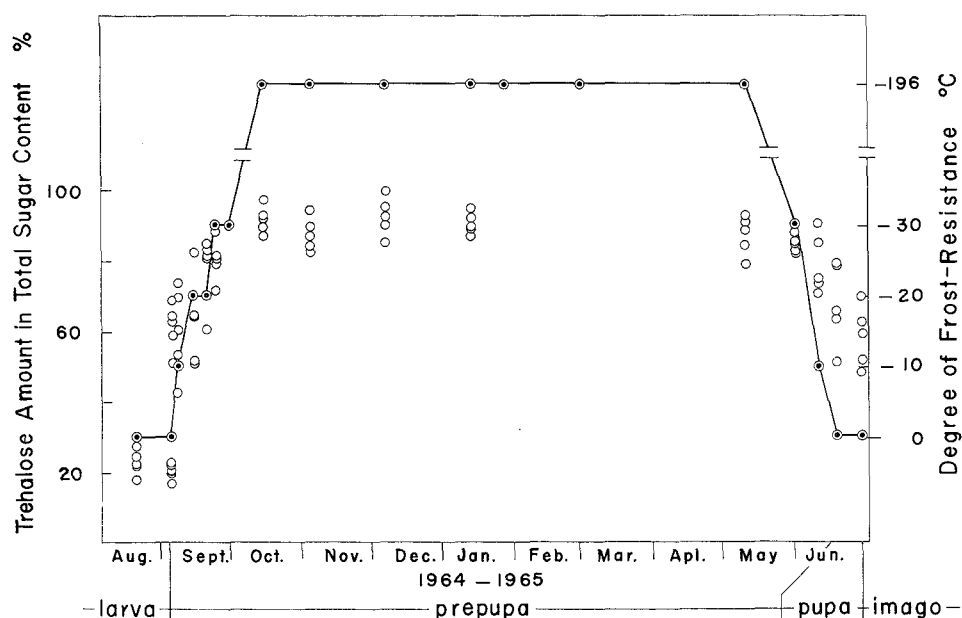


Fig. 2. Seasonal changes in the proportion of trehalose in total sugar content

- : Percentage of trehalose in total sugar content
 ● : Degree of frost resistance

About 90% of the total sugar content in the frost resistant prepupae was trehalose (Fig. 2). This persisted throughout the five month cold season. The percentage of trehalose in the total sugar in the insect also changed seasonally in parallel with the degree of frost resistance. Both the ability of frost resistance and the trehalose percentage reached its maximum on the 40th day after cocooning (Fig. 2), but the total sugar level reached its maximum on the 20th day after cocooning (Fig. 1).

With a few exceptions, it is known that almost all remarkably frost resistant insects accumulate glycerol or sorbitol in their bodies. The sawfly, however, contained no polyhydric alcohol during the overwintering period.

As mentioned above, a remarkable seasonal change of water content in the insect was observed (Fig. 3). The 5th larvae feeding on poplar leaves have a high water content, amounting to $79.2 \pm 2.1\%$ of the fresh body weight. After finishing feeding, the water content in the full grown larvae decreased to $68.1 \pm 1.1\%$. On the 3rd day after cocooning, the insects contained water amounting to $61.2 \pm 1.4\%$. On the 20th day after cocooning, the water content

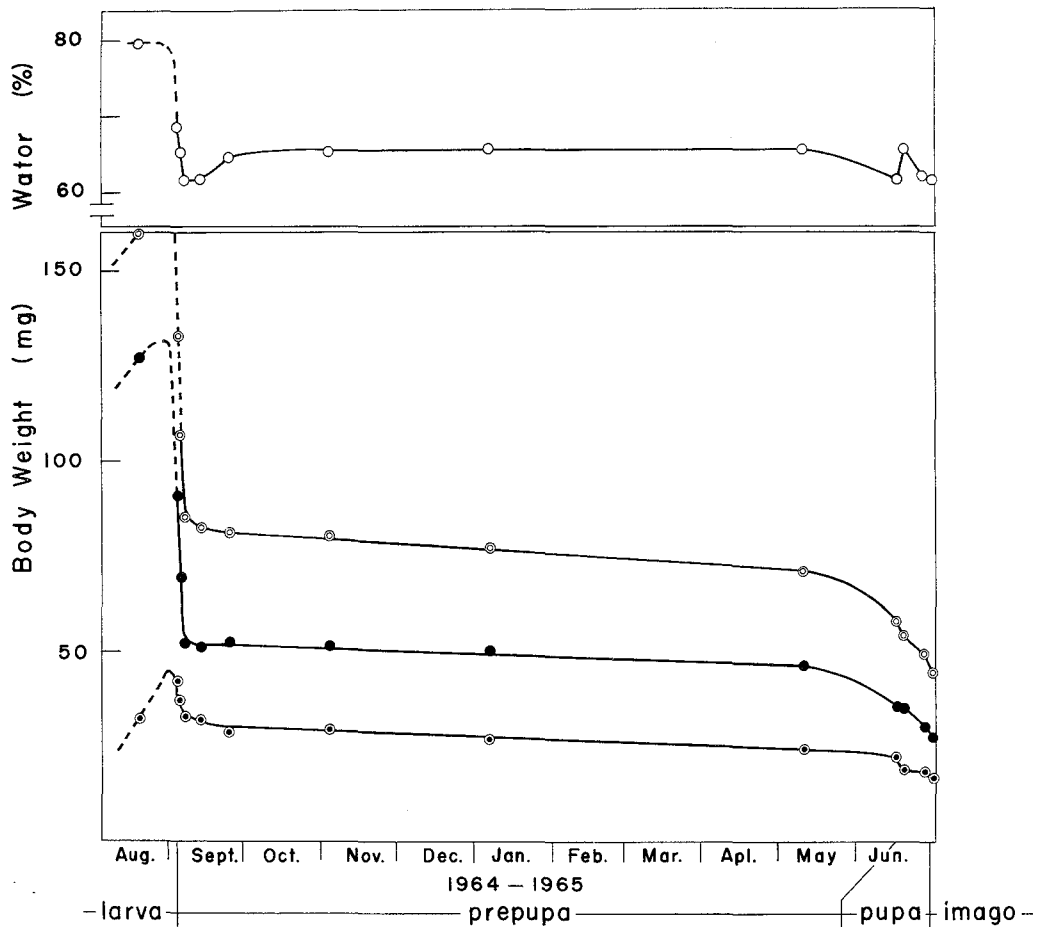


Fig. 3. Seasonal change in water content in a single insect

○: Water content (weight percentage based on fresh body weight) ⊙: Fresh body weight
●: Water content ⊙: Dry weight

was $64.8 \pm 1.7\%$. This water level persisted till early June of the following year. Immediately before pupation, the prepupae had a water content of $61.3 \pm 2.5\%$. The water content was $65.6 \pm 2.4\%$ in the pupae, and $61.8 \pm 2.0\%$ in the imagoes. The rapid decrease of fresh body weight during the transformation from larvae to prepupae may be mainly attributed to the water loss from the insects. The slight loss of dry matter from the insects during the same transformation was reasonably explained by the weight of cocoons spun. The average weight of the cocoon in 40 prepupae was 7.6 mg. The

fresh body weight of prepupae decreased slightly during the overwintering period. When the transformation into pupae occurred in prepupae in the middle of May of the following year, the fresh body weight rapidly decreased. During the transformation into imago, a considerable loss of fresh body weight also occurred. As described above, a remarkable change of water content occurred in the stages of both cocooning and pupation. In these stages, a remarkable change of the frost resistance also occurred in the insects. Since the sugar concentration in the insects is enhanced in accordance with the loss of water from the insects, the loss of water may be indirectly effective to the enhancement of the frost resistant ability in the insects.

Fat-body cells in the prepupa

As described above, the overwintering prepupae of this sawfly had such a high frost resistance that they could survive freezing at temperatures far below outdoor temperatures in severe winter. The prepupae, however, easily suffer frost injury by artificial rapid freezing even to -20°C . After freeze-thawing, these injured prepupae survived for a long period, but they were subnormal in resuming development. Judging from the important role of fat-body cells in the metamorphosis of the insect, the fat-body may assumably be one of the tissues responsible for such an indirect mode of freezing injury. The morphological structure of fat-body cells in the prepupae and the behavior of these cells during metamorphosis are, therefore, described here.

The prepupa, as known in many other insects²¹⁾, has two types of fat-bodies. One is the parietal layer, and the other is the visceral layer. The latter is arranged around the digestive canal and the former is situated along the body wall in the body cavity (Pl. II-1, 2, 4, 5). Each layer has nearly the same number of cells which are estimated to be about 2,700. There are some differences between these two types of fat-bodies (Table 1), especially in the deposit of uric acid in the cells.

The fat-body cells of the visceral layer are yellow colored spheres which are about 230μ in diameter (Pl. III-1). The fat-body cells of the visceral layer in the dorsal part are somewhat larger, about 250μ in diameter, than in the other parts of the body cavity. They are filled with oil drops. There are many pouches about 22 in number, on the surface of almost all individual fat-body cells in the visceral layer. These pouches are filled with many particles (Pl. III-4, 6, IV-1), and appear bright under a polarizing microscope (Pl. III-2). Since these particles show the so-called Maltese cross in polarized light, they presumably are spherulites (Pl. IV-2). When the fat-body cells in the visceral layer are placed in absolute ethanol and are stirred with

Table 1. Fat-body cells in the prepupa

	Fat-body cells in the visceral layer	Fat-body cells in the parietal layer
Color	yellow	orange
Diameter of a cell	200-250 μ	150-200 μ
Number of uric acid pouches in a cell	0*-27 (average in 100 cells: 22)	0
Number of the cells in a prepupa	2,500-3,000	2,500-3,000
Fresh weight percentage in an insect (average in ten prepupae)	22.7	10.5

* The fat-body cells in the visceral layer not containing the uric acid pouches are located in the vicinity of the ventral nerve cord

a glass needle, the particles depart from the pouches and scatter in the outside medium (Pl. IV-3). If these particles are suspended in absolute ethanol, and a small amount of distilled water is added they readily change from spherulites to single crystals (Pl. IV-4). These crystals were determined to be of uric acid⁴⁰⁾. The existence of uric acid in fat-body cells is known in many other insects³⁹⁾. During metamorphosis in the insect, these fat-body cells begin to decrease in size at the pupal stage, while the spherulites of uric acid in these cells show little or no change either in size or in number during the stages from full grown larva to imago. A few fat-body cells in the visceral layer which are located in the neighborhood of the ventral nerve cord contain no uric acid pouches. During the conventional procedure of paraffine wax embedding of fixed fat-body, all spherulites of uric acid readily dissolve in the medium and disappear from the pouches (Pl. II-4, III-5).

The fat-body cells in both the visceral and parietal layer are connected by fine canals with each other (Pl. III-3, IV-5). The fat-body cells are arranged continuously from the anterior to the posterior end in the body cavity in a folded thin sheet shape (Pl. II-5). Each fat-body cell in both layers is tightly covered with a thin membrane. When a fat-body cell is slightly flattened between a slide and a cover glass, the thin membrane separates from the protoplasmic surface (Pl. III-4).

The fat-body cells in the parietal layer are also connected by fine canals with each other (Pl. IV-5). These cells have no pouches filled with uric acid during the full grown larva to imago stage but are filled with oil drops and many tiny granules (Pl. IV-6). The fat-body cells in the parietal layer

are orange colored spheroids with a diameter of about $170\ \mu$. The arrangement of fat-body cells in both layers in the body cavity is schematically shown in Plate II-5.

In the utilization of fat-bodies during metamorphosis in the insect, a difference was observed between the parietal and visceral layer. The change in average cell volume within fat-bodies during metamorphosis from prepupa to imago is shown in Fig. 4. It seems that the fat-body cells in the parietal layer are mainly utilized for the formation of pupa during the prepupal stage,

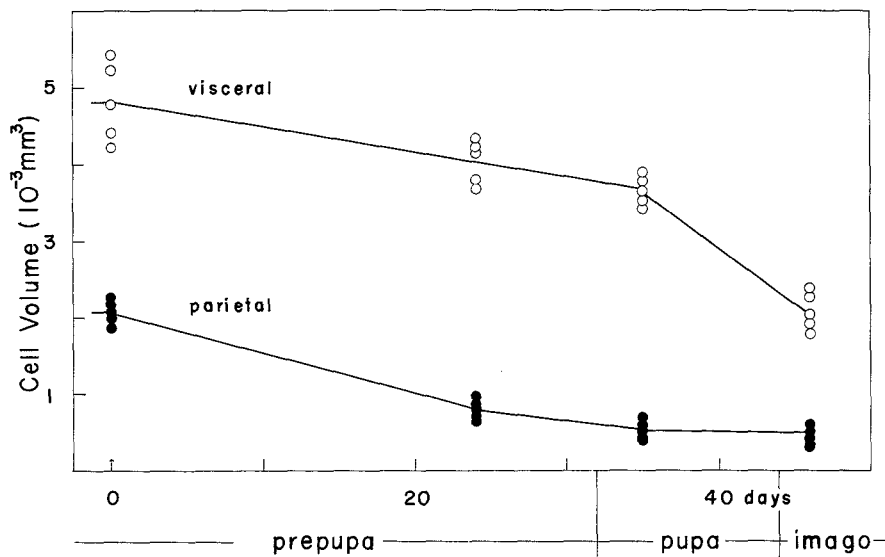


Fig. 4. Changes in volume of fat-body cells in accordance with the transformation of the sawfly at 25°C

○: Fat-body cells in the visceral layer

●: Fat-body cells in the parietal layer

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

and those in the visceral layer are mainly utilized for the formation of the imago during the pupal stage.

Freezing state of fat-body cells

To examine how fat-body cells freeze within the body cavity of an overwintering prepupa, frozen sections from the prepupa were observed under microscope. The freezing procedure used was the same as that employed to test the ability of frost resistance in the prepupae.

When the prepupae were cooled to -30°C at a cooling rate of about $2^{\circ}\text{C}/\text{min}$, extracellular freezing occurred in most of the fat-body cells except for a few in the visceral layer which froze intracellularly. The extracellularly frozen cells in the visceral layer contracted irregularly, and the oil droplets within them coalesced into larger drops. The uric acid pouches on the cell surface appeared brighter than the extracellular ice crystals under a polarizing microscope (Pl. V-2). There were no ice crystals within the fat-body cells (Pl. V-1). After keeping the same section in a cold room at -15°C for about one hour without a cover glass, all ice crystals sublimated and the freeze-dried fat-body cells alone remained on the slide glass (Pl. V-3). In such cells the uric acid pouches merely showed a bright appearance under a polarizing microscope (Pl. V-4). All fat-body cells in the parietal layer froze extracellularly within the prepupal body (Pl. V-5, 6). After thawing, the extracellularly frozen cells recovered their normal size and shape (Pl. VII-1, 2) except for the size of oil drops (Pl. VII-3, 4). The oil droplets within them coalesced into larger drops.

At a cooling rate of $2^{\circ}\text{C}/\text{min}$, a few fat-body cells in the visceral layer froze intracellularly, and the number of intracellularly frozen cells was observed to be less than 1% in the total fat-body cells in the visceral layer in a prepupa. These cells contracted very slightly, and many large ice crystals were seen within them (Pl. VI-1, 2). Almost all prepupae frozen in this manner could normally develop to imagoes after thawing. In the insects at the end of the prepupal stage, intracellular freezing readily occurred under the same freezing conditions; sometimes over 70% of the total cells in the visceral layer froze intracellularly. In the parietal layer, on the other hand, extracellular freezing alone occurred in fat-body cells. Prepupae frozen in this manner survived only for a few days after thawing.

When the prepupae were immersed directly in liquid nitrogen, intracellular freezing invariably occurred in all fat-body cells. These cells did not contract. Since numerous fine ice crystals were formed within them, the cells appeared dark under an ordinary light microscope except for the spaces occupied by oil droplets (Pl. VI-3, 5), and appeared bright under a polarizing microscope (Pl. VI-4, 6). Immediately after thawing, however, these cells appeared almost normal, because they were tightly covered with a thin membrane. Within the following few minutes in Ringer's solution, however, the thin membrane was separated from the protoplasm in all cells of both fat-body layers showing a destroyed structure of the protoplasm (Pl. VII-5, 6). The rapidly frozen prepupae invariably succumbed immediately after thawing.

Intracellular freezing in fat-body cells in the prepupae frozen with various cooling rates and the post-thawing injury in whole insect

The overwintering prepupae were separately frozen to -20°C by different treatments from A to E as shown in Table 2. These freezing treatments differed in cooling rates from $327^{\circ}\text{C}/\text{min}$ to $0.4^{\circ}\text{C}/\text{min}$. Freezing curves of these prepupae are shown in Fig. 5. In a cold room at -20°C , frozen sections were made from these frozen insects and observed under a microscope.

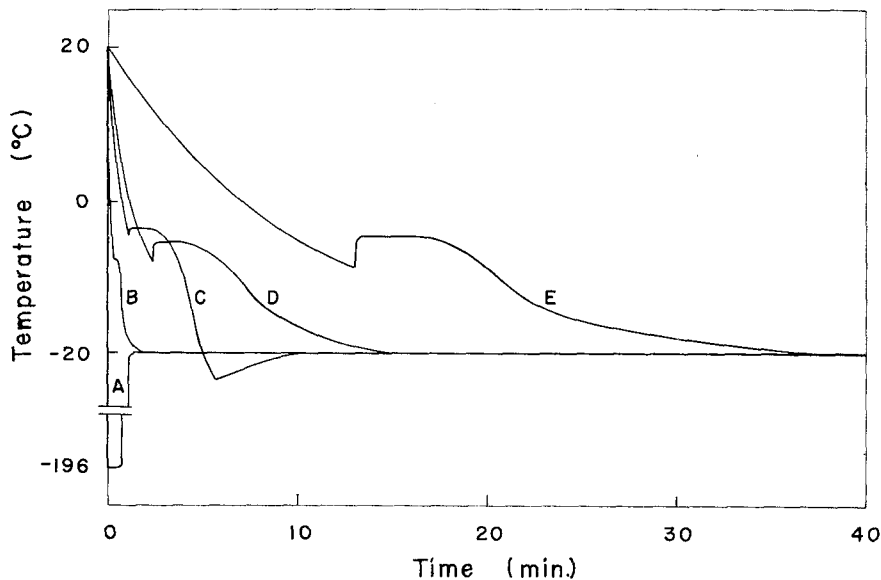


Fig. 5. Freezing curves of prepupae frozen with various cooling rates in the experiments indicated in Tables 2 and 3

When the prepupa was frozen with treatment A in which the cooling rate was as high as $327^{\circ}\text{C}/\text{min}$, all fat-bodies in both the parietal and visceral layers froze intracellularly (Pl. VIII). These frozen fat-body cells did not contract and appeared nearly intact as unfrozen fat-body cells in size and shape. Since numerous fine ice crystals were formed within these cells, the cells appeared dark in ordinary transmitted light (Pl. VIII-2, 3). Numerous fine ice crystals were also formed in other tissues. The diaphragm and the ventral nerve cord, therefore, appeared as dyed tissues under a microscope (Pl. VIII-1, XIII-3). The cells in these tissues other than fat-bodies were so small that it was impossible to observe whether these cells actually froze intracellularly or not.

In treatment B, the cooling rate was far slower than that of treat-

Table 2. Cooling rates and frequency of incidence of intracellularly frozen fat-body cells in the prepupae

Freezing * treatments	Cooling ** rates (°C/min)	Intracellularly frozen fat-body cells (%)***	
		Parietal layer	Visceral layer
A	327	100	100
B	15.4 ± 3.1	48 ± 8	100
C	4.0 ± 0.3	17 ± 3	98 ± 2
D	0.8 ± 0.1	0	17 ± 6
E	0.4 ± 0.0	0	0

* Final temperature was -20°C in all treatments. Details of the treatments are described in the text. Freezing curves of prepupae with the treatments from A to E are shown in Fig. 4

** Five prepupae were used in each treatment. Indicated cooling rates are the average cooling rates along the freezing curves from the freezing point of prepupae to -20°C

*** Five prepupae were used in each treatment. Three frozen sections cut from each prepupa were observed in a cold room at -20°C

ment A, however, it was far higher than the natural cooling rate to which outdoor insects are exposed. All fat-body cells in the visceral layer froze intracellularly with treatment B, and numerous small ice crystals were found within them (Pl. IX-1, 2). These cells were slightly contracted and deformed. Since these frozen cells were not so tightly packed with small ice particles as was seen in the cells frozen with treatment A, uric acid pouches were observed distinctly from the small ice particles within the cells (Pl. IX-2). In the parietal layer, on the other hand, about half of the fat-body cells froze extracellularly and the remainder froze intracellularly (Table 2). The amount of ice crystals within each intracellularly frozen fat-body cell was less in the parietal layer than in the visceral layer, and the cells in the parietal layer contracted in an irregular form (Pl. IX-3). No ice was observed within the muscle tissue. The individual muscle tissue as a whole was dehydrated and contracted in much the same manner as seen in an extracellularly frozen cell. Observed under a polarizing microscope, birefringent muscle tissue in frozen state appeared in a manner similar to that in freeze-dried states (Pl. XIII-2, 4). The birefringence observed in frozen muscle tissue was, therefore, assumed to result from the character of the muscle tissue itself, but not by the ice crystals formed.

In freezing treatment C, almost all fat-body cells in the visceral layer

froze intracellularly (Pl. X, Table 2). The number of ice particles within these frozen cells was apparently less than that in the cells frozen with treatment B, and a larger contraction of these fat-body cells was observed (Pl. X-2). These were some fat-body cells in the dorsal part around the gut which were larger than other fat-body cells. After freezing, a few of these large cells appeared dark as a result of a formation of a large number of ice particles within them (Pl. X-1). About 17% of the fat-body cells in the parietal layer froze intracellularly (Pl. X-3, Table 2), while the remainder froze extracellularly.

As the cooling rate became low in freezing treatment D, a less occurrence of intracellularly frozen fat-body cells was observed in the frozen insects (Pl. XI). About 17% of fat-body cells in the visceral layer froze intracellularly and the remainder froze extracellularly (Table 2, Pl. XI-1, 2). Almost all of these intracellularly frozen cells were the large fat-body cells located in the dorsal part around the gut (Pl. XI-1). All fat-body cells in the parietal layer froze extracellularly (Pl. XI-3).

When prepupae were frozen with treatment E in which the cooling rate was the slowest within the series of the experiments described above, all fat-body cells in both visceral and parietal layers were found to freeze extracellularly (Pl. XII-1). These fat-body cells were dehydrated and irregularly contracted (Pl. XII-2, 3). The spherical oil droplets within them coalesced into larger drops and were deformed. No ice was observed within the muscle tissues, the ventral nerve cord and the suboesophageal ganglion. The individual tissues as a whole were dehydrated and contracted as is seen in an extracellularly frozen cell. Since no darkening resulting from the formation of many fine ice crystals occurred in these tissues, the trachea within these tissues was clearly observed under a microscope (Pl. XIII-6, 7).

The observations above described are summarized in Table 2. With the increase in rate of cooling of the insect, an intracellularly frozen cells in the fat-body resulted. The fat-body cells of the visceral layer, especially in the dorsal part, underwent intracellular freezing more readily than that of the parietal layer.

A very interesting relation was observed in the frozen-thawed insects between the number of intracellularly frozen fat-body cells (Table 2) and the mode of injuries occurring at metamorphosis after thawing (Table 3). When the prepupae were frozen with a cooling rate of $327^{\circ}\text{C}/\text{min}$, intracellular freezing occurred in all fat-body cells. These frozen prepupae invariably died immediately after thawing. In the prepupae frozen at cooling rates of both 15.4 and $4.0^{\circ}\text{C}/\text{min}$ practically all the fat-body cells in the visceral layer froze

Table 3. Cooling rates in prepupae and their metamorphosis after freeze-thawing

Freezing * treatments	Cooling * rates (°C/min)	Survival after freeze-thawing (%)							
		Days after freeze-thawing	1	23		31		48	
		Developmental stage	prepupae	prepupae	pupae	prepupae	pupae	imagoes	imagoes
A	327		0						
B	15.4 ± 3.1		100	80		50			
C	4.0 ± 0.3		100	85		80			
D	0.8 ± 0.1		100	85	5	25	10	35	65**
E	0.4 ± 0.0		100	95		35	15	25	75

* The freezing treatments and the cooling rates are the same as those in Table 2

** About half number of the pupae could not shed their pupal skins and failed to appear on the wing

intracellularly while some of the fat-body cells also froze intracellularly in the parietal layer. These frozen prepupae survived for about one month after thawing, but could not pupate. When the prepupae were frozen with a cooling rate of $0.8^{\circ}\text{C}/\text{min}$, intracellular freezing occurred in about 17% of the fat-body cells in the visceral layer, while all the fat-body cells in the parietal layer froze extracellularly. After thawing, many of these prepupae were able to resume development to adults. Upon emergence, however, about half of the number could not shed their pupal skins and failed to appear on the wing. The prepupae which were frozen at a cooling rate of $0.4^{\circ}\text{C}/\text{min}$ completed metamorphosis normally after thawing. All fat-body cells of these prepupae were observed to freeze extracellularly.

In insects frozen at a relatively slow rate of cooling, intracellular ice crystals in the fat-body cells were observed usually in the center but not in the peripheral part of each cell (Pl. X, XI, XIV). This may have resulted from the concentration gradient of intracellular water brought about by partial dehydration from these cells during the freezing process. Since the small ice crystals formed in the periphery of the cells at the beginning of intracellular freezing are assumed to readily migrate to the loci where large ice particles form during slow cooling, these cells appeared as if the intracellular freezing was initiated spontaneously from the center of each cell (Pl. XIV-1, 2). However, observations of freezing sections from the prepupae suggest that the fat-body cell was inoculated by external ice crystals (Pl. XIV-3).

Injury in frozen insects cooled to liquid nitrogen temperature

The prepupae can survive liquid nitrogen temperature after previous slow freezing at -30°C at a cooling rate of $2^{\circ}\text{C}/\text{min}$ (Table 4). After thawing from liquid nitrogen temperature, some of the prepupae resumed development up to the formation of the imago, but all of them could not shed their pupal skins (Pl. I-10). Since this prefreezing method failed to produce an entirely normal adult, the following freezing treatments were tried. When prepupae were immersed in liquid nitrogen after very slow prefreezing to -30°C with a cooling rate of $0.5^{\circ}\text{C}/\text{min}$, 19% transformed into normal imagoes. Since both slow and very slow prefreezing treatments to -30°C are harmless in prepupae (Exp. 1 & 5 in Table 4), freezing injury may occur in prepupae at temperatures between -30°C and liquid nitrogen temperature. When prepupae were cooled down to -70°C after prefreezing to -30°C at a cooling rate of $2^{\circ}\text{C}/\text{min}$, 30% of these prepupae normally transformed to imagoes (Exp. 3 in Table 4). Most of the prepupae transferred into liquid nitrogen after the previous freezing treatment to -70°C described above suffered con-

Table 4. Survival at liquid nitrogen temperature

Exp. No.	Freezing treatments*	No. of prepupae used	Survival 10th day after thawing (%)	Insects developed to imagoes after thawing (%)	
				normal	abnormal
1	Prefreezing A**	10	90	90	0
2	Freezing at -196°C for 24 hrs. after prefreezing A	30	93	0	63
3	Freezing at -70°C for 18 hrs. after prefreezing A	10	100	30	30
4	Freezing at -196°C for 3 hrs. after the freezing treatment in Exp. No. 3	10	100	10	30
5	Prefreezing B***	53	91	91	0
6	Freezing at -196°C for 24 hrs. after prefreezing B	46	92	19	43

* Details of the treatments are described in the text

** The prepupae were frozen down to -30°C with a cooling rate of about $2^{\circ}\text{C}/\text{min}$, and then were kept at -30°C for 9 hours

*** The prepupae were frozen down to -30°C with a cooling rate of about $0.5^{\circ}\text{C}/\text{min}$, and then were kept at -30°C for 9 hours

siderable injury (Exp. 4 in Table 4). These results suggest that very slow prefreezing to -30°C is highly effective in protecting the insect against freezing injury occurring at temperatures between -30°C and liquid nitrogen temperature. It seems, therefore, that some unknown factors in the prefreezing insect prepared before immersing in liquid nitrogen affect the occurrence of frost injury. When freezing sections from prepupae treated with these prefreezing methods were observed under a microscope, it was found that the cooling rate during prefreezing affected the grain size of ice crystals formed within the prepupa. The grain size of ice crystals within a prepupa frozen down to -30°C at a cooling rate of $0.5^{\circ}\text{C}/\text{min}$ was about twice as large in diameter than those in a prepupa frozen to the same final temperature at a cooling rate of $2^{\circ}\text{C}/\text{min}$. If the formation of ice crystals of a larger grain size within a prepupa during the prefreezing process is a factor which would lead to the avoidance of freezing injury occurring upon the following immersion in liquid nitrogen, any treatment which produces larger grains of ice crystals in an insect body would decrease the same kind of injury. Based on this assumption, the following experiments were made.

Prepupae, previously frozen to -20°C at a cooling rate of about $1^{\circ}\text{C}/\text{min}$, were warmed to -5°C and kept at the same temperature for 18 hours. As described later, during the 18 hours at -5°C , many small ice crystals within

Table 5. Effect of prefreezing treatments on frost injury in the prepupae immersed in liquid nitrogen

Exp. No.	Freezing treatments *	No. of prepupae used	Insects developed to imagoes after thawing (%)	
			normal	abnormal
7	A **	20	75	0
8	B ***	20	80	0
9	Freezing at -196°C for 24 hrs. after the treatment A	20	55	25
10	Freezing at -196°C for 24 hrs. after the treatment B	20	75	0

* Details of the treatments are shown in the text

** The prepupae were frozen down to -30°C with a cooling rate of about $1^{\circ}\text{C}/\text{min}$ and kept there for 20 hours

*** The prepupae, previously frozen to -20°C at a cooling rate of about $1^{\circ}\text{C}/\text{min}$, were warmed to -5°C and kept there for 18 hours. These were then cooled down to -30°C with a cooling rate of $1^{\circ}\text{C}/\text{min}$, and kept there for 24 hours

the prepupal body recrystallized to large crystals. These prepupae were then cooled down to -30°C at a cooling rate of $1^{\circ}\text{C}/\text{min}$ and were kept at the same temperature for 2 hours. Finally, the prepupae were immersed in liquid nitrogen. After thawing from liquid nitrogen temperature, most of these prepupae transformed to normal imagoes (Table 5). On the other hand, when prepupae were immersed in liquid nitrogen after previous freezing down to -30°C at a cooling rate of $1^{\circ}\text{C}/\text{min}$, 25% suffered injury and transformed into abnormal imagoes after thawing. Since they could not shed their pupal skins, all of them died within several days. Both prefreezing treatments described above, of course were harmless to the prepupae (Table 5).

To clarify the difference of freezing states between prepupae separately subjected to these two prefreezing treatments, freezing sections were made from the frozen prepupae and observed under a microscope. As expected the grain size of ice crystals within the frozen prepupae which were kept at -5°C for 18 hours in the process of prefreezing treatment was larger than that in prepupae prefrozen without keeping at -5°C (Pl. XV, XVII). Cross sections of the head portion indicated that there were no ice crystals within the muscle tissues (Pl. XV). The muscle tissues in frozen prepupae which were once kept at -5°C during prefreezing (Pl. XV-2) were more deformed than that in prepupae frozen without keeping at -5°C (Pl. XV-1). This suggests the highly progressive migration of extracellular ice crystals in the frozen insect. The same results were also observed in sagittal sections from frozen prepupae (Pl. XVI-1, 2). Almost all fat-body cells were observed to freeze extracellularly in frozen prepupae with both prefreezing treatments (Pl. XVII-1, 2).

When freezing sections were kept in a cold room without a cover glass, ice crystals within these sections disappeared by sublimation. After sublimation, layers of concentrated haemolymph were found around the remaining tissues at the boundary between each ice grain which disappeared (Pl. XVIII-1, 2). The thickness of these layers were different between the prepupae frozen by the two treatments described previously. The layers of concentrated haemolymph appeared thicker in prepupae which were kept at -5°C for 18 hours during the prefreezing process (Pl. XVIII-4, 6) than that in prepupae frozen without keeping at -5°C (Pl. XVIII-3, 5).

Termination of prepupal diapause by body freezing

When the prepupae were kept at 2°C from the end of autumn, they remained in diapause up to the middle of March of the following year (Fig. 6). Almost all of them terminated diapause at the end of April. In 1965,

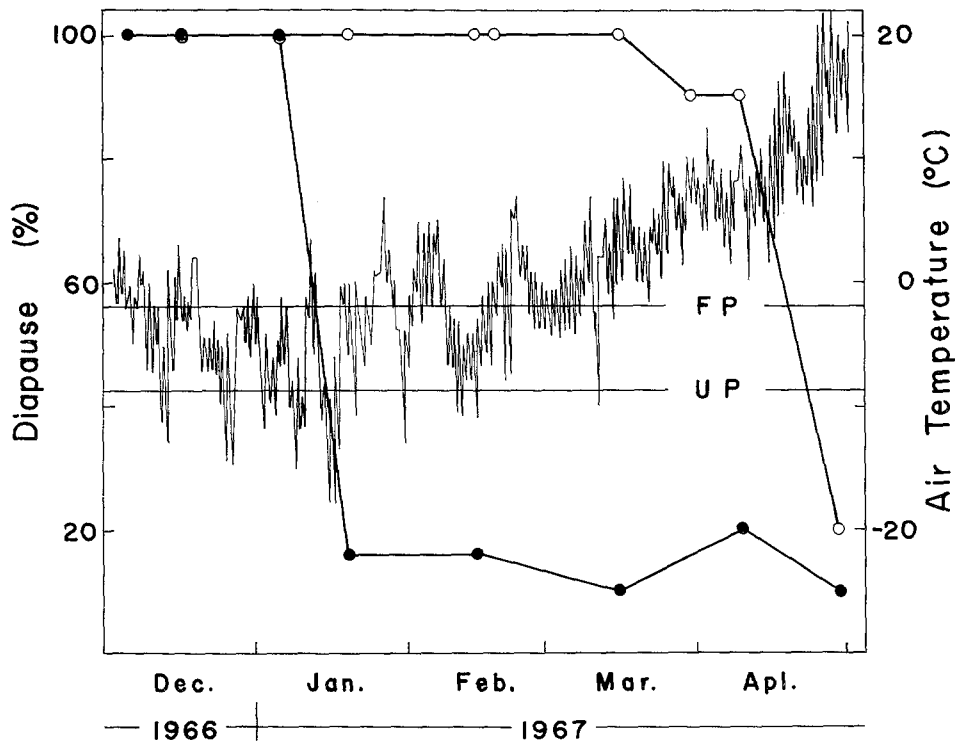


Fig. 6. Diapause in prepupae and environmental temperature

- : Percentage of diapause in prepupae which were kept at outdoor temperatures
- : Percentage of diapause in prepupae which were kept at 2°C
- FP: Freezing point of prepupae
- UP: Supercooling point of prepupae

on the other hand, some of the prepupae of this insect overwintering outdoors above the snow cover, which were frequently exposed to severe cold, terminated diapause sometime between 5th and 19th of January (Fig. 6). Before this time, the outdoor temperature had already dropped many times to temperatures below the supercooling point of the prepupa. This certainly resulted in the freezing of the insect body. However, none of these prepupae were released from the diapause until 5th of January.

With the expectation that freezing of the prepupa body would bring about the termination of diapause, following experiments were made. Groups of insect consisting of ten prepupae were slowly cooled separately to various temperatures from 0 to -30°C . Of these groups two were exposed to -5°C ,

Table 6. Termination of the prepupal diapause by body freezing. Dec. 5, 1966

Freezing duration (days)	1	4	7	14	21
Temperature (°C)	Number of prepupae terminating diapause				
0, unfrozen	—	—	—	—	0
— 5, supercooled	0	0	0	0	0
— 5, frozen *	0	0	0	0	0
—10, frozen	0	0	0	0	0
—30, frozen	5	7	8	6	6

Ten diapausing prepupae were used in each experiment

* Since the supercooled state of the prepupae is stable at temperatures above $-8.6 \pm 0.4^\circ\text{C}$, the prepupae were frozen at -5°C by external ice seeding

one of which was artificially frozen by external ice seeding, while another groups remained in a supercooled state. The other groups which were cooled to -10°C or to -30°C froze spontaneously, since the supercooling point of the prepupae is about -8.6°C (Table 6). No prepupae, however, terminated diapause after body freezing at a temperature higher than -10°C , even when the duration of freezing was prolonged for 21 days. On the other hand, when prepupae were frozen at -30°C for 24 hours, half of them terminated diapause. There was no relationship, however, between the duration of freezing and the percentage of termination of the diapause in the insects. Prepupae were separately frozen at temperatures between -5 and -30°C for 30 minutes or 24 hours (Table 7). It was found that the body freezing at

Table 7. Freezing temperatures and the termination of diapause in the prepupa. Feb. 2, 1967

Freezing duration (hrs)	unfrozen (°C)	Freezing temperatures (°C)					
		—5	—10	—15	—20	—25	—30
0.5	0	0	0	2	6	7	8
24	0	0	0	3	7	7	7

Number of insects which terminated diapause after body freezing in ten prepupae were indicated

The diapausing prepupae used in the experiment were kept at 2°C from late autumn of the previous year

temperatures below -15°C was apparently effective in terminating diapause. 25% of the prepupae terminated diapause after body freezing at -15°C . The body freezing at temperatures below -20°C resulted in the termination of diapause in 70% of the prepupae on an average. The same results were also seen in insects overwintering outdoors in (Fig. 6). During the winter of 1966–1967, the atmospheric temperature first dropped below -15°C in the middle of January, immediately after this drop some of the prepupae were observed to terminate diapause.

IV. Discussion

Accumulation of sugar and frost resistance in the poplar sawfly

Glycerol in insects have been studied in many species of overwintering insects in relation to their frost resistance⁷⁻⁹. With a few exceptions almost all of the remarkably frost resistant insects were shown to accumulate glycerol in their bodies^{3,8,9,11}. On the other hand, sugar in higher plants, mainly sucrose, was shown to be an important protective substance against frost injury¹⁷. It is of interest to note that a remarkable accumulation of trehalose which is the same non-reducing disaccharide as sucrose persists throughout the cold season in the poplar sawfly.

The process of increase in frost resistance in insects was interpreted as a protoplasmic change in tissue cells followed by an increase of some small molecular protective substances as additive factors⁹. Such changes in the protoplasm was conjectured to occur in an insect soon after entering diapause¹³. Since the prepupae of the poplar sawfly invariably enter diapause in early autumn, a protoplasmic change in tissue cells may well take place at the time of increase of sugar in the insect. With the concomitant increase of sugar mainly trehalose in the insect (Fig. 2), a remarkable increase in frost resistance was observed (Fig. 1). No accumulation of glycerol or of any other polyhydric alcohol was observed in the insect throughout the cold season. It seems, therefore, that the accumulation of trehalose may be an important factor which enhances the frost resistance in the poplar sawfly. Overwintering larva of a gallfly, *Euura nodus*, is remarkably frost-resistant but does not contain any polyhydric alcohol⁷. This gallfly may presumably accumulate sugar with the approach of the cold season. Sugar levels of this insect, however, have not been estimated as yet.

It was hitherto found that sugar in haemolymph in a variety of insects and other invertebrates is mainly trehalose^{41,42}. Sugar levels in haemolymph of most insect are, however, much lower than that in the poplar sawfly.

Extremely high sugar levels, reaching 92.9–184.0 mg per gram of fresh weight, were once found in overwintering adults of the solitary bees *Ceratina flavipes* and *C. japonica*⁴³⁾. This sugar consisted mainly of fructose and glucose as was observed in the blood sugar of honey bee⁴⁴⁾. The large amount of sugar in *Ceratina* bees were found to be useful in enhancing their supercooling ability but showed no influence in avoiding frost injury⁴³⁾. This means that a large amount of sugar in an insect does not always provide protection against frost injury.

Intracellular freezing in fat-body cells

It has been shown in various kinds of living cells that the larger the cell volume becomes, the occurrence of intracellular freezing tends to rise. This was explained by Mazur to be the result of an easier leaving of water from smaller cells which have a larger surface-volume ratio than from larger cells at the onset of extracellular freezing^{45,46)}. A remarkable example of such phenomenon was found in prepupae of the poplar sawfly as described in the present paper. Since fat-body cells in the parietal layer are arranged immediately below the body wall of the prepupa, when the prepupa is cooled, these cells may be cooled more readily than those in the visceral layer which are arranged in the center of the body cavity. When a prepupa is cooled at a rate of 4°C/min to –20°C, however, the intracellular freezing was observed to occur in all fat-body cells in the visceral layer, while most of the fat-body cells in the parietal layer froze extracellularly (Table 2, Plate X). Each cell in the visceral layer was larger in size than that in the parietal layer. Among the fat-body cells in the visceral layer, those arranged on the dorsal side of a body cavity were largest. These large cells show an especially high tendency to freeze intracellularly. These observations indicate that the occurrence of intracellular or extracellular freezing of fat-body cells in the prepupa largely depends upon the size of the cell when a moderately rapid cooling is applied to the prepupa. Since cells in tissues other than fat-bodies are, as a rule, smaller in size than a fat-body cell, almost all of them freeze extracellularly when fat-body cells, even a few of them, freeze intracellularly. In fact, it was observed that there were no ice crystals within the muscle tissues even when most fat-body cells froze intracellularly in the prepupa of the poplar sawfly. It was also observed in the freeze-substituted sections from larvae of the goldenrod gallfly, *Eurosta solidaginis*, that intracellular freezing hardly occurred in the small cells²⁸⁾.

A close correlation between the number of intracellularly frozen fat-body cells and the degree of injury occurring upon metamorphosis after thawing was observed in the prepupae (Tables 2, 3). It seems, therefore, that the

intracellular freezing is detrimental to the fat-body cells as seen in almost all living cells²⁵⁻²⁷). Salt, however, concluded that intracellular freezing was not fatal to the fat-body cells of the goldenrod gallfly, *Eurosta solidaginis*, judging from the following observations of frozen fat-body cells in a cold bath at -15°C to -25°C ²⁸). (1) Probing with a cold needle revealed the interior of the fat cells to be solid in the sense of being firmly granular or mealy. (2) Bits of the cell contents raised to the warmer surface of the oil bath changed, upon melting, from a whitish, irregular mass to a light amber liquid sphere. (3) The fat cells retained their subspherical shape when frozen and, as nearly as could be judged, their original size, showing that no appreciable amounts of water were removed as would be expected if extracellular freezing alone had occurred. After thawing, larvae of this insect could resume their normal development. It seems, however, difficult to prove unfatal intracellular freezing in a cell from the survival of frozen-thawed insects. Freezing sections can be made easily from frozen prepupae of the poplar sawfly with a microtome at -15°C regardless of whether cells within the insects froze intracellularly or extracellularly²³). This suggests that these cells are as hard as ice crystals at such temperatures. A high degree of stiffness in a fat-body cell, therefore, does not always mean an occurrence of intracellular freezing in itself. The second and the third observations above described may indicate an occurrence of intracellular freezing in fat-body cells of the goldenrod gallfly. However, since the observations were made with larvae injected with fast green dye in Ringer's solution, the freezing condition in the fat-body cells might differ from that in an intact larva without such treatment, even when the cooling rates were the same. Moreover, the present experiment on the poplar sawfly frozen at various cooling rates revealed that the occurrence of intracellular freezing in a fraction of fat-body was not necessarily fatal to the insect as a whole. Such frozen insect could resume development even to adult after thawing.

An electron microscopic study of insect fat-body cells indicated that they contained RNA granules which were complicated in structure and were important in the synthesization of protein during metamorphosis⁴⁷). Judging from the complicated structure of fat-body cells, intracellular freezing may be assumed to be injurious to fat-body cells as seen in almost all living cells. Moreover, it is known that fat-body cells are not only the preserving place of some materials but also the site of dynamic metabolism^{48,49}). It was revealed in the present work that in a prepupa of the poplar sawfly, the fat-body cells in the parietal layer were mainly utilized for the formation of the pupa and those in the visceral layer were mainly consumed for the formation of the imago (Fig. 4). It is clear in Tables 2 and 3 that the metamorphosis to

imagoes from the frozen-thawed prepupae increases. When practically all the fat-body cells in the visceral layer are frozen intracellularly, no adults appear on the wing. It seems, therefore, reasonable that failure to emerge into the adult stage in the frozen-thawed insects may perhaps be explained to be the result of death of the fat-body cells in the visceral layer by intracellular freezing.

Except for the case of very rapid cooling, when an insect is cooled to a certain low temperature, if the insect is released from a supercooled state at a relatively high subzero temperature, the rate of cooling at the time of ice formation within the insect body becomes low. As mentioned before, with the increase in rate of cooling of an insect, the number of intracellularly frozen cells in the cooled insect increases. The fact that prepupae of the poplar sawfly have a relatively high supercooling point of -8.6°C may, therefore, be useful in preventing the occurrence of intracellular freezing in the insects.

Mechanism of frost injury in the insect cooled to a very low temperature after prefreezing

The prepupae of the poplar sawfly could survive freezing down to liquid nitrogen temperature provided that they were previously frozen at -30°C . After rewarming from the super-low temperature, some of them were able to resume development to normal pupae. Upon emergence, however, they could not shed their pupal skins and failed to appear on the wing. Since the mode of freezing injury in these prepupae was similar to that observed in the frozen prepupae cooled rapidly to -20°C , a similar explanation may be applicable. When prepupae are immersed in liquid nitrogen after prefreezing at -30°C , some fat-body cells in the visceral layer suffer fatal injury with or without apparent intracellular ice formation, but the cells in the parietal layer are not damaged. After thawing, the prepupae can not complete normal development, because they have an insufficient amount of uninjured fat-body cells in the visceral layer to be consumed for adult transformation. They are too weak in movement to shed their thin pupal skins by themselves, resulting in a failure to appear on the wing.

It has been indicated that an application of prefreezing to a frost resistant insect at temperatures below a certain low temperature is necessary to enable it to survive freezing in liquid nitrogen¹⁸⁾. Since larger amounts of water remained within cells at a higher prefreezing temperatures, intracellular freezing may be assumed to occur readily in insects prefrozen at a higher temperature in the following process of cooling to the liquid nitrogen temperatures. It

was demonstrated in the slug caterpillar, *Monema flavescens*, that the amount of water crystallizing at -30°C was nearly all of the freezable water in the insect, and ice formation showed but very small increase at lower temperatures⁵⁰⁾. Under such conditions, the occurrence of intracellular freezing during subsequent cooling down to liquid nitrogen temperature may be very difficult or impossible. Since the prefreezing temperature of prepupae of poplar sawfly was usually -30°C in all experiments carried out to test survival at liquid nitrogen temperature in the present study, the amount of freezable water remaining within cells of the insect may be little and moreover may be quite the same in all experiments. The sublethal frost injury in an entire insect frozen in liquid nitrogen after the prefreezing can not, therefore, be explained by the occurrence of intracellular freezing in some cells within the prepupae. Moreover, since the respective prefreezing treatments themselves were entirely harmless to the paepupae, no injury to the cells during the prefreezing process may be expected.

When an insect is rapidly immersed in liquid nitrogen after almost the entire amount of the freezable water in the insect is frozen by the prefreezing process, a drastic mechanical stress may be caused in the insect by rapid thermal contraction of the insect body. In fact, big insects such as the pupae of the *cecropia* silkworm were observed to be burst at the time of immersion in liquid nitrogen⁵¹⁾. With the prefreezing treatment, an insect body is filled with many ice crystals and the cells in the insect contract by extracellular freezing, and between them a thin layer of concentrated haemolymph may be formed. The mechanical stress occurring in the insect by thermal contraction at the time of immersion in liquid nitrogen may be emphasized by a variation in thermal contractility of these components within the frozen insect. When the contraction increases in the insect, tissue cells may suffer stress from various directions by ice crystals surrounding them. Tissues within a frozen insect may be sometimes deformed as a whole by the movement or deformation of ice crystals to release the stress. Most of the tissues, however, may suffer little mechanical injury since they consist of many minute cells and the stress within the tissues may be released by a slight movement or deformation of each cell. On the other hand, fat-body cells may not be able to avoid the increasing mechanical stress due to thermal contraction in a frozen insect, since they are far larger in size than the ice grains surrounding them. As described above, almost all of freezable water is deprived from extracellularly frozen fat-body cells at temperatures below -30°C and these frozen cells were observed to become rigid. These cells may, therefore, tend to be broken by mechanical force. Since the fat-body cells of the

visceral layer are larger in size than those of the parietal layer, the injury produced upon the transference of the insect from -30°C to the liquid nitrogen temperature may occur more readily in the former than in the latter. This is perhaps the reason why the sublethal injury sustained by the surviving prepupa at liquid nitrogen temperature gives rise to failure to complete emergence.

If the mechanical stress to the fat-body cells is decreased during the process of cooling from a prefreezing temperature to the final temperature of -196°C , sublethal frost injury may be avoided or at least decreased. The stress may be released by deformation of the frozen insect body. Such deformation is probably occurs by the movement of ice crystals which occupy almost all spaces in the frozen insect body. Therefore, if ice grains within a frozen insect can move smoothly, the mechanical stress in the fat-body cells may effectively be decreased and the insect may not suffer frost injury.

The mobility of ice grains in the frozen insect may be controlled by the shape and number of ice grains adjoining each other, and a relative distance between them. By the use of two modes of previous temperature treatment of prepupae before the immersion in liquid nitrogen, the relation between the degree of frost injury and ice grain condition in the frozen insect was determined. The result obtained revealed that a slow previous cooling with an intermediate maintenance of the frozen insect at -5°C for 18 hours produced more globular and larger ice grains in the insect and smaller frost injury in frozen-thawed insect from liquid nitrogen temperature than the same previous treatment without holding at -5°C (Table 5). In the former treatment ice grains produced in the insect were not only more globular but also smaller in number than those produced by the latter treatment. An easier movement of ice grains may therefore be expected in the frozen insect by the former treatment than that of the latter one. The layer of concentrated haemolymph produced around the ice grains in insects may facilitate the easy movement of ice grains, because it may play a role of a lubricant in movement of ice grains and may provide spaces in which ice grains may move. The amount of concentrated haemolymph within a frozen prepupa may be the same in any previous temperature treatment at the same final temperature. The layer of concentrated haemolymph surrounding ice grains, however, may become thicker with the formation of a smaller number of larger ice grains in the frozen insect, since the total surface area of all ice grains within a frozen insect becomes larger with the formation of a larger number of smaller ice grains. In fact, the layer of concentrated haemolymph around ice grains in the insect prefrozen by slow cooling including an intermediate maintenance at -5°C

was observed to be thicker than that in the insect prefrozen without such maintenance. (Pl. XVIII) Also for this reason, the movement of ice grains may be easier in the insects prefrozen by a temperature treatment to produce larger ice grains than in the insects prefrozen by a treatment to produce smaller ice grains within the insect. All of these observations seem to support the hypothesis that frost injury in the prefrozen insect occurring at the time of immersion in liquid nitrogen may be the result of the death of fat-body cells caused by mechanical stress which arises in the cells during the process of cooling to very low temperatures.

A few prepupae could also be kept alive without any injury in liquid nitrogen with a gradual three-step freezing method (Table 4). This method was, however, not so effective to protect prepupae from frost injury, as compared against the slow prefreezing treatment with a maintenance at -5°C (Table 5). The gradual three-step freezing method may somewhat lower the mechanical stress brought about by a thermal gradient within a frozen prepupa arising from the immersion in liquid nitrogen, but may not be effective in avoiding the stress caused by differences in thermal contraction of various components in the prepupal bodies. It was found in pupae of the *cecropia* silkworm that a few of the glycerol injected pupae could be kept alive in liquid nitrogen by the gradual three-step freezing method⁵²⁾. After thawing from the super-low temperature, they could transform into imagoes although all of them failed to shed their pupal cuticles. On the other hand, no pupae without glycerol injection could survive freezing at liquid nitrogen temperature even by the gradual three-step freezing method. Freezing at -70°C was harmless in this insect. These results suggest the occurrence of mechanical injury in this insect by the same reason as proposed for prepupae of the poplar sawfly.

By freezing to super-low temperatures, almost all types 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^{9,13)}. One of the reasons why the present poplar sawfly can completely remain uninjured may be attributed to the application of the special freezing method to produce large globular ice grains within the insect during the prefreezing process. The other reason may be the possession of a large amount of trehalose by the prepupa. It is interesting to note that an addition of sucrose to the medium is quite effective to protect blood cells from freezing injury occurring at the time of rapid cooling to liquid nitrogen temperature but glycerol is not effective to the same kind of freezing injury in blood cells⁵³⁾. Since trehalose is the same disaccharide as sucrose, a similar type

of protective effect against frost injury in living cells may be expected.

A similar sublethal frost injury was also observed in other insects. When the overwintering prepupae of the slug caterpillar, *Monema flavescens*, were immersed in liquid nitrogen after prefreezing at -30°C , and then 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 emergence¹³⁾. The cause of such sublethal frost injury in the slug caterpillar may be the same as that in the poplar sawfly discussed above, although the role of fat-body cells during metamorphosis has not been determined as yet in the slug caterpillar. It was reported that overwintering pupae of a butterfly, *Papilio machaon hipocrates* could survive liquid nitrogen temperature for 2 days after prefreezing at -30°C . About half of them were able to resume their development at 20°C after thawing. In these insects, however, the transformation from the pupa to the imago was restricted to the anterior half of the pupal bodies. The abdomen behind the third or fourth segment, remained in the pupal state, and was filled with fat-bodies. They survived for some ten days with an active heart beat even after the anterior halves died⁹⁾. Such a half-imago formation may be assumed to be the result of freezing injury in the prothoracic gland in the frozen pupae which supplies moulting hormone within the insects at the time of transformation to imago. However, it may be noted that the abnormal adult butterflies from the frozen-thawed pupae were incomplete not only in moulting but also in the formation of normal internal tissues of an imago. In the butterfly, it seems very probable that the half-imago formation may be the result of the frost injury in some fraction of the fat-bodies.

In a freezing susceptible pupae of a butterfly, *Papilio xuthus*, half-imagoes were invariably produced by a body freezing, even when they were rapidly thawed within a few minutes immediately after the initiation of body freezing⁵⁴⁾. Thus it may be said that the half-imago formation is not a specific injury produced by the freezing of the insect at super-low temperatures.

Termination of diapause by freezing

In the present study an occurrence of immediate termination of diapause by body freezing in prepupae of the poplar sawfly was clearly indicated. Since the nature of diapause in the poplar sawfly is not yet known, the mechanism of termination of diapause in this insect is merely speculated from data obtained from other diapausing insects. It was shown by Fukaya in the larval diapause of the rice stem borer, *Chilo suppressalis*, that the corpora allata was highly active during diapausing period, and the diapause was

characterized by the temporal failure in the activities of the brain-prothoracic gland system accompanied by the high activity of the corpora allata^{55,56}). This view was supported by the experimental results in some other insects⁵⁷⁻⁵⁹). It is well known that living organisms are, as a rule, more freezing-susceptible in an actively living stage than in a dormant stage⁶). Therefore, immediate termination of diapause by freezing in prepupae of the poplar sawfly may be explained to be the result of the inactivation of the corpora allata due to freezing damage in some cells of the organ.

After body freezing at -10°C , no prepupa of the sawfly terminated diapause, even when the duration of body freezing was prolonged for 21 days. On the other hand, immediate termination of diapause occurred in prepupae by body freezing below -15°C for only 30 minutes. Even when the duration of freezing was prolonged to 24 hours, the effect on terminating diapause was the same as in the case of freezing for 30 minutes (Table 7). About 25% of the prepupae terminated diapause after body freezing at -15°C . The body freezing at temperatures below -20°C resulted in the termination of diapause in 70% of the prepupae on an average. It was demonstrated by SHINOZAKI in the slug caterpillar, *Monema flavescens*, that the amount of ice crystallized within freezing prepupae increased very rapidly until the freezing temperature reached about -15°C , below which the amount of ice increase slowed down. He also reported that the amount of water crystallizing at -15°C was more than 80% of the total water content⁵⁰). Since the degree of dehydration from extracellularly frozen tissues would mainly increase in proportion to the amount of ice crystallized within the insect, tissues frozen below -15°C may lose more than 80% of the total water content. Such a severe dehydration from the highly active cells in the corpora allata may presumably result in a fatal injury to the cells.

Summary and Conclusion

1. The Japanese poplar sawfly, *Trichiocampus populi* OKAMOTO, was examined in the developmental stages from the full grown larva to the adult with regard to its frost resistance and related properties.

The overwintering prepupae of this insect were found to survive liquid nitrogen temperature after previous freezing at -20°C . Such high levels in frost resistance persisted throughout the five months cold season. On the other hand, none of larvae, pupae nor adults could survive freezing even at a temperature of -10°C for 30 minutes.

Almost all of the remarkably frost resistant insects are known to contain glycerol or sorbitol within their bodies. The present poplar sawfly, however,

has neither glycerol nor any other polyhydric alcohol throughout the overwintering season. Instead, a remarkably large amount of sugar, estimated to amount to 5% of fresh body weight, was found in the prepupae of this insect. The main part of sugar in the prepupae was shown to be trehalose. A remarkable correlation was observed in this insect between frost resistance and sugar levels.

Since some of prepupae were overwintering in the twigs remaining uncovered by snow during the coldest season, they were generally exposed to severe cold. As expected from their supercooling point which is as high as $-8.6 \pm 0.4^\circ\text{C}$, the prepupae in nature were, indeed, observed to freeze and thaw many times during the winter. Since they survived freezing at temperatures far below the climatic winter temperatures, the prepupae may avoid frost injury during winter.

2. The overwintering prepupae can survive a slow freezing down to extremely low temperatures. The prepupae, however, suffer frost injury by artificial rapid freezing even to -20°C . With the increase in rate of cooling of the prepupae, an increase in the degree of frost injury in the prepupae was observed. A clear correlation was observed in the frozen-thawed insects between the number of intracellularly frozen fat-body cells and the mode of injury in the entire insect occurring upon metamorphosis after thawing. Fat-body cells play a very important role in the metamorphosis of the insect, and more than a half volume of the fat-body cells in the prepupae is utilized during the metamorphosis to the imago. The variety seen in the mode of injury in an entire insect resulting from the freezing at various cooling rates may perhaps be explained to be the result of the variety in the number of fat-body cells damaged by intracellular freezing.

A sublethal frost injury was also observed in the prepupae frozen down to -196°C and thawed. After rewarming from the super-low temperature, they can resume development and transform into pupae. Upon emergence, however, they can not shed their pupal skins and fail to appear on the wing. Such sublethal frost injury could be prevented by maintaining the frozen prepupae at -5°C for 18 hours in the step of the prefreezing process before immersion into liquid nitrogen. The maintenance of insects at -5°C during the prefreezing process was highly effective to produce a small number of large, globular ice grains in the frozen insect. The following mechanism of the sublethal frost injury was, therefore, assumed.

When a prepupa is rapidly immersed in liquid nitrogen after prefreezing, the tissue cells may suffer drastic mechanical stress caused by the difference in the degree of thermal contraction of the components in the frozen insect

body. Among the tissue cells in the insect, fat-body cells are the largest and are far larger than the other components in the frozen insect. Fat-body cells may, therefore, be the most susceptible part to the mechanical stress. It seems very probable that some fat-body cells may suffer fatal injury at the time of immersion in liquid nitrogen. Since the fat-body cells play a very important role in the completion of metamorphosis in the prepupae, the fatal injury in some of the fat-body cells may result in failure to complete metamorphosis. The prefreezing treatment with a maintenance of the frozen insect at -5°C , may provide a favorable arrangement of fat-body cells, ice crystals and the surrounding layer of concentrated haemolymph in the insect to decrease the mechanical stress arising between them.

3. Immediate termination of diapause occurred in the prepupae of the poplar sawfly by body freezing below -15°C for 30 minutes. No prepupa, however, terminated diapause after body freezing at -10°C , even when the duration of body freezing was prolonged up to 21 days. In some insects the corpora allata is known to be highly active during diapause, and diapause is characterized by the temporal failure in the activities of the brain-prothoracic gland system accompanied by a high activity of the corpora allata. In addition, almost of all living organisms are known to be more freezing-susceptible in an active stage than in a dormant stage. Therefore, the immediate termination of diapause in prepupae of this sawfly may be explained to be the result of the inactivation of the corpora allata by a severe dehydration due to freezing at temperatures below -15°C .

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Plate 1. The Japanese poplar sawflies during the stages from last instar larvae to adults

1. The 5th instar larvae feeding on poplar leaf. $\times 1.2$
2. A 5th instar larva. $\times 2.5$
3. A full-grown larva after cessation of feeding on. $\times 1.2$
4. A full-grown larva found in a small hole in the bark of dead tree.
 $\times 1.2$
5. A cocoon spun by a larva within a stem of a herbaceous plant. $\times 1.2$
6. An overwintering prepupa. $\times 4$
7. A prepupa several days before pupation. $\times 4$
8. A pupa. $\times 4$
9. An imago $\times 4$
10. An abnormal adult which failed to shed its pupal skin as a result of body freezing at liquid nitrogen temperature during prepupal stage.
 $\times 4$
11. A diapausing prepupa after being reared at 25°C for 30 days. The prepupa showed reduction of body weight and finally died while remaining in the prepupal stage. $\times 4$

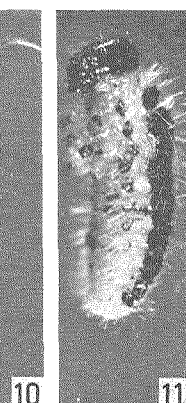
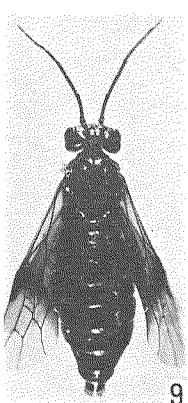
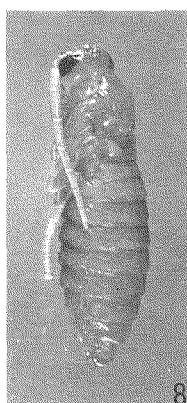
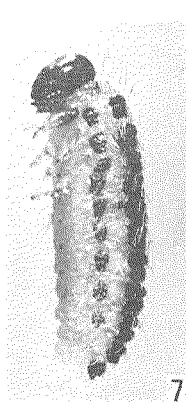
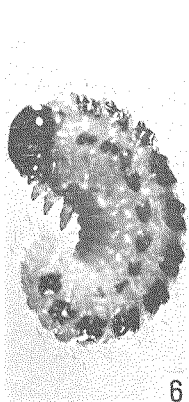
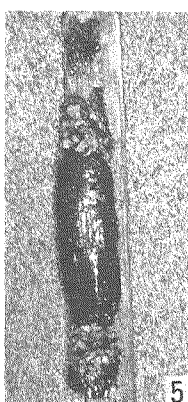
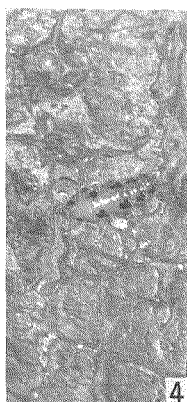
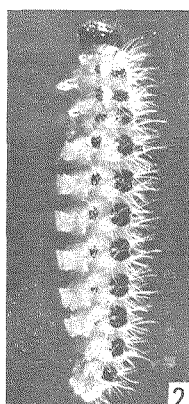
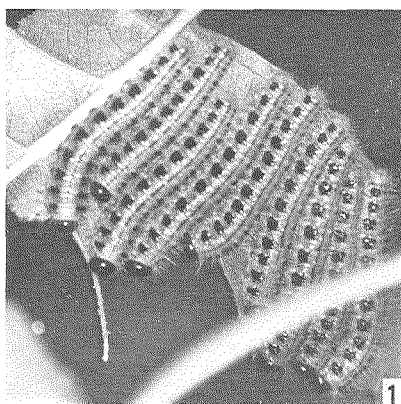


Plate II. Fat-bodies in a prepupa

1. The median plane. The plane was made from a rapidly frozen prepupa at -20°C
P, Fat-body in the parietal layer; V, Fat-body in the visceral layer;
D, Digestive canal. $\times 8$
2. A cross plane of the abdomen. The cross plane was made from a rapidly frozen prepupa at -20°C
P, Fat-body in the parietal layer; V, Fat-body in the visceral layer;
D, Mid gut. $\times 15$
3. A frozen-dried cross section of the abdomen under a polarizing microscope. Each fat-body cell in the visceral layer (V) contains several pouches filled with many spherulites of uric acid which show birefringence. No such pouches are observed in fat-body cells in the parietal layer (P). D, Mid gut. $\times 20$
4. Cross section of the abdomen with a paraffine-embedded method. $\times 15$
5. Diagramatic representation of the cross section of abdomen
A, Dorsal vessel; B, Pericardial cells; C, Dorsal longitudinal muscle;
D, Ventral longitudinal muscle; E, Fat-body cells in the parietal layer;
F, Ventral nerve cord; G, Digestive canal; H, Fat-body cells in the visceral layer; I, Trachea; J, Dorsal diaphragm; K, Spiracle; L, Ventral diaphragm

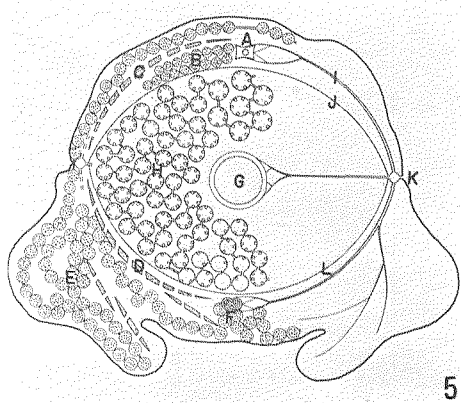
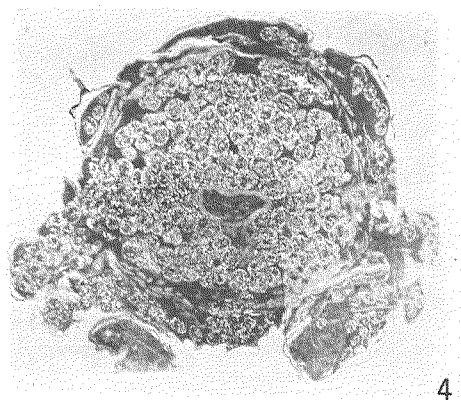
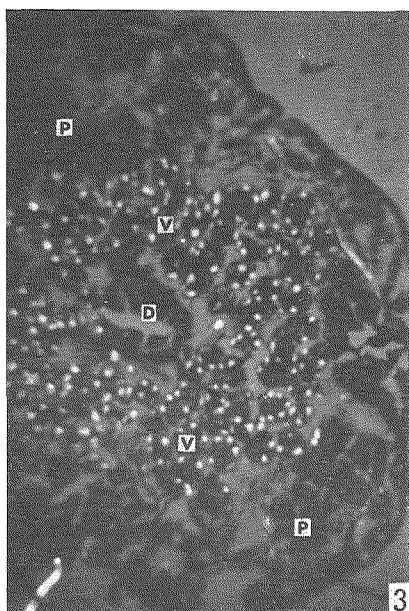
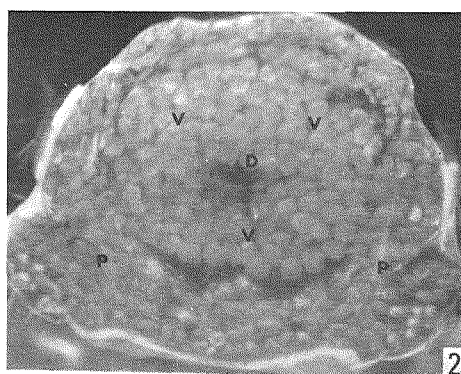
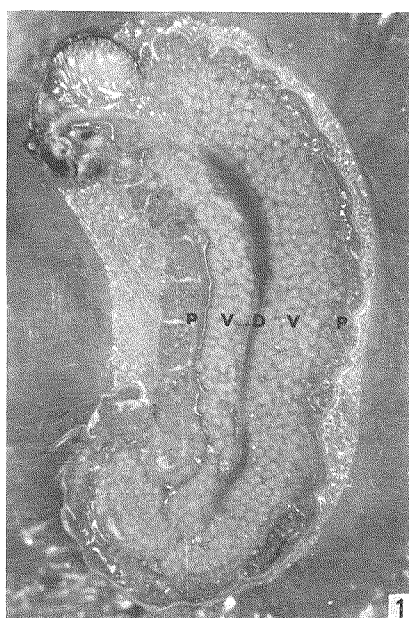


Plate III. Fat-body cells in the visceral layer

1. Under an ordinary microscope. There are several pouches filled with many spherulites of uric acid in the surface layer of each cell. $\times 10$
2. The same as 1 under a polarizing microscope. Spherulites of uric acid show birefringence. $\times 100$
3. Flattened fat-body cells showing fine canals connecting the fat-body cells with each other. $\times 100$
4. A part of a fat-body cell in the visceral layer showing a thin surface membrane separated from the protoplasm by slightly compressing the cell. $\times 350$
5. Section of a fat-body cell obtained by paraffine embedded method and stained with eosin-hematoxiline. The numerous empty circles indicate the spaces occupied by oil drops before the treatment
U, Pouch of uric acid after all spherulites of uric acid are dissolved by the treatment. $\times 350$
6. Frozen-dried section of the fat-body cells, showing pouches filled with uric acid (U) within the cell. $\times 350$

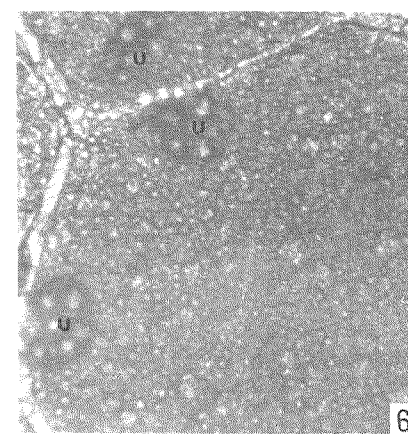
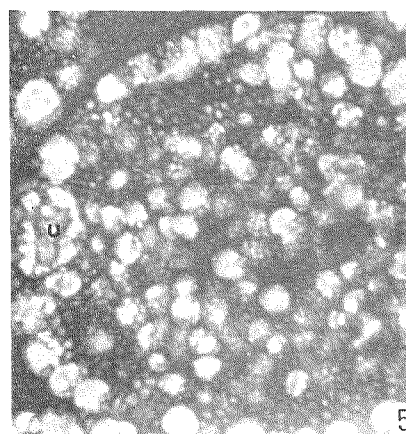
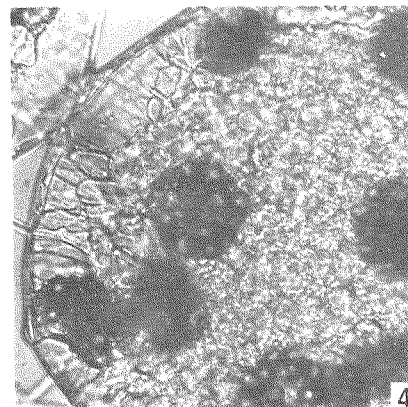
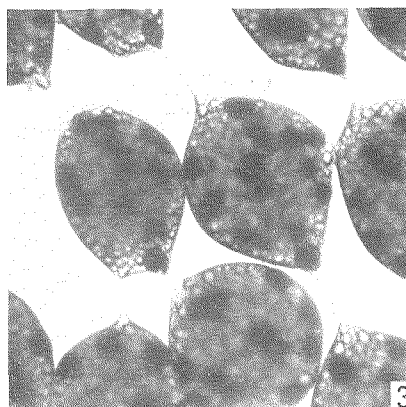
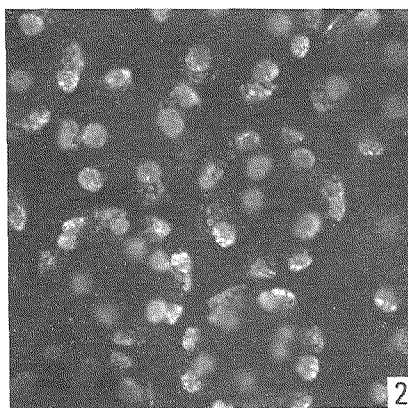
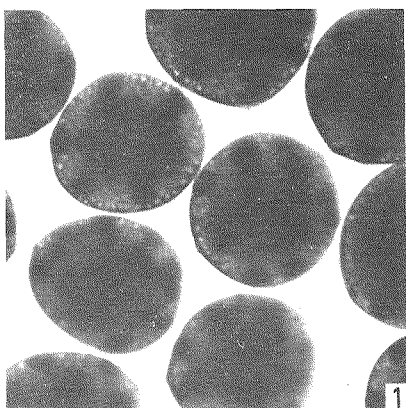


Plate IV.

1. A pouch filled with many spherulites of uric acid in the frozen-dried section of fat-body cells in the visceral layer. $\times 800$
2. The same as 1 under a polarizing microscope. Spherulites show birefringence. $\times 800$
3. Spherulites of uric acid separated from fat-body cells in the absolute alcohol, under a polarizing microscope. $\times 200$
S, A spherulite magnified, showing the Maltese cross in polarized light. $\times 800$
4. Crystals of uric acid converted from spherulites by adding a small amount of water to the spherulite suspension in absolute alcohol. $\times 100$
5. Fat-body cells in the parietal layer. No pouches of uric acid are observed in these cells. $\times 100$
6. Section of a fat-body cell in the parietal layer obtained by the paraffine embedded method and stained with eosin-hematoxiline. The numerous empty circles indicate the spaces occupied by oil drops before the treatment. $\times 350$

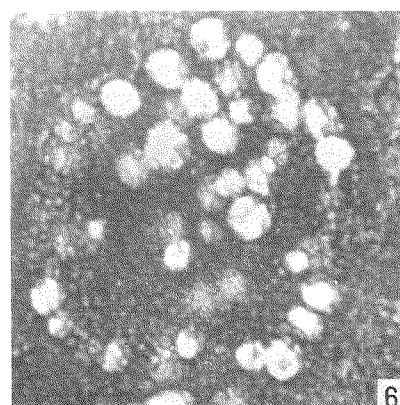
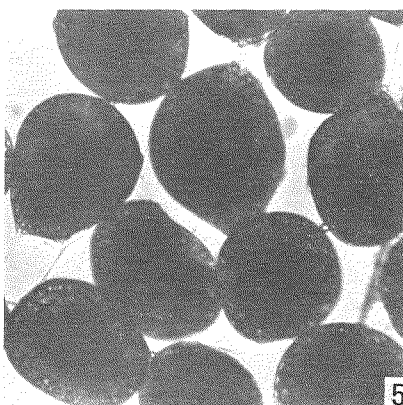
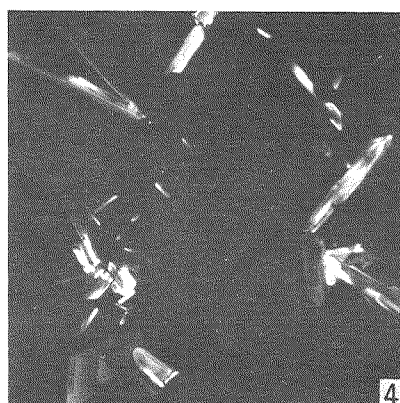
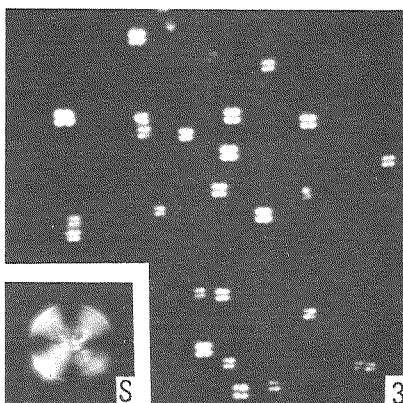
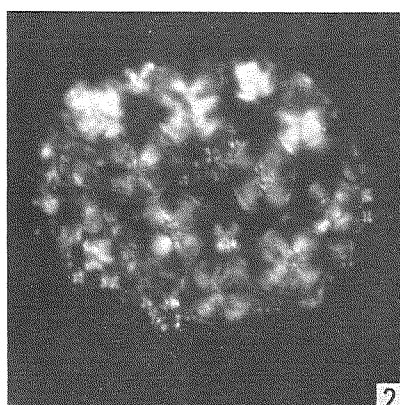
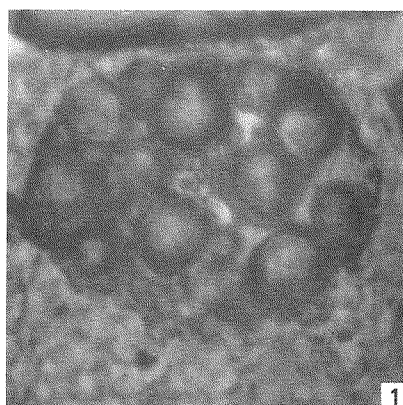


Plate V. Extracellular freezing in fat-body cells, photographed at -15°C . A prepupa was frozen to -30°C at a cooling rate of about $2^{\circ}\text{C}/\text{min}$ and was kept there for 24 hours before it was sectioned. The freezing section is $25\ \mu$ in thickness. $\times 100$

1. Extracellularly frozen fat-body cells in the visceral layer (V)
2. The same as 1 under a polarizing microscope showing birefringence in extracellular ice crystals and in spherulites of uric acid in the cells
3. The same as 1 after sublimation of ice at -15°C
4. The same as 3 under a polarizing microscope. Only spherulites of uric acid in the cells can be seen.
5. Extracellularly frozen fat-body cells in the parietal layer (P)
6. The same as 5 under a polarizing microscope

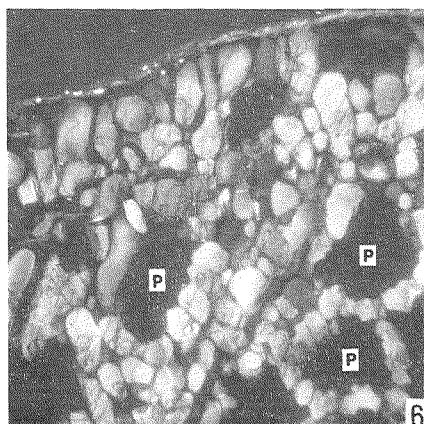
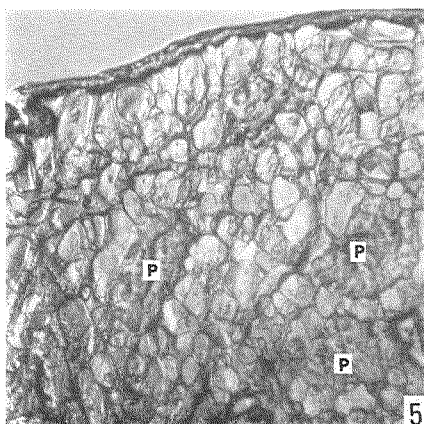
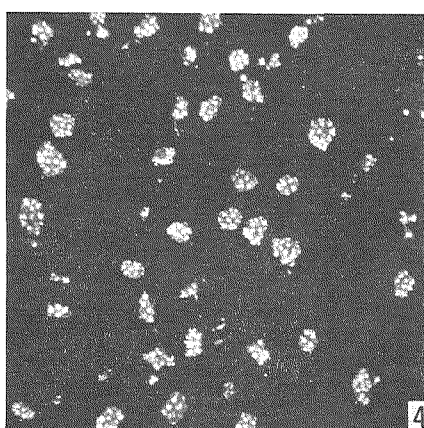
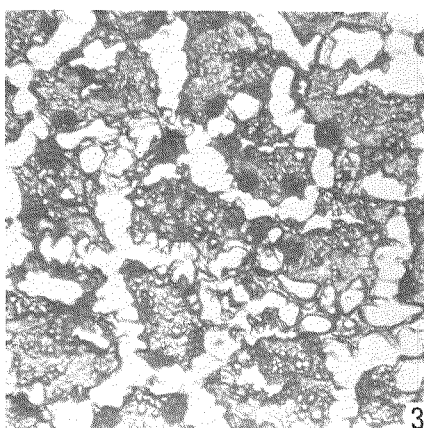
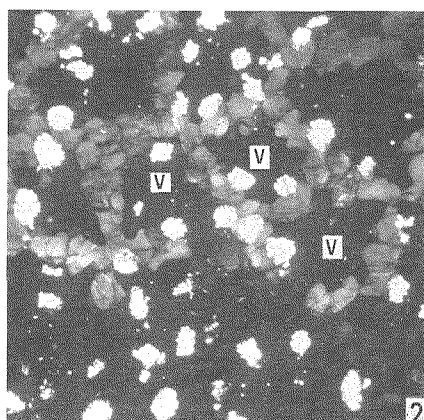
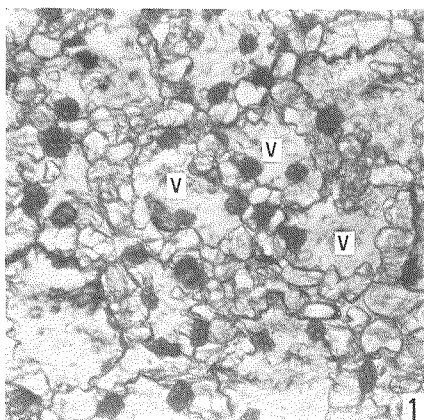


Plate VI. Intracellular freezing in fat-body cells, photographed at -15°C . $\times 100$

1. Intracellularly frozen fat-body cells in the visceral layer. Frozen sections were obtained from the prepupa treated in the same manner as indicated in Plate V
2. The same as 1 under a polarizing microscope
3. Intracellularly frozen fat-body cells the visceral layer from the frozen prepupa immersed directly in liquid nitrogen. Ice crystals within these cells are so small that the cells appear dark except for the spaces occupied by oil drops
4. The same as 3 under a polarizing microscope
5. Intracellularly frozen fat-body cells in the parietal layer. The prepupa was frozen in the same manner as indicated in 3
W, Body wall; M, Muscle tissues
6. The same as 5 under a polarizing microscope

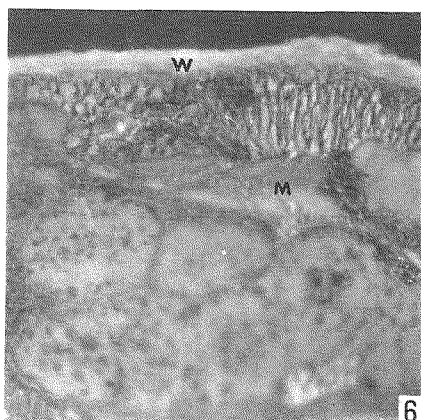
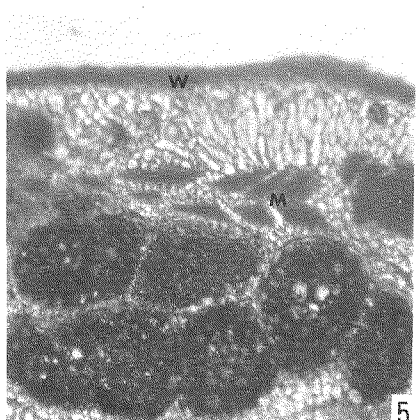
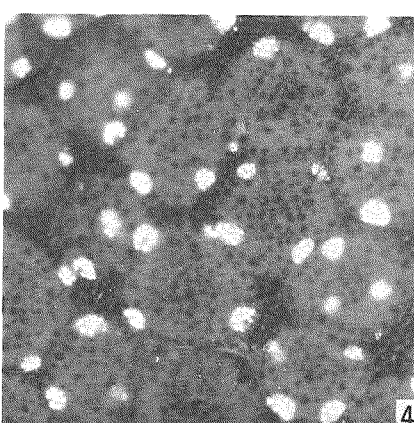
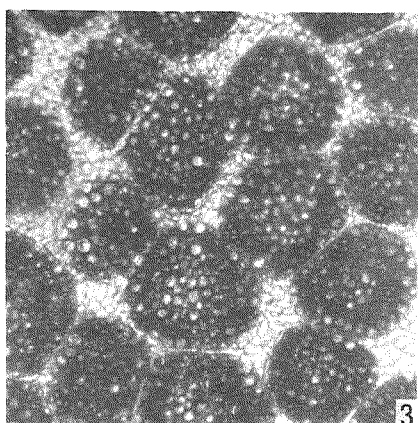
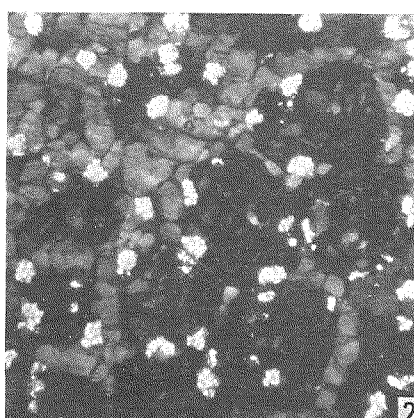
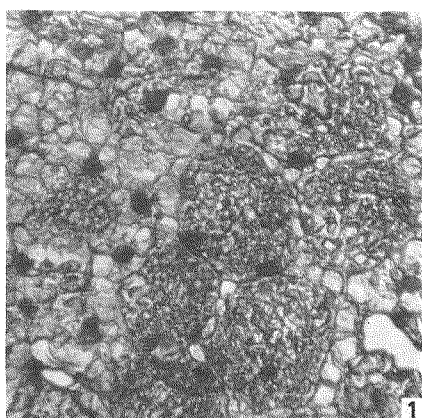


Plate VII. Fat-body cells before and after freeze-thawing. $\times 80$

1. Fat-body cells in the visceral layer
2. Fat-body cells in the parietal layer
3. Fat-body cells in the visceral layer after thawing from an extracellular freezing. The oil drops within the cell coalesced into larger ones
4. Fat-body cells in the parietal layer after thawing from an extracellular freezing. The oil drops within the cell coalesced into larger ones
5. Fat-body cells in the visceral layer from the prepupa immersed directly in liquid nitrogen, and thawed at room temperature. Destroyed cell structure with the thin surface membrane separated from the protoplasm
6. Fat-body cells in the parietal layer after freeze-thawing at and from liquid nitrogen temperature. Destroyed cell structure is apparent

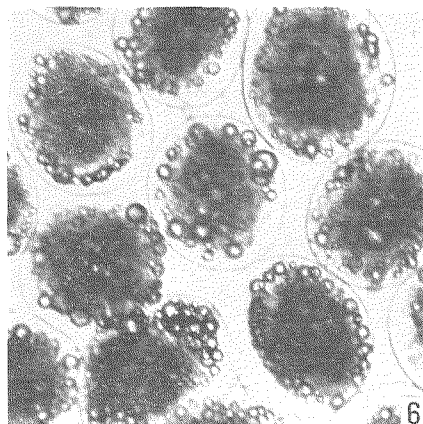
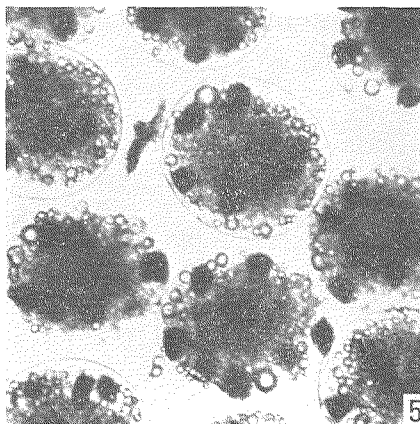
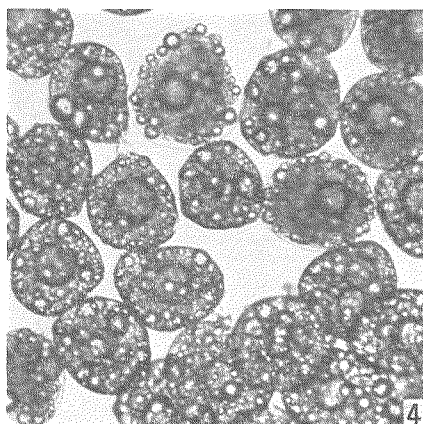
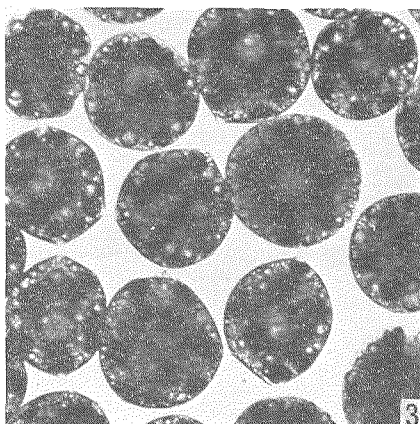
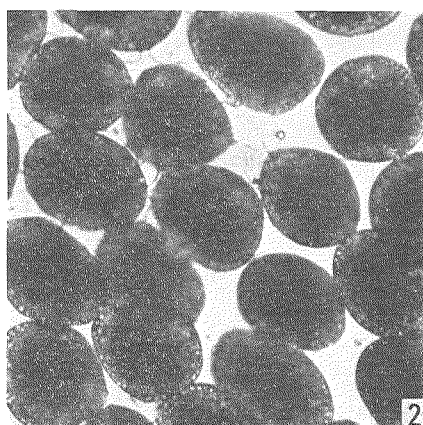
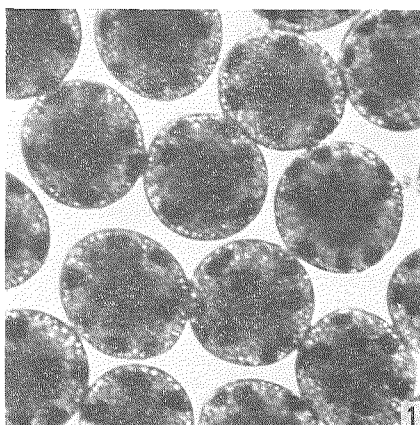


Plate VIII~XII. Freezing states of prepupae frozen to -20°C at various cooling rates. Frozen sections are 20μ in thickness

Plate VIII. Frozen sections from the insect cooled at a rate of $327^{\circ}\text{C}/\text{min}$

1. Cross section of the abdomen

D, Mid gut; M, Muscle tissues; N, Ventral nerve cord; V, Fat-body cells in the visceral layer; P, Fat-body cells in the parietal layer. Darkening of all fat-body cells indicates the formation of very fine ice crystals within these cells. $\times 33$

2. Details of frozen fat-body cells in the visceral layer. $\times 250$

3. Details of frozen fat-body cells in the parietal layer. $\times 250$

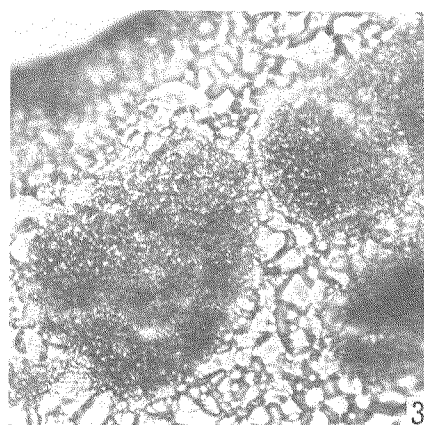
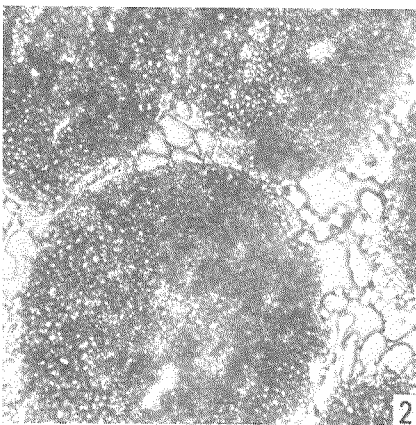
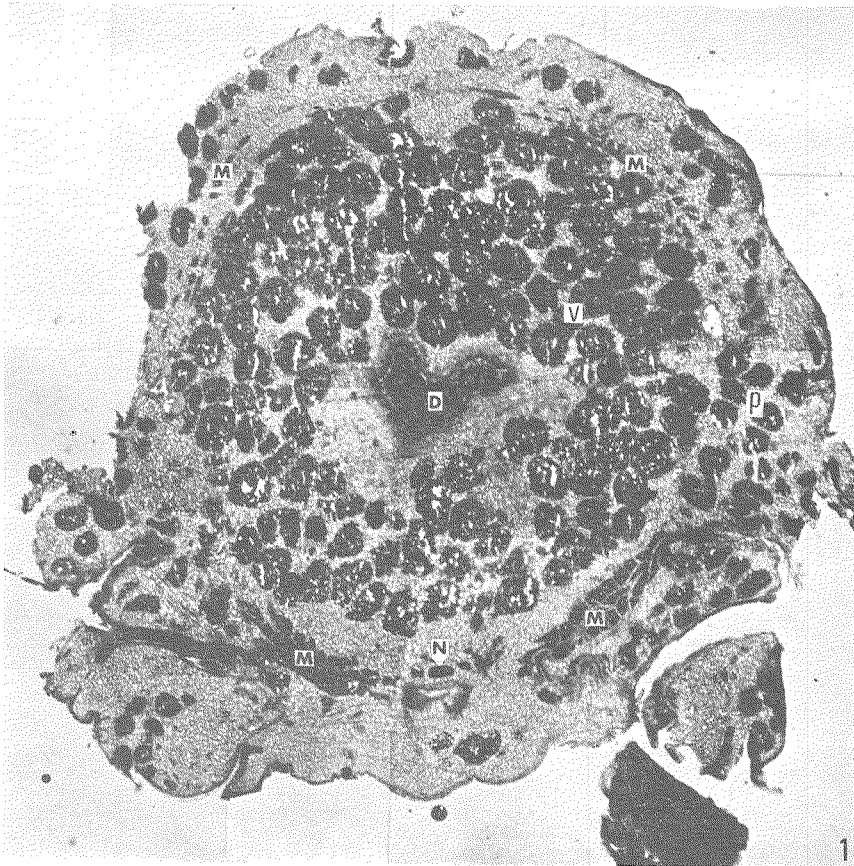


Plate IX. Frozen sections from the insect cooled at a rate of 15.4°C/min

1. Cross section of the abdomen. D, Mid gut. All fat-body cells in the visceral layer freeze intracellularly, while about half of the fat-body cells in the parietal layer freeze extracellularly. $\times 33$
2. Details of an intracellularly frozen fat-body cell in the visceral layer U, Pouches of uric acid. $\times 250$
3. Details of an intracellularly frozen fat-body cell in the parietal layer $\times 250$

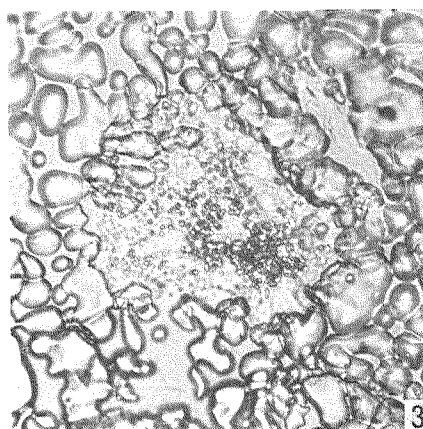
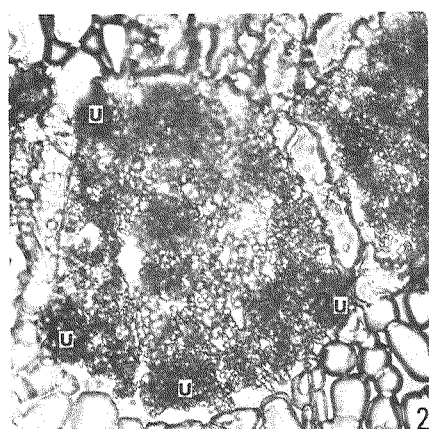


Plate X. Freezing sections from the insect cooled at a rate of $4.0^{\circ}\text{C}/\text{min}$

1. Cross section of the abdomen. D, Mid gut. Practically all fat-body cells in the visceral layer freeze intracellularly. The number of ice particles within these frozen cells is apparently less than that in the cells indicated in Plate IX-2. About 17% of the fat-body cells in the parietal layer freeze intracellularly and the remainder freeze extracellularly. $\times 33$
2. Details of an intracellularly frozen fat-body cell in the visceral layer U, Pouches of uric acid. $\times 250$
3. Details of an intracellularly frozen fat-body cell in the parietal layer $\times 250$

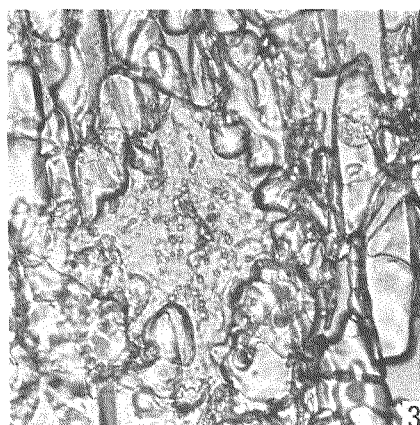
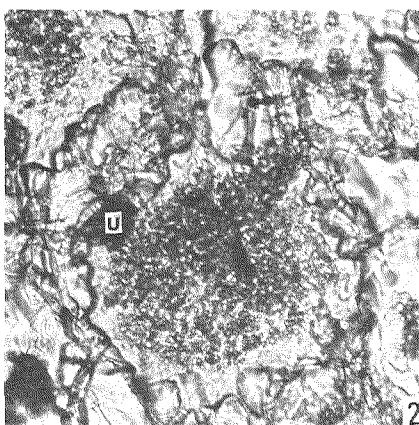
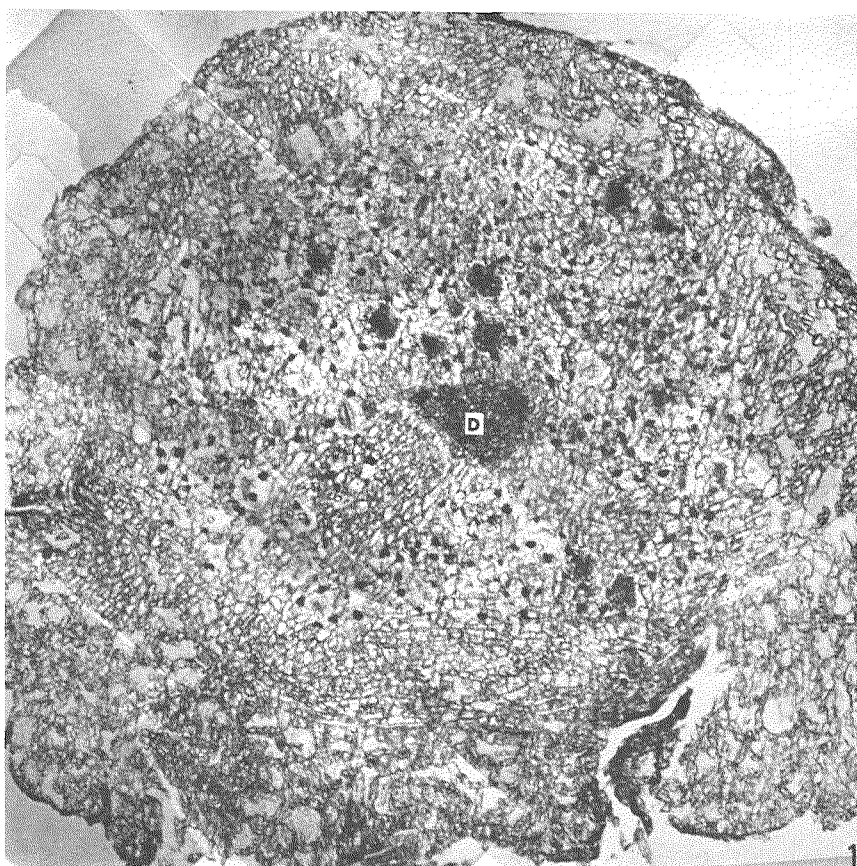
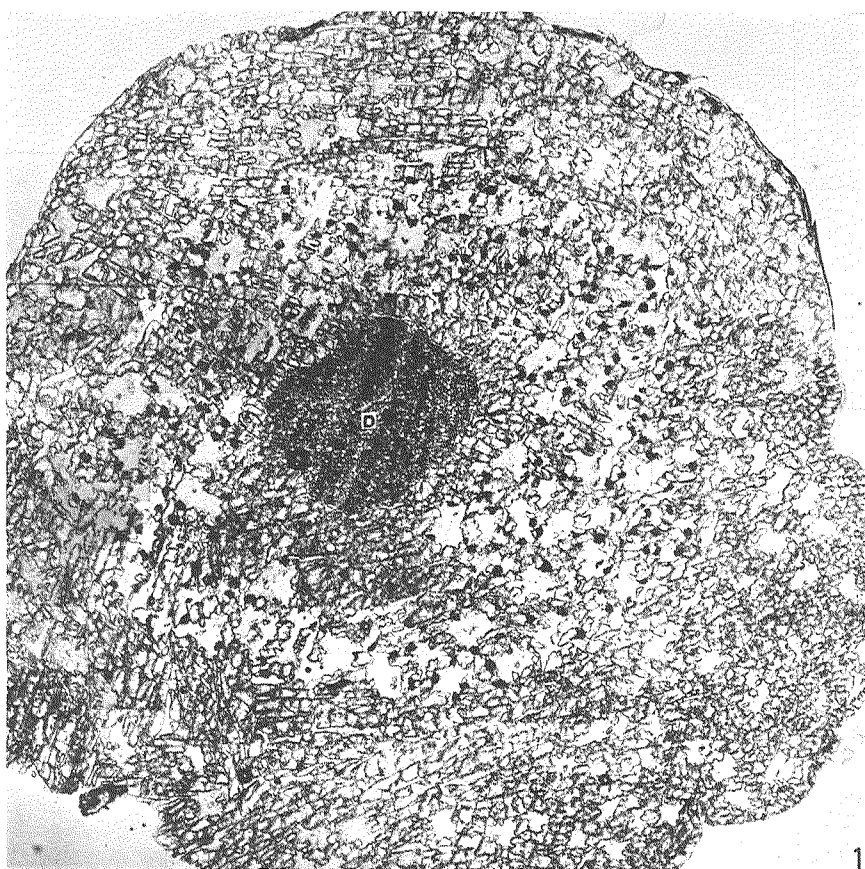
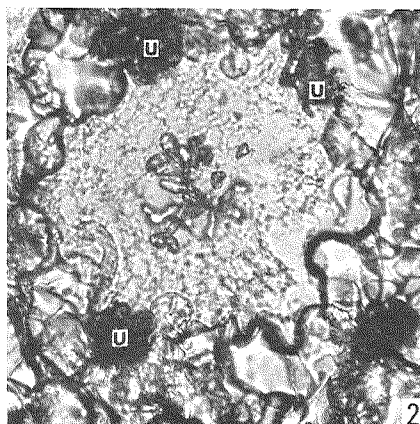


Plate XI. Frozen sections from the insect cooled at a rate of 0.8°C/min

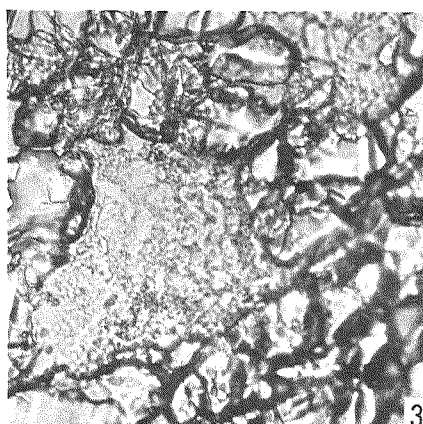
1. Cross section of the abdomen. D, Mid gut. Most of the fat-body cells in the visceral layer freeze extracellularly. All fat-body cells in the parietal layer freeze extracellularly. $\times 33$
2. Details of an intracellularly frozen fat-body cell in the visceral layer U, Pouches of uric acid. $\times 250$
3. Details of an extracellularly frozen fat-body cell in the parietal layer $\times 250$



1



2



3

Plate XII. Frozen sections from the insect cooled at a rate of $0.4^{\circ}\text{C}/\text{min}$

1. Cross section of the abdomen. D, Mid gut. All fat-body cells in both visceral and parietal layers freeze extracellularly. $\times 33$
2. Details of extracellularly frozen fat-body cells in the visceral layer U, Pouches of uric acid. $\times 250$
3. Details of an extracellularly frozen fat-body cell in the parietal layer $\times 250$

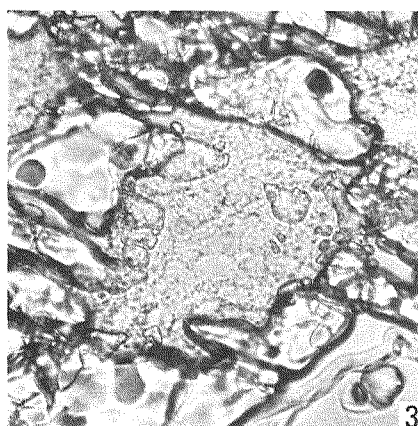
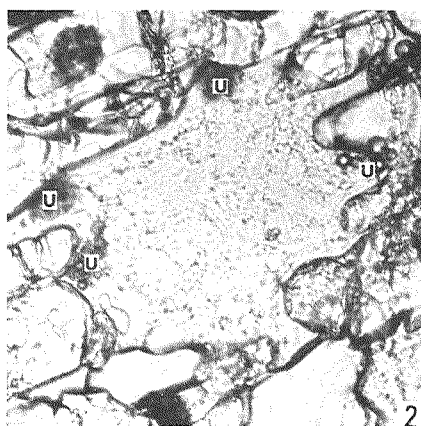
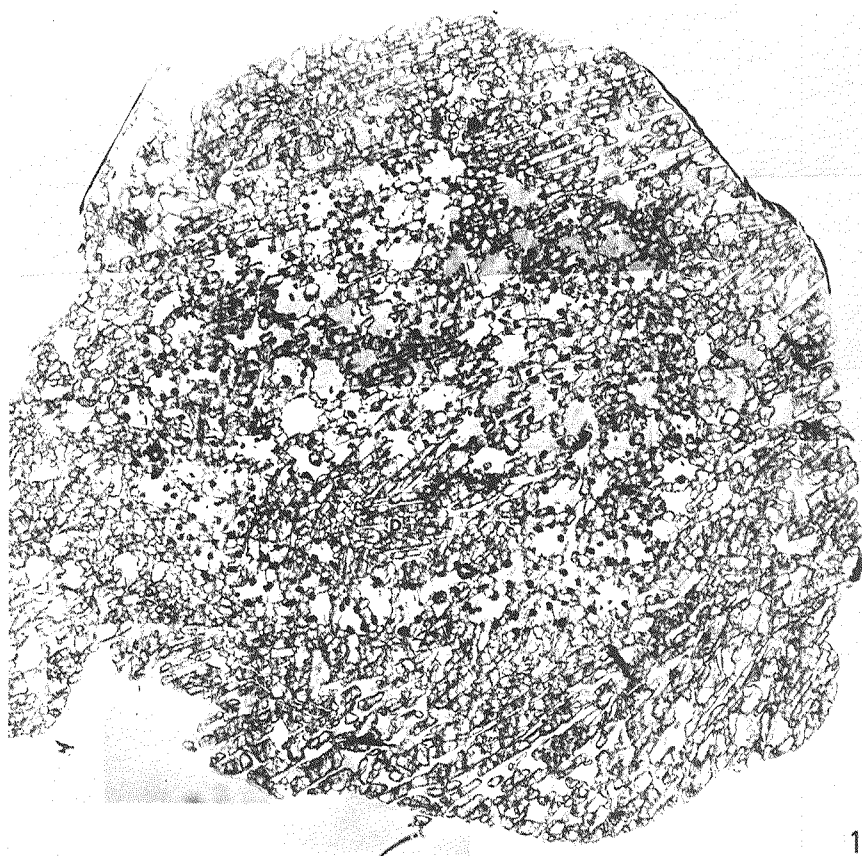


Plate XIII. Freezing state of muscles and nerve tissues at -20°C . 1 to 4 show that no ice crystals form within muscle tissues

W, Body wall; V, Fat-body cells in the visceral layer; P, Fat-body cells in the parietal layer; Muscle tissues are along the X-Y line

1. Part of the frozen section including muscle tissues under an ordinary microscope. The frozen section was cut from an insect cooled at a rate of about $0.8^{\circ}\text{C}/\text{min}$. $\times 100$
2. The same as 1 under a polarizing microscope. $\times 100$
3. The same as 1 after sublimating ice crystals as seen under an ordinary microscope. $\times 100$
4. The same as 3 under a polarizing microscope. $\times 100$
5. Frozen cross section of the ventral nerve cord from a prepupa cooled at a rate of $327^{\circ}\text{C}/\text{min}$. The ventral nerve cord appears dark as a result of formation of numerous fine ice crystals within the tissue $\times 350$
6. Frozen cross section of the ventral nerve cord from a prepupa cooled at a rate of $0.4^{\circ}\text{C}/\text{min}$. Since no ice is observed within the ventral nerve cord, the trachea can clearly be seen within the tissue. $\times 350$
7. Frozen section of the suboesophageal ganglion from a prepupa cooled at a rate of $0.4^{\circ}\text{C}/\text{min}$. No ice crystal forms within the tissue. $\times 350$

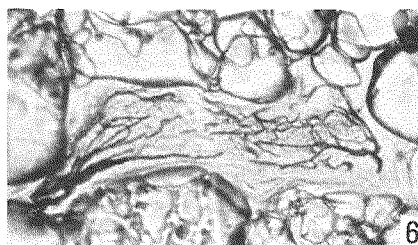
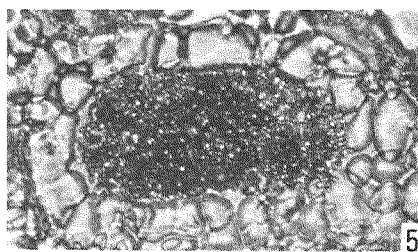
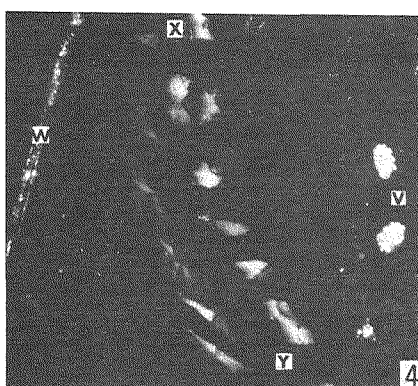
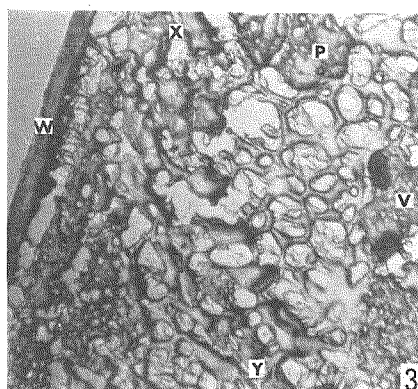
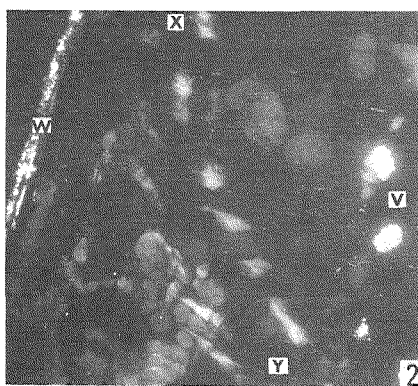
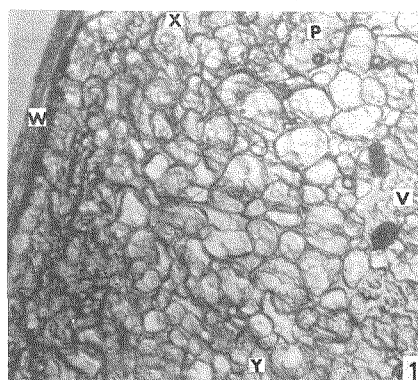


Plate XIV. Localization of ice crystals in fat-body cells frozen intracellularly
× 250

1. Intracellularly frozen fat-body cells in the visceral layer. Intracellular ice crystals are found only in the center of the cell. U, Pouches of uric acid
2. Intracellularly frozen fat-body cells in the parietal layer. Intracellular ice crystals are also found in the center of the cell
3. Intracellularly frozen fat-body cells in the visceral layer. Localization of intracellular ice crystals around the point indicated by an arrow may suggest possible inoculation with the external ice crystals. U, Pouches of uric acid
4. Intracellularly frozen fat-body cells in the visceral layer. Fat-body cells are connected with each other by fine canals (arrows). Within the fine canal, no ice crystals are observed. U, Pouches of uric acid

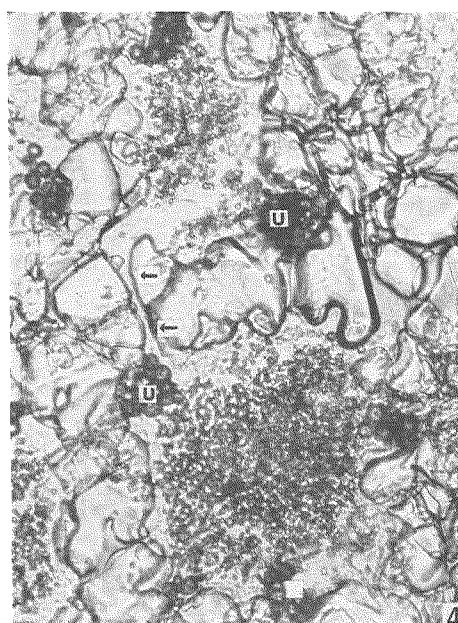
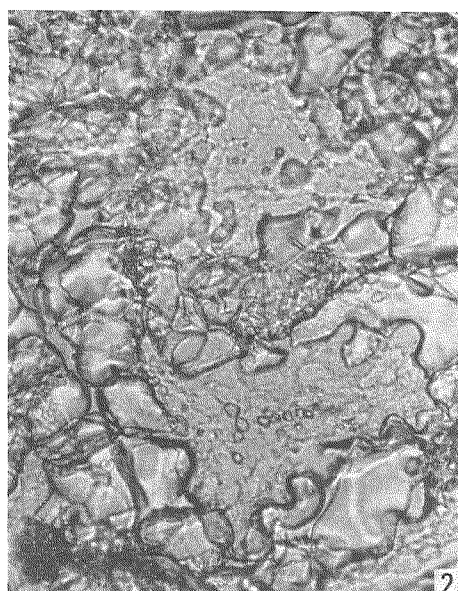
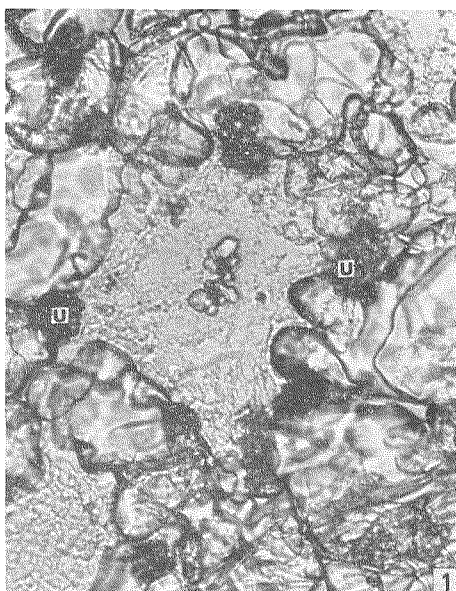


Plate XV.~XVIII. Comparison of freezing states in the prepupae frozen by two methods, A and B. $\times 38$

- A. Prepupae were frozen down to -30°C at a cooling rate of about $1^{\circ}\text{C}/\text{min}$ and then were kept at the same temperature for 20 hours. Frozen sections were made from the frozen prepupae at -20°C
- B. Prepupae, previously frozen to -20°C at a cooling rate of about $1^{\circ}\text{C}/\text{min}$, were warmed to -5°C and kept at the same temperature for 18 hours. These were then cooled down to -30°C at a cooling rate of $1^{\circ}\text{C}/\text{min}$ and kept at the same temperature for 24 hours. Frozen sections were made at -20°C

Plate XV. Cross sections of the head part

- 1. From the prepupa frozen with treatment A
- 2. From the prepupa frozen with treatment B

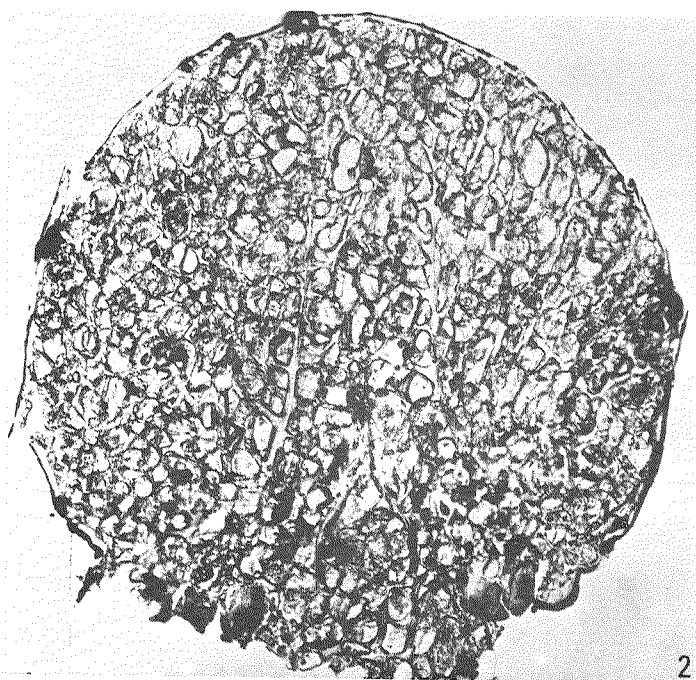
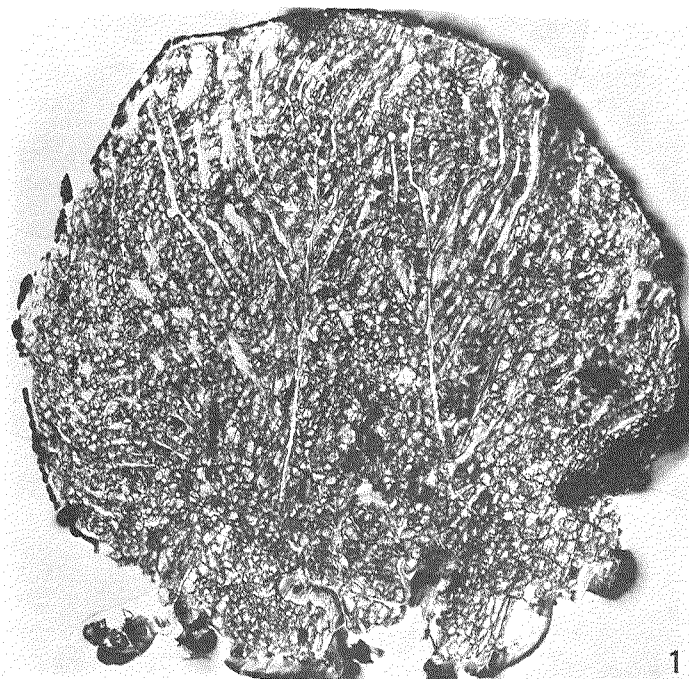


Plate XVI. Sagittal sections of the head and thorax

1. From the prepupa frozen with treatment A
2. From the prepupa frozen with treatment B

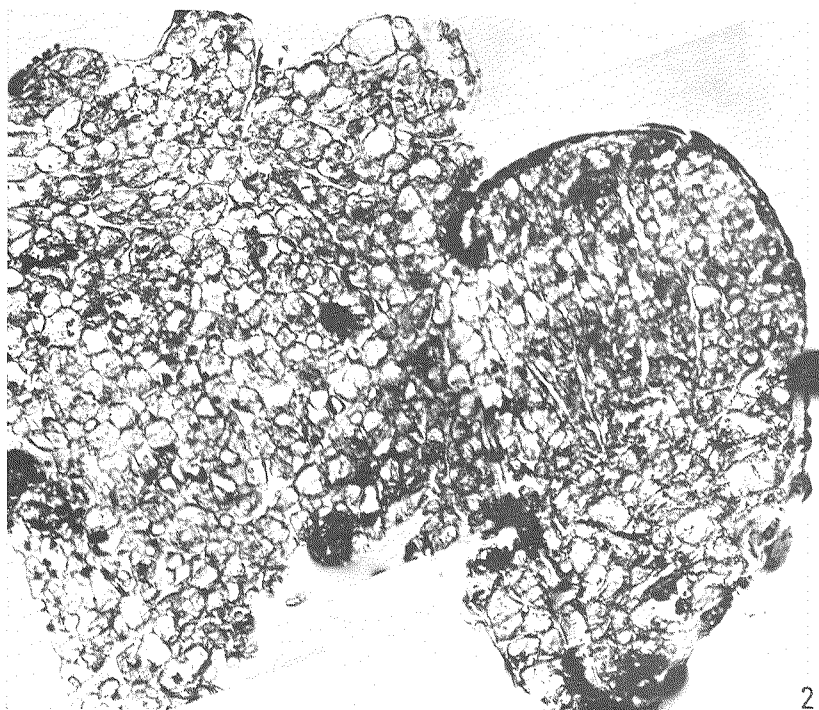


Plate XVII. Cross sections of the abdomen in frozen prepupae. D, Mid gut

1. From the prepupa frozen with treatment A
2. From the prepupa frozen with treatment B

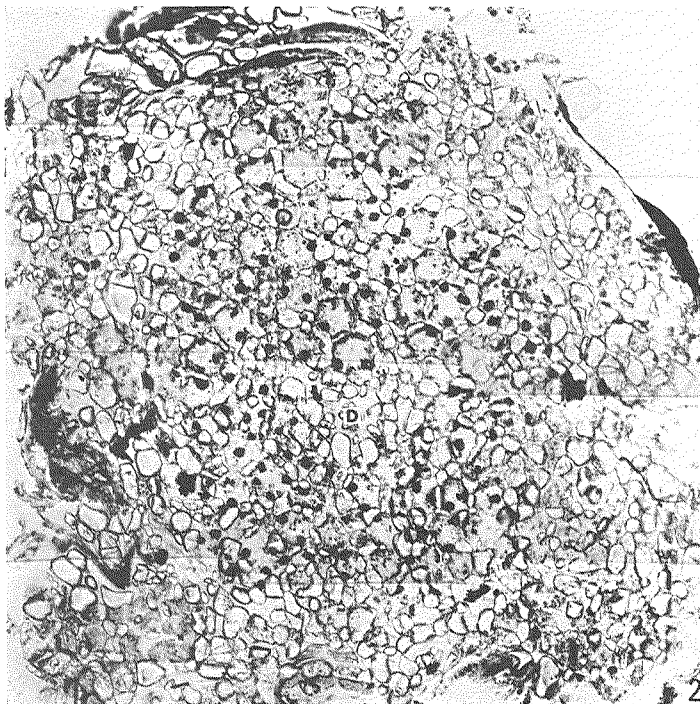
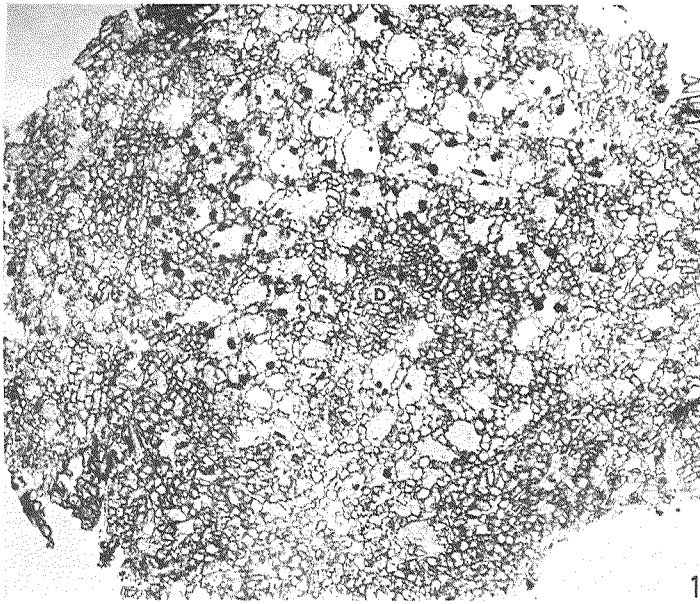


Plate XVIII. Frozen sections from the prepupae showing concentrated haemolymph
× 100

1. A part of the abdomen in the prepupa after treatment A in Plate XV. W, Body wall; P, Fat-body cells in the parietal layer. Many extracellular ice particles form around the fat-body cells in the parietal layer
2. The same as 1 after sublimating ice crystals. Concentrated haemolymph is seen remaining between the spaces once occupied by ice grains before sublimation. W, Body wall; P, Fat-body cells in the parietal layer
3. A part of the abdomen in the prepupa after treatment A, under phase contrast microscope. V, Fat-body cells in the visceral layer; P, Fat-body cells in the parietal layer
4. A part of the abdomen in the prepupa after treatment B, under phase contrast microscope. V, Fat-body cells in the visceral layer; P, Fat-body cells in the parietal layer; T, Trachea
5. A part of the head in the prepupa after treatment A, under phase contrast microscope. W, Body wall. Net work remaining between the empty spaces (E) once occupied by ice particles containing muscle tissues surrounded by concentrated haemolymph
6. A part of the head of prepupa after treatment B, under phase contrast microscope. W, Body wall; E, Empty spaces once occupied by ice particles. Layers of concentrated haemolymph containing muscle tissues are thicker than those indicated in 5

