Amount of Ice Formed in the Prepupa of Slug Moth and its Periodicity*

by

Jutarō SHINOZAKI**
Biological Institute, Ibaraki University

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I. Introduction

Some cold hardy insects have long been known to survive body freezing. It seems to be an interesting problem to determine quantitatively, how much ice is really formed in the insect frozen at various low temperatures, and to establish the relationship between the graded freezing temperatures and the amounts of ice formed in the insect body. If the ice-amount-temperature relationship was established, the amount of ice formed in the insect can be easily obtained, if only its freezing temperature is measured. This relationship will also be of ecological significance, because the relation may suggest how much ice forms in the insect under the natural circumstance changes with the rise or fall of the atmospheric temperature. Since frost injury in living organisms has been believed to be brought about as a result of the loss of water separated as ice upon their freezing, the quantitative determination of ice formed in the frozen insect body may yield one of the most important clues to solve the frost-resistance mechanism in the insect. In view of such considerations, in the present study, the calorimetric determination of the amount of ice in the prepupae of a "slug moth", *Monema flavescens* was carried out. This insect is one of the representatives of the cold hardy insects; it can survive body freezing at −20°C or below[1,2]. The determination of the amount of ice formed in the insect may also be useful to analyse and to interpret more precisely the freezing curve of the same insect which has been presented in previous reports[3,4].

The periodicities in the cold-hardiness*** and in some physiological properties have been found in various insects. The cold-hardiness increases gradually in autumn and reversely it decreases in spring. The undercooling point in the cold hardy insects

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*** Cold-hardiness has been in a wide sense interpreted to be the ability of an organism to withstand low temperatures[5-8], including the frost-hardiness or the freezing-tolerance and the ability to endure supercooling in freezing-susceptible insect *etc.*[9]

reaches minimum in the coldest season[2,3,7,10,11] and the lowest freezing point of the blood is attained in the winter with corresponding rise in the solute concentration of the blood[2,3,12]. Besides, in some overwintering insects, there is an actual increase of glycerol content in cold season[13-17]. All these hold true in the case of the prepupa of the slug moth. The periodicity of the amount of ice formed in insects, however, has not been studied yet.

In the present study, monthly ice-determinations in the prepupa of the "slug moth" were executed from autumn to spring in 1958 and in 1959. In the insect the total water contents in the body and the freezing points of the blood were measured in parallel with the ice determinations, to make clear the correlation between the amount of ice formed and the freezing points of the blood or the water content. By determining the seasonal variation in the amount of ice, the amounts of ice formed in the prepupae of various grades of cold-hardiness can also be determined. This may to some extent contribute to an explanation of hardening and dehardening processes.

In the earlier stage of the ice determination in insects, some investigators held the opinion that the cold-hardiness of the insect was determined by the amount of unfreezable or "bound water", which was estimated as the difference between the amount of total water and that of water frozen at a definite temperature, say $-20°C$[18-21], though this view was recently objected to by the several authors[6,22-24]. This problem, too, will be briefly dealt with in the present study.

II. Materials and Methods

The common "slug moth" of Japan, *Monema flavescens* mainly in prepupal and partly in pupal stages was used as material. This species passes the winter as prepupa in a cocoon attached to the twigs of such trees, as cherry, plum (or "ume"), persimmon, zelkova, chestnut, maple and so on, which provide leaves to the caterpillars for food. Consequently, this species is exposed to severe cold on the twigs. A sufficient number of individual prepupae with the cocoons was collected from these trees at the beginning of each month from September to December, because the number of individuals satisfying the demand of the present experiments covering the whole period was unobtainable at one time owing to the scarcity of this species in Mito vicinity. The full-grown larvae spin the cocoons mainly during one month extending from the middle of August to that of September. The caterpillars captured in this season were reared in the laboratory, till they spun their cocoons. These were also employed as the material. As the prepupae of this species are sold in fishing tackle shops as bait for catching "tanago" (*Acheilognathus* sp.), these were mainly made use of for the experiments after January. These materials were of course collected from nature. The prepupae were kept under near-outdoor conditions in a cage. To minimize the effect of the difference of the source of materials on the results, the measurements were made with individuals selected at random from the materials obtained at different times, from different trees, in different places, and from the shop. The pupae which had

pupated under laboratory conditions in May and early June were also subjected to the ice determination. The weight of the prepupae used ranged from 250 to 500 mgm.

Measurements of the amount of ice formed in the organism have been carried out by the dilatometric method[20,21,25], the calorimetric method[18,22,25-29], the flotation method[24], and the dehydration-melting point method[23]. In the present study, the calorimetric method was preferred to the rest, for fear that the prepupa should contain air in its tracheal system, because, if the materials contain air bubbles in the body, the dilatometric and the flotation methods should be accompanied by gross errors in the measurements. The dehydration-melting point method can be properly employed only for the fluid material.

Let consideration be given to the quantity of heat required to warm a frozen solution from a certain subzero temperature (t_e) to a temperature (t) above zero with thawing of ice. If the quantity of heat absorbed in this process is determined, the amount of ice existing in the frozen solution at t_e could be evaluated. Since this process takes place under constant atmospheric pressure, the quantity of heat absorbed must be equal to the difference between the values of the enthalpy at the beginning and at the end of the process according to the first law of thermodynamics. Since the enthalpy is a quantity depending only on the state, the quantity of heat absorbed must be constant independently of the intermediate path by which the process takes place, provided that the initial and the final states are fixed. This process may occur actually along the following path. When some quantity of heat is added to the frozen solution at t_e, a small fraction of ice will thaw and then the whole system will attain to a new equilibrium state at $t_e+\Delta t$ and so forth. Such processes are repeated and all ice melts away finally at the freezing point* (t_f) of the solution. Then the ice-free solution is warmed from t_f to t. From the above explanation, it is clear that the quantity of heat absorbed during this actual process is equal to the amount of heat absorbed in the imaginary path, in which the frozen solution is warmed in the first place from t_e to 0°C without the fusion of ice, then ice melts away at 0°C, and finally the solution is warmed from 0°C to t. To estimate the amount of ice, it may be convenient to think the process takes place according to the imaginary path. Such is also the case with the frozen insect. Since it is recognizable that the frozen insect can be warmed up to 0°C without melting ice in it, only the quantity of heat demanded to thaw and to warm the frozen insect is required, but not the freezing point of the blood in the insect, in order to determine the amount of ice formed in it. Now, the equation for calculating the amount of ice in the insect body could be derived on the basis that the quantity of heat which is lost by the introduction of the frozen insect from water in the calorimeter, the calorimeter vessel itself and its appurtenances until the temperature equilibrium is reached, is equal to the amount of heat required to thaw and to warm the frozen insect to the final temperature in the calorimeter. Then,

* The freezing point of the solution means the temperature, above which ice can never crystallize from it.

the equation is:

$$X=\frac{W\int_{t_2}^{t_1}C_w dt+w(t_1-t_2)-A}{H_0-\left[\int_{t_e}^{0}C_w dt-\int_{t_e}^{0}C_i dt\right]} \qquad (1)$$

and

$$A=(h+C_d M_d)(t_2-t_e)+M_w\left[\int_{t_e}^{0}C_w dt+\int_{0}^{t_2}C_w dt\right] \ *$$

where X = mass of ice formed in an insect,
W = mass of water in the calorimeter vessel,
M_d = mass of dry matter in insect,
M_w = mass of total water in insect,
t_1 and t_2 = initial and final temperatures in calorimeter vessel,
t_e = freezing temperature of insect (negative value),
H_0 = heat of fusion of ice at 0°C (79.60 cal./gm.),
C_w = specific heat of water,
C_i = specific heat of ice,
w = water equivalent of the calorimeter (1.47),
h = heat capacity of the insect container which will be mentioned later in detail,
C_d = specific heat of dry matter in insect (0.32 cal./gm./deg.).

This equation is essentially the same as the ones used by GREATHOUSE[26], DITMAN *et al.*[22], and others. Let X' denote the amount of ice in per cent of the total body water, then one gets

$$X'=100\frac{X}{M_w} . \qquad (2)$$

Hereafter, the relative amount of ice (X') will be simply referred to as the amount of ice.

The values of $\int_0^t C_w dt$ in equation (1) may be easily obtained, provided that the values of the mean specific heat between 0°C and t ($t>0$), which are expressed by $\bar{C}_w=\left(\int_0^t C_w\,dt\right)/t$, have been previously estimated. As these values have been presented by ROTH[30], a chart or table for the mean values can be easily constructed. It seems, as far as the author is aware, that there is no value available for specific heat of undercooled water. In the present study, it was necessary to use the values, which were obtained by extrapolating from 0° to about −35°C the values calculated by the

* The value in the parenthesis of second term of this formula is equal to $\int_{t_e}^{t_2}C_w dt$, but it was more convenient to calculate $\int_{t_e}^{0}$ and $\int_0^{t_2}$ individually than to do $\int_{t_e}^{t_2}$, because the chart of C_w (specific heat of water) for the temperatures above and below zero was constructed separately, as will be noted later.

equation given by ROTH for the specific heat of water between 0° and 40°C. The estimation of $\int_{t_e}^{0} C_w dt$ for the undercooled water was made in the same manner as in temperatures above zero.

Specific heat of ice was evaluated from the formula given by DICKINSON and OSBORNE (cited from DORSEY[31]), neglecting correction for the impurity of ice. The values used were the ones for pure ice, because, even if the impurity of ice is relatively much, the correction is fairly small, excluding the correction at subzero near 0°C[31]. Another reason, why this correction was disregarded, is that scholars are ignorant as to the impurity of ice formed in the insect body. These values for specific heat of ice are the same as those adopted by SAYRE[25]. The values of $\int_{t_e}^{0} C_i dt$ were evaluated in the same manner as in water. Specific heat of dry matter (C_d) and heat capacity of the insect container (h) may change with the temperature, but, since the magnitude of the terms including both of the values in the equation (1) is very small as compared with the terms relating to specific heats of water and of ice, even if both of the values were regarded as practically constant, the result will not be affected very much.

The method of the ice determination was the same as used by the author in determining specific heat of the insect[32]. The main part of the calorimeter consists of four copper vessels inserted inside of each other according to sizes, avoiding contact with each other by fixing them with cork disks and bakelite rings; it is enclosed in a water jacket. The water jacket, through which tap water is constantly circulated, serves to keep the temperature of the parts surrounding the calorimeter vessel practically uniform. The innermost smallest vessel with a lid is used as the calorimeter vessel. The water equivalent (w) of the calorimeter was measured from the drop of the temperature in the calorimeter caused by the introduction of a known mass of ice. Its mean value of 12 measurements was 1.47 ± 0.21.

Since the naked prepupa cannot be easily handled for treatments, an insect container was employed. This container served to sink the frozen insect in water within the calorimeter. The container made of nickel-plated copper consists of two small cylinders, the one of which with smaller diameter and longer height fits inside the other. A frame of nickel-plated copper wire is soldered at one end of each cylinder to form the lid and the bottom when the one piece is inserted in the other; the slenderer one bears a bail to hang the whole container. The overall size of the container is about 9 mm. in diameter and 13 mm. in height. Heat capacity of the insect container was calculated from the mass of copper and nickel composing it and their specific heats. Four containers were used in the present study; their heat capacity ranged from 0.105 to 0.123.

Specific heat of the dry matter in the prepupa was measured by essentially the same methods as described in a previous paper[32]. Some 50 or 60 of the dried prepupae were ground in a mortar grinder and the obtained powder was again dried as completely as possible in the thermostat at 105°C. After the dried powder was wrapped

in aluminium foil of known mass, its weight was determined; it was put in the insect container and then cooled in small chamber at 0°C. The aluminium foil served to prevent contact of the dry matter with water, lest the dry matter, when absorbing water, might evolve some quantity of swelling heat. Specific heat of the dry matter was evaluated from the fall of temperature in the calorimeter, when the former cooled to 0°C was introduced in the latter, using the relation

$$C_d = \frac{W\int_{t_1}^{t_2} C_w dt + w(t_1 - t_2) - [mC_a + h]\, t_2}{M_d t_2} \qquad (3)$$

where m = mass of aluminium foil,

C_a = specific heat of aluminium.

The mean of 15 measurements gave the value of 0.32±0.03 cal./gm./deg.

The prepupae of the slug moth during the overwintering period remain unfrozen over a relatively long period above −15°C, but they freeze within 40 minutes at −20°C[33]. It was, therefore, necessary to expose the prepupa previously at about −25°C for an ample period of time in order to freeze it. The weighed animal was put in the insect container and then the container was put into the small vessel of copper with the lid which was immersed up to its neck in a freezing mixture at about −20° to −25°C in a Dewar jar. The prepupa was frozen and kept for several hours in it. The prepupa frozen in such a way had to be equilibrated with a desired temperature (t_e), before it was dropped into the calorimeter. Since unfortunately the thermostat desirable for the subzero temperature was not available, the frozen animal in the container was hung in the cooling tube (Fig. 1), which was immersed into a freezing mixture at the desired temperature (t_e) in an one-liter Dewar flask. The temperature in the Dewar flask increased, though very slowly, under the influence of the room temperature. To know whether or not the temperature equilibrium between the frozen prepupa and the surroundings was reached, the temperature of the frozen prepupa was read by a thermocouple at arbitary time intervals. The tip of the thermocouple was inserted into the oral pit formed by the retraction of the head into the body cavity. When the freezing temperature (t_e) was considerably low, the prepupa previously frozen in the container was transferred as quickly as possible from the copper vessel in the Dewar jar to the cooling tube in the Dewar flask at the temperature, t_e, within about fifteen seconds, but when the freezing temperature was slightly below the freezing point of the blood, the frozen prepupa was transferred at a relatively slow rate, allowing ice in the prepupa to thaw to some extent. These procedures served to shorten the time required to equilibrate the frozen prepupa with the freezing temperature, t_e. When the temperature of the insect had become almost constant, it was considered that the temperature equilibrium was established between the two. The temperature equilibrium was reached within 40 minutes, but the insect was kept in the cooling bath for at least one hour to make sure of the equilibrium, before being introduced into the calorimeter. The ice determination was carried out within

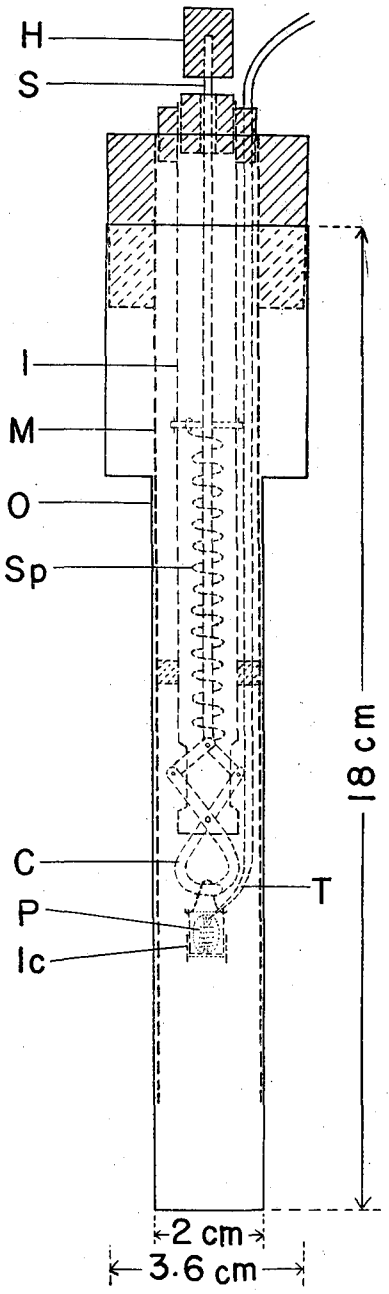


Fig. 1. Cooling tube. This consists of three tubes : outer tube (O) of copper with bottom, middle tube (M) of copper without bottom, and inner tube (I) of duralumin provided with the small crampons (C) used to hang the insect container (IC) including the frozen prepupa (P). The tip of thermocouple (T) is in contact with the prepupa, passing between middle and inner tubes. The crampons are closed by the tension of spring (SP) at a standstill and opened by pushing the head (H) of shaft (S), which passes through the center of the inner tube to connect with crampons. In the introduction of frozen prepupa into the calorimeter, the combination of middle and inner tubes is drawn out, leaving only outer tube in cooling bath, and the frozen insect with its container could then be dropped into calorimeter by pushing the head (H). The middle tube here serves largely to eliminate the effect of room temperature on the frozen prepupa.

the temperature range from the freezing point of blood to about −35°C; a mixture of shaved ice with sodium chloride was used above −10°C and with calcium chloride below −10°C. The temperature of the freezing mixture of ice and calcium chloride could hardly be lowered below −40°C in the Dewar flask under the influence of the room temperature. The incorrectness of specific heat of undercooled water, which was obtained by extrapolating the values above zero to below, becomes larger with the decrease of the temperature; and the formula concerning specific heat of ice proposed by DICKINSON and OSBORNE (cited from DORSEY[31]) gives only the values down to −40°C. Consequently, the ice amount determination below −40°C is not easily carried out and does not appear to give a valid result.

The temperature of water in the calorimenter as well as that of the frozen prepupa was measured with a copper-constantan thermocouple (0.2 mm. in diameter). A deflection of *ca.* 10 mm. on the galvanometer scale corresponded to one centigrade. The temperatures of the calorimeter water and of the frozen animal could be read on the same scale within a few seconds by the change-over of the switch. The calibration curve for the thermocouple was checked with a standard thermometer graduated to one tenth of a degree each day when the ice determination was made. By the exercise of extreme care, the temperatures could be measured within the limit of error of ±0.03°C.

After the temperature of the frozen prepupa in the cooling tube had reached approximately that of the surroundings, about 15 cc. of pure water, the temperature of which had been equilibrated with that of circulating water in the water jacket, was poured into the smallest calorimeter vessel and its exact quantity was estimated by weighing. Then, the vessel was fixed in the calorimeter, and the stirrer and the thermocouple were set. Since the room temperature was higher in most cases than that of the circulating water, the temperature of the water in the calorimeter was one to two degrees higher than that of the circulating water, even after the procedures noted above.

The temperature change in the calorimeter caused by the temperature gradient between the calorimeter and the water jacket was measured every thirty seconds during the first four minutes, mixing water in the calorimeter by stirring, prior to the introduction of the frozen prepupa into the calorimeter. The final measurement of the temperature (t_e) of the frozen insect was made by the change-over of the switch at four and a half minutes after the start of the ice determination and then, returning the switch to the original circuit, the temperatures in the calorimeter at five minutes and at five and a half minutes were again read. As soon as the reading of the temperature at five and a half minutes has been done, the lid of the calorimeter was quickly opened and the frozen prepupa with its container was dropped into the calorimeter by pushing the head (*H*) of the cooling tube (*cf.* Fig. 1). Then the lid was again put on promptly. This procedure was completed in most cases within about ten seconds. The cooling tube served for transference of the frozen animal from the

Dewar flask to the calorimeter with minimum exposure to room temperature and besides the middle tube was useful as a protector for the frozen prepupa against the rapid temperature change. When the frozen prepupa was dropped into the calorimeter, both it and the calorimeter may be influenced by the room temperature to some extent, possibly introducing a slight error into the result. The instant at which the prepupa was dropped in the calorimeter was exactly recorded and as will be shown later, this made it possible to estimate the exact initial temperature (t_1), *i.e.* the temperature at the instant of the introduction of the frozen insect into the calorimeter. The temperature within the calorimeter falls quickly due to the absorption of heat by the frozen prepupa introduced. This rapid decrease in the temperature was determined at intervals of fifteen seconds during six to eight or nine minutes; thereafter the following linear and very slow change in the temperature of the calorimeter was recorded every thirty seconds for about three minutes or longer. To determine exactly the initial (t_1) and the final (t_2) temperatures in the calorimeter, a curve in Fig. 2 was drawn by plotting the temperature against the time. The initial temperature (t_1) could be read from the point on the curve corresponding to the instant at which the frozen insect was introduced into the calorimeter, being indicated by the arrow in Fig. 2. The apparent final temperature (t_2') could be determined from the value at the moment, when the temperature of the prepupa became equal to that of the calorimeter water. During the rapid decrease of the temperature in the calorimeter, the transfer of heat from and to the calorimeter simultaneously occurred according to the gradient of temperature between the calorimeter vessel and the water jacket, and consequently the apparent final temperature (t_2') must be corrected in order to find the exact value of the final temperature (t_2). This correction was made by the ordinary method on the basis of NEWTON's law of cooling.

After the amount of ice in the prepupa was calorimetrically measured, it was dried in the thermostat at about 105°C to estimate the contents of water (M_w) and the dry matter (M_d). Thus, all the unknown quantities in equation (1) were evaluated except the amount of ice (X) formed in the prepupa; accordingly the amount of ice could be computed by use of this equation.

To determine whether or not there is a parallel relationship between the periodicity of the quantity of ice formed in the prepupa and that of the freezing point of its blood, the freezing points were measured before and after the period of the ice determination extending over two weeks or more in the middle of every month from fall to spring. The method for determining the freezing point of blood was the same as that described in the previous papers[2,3]. The measurement of the freezing point was individually made with a thermocouple. Samples of about 0.2 cc. of the blood, which were taken from a small hole in the anterior body wall (having been pierced by a needle) by giving a light pressure on insect body with the finger tip, were individually placed in small test tubes. The tube was about 5 mm. in outer diameter and 30 mm. in height and was provided with a small aperture at the lower lateral side, through

which ice was inoculated into the blood to prevent excess supercooling. Owing to its surface tension the blood would not flow from the small aperture. The terminal portion of the thermocouple ran through the glass tube with a conical shaped end. When the tip of the thermojunction was inserted into the center of the blood in the tube, the conical end of the glass tube served as the stopper of the tube. Accordingly, the thermocouple was not only useful in the measurement of the temperature, but also served as the holder of the tube. The small test tube was then put into a larger protective tube, which was provided with a steel wire for the inoculation of ice, and the whole was placed in the freezing mixture at about −5°C. To the lower end of the steel wire, a U-shaped frame was soldered firmly at a right angle and was wrapped around with absorbent cotton soaked with water to be frozen. When this steel wire was pulled upwards, the ice on the cotton surface came in contact with the supercooled blood through the side aperture of the tube, resulting in the inoculation of ice. The protective tube was shaken by a motor from side to side in the cooling bath to roll the small lead particles in the blood, moving of which rendered the temperature in the blood homogeneous. The ice inoculation could be made during the shaking. As the blood began to cool immediately after the sampling, it was evident that its freezing point was scarcely affected by evaporation and by chemical changes perhaps caused by enzyme action.

III. Results

Freezing Point of the Blood

It is clear, on the basis of RAOULT's law, that the amount of ice formed in the solution at a given subfreezing temperature depends upon its molal concentration and accordingly upon its freezing point. Hence, there should be an intimate relationship between the amount of ice formed in the insect body and the freezing point of its blood. If periodicity is found in the freezing point of the blood of the prepupa, periodicity in the amount of ice formed in the prepupa would also occur. Freezing points of the blood of the prepupa and the pupa measured from January to May 1958 and from September 1958 to June 1959 are given in Table 1. In the fall, the freezing point of the blood was about −0.9°C, but, as the weather grew colder in November, the value dropped rapidly to −1.8°C. The freezing point decreased somewhat further in December, reaching the lowest value of −2.0° to −2.1°C from January to the beginning of March. In April the freezing point began to rise rapidly and in May attained to approximate −0.9°C; even after the metamorphosis to pupa the freezing point of the blood remained unchanged. The freezing points of the blood of the prepupa in fall and in spring were equal to each other; this observation has already been reported[2,3,12].

Water Content of Prepupa

The prepupa on which the amount of ice formed in the body had been measured,

Table 1. Freezing point of blood, and total water content of prepupa and pupa.

Freezing point of blood				Total water content			
Date	Stage	No. of exps.	0.95 confidence interval of mean of f.p.* °C	Month	Stage	No. of exps.	0.95 confidence interval of mean of water content %
1958				1958			
Jan. 20	Prepupa	3	−2.19±0.45	Feb.	Prepupa	27	61.4±0.8
27		7	−1.97±0.09	Mar.		38	63.1±1.1
28		4	−2.00±0.21	Apr.		24	63.3±0.6
Mar. 11		7	−1.88±0.11	May		21	66.1±1.3
Apr. 17		2	−1.02				
22		4	−0.69±0.05	May	Pupa	18	66.7±1.9
May 13		3	−0.76±0.23				
1958				1958			
Sep. 20	Prepupa	8	−0.89±0.06	Sep.	Prepupa	37	61.0±1.0
Oct. 2		5	−0.95±0.12				
10		6	−0.87±0.07	Oct.		48	60.8±0.7
29		7	−1.05±0.14				
Nov. 9		7	−1.08±0.12	Nov.		57	60.4±0.6
28, 29		11	−1.83±0.20				
Dec. 7		7	−1.94±0.10	Dec.		60	59.4±0.3
26		7	−1.93±0.18				
1959				1959			
Jan. 9		9	−1.97±0.17	Jan.		60	59.7±0.5
29		8	−2.00±0.14				
Feb. 6		8	−2.18±0.16	Feb.		53	59.5±0.4
23		10	−2.04±0.17				
Mar. 5, 6		10	−2.09±0.14	Mar.		57	59.7±0.6
21		8	−1.78±0.24				
Apr. 6		9	−1.55±0.24	Apr.		54	60.0±0.6
21		8	−1.05±0.11				
May 5		7	−0.89±0.04	May		44	59.4±0.5
20		7	−0.86±0.06				
29		6	−0.85±0.08				
Jun. 9		6	−0.84±0.09	Jun.		37	59.5±0.6
Jun. 11	Pupa	5	−0.81±0.05	Jun.	Pupa	39	60.5±0.8
24		4	−0.84±0.10				

* f. p. ··· Freezing point.

was put into a weighing bottle and dried overnight in a thermostat at about 105°C for the measurement of the water content. The seasonal variation of the water content was estimated as the average of the whole for the individuals used every month in the ice determinations. The change in the water content from fall to mid winter has been published in previous papers[2,3]; the high water content (80 %) of the larva on

spinning the cocoon decreased rapidly to a lower value (65%) and then gradually approached to the constant level (about 60%). Furthermore, no measurements of the water content from winter to spring were made hitherto. The results are given in Table 1. The values were almost constant (about 60%) throughout the overwintering period and even after pupating the values remained unchanged. From winter to spring in 1958, the water content of the prepupa appeared to increase, but the increase may be due to differences within the lot of material used; materials obtained from different sources and at different times, as mentioned above, may probably be heterogeneous in their physiological conditions.

Quantity of Ice Formed in Insect Body

The ice determination in the prepupa was first carried out preliminarily each month from February to May 1958 and then repeated precisely in next overwintering period, *i.e.* from September 1958 to June. 1959. The curve shown in Fig. 2 is an example of values obtained by such ice determination. The initial (t_1) and the final (t_2) temperatures can be estimated from the analysis of the curve according to the aforementioned

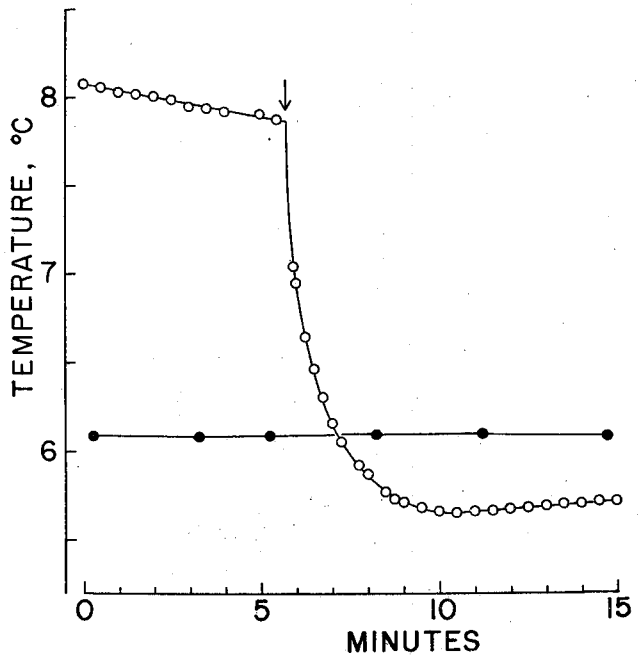


Fig. 2. Temperature-change in the calorimeter in determining the amount of ice formed in the frozen prepupa (Exp. 719; Jan. 20, 1959). Open circles represent the temperatures in the calorimeter vessel and solid circles those in the water jacket. The arrow shows the instant, at which the frozen insect was dropped into the calorimeter. Weight of the prepupa used is 506 mgm., and its water content 306 mgm. Freezing temperature t_e is -33.5°C, and the amount of ice was estimated to be 92.1 per cent of the total water content.

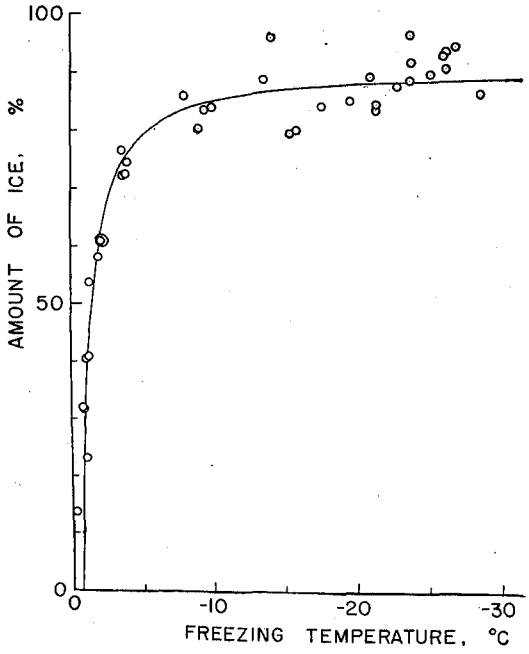


Fig. 3.

Relative amount of ice formed in the frozen prepupa in relation to the freezing temperatures. The curve was drawn from regression equation (4). The data were individually obtained with the prepupae soon after spinning the cocoon. Determinations were made in September 1958.

The explanation in Fig. 3 is applicable to Figs. 4 to 13.

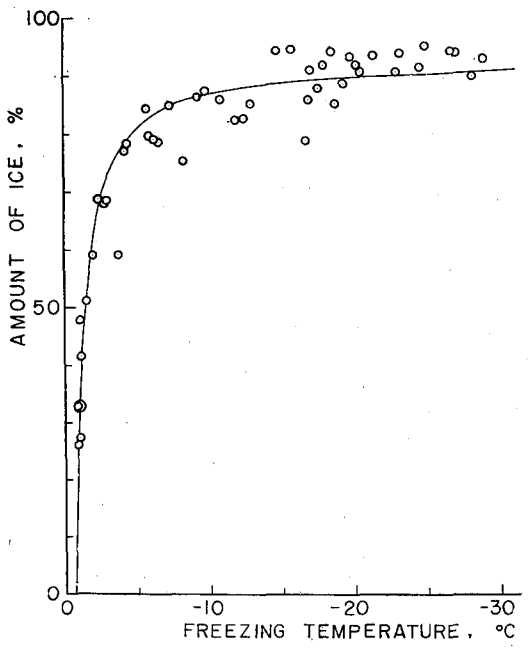


Fig. 4.

Relative amount of ice in the prepupa. Determinations were made in October 1958.

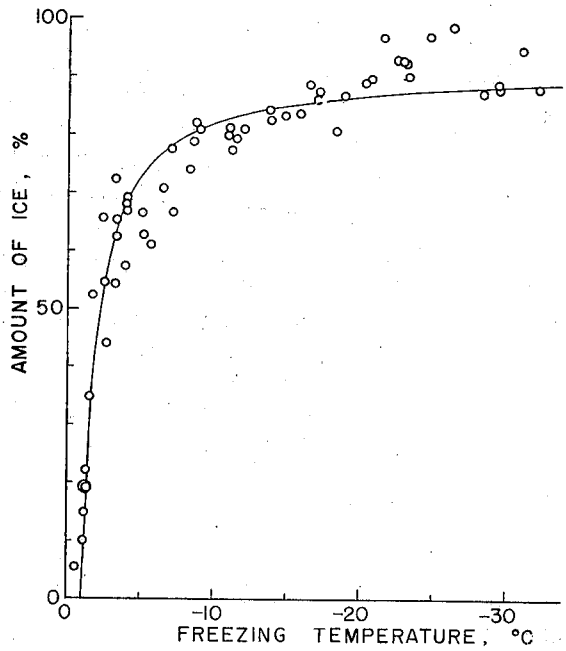
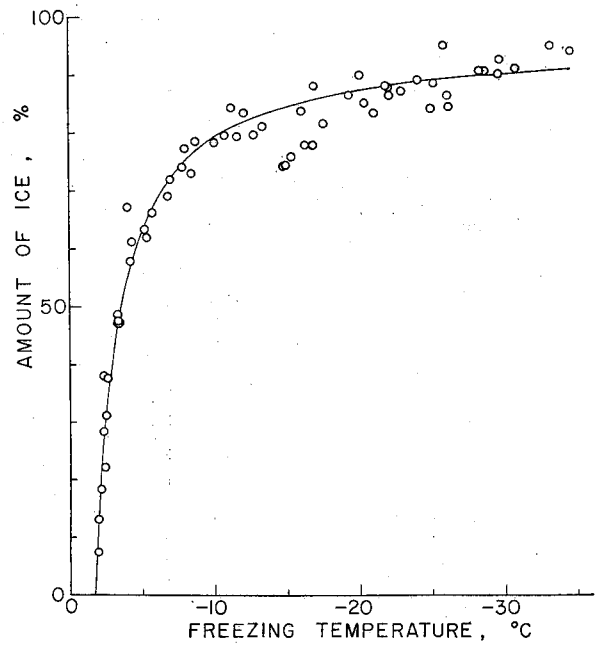


Fig. 5.

Relative amount of ice in the prepupa. Determinations were made in November 1958.

Fig. 6.

Relative amount of ice in the prepupa. Determinations were made in December 1958.



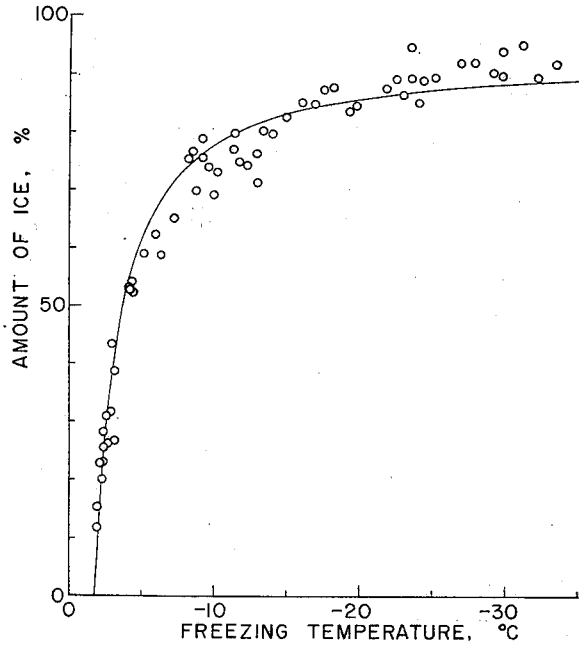
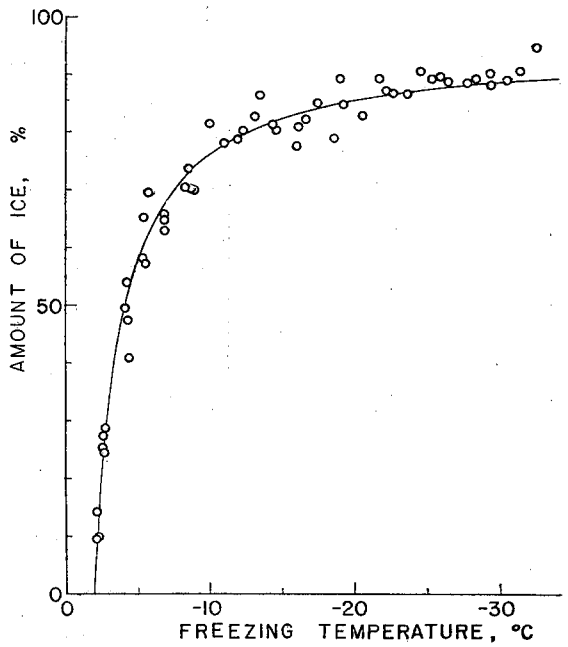


Fig. 7.

Relative amount of ice in the prepupa. Determinations were made in January 1959.

Fig. 8.

Relative amount of ice in the prepupa. Determinations were made in February 1959.



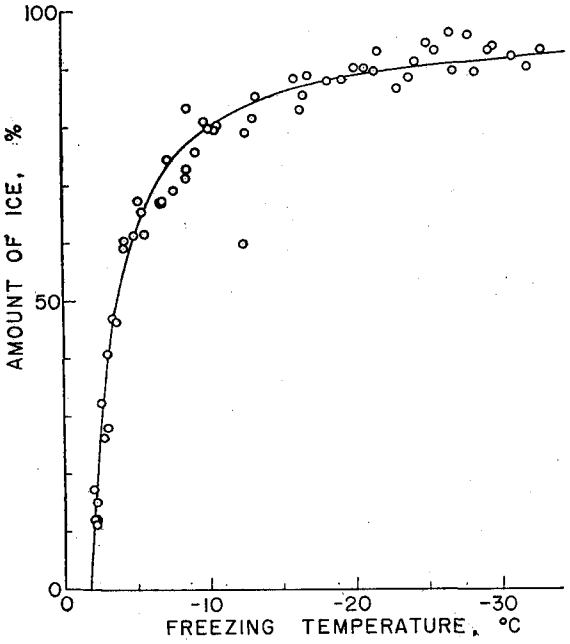
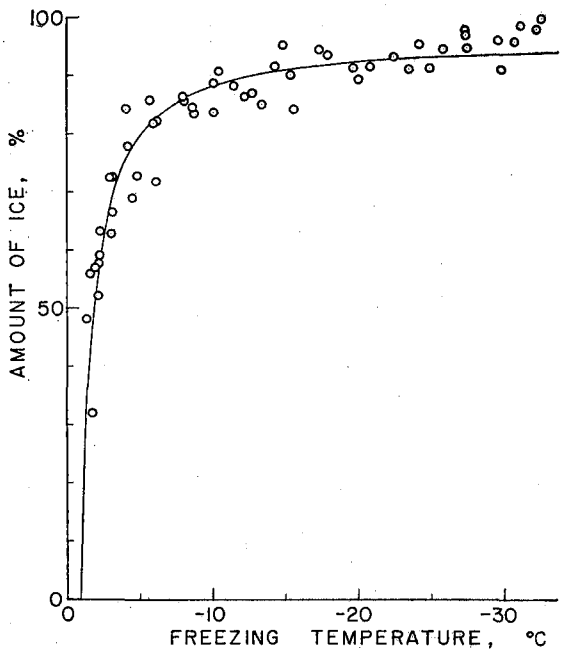


Fig. 9.

Relative amount of ice in the prepupa. Determinations were made in March 1959.

Fig. 10.

Relative amount of ice in the prepupa. Determinations were made in April 1959.



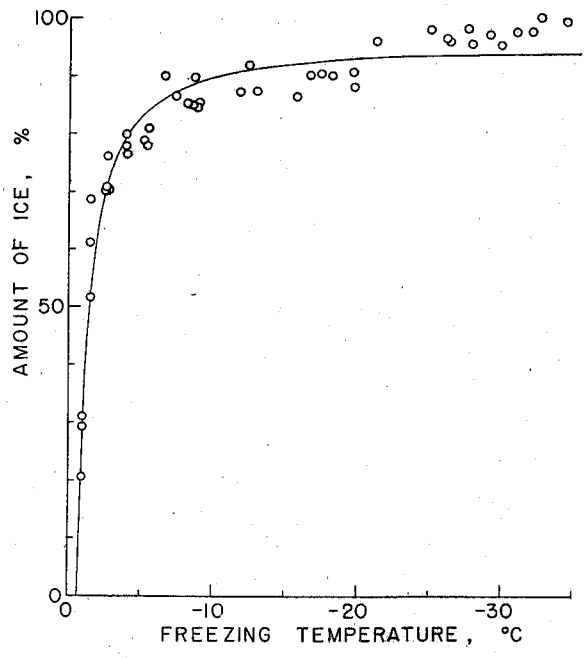


Fig. 11.

Relative amount of ice in the prepupa. Determinations were made in May 1959.

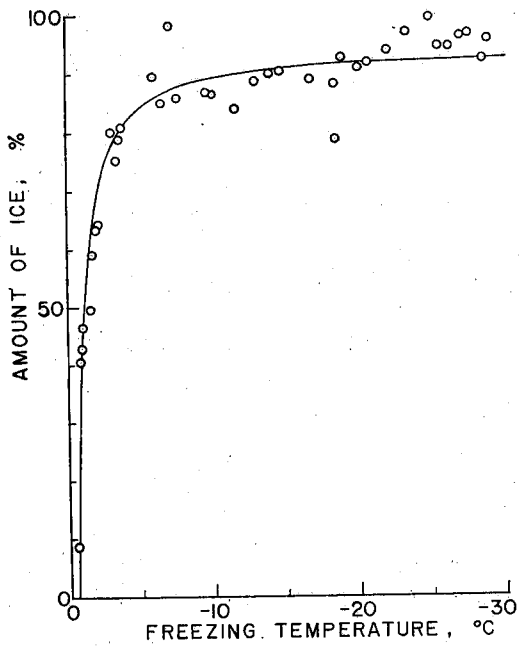


Fig. 12.

Relative amount of ice in the prepupa soon before pupating. Determinations were made in early June 1959.

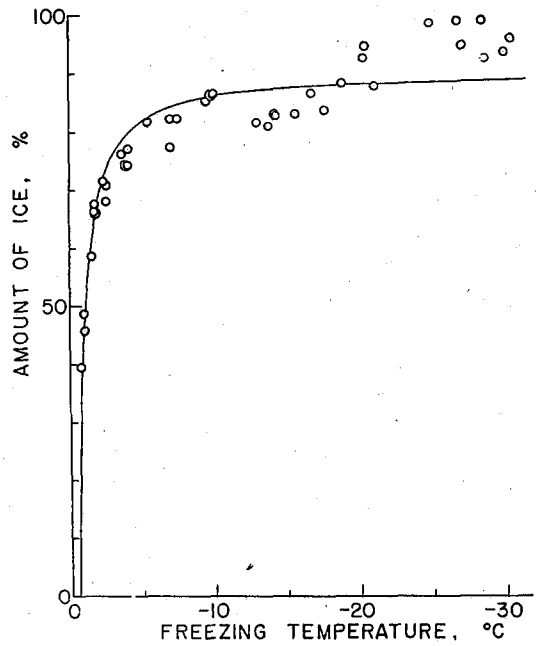


Fig. 13. Relative amount of ice in the pupa. Determinations were made in mid-June 1959.

method. Substituting these values and other necessary values into equation (1), one can evaluate the amount of ice formed in the prepupa. The amounts of ice formed in the frozen insects each month from September 1958 to June 1959 were plotted against the graded freezing temperatures, with which the frozen prepupae were equilibrated, in Figs. 3 to 13 respectively. The curves in these figures were drawn according to the empirical formula or the regression equation,

$$X' = a + \frac{b}{t} \qquad (4)$$

where X' = quantity of ice in per cent of the total body water of the insect,
t = freezing temperature (negative value),
a and b = constants.

This equation represents a hyperbola, and its theoretical basis will be considered later. The constants "a" and "b" can be estimated as follows: the graph obtained by plotting X' as ordinates against the reciprocals of the freezing temperatures, $1/t$, will be a straight line and the "b" could easily be found, for the slope of this line is equal to "b". The "a" could be evaluated as the intercept of the ordinate or X'-axis by this straight line (Fig. 14). In practice, the constants "a" and "b" of the equation expressing this straight line were estimated by the method of least squares. The values of "a" and "b" are given in Table 2. To examine whether there is a periodicity in the

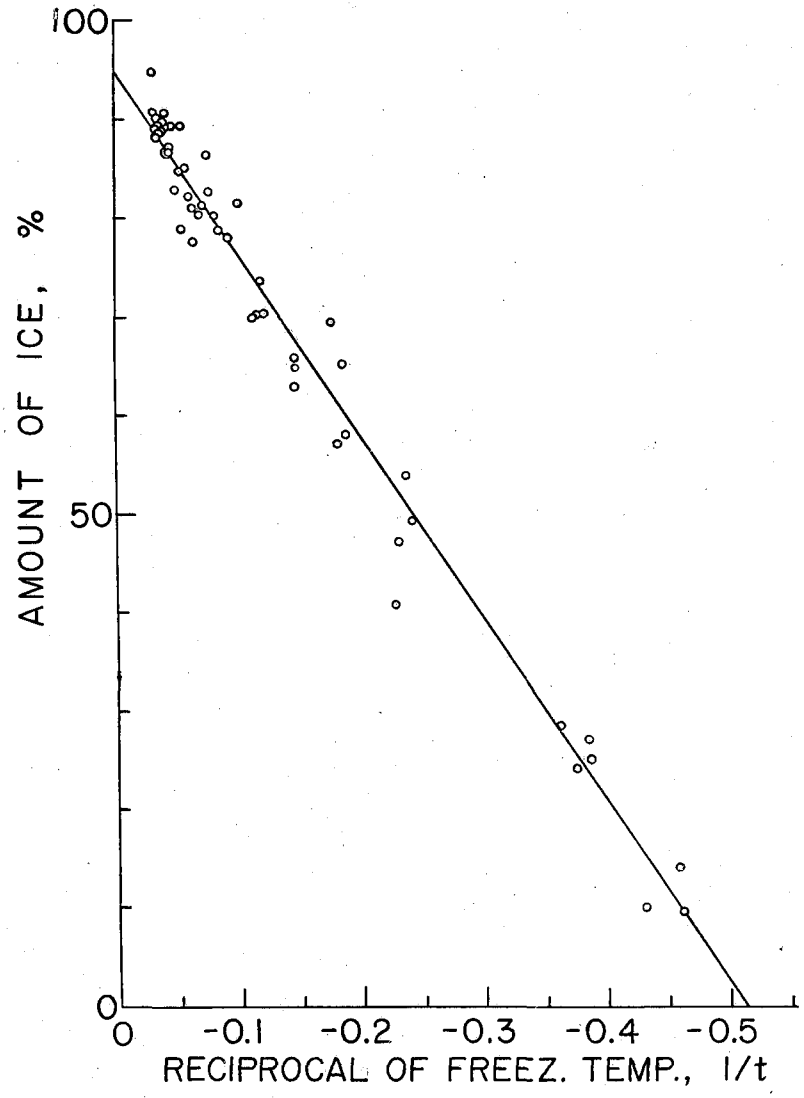


Fig. 14. Amounts of ice formed in the frozen prepupae are plotted as ordinates against the reciprocals of the freezing temperatures. Determinations were made in February 1959. For details, see text.

Table 2. The values of "a" and "b" in the regression equation, $X'=a+b/t$, and the values of the root of the variance from the regression.

Month	No. of exps.	a	b	$-b/a$ *	$\sqrt{V}$**
1958					
Feb.	32	90.4	140.1	− 1.55	3.45
Mar.	38	88.9	102.3	− 1.15	6.20
Apr.	24	93.7	138.5	− 1.47	6.31
May	21	89.1	43.4	− 0.49	5.52
May (Pupa)	18	90.9	39.4	− 0.43	4.27
1958					
Sep.	35	90.9	57.4	− 0.63	4.93
Oct.	43	92.9	59.5	− 0.64	3.35
Nov.	52	91.3	92.5	− 1.01	5.11
Dec.	53	95.2	160.0	− 1.68	2.88
1959					
Jan.	57	94.1	164.0	− 1.74	3.80
Feb.	51	94.9	185.3	− 1.95	2.96
Mar.	55	97.2	172.9	− 1.78	3.52
Apr.	49	96.9	85.5	− 0.88	3.52
May	42	96.5	66.7	− 0.69	3.60
Jun.	33	94.6	51.3	− 0.54	3.48
Jun. (Pupa)	39	90.7	43.1	− 0.48	4.94

* This ratio is equivalent to the freezing point of the blood, as will be indicated later in the discussion.

** V represents the variance from the regression and this will be discussed later in detail.

amount of ice formed in the insects at graded subfreezing temperatures or not, the curves represented by the regression equations are collated in Figs. 15, 16 and 17 respectively, dividing them into three groups from February to May 1958, from September 1958 to February 1959, and from February to June 1959. From these figures, it may be seen that the amounts of ice tend to decrease in fall as weather becomes colder and it reaches its minimum in severe cold season, in other words, the ice-amount-temperature curves tend to move right-down from autumn to winter, and all the situations are reversed from winter to spring. These curves have apparently a tendency to be divided into two groups of the mild seasons of autumn and spring and the cold season of winter. The data obtained from winter to spring in 1958 and

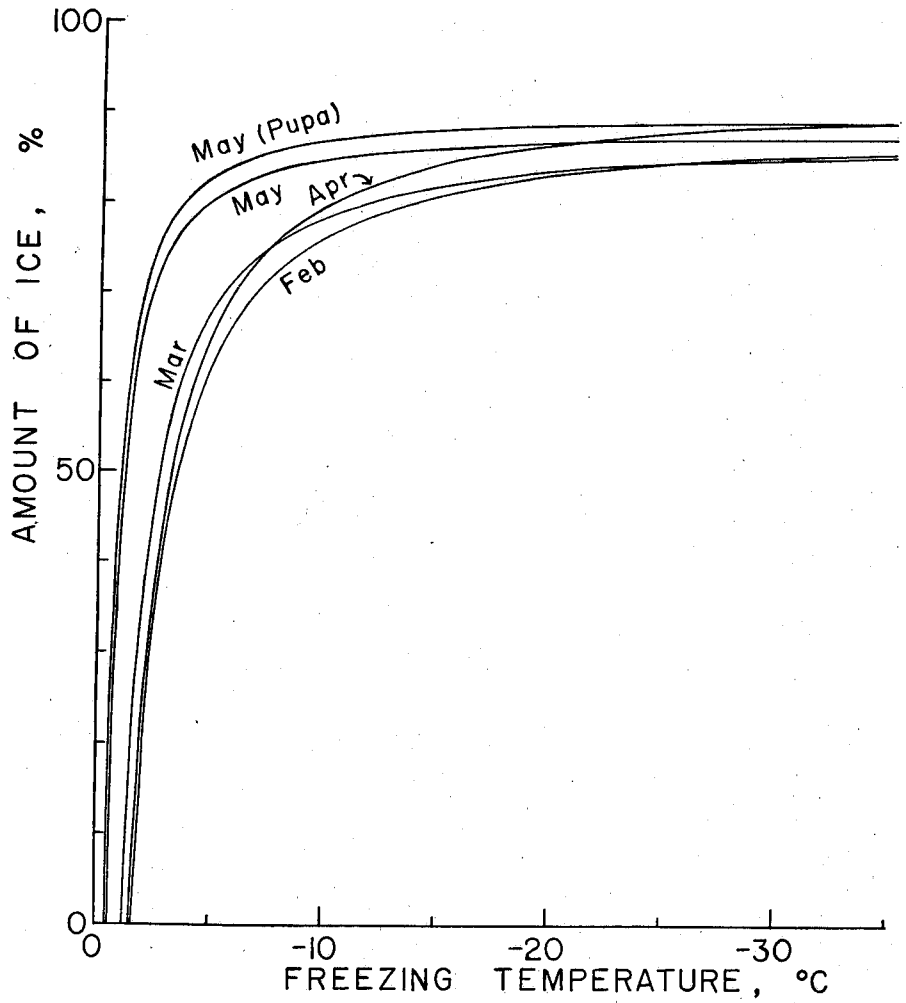


Fig. 15. Curves drawn from regression equation (4) from February to May 1958.

in 1959 agree fairly well with each other in respect to the tendency of the periodicity in the quantity of ice. There is no difference in the amount of ice between the prepupa and the pupa in the spring seasons of the two years. But it remains to be solved, whether the differences in the amount of ice month by month in Figs. 3 to 13 and 15 to 17 are statistically significant or not, for the experimental data of each month considerably deviate from the regression curve. The statistical analysis concerning this difference will be discussed later.

Amount of Ice Formed in Prepupa Injected with Glycerol

Change in the form of the freezing curve of the prepupa is caused by the injection

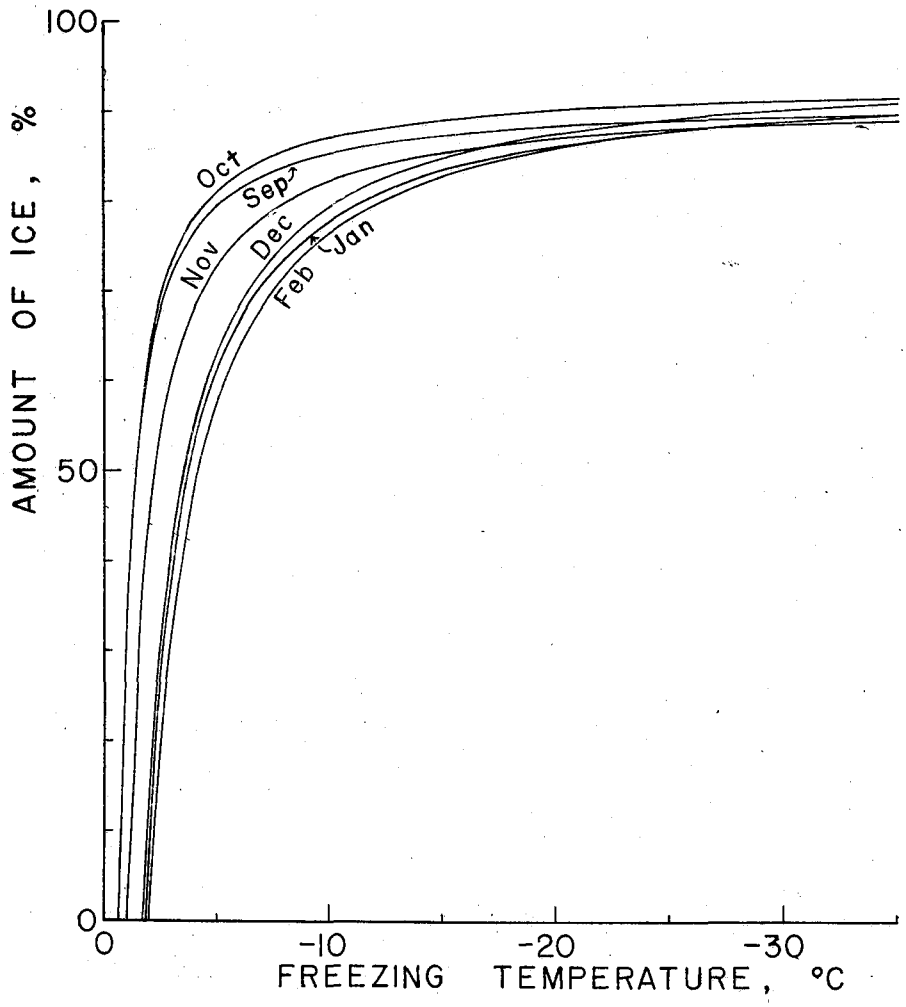


Fig. 16. Curves drawn from regression equation (4) from September 1958 to February 1959.

of glycerol into its body cavity[4]. The decrease in the growth rate of ice crystal in the blood and that in the amount of ice formed in it which were brought about by the injection of glycerol, may be pointed out as a cause of the change in the shape of the freezing curve. To ascertain the correctness of this view, ice determination in the prepupa injected with glycerol was made in March 1958. Since the freezing point of the blood begins to change in March as the weather becomes warm, the amount of ice may change according as the experiments are made in earlier or later days in March. To compensate for the difference in the amount of ice, which would result from such difference in dates of the experiments, ice determinations for prepupa injected

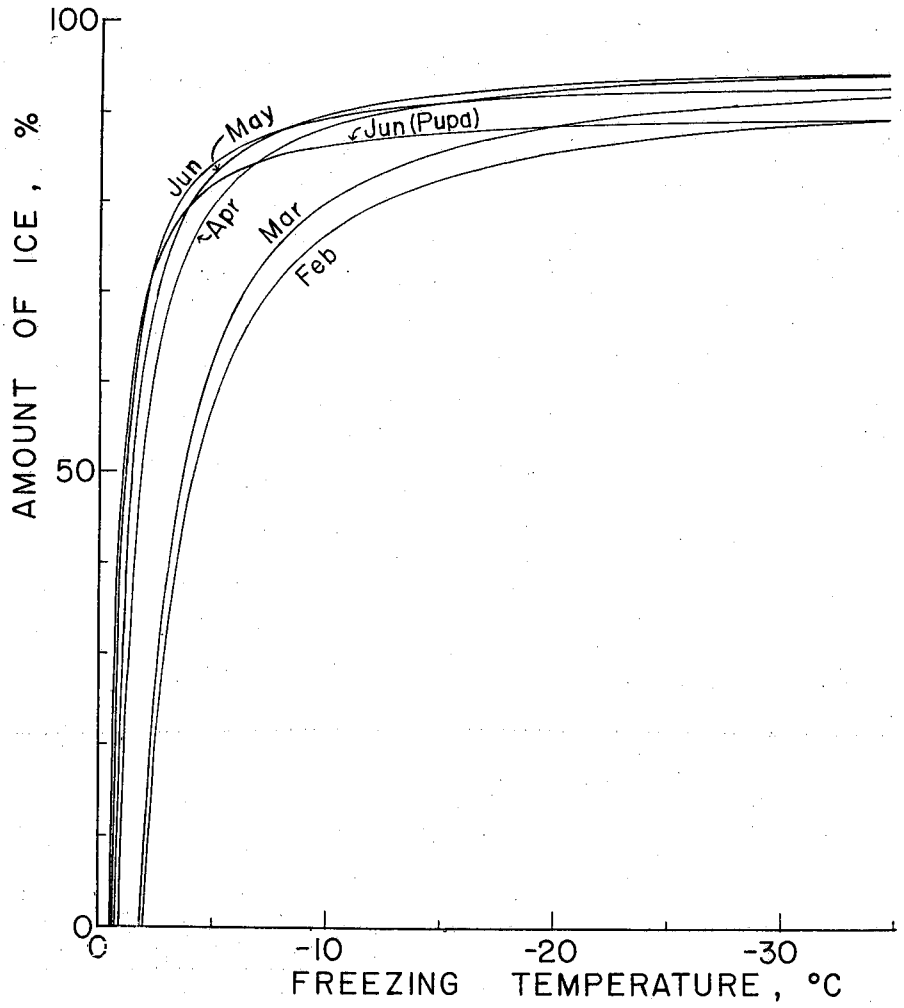


Fig. 17. Curves drawn from regression equation (4) from February to June 1959.

with glycerol and for normal prepupa were made on the same day or alternately every other day.

The results are shown in Fig. 18. The amount of ice formed in the prepupa injected with glycerol was clearly smaller than that in the normal. The expected values of the amount of ice, which can be estimated on the basis of the additional lowering of the freezing point of the haemolymph by the injection of glycerol, are also plotted in this figure. These values agree with the measured values within the limit of the deviation of the latter. In this figure, the amounts of ice formed in the prepupa in which the blood has been exchanged as completely as possible for 0.2 M

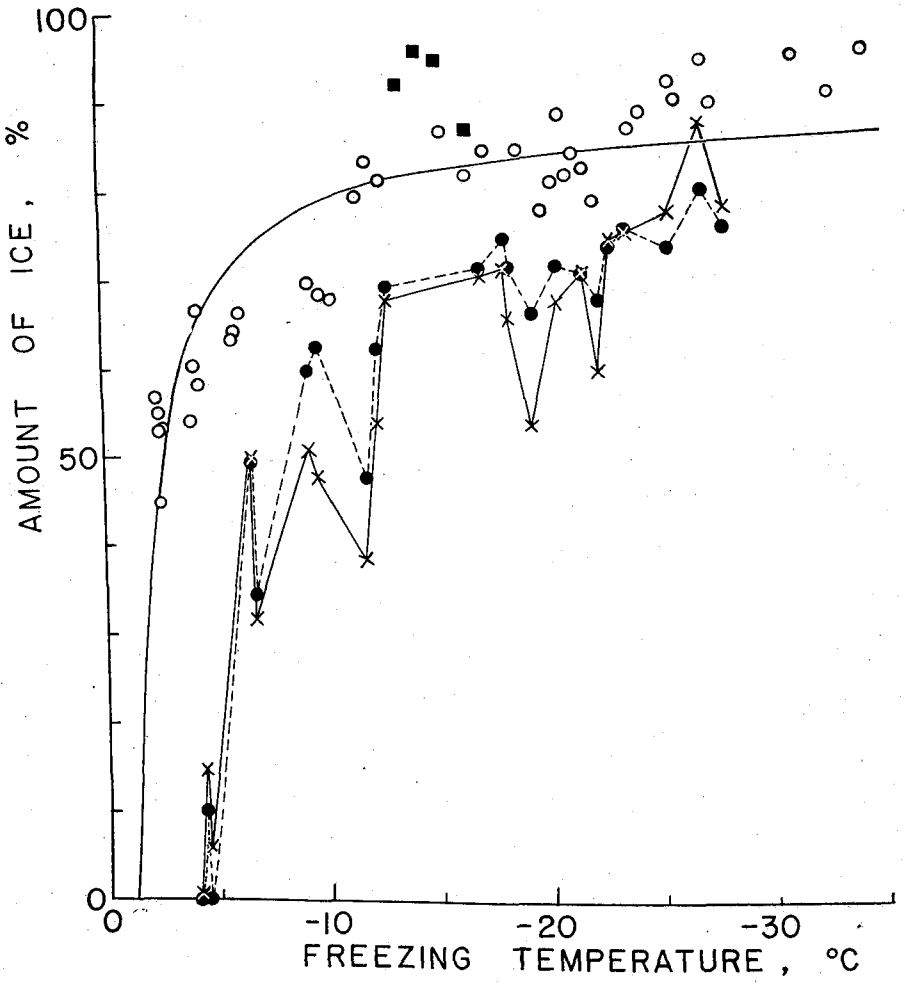


Fig. 18. Amounts of ice formed in the prepupa injected with glycerol (crosses), those in the normal prepupa (open circles), and those in the prepupa (squares) the blood of which has been exchanged with 0.2 M NaCl. The solid circles indicate the expected values of the amount of ice calculated on the basis of the increase in the concentration of the blood caused by the injection of glycerol. Determinations were made in March 1958.

NaCl (Δ=0.7°C), are also plotted. A tendency of increase in the amount of ice is found, although it can not be decided definitely owing to the small number of the determinations.

Congealment of Oil Extracted from Prepupa

If oil contained in the prepupa congeals in the freezing of its body, excess heat

is required to fuse the congealed oil at the time of warming of the frozen prepupa, and this may introduce error in the calorimetric ice determination. To know the behavior of the oil at low temperature, the following measurement was made.

Table 3. Congealment of the oil extracted from prepupa at low temperature.

Exp. no.	Amount of oil used in mgm.	Undercooling point °C	Rebound point °C	Extent of rebound °C
178	103	− 26.9	− 23.7	3.2
179	113	− 26.5	− 23.1	3.4
180	130	− 25.8	− 20.8	5.0
181	105	− 24.0	− 19.8	4.2
182	114	− 26.6	− 21.8	4.8
Mean*	113	− 26.0±1.2	− 21.8±1.6	4.1±0.65

* 0.95 confidence interval of mean.

Oil was extracted from 120 prepupae (38.25 gm.) by SOXHLET's extractor, using petroleum ether as solvent, but the oil content was not measured quantitatively. The oil extracted thus was yellow and transparent. The measurements of the undercooling and the rebound points of oil were made by the same procedure as that of the freezing point of the blood. The obtained data are given in Table 3. The undercooling point of the oil was somewhat lower than that of the intact prepupa in the overwintering period, being −20° to −24°C[2,3,5,34]. The extent of rebound in its congealment was not very large. The oil lost transparency and became cloudy when congealed, but after thawing, it became transparent again.

IV. Discussion

Experimental Methods for Ice Determination and Errors due to Them

The amount of ice formed in frozen insect has been measured by many authors, using several methods. Of these methods, the calorimetric method was employed in the present study for the reason mentioned briefly above. This method is based on the two facts that the heat capacity of the frozen animal is less than that of the unfrozen one, owing to the difference between specific heats of ice and of water, and that ice absorbs the latent heat on melting into water.

In this method, the amount of ice formed in the prepupa can be estimated from the quantity of heat required to warm it, involving the fusion of ice in it. If a solution frozen at a subzero temperature (t_e) is warmed to produce an ice-free solution at a temperature (t) above zero, the number of calories absorbed by the solution is constant quite independently of the intermediate path as was indicated above in the section on method. Hence, it is evident that the amount of ice in the prepupa can

be estimated from the quantity of heat absorbed by the frozen prepupa while it is warmed from t_e to 0°C without thawing of ice and then, after melting of ice in it at 0°C, its temperature is increased from 0°C to t. From such considerations, equation (1) was derived. But many of the authors who have used the calorimetric method for ice determination, adopted in the formula for estimating the quantity of ice, the freezing point of the body fluid as the temperature at which ice formed in the organism completely melts away[6,18,22,25,26,35]. This makes the methods used by them somewhat inconvenient as compared with the present one. THOENES[29] and ROBINSON[19] derived formula for ice determination, though relatively simple, on the same ground as the present study.

Criticism of the validity of the calorimetric method in the ice determination was made by SALT[23]. He made objections that this method involves a large element of uncertainty as to the thermal properties of the nonaqueous fraction, and that the use of the mean values for such values as specific heats of ice and water, which vary appreciably with temperature changes, is hard to think valid. Even though specific heat of the dry matter was found, it is hardly considered that the thermal properties of the nonaqueous fraction were made clear, because specific heat of the nonaqeous fraction may be changed by its possible interaction with water. However, the specific heat of the dry matter is relatively small and the fraction of the dry matter is also far smaller than that of water; therefore, the slight extent of incorrectness in specific heat may scarcely influence the results. In the present study specific heat of the dry matter in the prepupa of the slug moth was estimated as the mean of 15 measurements to be 0.32 ± 0.03 (cal./gm./deg.). The value of 0.49 was reported by DITMAN. *et al.*[6] as the specific heat of the dry matter of the prepupa and the pupa of the corn earworm (*Heliothis armigera*); the value of 0.3 was reported in the intertidal algae by KANWISHER[28]. The value of 0.43 could be estimated as that in the larva of the codling moth (*Carpocapsa pomonella*) by calculating from the specific heat of 0.75 for the intact body and the mean water content of 55.9 per cent given by SIEGLER[21]. The specific heat of 0.330 in dried muscle obtained by ROSENTHAL (1878) was cited by BĚLEHRÁDEK[36]. These specific heats are much smaller than that of water in every case. Using 0.32 as specific heat of the dry matter in the prepupa of the slug moth and 60.0 per cent* as the water content, specific heat of the whole body can be evaluated to be 0.73. A substantial agreement is found between the specific heat of the whole body calculated thus and that (0.72) measured directly on the intact prepupa[32]. This agreement indicates the correctness of specific heat of the dry matter. Since heat capacity of the intact prepupa can be considered as the sum of those of the dry matter and water in it, it may be recognized as valid that the terms having relation to the body water and to the dry matter are involved separately in equation (1). As was mentioned before in detail, specific heat of water above zero can accurately be

* This value is the mean of the water content of the prepupa from September 1958 to June 1959.

evaluated from the equation proposed by ROTH[20] and that of ice from the formula by DICKINSON and OSBORNE (cited from DORSEY[31]). As far as the present author is aware, there are no available values for specific heat of undercooled water. Such values can be obtained by the extension to below zero of ROTH's equation concerning specific heat of water for 0° to 40°C. The values so estimated are somewhat smaller than those of SAYRE[25] for water below zero evaluated by extending the curve for above zero to the range of 0° to −30°C as a straight line. The value (1.0097) at −5°C estimated from ROTH's equation was somewhat lower than the value (1.0155) given by BARNES for −5°C (cited from Smithonian Phys. Tab., 1933). A departure of values of specific heat obtained thus from the true value may be slight if any. It is also pointed out by SALT[23] that the calorimetric method is suitable for the ice determination of a large specimen, but does not seem to be sufficiently precise for an entomological work. A Dewar flask was hitherto used as the calorimeter. Since glass is not a good conductor of heat, a relatively long time is required for establishment of equilibrium among the temperatures of water in the calorimeter vessel, of the vessel itself and of the dropped frozen insect. If too much time is required for the measurement, the excess heat would be transferred from or into the calorimeter and thus the result of the ice determination of the insect would become probably less precise. Since the capacity of the Dewar flask hitherto used as the calorimeter vessel was relatively large and accordingly the amount of water in it was large, the ice determination had to be made with a set of a certain number of individual specimens, weighing usually 1 to 2 grams in total[6,18,21,22]. In the present study, a small copper vessel (2.6 cm. in diameter and 3.5 cm. in height) was employed as the calorimeter vessel, with about 15 cc. of distilled water put into it. Since the heat capacity of the calorimeter containing water is relatively small, a detectable change of temperature can be easily caused by introducing a frozen insect. The equilibrium of temperature in the calorimeter after the introduction of a frozen insect is rapidly reached, for copper is a good conductor of heat. Thus, the amount of ice in the prepupa was estimated individually and could be easily determined even in the prepupa of 200 mgm., using this calorimeter. The presently used calorimetric method, therefore, seems to be fairly precise for an entomological study.

As mentioned before, the materials used in this work were prepupae obtained at different times from several sources. Part of the deviation of the results may have been brought about from the heterogeneity in the materials used. As results obtained with the set of many individuals are the mean in a sense, the deviation of the data in this case must be relatively small. On the other hand, results obtained individually will indicate large fluctuation resultant from the differences of individuals. This may be a cause of the fluctuation of the data obtained in the present study.

As will be described later, the amount of ice crystallized in an insect body altered with the change of the freezing point of the haemolymph; consequently it was desirable that the monthly ice determinations, especially in November and in April when the

freezing point of blood varied, should be completed in a short period of time. It took, however, two weeks or more for the completion of one set of ice determinations each month, because ice determinations of only four specimens per day were possible on account of the requirement of much time to equilibrate the frozen insect with the surrounding freezing temperature. So it is apprehended that in November, when the freezing point lowers, a larger amount of ice may be formed in the prepupa at the beginning than at the end of the experimental period at a given freezing temperature; then, in April, when that freezing point rises, this relation is reversed.

The accuracy of the calorimetric method depends largely upon the accuracy of the temperature measurements. Error in temperature measured with the thermocouple may be produced by the fluctuation of reference temperature of ice-water mixture in which the reference junction of the thermocouple is immersed or by the local thermo-electromotive force between copper and brass caused by possible small temperature difference, *etc.* The calibration of the thermocouple was made with a standard mercury thermometer graduated to 1/10 degree in centigrade and the temperature was measurable to 1/100 degree in centigrade with the aid of a lens. Again, this may bring some error into the temperature measurement. Such error was detected to be within ± 0.03°C. The initial (t_1) and final (t_2) temperatures in the calorimeter were measured in a relatively short time, as was mentioned already. Then the errors of t_1 and t_2 arise to the same direction and are probably almost equal to each other, for it is supposed that, even if an error is introduced by certain causes, these causes may be unaltered in such a short time. Thence, the temperature difference between t_1 and t_2 may be precisely determined. In the ice determination, the correctness of this difference is more important than the correctness of the values of t_1 and t_2 themselves. The error of 1/100 degree centigrade in this difference results in an error of only about 1 per cent in the amount of ice. The error in measurement of the freezing temperature (t_e) of the prepupa will be considered later.

In the experiments, to equilibrate the temperature of the prepupa frozen previously at about -20°C with the freezing temperature (t_e) of the cooling tube, a relatively long time is required, especially when the freezing temperatures fairly differ from -20°C. One may consider that the equilibrium between the temperature of the prepupa and the freezing temperature (t_e) has been established, when the temperature of the frozen prepupa becomes constant. But since the equilibrium state is approached asymptotically at a very slow rate, the fact that its temperature has become constant does not mean the establishment of an exact equilibrium. In the present experiment, after its temperature became constant, the frozen prepupa was left in the freezing tube for some time, in order to establish the highest equilibrium state attainable. If the establishment of the equilibrium is not sufficient, the amount of ice formed in the prepupa tends to be estimated either somewhat greater at the temperature slightly below the freezing point of the blood or somewhat less at a fairly low temperature.

The fat content of the insect in the overwintering period is relatively high. Sup-

posing that the freezing of water and the congealment of oil occur simultaneously in the prepupa, on rewarming the frozen insect an additional heat must be consumed to melt the congealed oil, and the temperature fall in the calorimeter increases owing to this heat consumption. In such a case, the amount of ice formed in the prepupa will be estimated somewhat greater.

Of the insects whose fat content was measured by SACHAROV[20], the adult of *Scoliopteryx libatrix* contains the greatest quantity of fat, being 18.18 per cent of the live weight or 56.31 per cent of the dry weight. The larva of *Bracon cephi* has the fat content of 42 per cent of the dry matter in fall and that of 35 per cent in winter[13]. So the fat content in this species may be considered to be about 40 per cent of the dry matter in overwintering period. Although the fat body in the prepupa of the slug moth is well developed and a relatively large amount of fat is extracted with SOXHLET's extractor, the prepupa will hardly exceed the aforementioned species in fat content. If the value of 40 per cent of the dry matter is adopted as the fat content, its proportion to the live weight in the prepupa can be estimated as 16 per cent, because the water content is 60 per cent. The prepupa whose live weight is 0.35 gram, must contain 0.056 gram of oil. The heat of fusion of tristearin is 45.6 calories per gram (cited from Int. Crit. Tab., 1929). If the heat of fusion of oil contained in the prepupa is assumed to be equal to this value and the oil in the prepupa congeals completely on cooling at about −30°C, the congealed oil absorbs about 2.55 calories because of its melting on rewarming the frozen prepupa. About 0.032 gram of ice is melted by this amount of heat and this corresponds to about 15 per cent of the total body water. This will lead to the wrong conclusion that the amount of ice estimated here is 15 per cent greater than the amount of ice formed actually in the prepupa. This is not necessarily valid, for the discrepancy may be attributable to the value calculated on the assumed value of the fat content. However, if this were true, values greater than 100 per cent of the total body water would be frequently evaluated as the amount of ice formed at very low temperatures, but this was not the case. Besides, the lack of the congealment of oil may be also supported by the following facts. According to the writer's previous reports[2,4], it seems very likely that when a prepupa is subjected to freezing, ice forms at first in the blood but not in tissue cells and that the fat cells containing the oil are very easily dehydrated from outside with the other tissue cells as the freezing proceeds. Moreover, the undercooling point (−26°C) of the oil extracted is very low and the oil in the fat cells is in a form of minute globules. Under these conditions favourable to keep a stable supercooled state in the oil, it seems very probable that the oil in the insect body hardly congeals simultaneously with the body freezing. A low undercooling point (−25°C) of the oil was also found in the cold-hardy larva of *Synchroa punctata*[7]. In *Eurosta solidaginis*, the oil was not solidified even by the cooling to a temperature as low as −50°C, either *in situ* or after squeezing it out of the fat cells into haemolymph[37].

Amount of Ice Formed in Prepupa

The regression curves in Figs. 3 to 13 are drawn from equation (4), values for "a" and "b" of which are given in Table 2. The points in each figure fluctuate considerably from each regression line. A part of the causes of these fluctuations may be found in such facts, as the lack of the correct value available for the specific heat of undercooled water, the slow change of the freezing temperature (t_e) in the cooling tube, the effect of the room temperature on the frozen animal and on the calorimeter, the error in the measurement of the temperature, *etc.* But the main cause may be ascribed to the deviations of the freezing points of the blood of the prepupa month by month, as indicated in Table 1. This point will later be discussed in detail.

Equation (4), which is empirically obtained and represents a hyperbola, may be derived from theoretical grounds in the following way. Since the total sum of the mass of the various solutes contained in the prepupa is constant regardless of the quantity of solvent water therein, the concentration of the body fluid should be inversely proportional to the quantity of the solvent water. According to RAOULT's law, the freezing point of the body fluid is proportional to its concentration. Thus, one obtains:

$$t_f = \frac{k}{M_w - u} \tag{5}$$

where t_f = freezing point of body fluid,
M_w = total mass of water in a prepupa,
u = mass of unfreezable water,
k = constant,

and

$$t = \frac{k}{M_w - u - X} \tag{6}$$

where t = freezing temperature,
X = mass of ice formed in a prepupa at a temperature t.

Combining (5) and (6), and rearranging, one gets

$$\frac{X}{M_w} = \frac{M_w - u}{M_w}\left(1 - \frac{t_f}{t}\right), \tag{7}$$

or

$$X' = 100\frac{X}{M_w} = 100\left(1 - \frac{u}{M_w}\right)\left(1 - \frac{t_f}{t}\right), \tag{7'}$$

where X' = amount of ice formed within prepupa in per cent of total water.

Comparing equation (4) with (7'), the following relations can be found

$$a = 100\left(1 - \frac{u}{M_w}\right) \tag{8}$$

and

$$b = -100\left(1 - \frac{u}{M_w}\right) t_f . \qquad (9)$$

Consequently, the constant "a" in equation (4) corresponds with the per cent of total freezable water relative to the total body water contained in the prepupa, and "b" is equal to the negative value of the product of "a" by t_f, *i.e.* $t_f = -b/a$. Unfreezable water was introduced, in order to make the theoretical equation coincident formally with equation (4); it will be discussed later in detail. A similar relationship to that given by equation (4), between the amount of ice formed in the insect and its freezing temperature, was found in a chironomid larva by SCHOLANDER *et al.*[24] The amount of ice formed in the chironomid larva increases in a hyperbolic fashion with decreasing temperatures, *i.e.*, if the ratio of the dry matter to the amount of unfrozen water could be regarded as equivalent to the concentration, the relation between this ratio and the graded temperatures below freezing point is linear. At comparatively higher concentrations, the freezing point depression tends to depart from RAOULT's law. In other words, the extent of the freezing point depression at high concentrations becomes somewhat larger than the value expected from the linear relationship between the two. This is a fact in regard to the solutions of various crystalloids and colloids. Although such departure from RAOULT's law may also be found in frozen insects, it may perhaps be masked by the large extent of the fluctuation of data from the regression line. The data on insects presented by DITMAN *et al.*[22], on lichen by SCHOLANDER *et al.*[24], and on intertidal molluscs and algae by KANWISHER[27,28] may also be described by equation (4), as is inferred from the shape of the curves presented by them. The dehydration-melting point method for ice determination developed by SALT[23] is also principally based upon RAOULT's law; further SALT's curve expressing the relation of the melting point of the blood from *Loxostege sticticalis* larva to the water-loss, which is equivalent to the amount of water frozen in the blood, shows similarity to the graph of equation (4)[23,38]. All the results published by these authors show that 50 per cent or more of water contained in the organism freezes at a few degrees below the freezing point of their body fluids, 80 per cent or more water at about −10°C, and that as the temperature falls further below −10°C, the amount of ice increases gradually, but several per cent of water remains unfrozen even at −30°C. Similar relationship was also found in gelatin gels by MORAN[39]. It is clear from the present study and the works cited here that the amount of ice formed in the living matter is a function of the subfreezing temperatures. DITMAN *et al.*[22], however, interpreted differently similar results of their own. They made the ice determinations with samples of several individuals, and measured the amount of ice formed in insects, taking precautions not to break, as far as possible, the undercooling state of the insects. These points differ greatly from the experimental procedures used in the present study. From the results measured in the several species of insects, DITMAN *et al.*[22] pointed out that three temperature ranges could be distinguished according to the amount of ice formed in the insects as follows: above a certain temperature A, no water in the insects is frozen;

in the temperature range below B, a maximum amount of water is frozen and the percentage of ice to body water is almost constant; and in the temperature range between A and B, which corresponds to the undercooling range of each species of insects, the various amounts of water in the insects are frozen. They explained these phenomena as follows. If the insect is once frozen at any temperature, it is always frozen to the maximum extent. The variations of the amount of ice formed in the samples at any temperature in the range from A to B appear largely dependent upon the fact that some individuals were frozen to maximum extent, while others had no water at all frozen in them. In the temperatures below B, the maximum extent of freezing occurs in all individuals and accordingly the uniformity in the amount of ice can be found as natural consequence in this temperature range. It seems possible that some individuals are frozen and others are not in the temperature range from A to B, as is judged from their experimental procedures. Generally speaking, the water in the insect could be regarded as being frozen to nearly maximum extent in relatively low temperature range. However, judging from the fact that the amount of ice increases in a hyperbolic fashion as the temperature falls, the opinion of DITMAN *et al.*[22], that the quantity of ice in a frozen insect is produced to maximum extent regardless of the freezing temperature, is hardly acceptable. This opinion can also be disapproved from the results, which were obtained in individual determinations of quantity of ice in the prepupa of the slug moth and in the chironomid larva. It can be indicated from a simple calculation based on RAOULT's law that in the insects, the blood of which has the freezing point of *ca.* −1°C, about 90 per cent of body water freezes at −10°C, 93 per cent at −15°C, and 95 per cent at −20°C, assuming that no unfreezable water is contained. The opinion of DITMAN *et al.* may have been influenced by this apparent constancy in the amount of ice below −10°C corresponding approximately to the temperature, A. Thus, taking into consideration the fact mentioned above, one can interpret all the results presented by DITMAN *et al.*[22] on the principle that the amount of ice formed in an insect is a function of the temperature.

If the constants "a" and "b" in equation (4) are determined by experiments, the amount of ice formed in a frozen insect can easily be estimated by equation (4), when only the freezing temperature of the insect is measured. When the ratio, $-b/a$, which is equivalent to the freezing point (t_f) of the blood (Table 2), is compared with the measured freezing point shown in Table 1, the former is found to be higher than the latter in each month without exception. This may be interpreted as follows. When the prepupa which has been frozen previously at −20°C or below is brought to a freezing temperature slightly below the freezing point of the blood, it may take a relatively long period of time to establish an equilibrium between ice and liquid phases in the insect body, as the heat of fusion of ice is large. Thence, although the frozen material remains for a fairly long time at temperatures near the freezing point of the blood, it may be possible that the amount of ice is estimated somewhat

larger than that in the equilibrium, unless the period of time is sufficient to allow attainment of true equilibrium. This may appear to cause the corresponding elevation in the freezing point. This is the reason why the ratio, $-b/a$, is always larger than the value of the freezing point of the blood measured actually. However, the ratios $-b/a$ computed from the data obtained in the period of September 1958 to June 1959 do not depart very remarkably from the respective values of the freezing points measured monthly, and the seasonal variations of both are completety parallel, whereas the ratios from February to May 1958 depart to some extent from the measured freezing points. This may be due to the deficiency of data on the quantity of ice at the temperatures near the freezing point of the blood in the latter case.

The relative error in the estimation of amount of ice resulting from the error (dt) in the freezing temperature is given by

$$\frac{dX'}{X'} = \frac{t}{t(t-t_f)}\, dt\,, \qquad (10)$$

which is derived by differentiating the logarithm of equation (7'). It is clear from this equation, that the nearer the freezing temperature (t) is to the freezing point (t_f), the greater becomes the relative error in the amount of ice, assuming that the error, dt, is almost constant within the range of the freezing temperatures. If the freezing temperature falls several degrees below the freezing point, the slight error in the freezing temperature is negligible for the preciseness of the results*.

Participation of Water Drawn from Tissue Cells in Freezing of Blood

In spite of the fact that the cells are always killed by intracellular freezing**, the prepupa of the slug moth survives body freezing at a low temperature of −20°C or below[1,2,5,12,33,40,41]. When the tissues and the organs which are excised from the body and immersed in its blood are exposed to a temperature as low as −20°C, the freezing occurs only in the blood and not in the interior of the cells[2,12]. When a part of the blood is squeezed from the body or exchanged with an equivalent amount of isotonic NaCl solution, or when glycerol is injected into the body cavity, such changes of the amount or of the constitution of the blood in the prepupa are always reflected in change in the shape of the freezing curves[4]. The extracellular formation of ice in some frozen intertidal animals was histologically demonstrated by KANWISHER[42]. That amoeba and the muscle fibers of frog were also frozen extracellularly without

* Adopting −2.0°C as t_f and 0.1° as the absolute value of the error of the freezing temperature (dt), the following table can be constructed:

Freez. Temp. (t) °C	− 2.1	− 2.5	− 3.0	− 5.0	− 10.0
dX'/X'	0.95	0.16	0.07	0.01	0.002

** It was recently reported by SALT[37] that the fat cells of cold-hardy *Eurosta* larvae could survive intracellular freezing, but further study seems to be required to ascertain whether or not this was really the case.

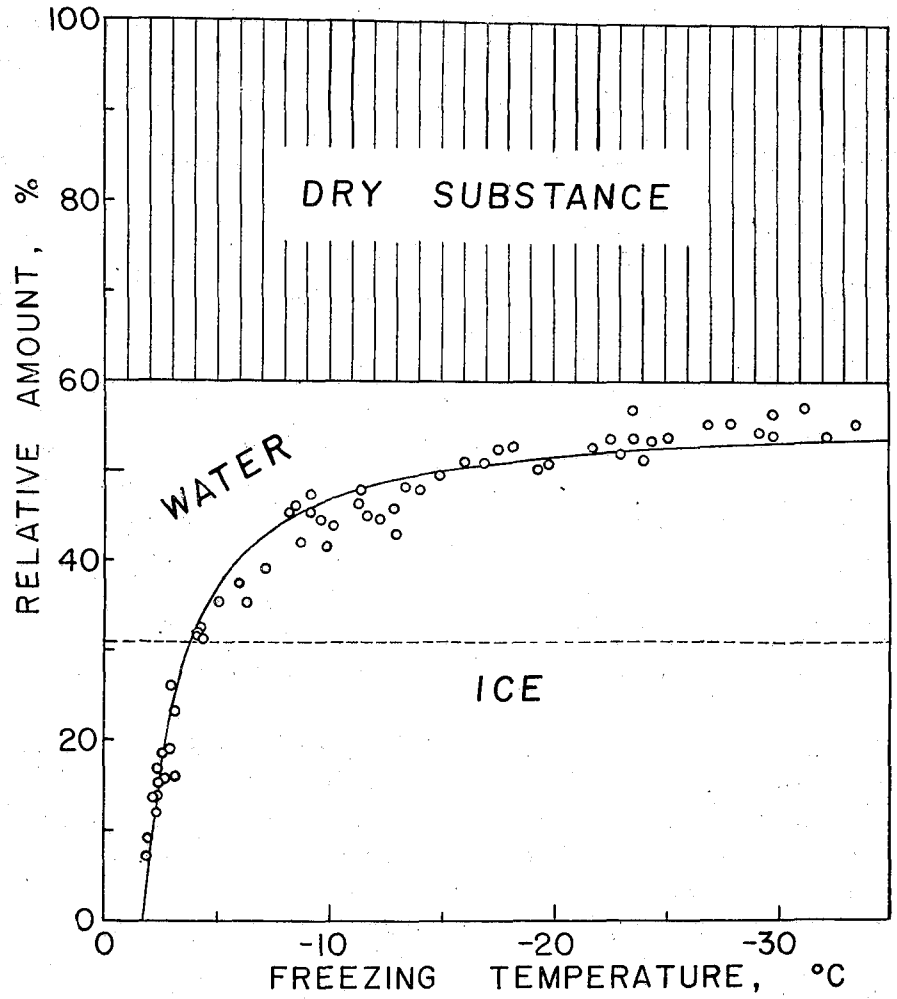


Fig. 19. Relationship among the relative amounts of the dry substance, of unfrozen water, and of ice in the prepupa at graded subzero temperatures. The horizontal broken line at 30.8 per cent indicates the relative amount of water contained originally in the blood. This value alone was measured on January 28, 1961. Data except the water content in the blood were obtained in January, 1959. This figure is the same as Fig. 7, excluding the use of percentage of the total body weight for the total water content.

ill effects, was found by CHAMBERS and HALE[43]. On relatively slow cooling, the egg cell of a sea-urchin freezes also extracellularly in sea water, with shrinking in its volume; after thawing it shows normal fertilizability[44]. Frost-hardy plant cells resist ice-penetration into the cell interior, unless they are cooled very rapidly[45]. KISTLER[46] pointed out, as to the process operating in extracellular freezing, that when ice grows, it dehydrates the colloidal solution in its immediate vicinity and becomes coated with a protective layer of dehydrated colloid, which prevents the cell surface from coming into direct contact with ice. On the basis of these facts, a probable freezing process in the prepupa of the slug moth is as follows. At the first place the ice formation occurs always in the blood. As the extracellular ice mass grows, the blood is concentrated and accordingly more and more water is drawn from the tissue cells into the blood by osmosis. Thus the water drawn from the cell interior may also participate in extracellular freezing. It may be considered that this is highly possible, according to the quantitative relationship among the amount of water existing originally in the blood, that of total body water, and that of ice formed in the body. The relation among the relative amounts of the solid, of ice formed, and of unfrozen water in the prepupa at graded subfreezing temperatures is shown in Fig. 19, which is drawn from the data obtained in January 1959 as an example. In this figure, the amount of water originally contained in the blood is also indicated. From the difference in the body weight before and after as much as possible blood was pressed out, the quantity of blood was estimated to be 40.8 ± 2.3 per cent of the total body weight. At the same time the water content of the blood was 75.5 ± 0.8 per cent. Accordingly, the amount of water contained originally in blood can be evaluated to be 30.8 per cent of body weight*. It is evidently recognized from this figure that, as the freezing temperature lowers below about -4°C, more water than that contained originally in blood freezes, indicating the participation of water drawn from cells into extracellular freezing. Speaking more strictly, if the body freezing is initiated even at a higher freezing temperature than -4°C, blood is concentrated by separation of ice from it and water extracted by osmosis from cells is also frozen extracellularly; at -4°C the amount of ice formed in the body becomes equivalent to that of water contained originally in the blood. Similar relations are found in other months than January as well.

Statistical Analysis of Experimental Data

As was mentioned already, though the amount of ice formed in the prepupa can be described by the regression equation (4), the plots of data deviate considerably from

* To check the validity of the amount of water in the blood obtained here, the total water content,—which was calculated from the water content in the blood, the amount of blood, the water content of body tissues excluding the blood (51.2 ± 2.2%), and the amount of body tissues (59.2 ± 2.3%),—was compared with the total water content measured actually. Both the calculated and measured values agreed fairly well with each other, indicating the former to be 61.1 ± 1.7 per cent and the latter 62.0 ± 1.4, and no significant difference between both was found by the statistical test. All the values of these amounts in the text and in the foot-notes indicate 0.95 confidence interval of the means.

the regression lines each month illustrated in Figs. 3 to 13. Consequently, the method of least squares was applied to the evaluation of the constants "a" and "b" in these equations. The values of "a" and "b" are given in Table 2. The root of the variance (V) from the regression is also shown in this table. If the variance of an estimated X' be denoted by $V(X')$, the relation follows, according to the statistical law,

$$V(X') = V\left\{1+\frac{1}{N}+\frac{\left(\frac{1}{t}-\frac{\bar{1}}{t}\right)^2}{\sum\left(\frac{1}{t}-\frac{\bar{1}}{t}\right)^2}\right\}, \tag{11}$$

where N = number of measurements,

$\frac{\bar{1}}{t}$ = monthly mean of $1/t$.

If N is considerably large as in the present study, one gets

$$V(X') = V. \tag{12}$$

Consequently, 0.95 confidence limit of the regression of X' on $1/t$ is approximately expressed by

$$X' = a+\frac{b}{t} \pm 2\sqrt{V}, \tag{13}$$

assuming that N is at least larger than 30, because t (in t-distribution) is approximately equal to 2.0 in the range for $N \geqq 30$ at $\alpha = 0.05$.

The ice-amount-temperature curves of three groups, illustrated in Figs. 15 to 17 respectively, show apparently that the amount of ice formed in the insect body at the same freezing temperature decreases step by step from fall to winter and all conditions are reversed from winter to spring. As has been mentioned previously, since the fluctuation of the data from these curves is fairly great, a statistical examination of the data is necessary in order to determine whether the prepupae show certainly a definite periodicity in the amount of ice formed in their bodies. Thus a test of significance of the difference among the monthly means of the amount of ice was made according to the method of SNEDECOR[47]. It is nonsense that the means in every month, which are calculated by dividing simply the total of the amount of ice by the number of measurements, should be directly compared with each other, because, even if there is difference among these means, this difference may be brought about by that among the means of the reciprocals of the freezing temperatures in every month. Thus, the significance of the difference should be tested on the basis of the adjusted means of the amount of ice evaluated, after allowance is made for the difference of the reciprocals of the freezing temperatures. The test of significance of the difference among the adjusted monthly means of the amount of ice is illustrated in Table 4. Since calculated F is highly significant, it may safely be concluded that the difference among the monthly means of the amount of ice cannot be explained by the differences among the means of the reciprocals of the freezing temperatures: after these means

Table 4. Analysis of covariance and test of significance of differences among adjusted monthly (lot) means.

Source of variation	Degrees of freedom	Sums of squares and products			Error of estimate		
		$\sum\left(\frac{1}{t_d}\right)^2$ (1)	$\sum\frac{x}{t_d}$	$\sum x^2$ (2)	Sum of squares (3)	Degrees of freedom	Mean square
Total	508	26.1944	1,954.9991	228,322.81	82,412.94	507	
Lots (Months)	10	0.9156	− 55.8621	15,515.09			
Within lots (Error)	498	25.2788	2,010.8612	212,807.72	52,849.07	497	106.34
For test of significance of adjusted means					29,563.87	10	2,956.39**

(1) $\frac{1}{t_d}=\frac{1}{t}-\frac{\bar{1}}{t}$, where $\frac{\bar{1}}{t}$ is the mean of $1/t$.

(2) $x=X'-\bar{X}'$, where $\bar{X}'$ is the mean of X'.

(3) $\sum x^2-\frac{\left(\sum\frac{x}{t_d}\right)^2}{\sum\left(\frac{1}{t_d}\right)^2}$

$F=2{,}956.39/106.34=27.80$, Degrees of freedom$\cdots n_1=10,\ n_2=497$.

** $\alpha=0.01$.

are adjusted to the basis of a common reciprocal of the freezing temperature, the means still differ significantly. This difference, therefore, may be explained by the seasonal variations of such a physiological property of the insect as the freezing point of the blood during the overwintering period; it will be shown later that this is the case.

The adjusted monthly means can be evaluated from

$$\bar{X}'-b\frac{1}{t_d}\ , \tag{14}$$

where $\bar{X}'$ = monthly mean of amount of ice,

$\frac{1}{t_d}$ = deviation of $\frac{\bar{1}}{t}$ (monthly mean of $1/t$) from $E(1/t)$ $(=-92.849/509=1/-5.50=-0.182)$, which shows the mean of $1/t$ in all of the experiments of the whole period or the common reciprocal of the freezing temperatures,

b = error regression coefficient.

The value of "b" can be computed from the figures given in Table 4,

$$b=\frac{\sum\frac{x}{t_d}}{\sum\left(\frac{1}{t_d}\right)^2}=\frac{2010.86}{25.28}=79.55\ . \tag{15}$$

The monthly means of the amount of ice ($\bar{X}'$) and their adjusted means are plotted

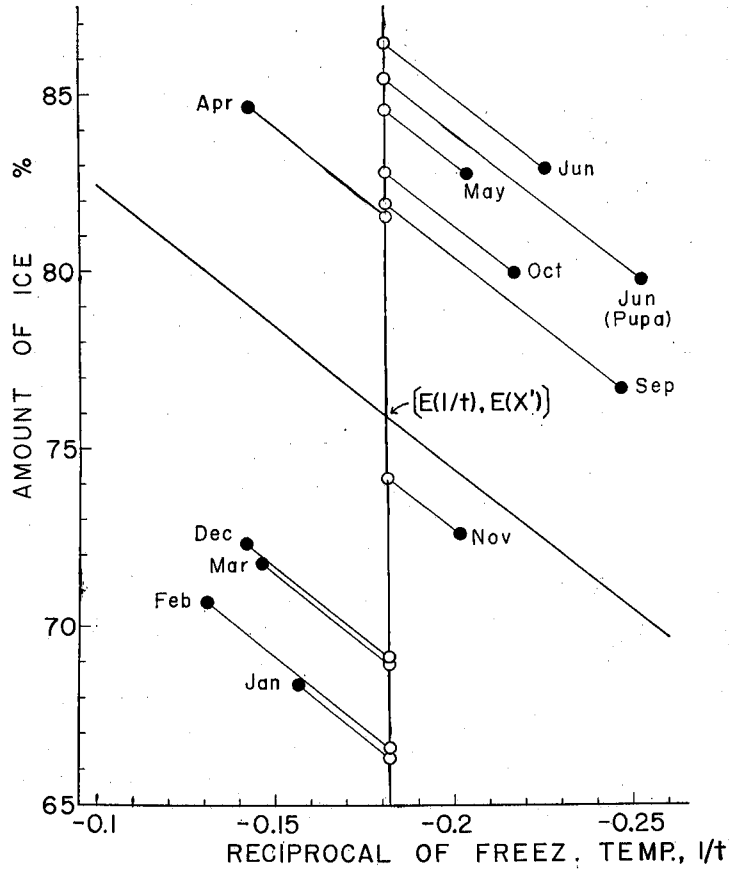


Fig. 20. Average regression of the amount of ice against reciprocals of freezing temperatures in ten months or eleven lots*. The solid circles represent the lot means and the open circles thier adjusted means.

* Though the ice determinations were made over ten months, in June the ice determinations in two lots of the prepupa and the pupa were made and consequently the number of the lots is more by one than that of the months.

in Fig. 20 with the straight line representing the regression equation within lots (months). This equation is

$$\begin{aligned}\bar{X}' &= E(X')+b\left[\frac{\bar{1}}{t}-E\left(\frac{1}{t}\right)\right]\\ &= 75.94+79.55\left(\frac{\bar{1}}{t}+0.182\right)\\ &= 90.42+79.55\frac{\bar{1}}{t}\ ,\end{aligned} \tag{16}$$

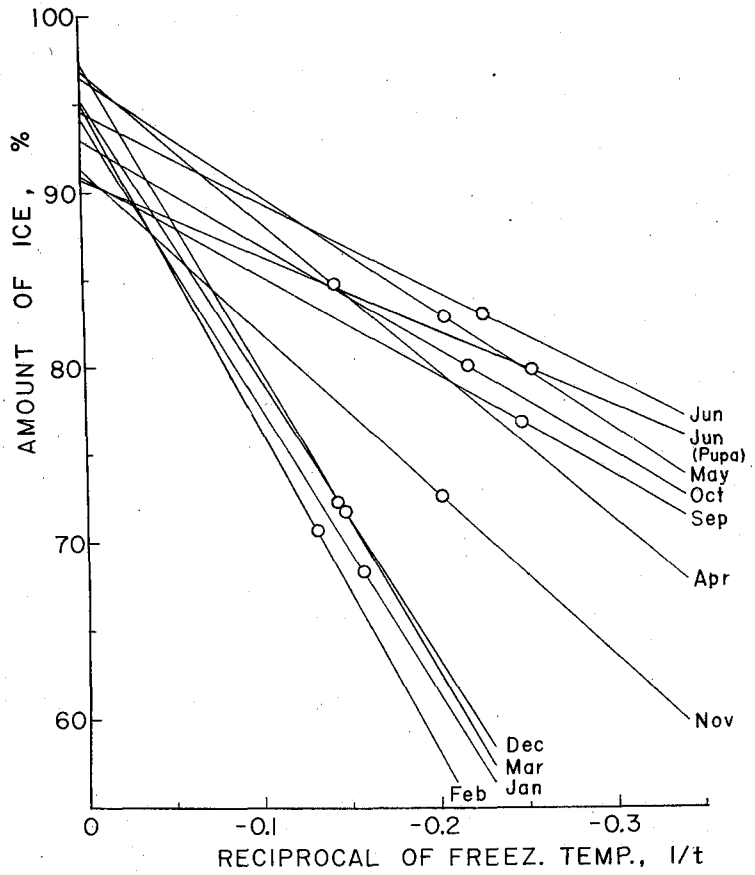


Fig. 21. Regression of the amount of ice against the reciprocals of the freezing temperatures in eleven lots of insects. The open circles represent the lot means.

where $E(X')$ = mean of the amount of ice in all of the experiments (38,652.5/509=75.94).

Fig. 20 indicates clearly that the differences among the means of the amount of ice each month are significant.

By plotting of the amounts of ice (X') formed in the frozen prepupa against the reciprocals of the temperatures ($1/t$), a straight line is obtained, as shown in Fig. 14; all the lines which are represented by the regression equations from September 1958 to June 1959 are collectively drawn in Fig. 21. In this figure, the monthly means are also indicated. Although the points, at which the lines cross the ordinate at $1/t=0$, *i.e.* the values of "a" lie scattered between 91 and 97 per cent, it may be considered valid that this difference is mainly caused by the experimental errors; further the differences among the values of "a" may not be very large, because the

variance of the amount of ice from the regression is fairly large. These lines are obviously divided into two groups according to their slopes; one is the group with steep slope in winter and the other the group with gentle slope in mild seasons of autumn and spring. The slopes of the lines in November and in April lie between these two groups and it is interesting that in the changing of the season from autumn to winter and from winter to spring, the transitional feature is found in the amount of ice. From this fact, it is again clearly evident that a greater quantity of ice is usually fromed in the insect body in mild seasons than in cold season at any given freezing temperature.

A few conclusions derived from statistical analysis will be added here. There is a significant difference between the adjusted mean of the amunt of ice in mild seasons and that in cold season. The differences among the regression coefficients within lots are also significant and the difference between the coefficients of mild seasons and of cold season is also significant.

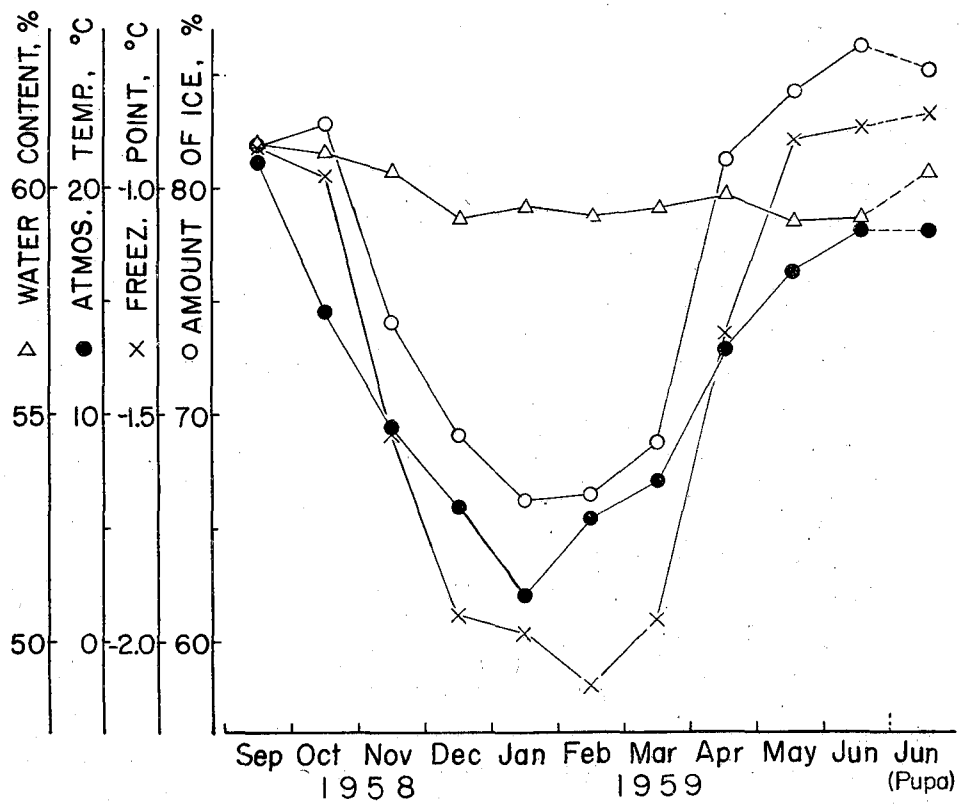


Fig. 22. The seasonal variations of the monthly means of the atmospheric temperature, of the freezing point of blood, of the water content, and the adjusted monthly mean of the amount of ice.

Periodicities of Amount of Ice Formed in Insect Body and of Freezing Point of Blood

As described above, seasonal variations have been clearly found in the freezing point of the blood and in the amount of ice formed in the prepupa of slug moth. The periodicity of the freezing point of the blood in this species has already been reported briefly in previous papers[2,3,12]. In addition to these physiological properties, the undercooling point[2,3,12], the velocity of growth of ice crystal through the blood[48,49], and the intensity of the prepupal diapause[5] *etc.* show the seasonal variation, too. It may be most probable that seasonal variation in the atmospheric temperature causes the periodicity in the physiological properties of the insect. The monthly means of the atmospheric temperature*, of the freezing point of the blood and the adjusted monthly means of the amount of ice are plotted against the months in Fig. 22, with the monthly means of the water content. There is a clear parallelism among the periodicities of the atmospheric temperature, of the freezing point and of the amount of ice, *viz.* in a cold winter both the freezing point and the amount of ice take the minimum. This fact indicates that there is an intimate relationship between them. The combination of equations (7′) and (8) gives the relation,

$$X' = a\left(1 - \frac{t_f}{t}\right). \qquad (17)$$

As is evident from this equation, the freezing point of the blood is a primary factor in determining the amount of ice formed in the insect and the amount of ice (X') decreases in proportion to the fall of the freezing point (t_f) of the blood at a difinite freezing temperature (t). That this relation is satisfied experimentally is clear from Fig. 23, in which the adjusted monthly means of the amount of ice are plotted against the corresponding values of the freezing point of the blood in every month. The straight line in this figure is drawn from equation (17), in which the values of 98.1 and −6.50, estimated by the method of least squares from the data in the figure, are substituted for "a" and "t" respectively. The value of −6.50 corresponds obviously with the freezing temperature of the insect. As was previously mentioned, the adjusted means of the amount of ice in every month can be estimated as the amount of ice which may be formed in the insect body when the freezing temperature is −5.50 or its reciprocal −0.182. Therefore, it is expected that these two values of the freezing temperature coincide with each other, but there is found a difference of 1°C between them. This difference may partly be attributable to the discrepancy which may exist between the freezing points of the individuals used for the determination of the freezing point itself and the freezing points of those employed for the ice determination each month, and further to the fact that the value of "a" each month differs from 98.1, but mainly it is attributable to the experimental errors which may be introduced for

* The author is indebted to the Mito Meteorological Observatory for supplying the data of monthly mean temperature.

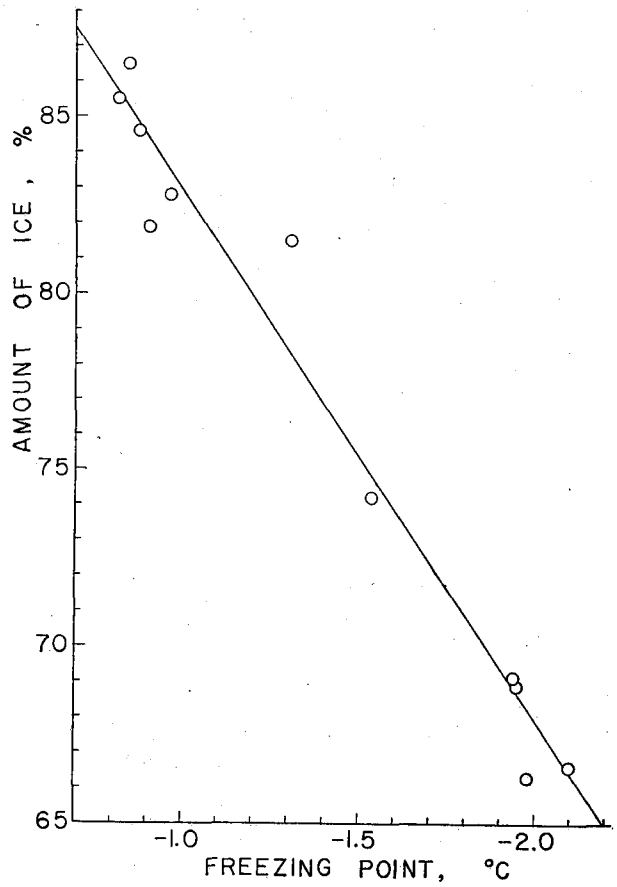


Fig. 23. Relation between the monthly means of the freezing point of the blood and the adjusted monthly means of the amount of ice. The straight line was drawn from equation (17).

the causes mentioned above. Thus it is highly probable that the relatively large fluctuations in the amount of ice each month may mainly result from the probable deviations of the freezing point of the blood of different individuals. The amount of ice in the prepupa, in which glycerol has been injected, is much smaller than that in the normal prepupa*; this decrease may be attributed to the additional fall of the freezing point of the blood after the injection of glycerol (*cf.* Fig. 18). This fact shows also that the freezing point of the blood is the essential factor determining the amount of ice formed in the insect body.

The correlation coefficients between the monthly means of atmospheric temperature and those of the freezint point of the blood and between the monthly means of the

* This decrease in the amount of ice is significant at 1 per cent level by the statistical test.

freezing point and the adjusted monthly means of the amount of ice are calculated to be 0.954 and 0.983 respectively, as are given in Table 5. Let ρ denote the correlation coefficient in population. The null hypothesis, $\rho=0$, can be rejected at the 1 per cent level of significance in both cases. Thence, it will be clear that the correlations in both cases are very close. From these statements, it is undoubtedly to be concluded that the periodicity of the amount of ice formed in the insect body depends surely on the seasonal variation of the freezing point of the blood. The periodicity in the freezing point of the blood is, too, perhaps brought about by a direct or indirect influence of seasonal variations in atmospheric temperatures.

Table 5. Correlation coefficient between the monthly means of the atmospheric temperature and those of the freezing point of the blood, and that between the monthly means of the freezing point and the adjusted monthly means of the amount of ice.

	Correlation coefficient in sample r	0.95 fiducial limits of correlation coefficient in population ρ	
		Upper limit	Lower limit
Betweem atmos. temp. and f.p.	0.954	0.988	0.828
Between f.p. and ice-amount	0.983	0.996	0.932

There have been only a few studies regarding the periodicities in the freezing or undercooling point of insect. PAYNE[7,10] pointed out that the insects not exposed usually to low temperatures, such as lake dwellers and certain stored-products pests, did not exhibit periodicity in their freezing* and undercooling points, whereas those of the insects exposed normally to temperature extremes changed periodically with the seasons, though it has recently been made clear that there were some insects possessing a constant undercooling point in spite of the exposure to different environmental temperatures (*e.g. Phylosamia* pupa, Tanno, unpublished). Further she reported that there was a linear relationship between average monthly temperatures and the undercooling points of a oak borer, *Synchroa punctata*. Similar relationships were also found in the prepupa of the slug moth by ASAHINA[5] and in the present study.

From the previous statements, the seasonal change in the freezing point of the blood, which results from the fluctuation of atmospheric temperature, appears to have influence on the periodicities of other physiological properties. It goes without saying that a change of the solute concentration in the blood leads to an alteration of its freezing point. In the change of concentration of the blood there are two conceivable ways; the one is the decrease in the moisture content of the body and the other the

* Though the method for determining the freezing point is not indicated clearly in her papers, the freezing point used by her is thought to exhibit the rebound point of insect body.

increase of the solute concentration resulting from chemical reactions. It is obvious from Fig. 22 that the freezing point of the blood decreases from the comparatively higher value in fall to the lowest in winter and rises again in the succeeding spring to the same level as in the fall, being accompanied by corresponding increase or decrease of the solute concentration of the blood, whereas the moisture content of the whole body is practically constant throughout the overwintering period. Then, since the correlation coefficient in the sample (r) between the freezing points and the water contents eaeh month is calculated to be 0.483 and degrees of freedom = 9 in this sample, the null hypothesis, $\rho=0$, cannot be rejected by the test of significance. It is, therefore, concluded that there is no correlation between the water content and the freezing point or the concentration of the blood. From these facts, in the prepupa of the slug moth the increase of solute concentration in the blood is not attributable to the loss of moisture content, but to the increase in the number of molecules or ions of solutes. That the latter is the case is shown by the fact that the freezing point of the blood changes considerably with the season in spite of the small fluctuation of moisture content in the insect (*cf.* Fig. 22). SALT[50] also pointed out the same. There exists the possibility that the depression of the freezing point of the blood may be caused by increase in the fraction of so-called "bound water". This point will be discussed later.

In recent years, it has been indeed shown that glycerol occurs in a considerable amount in the body fluid of various insects during cold season, but does not occur during other seasons, though this fact is not applicable to all insects[13-17,51-53]. According to SALT[13,53], glycerol content of *Bracon cephi* attains to a very high concentration in winter, lowering the melting point of the haemolymph to a fairly low value and simultaneously the undercooling point of the intact larva to a very low value. It was reported by TAKEHARA and ASAHINA[15,17] that the glycerol content in the prepupa of the slug moth increased rapidly with the optimum temperature of glycerol formation at 10°C from the middle of October to that of November, to climb from the zero level to the maximum constant level at which the glycerol content remained during cold winter; then when the prepupa was incubated at 10° or 20°C in March, its content decreased to the zero level, whereas the rise and the fall of the glycogen content were completely contrary to the seasonal variations in the glycerol content; the glycogen content fell practically to zero in winter, indicating plainly that the source of glycerol was glycogen. On the other hand, total sugar content remained almost constant from October to March. Such a rise and fall in the glycerol content will inevitably give rise to the depression of the freezing point. Indeed the periodicity in the freezing point of the blood found in the present study indicates complete correlation with periodicity in the glycerol content found by TAKEHARA and ASAHINA. Moreover, the difference between the freezing points of the blood in autumn and in winter can be quantitatively accounted for by the increase of glycerol content[15,17].

The seasonal variation in atmospheric temperatures causes the periodicity of the

freezing point of the blood in the prepupa as a result of the change in solute concentration of the blood; the latter is accompanied by the periodicity of the amount of ice formed in the insect body at graded freezing temperatures. The decreasing ice formation in the prepupa in severe winter appears to be favourable in protecting tissue cells against frost injury, because the frost-injury may be explained to occur primarily as a result of the loss of liquid water on the freezing, on the ground of any one of the theories regarding frost injury*.

Unfreezable Water

It is very probable that unfreezable or bound water exists really, as is suggested by THOENES[29], LUYET[55], and others. There are a few definitions regarding bound water, but none of them is satisfactory. For these definitions, the paper of SAYRE[25] should be consulted.

Solving equation (8) for $100\,u/M_w$ or the percentage content of the unfreezable water, one gets the relation,

$$100\frac{u}{M_w} = 100-a\,. \tag{18}$$

From this equation, the relative amounts of unfreezable water of the prepupa each month can be easily estimated to be 3 to 10 per cent of the total body water, referring to the values of "a" in Table 2. It is not easy to judge, whether the values obtained here as unfreezable water are valid or not, because the correct value of "a" in every month can hardly be estimated owing to the large fluctuation in the data. Accordingly, periodicity in the amount of unfreezable water, if any, cannot be found.

It was found by MORAN[39] that after gelatin gels of 12 to 40 per cent were frozen for a time at a given subzero temperature, the concentration of the unfrozen portion of the gel could be determined by the freezing temperature alone, irrespective of the initial concentration. This means that the amount of ice formed in the gel of known concentration is determined by the freezing temperature alone. The ice-amount-temperature curve given by him was similar in shape to those of insects and other organisms drawn by several authors. He confirmed also that gelatin gel of 65 per cent never froze even at the temperature of liquid air, suggesting the existence of bound water. SALT[23] attempted to explain from several points of view MORAN's results, why the unfrozen portion of the gelatin gel does not further freeze at a given freezing temperature, though that freezing temperature is below the freezing point of the unfrozen portion; SALT finally reached the opinion that the amount of bound water was not constant, but varied with temperature. That is to say, water in the unfrozen portion of gelatin gel is almost completely bound to the gelatin molecules at a given temperature, and as the temperature lowers further, a part of the water bound is liberated to freeze. Thus, in the derivation of equation (7′), the amount of unfreezable

* None of the theories explains completely the frost injury in an organism. For the theories, see the recent reviews by SALT[9] and ASAHINA[54].

water was assumed to be constant independent of freezing temperature, but that in the insect may vary with temperatures.

By THOENES[29], ROBINSON[18], SACHAROV[20], GREATHOUSE[26], and others ice determinations have been made on the basis of the opinion that the cold-hardiness depends upon the amount of unfreezable water in the organisms. They determined the amount of bound water on the assumption that bound or unfreezable water contained in an organism is by no means frozen even at a definite low temperature of −20°C or below. This assumption seems to be supported by the fact that the amount of ice formed in the organism is practically constant below −20°C. But, strictly speaking, the assumption is not valid, because the temperature-ice-formation curves as determined by equation (7′) represent a hyperbola, so that the large fraction of body water is crystallized into ice at several degrees below the freezing point and the amount of ice formed approaches asymptotically to a level not far from complete freezing, as the temperature falls further. The increase in the amount of ice with the lowering of the temperatures in the range of relatively low temperatures is so slight that it may be masked by the relatively large fluctuation of the data. Concretely speaking, if the freezing points (t_f) of body fluid are −1.0° and −2.0°C, the amounts of ice in per cent of total body water are about 87.5 and 75.0 per cent respectively at −8°C; 95.0 and 90.0 at −20°C; and 96.7 and 93.3 at −30°C, provided that no unfreezable water (u) is contained in body fluid. This indicates that the amount of ice formed in an insect body is practically unvaried below −20°C. From this fact it is clear that the above assumption on unfreezable water is not valid. It will be clear that the difference in the amounts of ice in the above two cases at low tempratures of −20° and −30°C is not attributable to the difference in the amount of unfreezable water, but to the difference of the freezing points. Hyperbolic features of the change in the amount of ice with the freezing temperatures are experimentally presented by the results of DITMAN *et al.*[22], SCHOLANDER *et al.*[24], SALT[23], and KANWISHER[27,28] as well as by the present results. As mentioned before, it is not valid to regard the water unfrozen at −20°C as bound or unfreezable water, simply because of the constancy of the amount of ice in such a range of relatively low temperatures, as below −20°C. Therefore, the values of bound water, which have been estimated by THOENES[29], ROBINSON[18], SACHAROV[20], SAYRE[25], SIEGLER[21], GREATHOUSE[26], DITMAN *et al.*[6], DITMAN *et al.*[22], and others as the difference between the amount of total body water and that of water frozen at a given low temperature (−20°C), are hardly proven to be correct on the ground of the previous explanation. KANWISHER[27,28] found that there are differences between the amount of ice formed in the intertidal animals or algae and that of ice formed in an equal amount by weight of sea water at any subfreezing temperature. He attributed these differences to unfreezable water contained in the intertidal organisms. Since the body fluids of the intertidal invertebrates and algae are considered to be isotonic to sea water, the difference given by him appears to be somewhat too great. On the basis of the similarity between the water-loss-melting-point curve (equivalent

to the ice-amount-temperature curve) of the blood of *Loxostege sticticalis* and that of the sodium chloride solution, it was pointed out by SALT[23] that in the blood, solutes of low molecular weight are dominant whilst bound water is not very plentiful. This may be the case as well in the prepupa of the slug moth. Although there is no denying the fact that bound water exists actually in biological materials, the greater fraction of the measured values of the so-called "bound water" obtained hitherto should rather be considered as the amount of water in unfrozen state at a given temperature. It may be, therefore, nonsense to state that the cold-hardiness of such an organism as an insect depends on the amount of so-called "bound water".

It has recently been proven by ASAHINA and AOKI[1,41], and by ASAHINA[40,56] in animals, including the prepupa of the slug moth, as well as by SAKAI[57,58] in plants, that some organism frozen previously at a given low temperature can survive exposure to an extremely low temperatures. They found that the prefreezing at −20°C is not very effective for enabling the organism to survive immersion in liquid oxygen, while the prefreezing at −30°C is. They explained this phenomenon from the point of view that some extent of freezable water still remained intracellularly after the prefreezing at −20°C, whereas freezable water almost crystallizes into ice outside the cell during the prefreezing at −30°C. But the difference between the amounts of ice formed in the organism at −20° and at −30°C is only a few per cent, as is clear from the data given in the present study. Viewed from this fact, it is an interesting problem that such a small difference in the amount of freezable water is responsible for the occurrence of intracellular freezing at a very low temperature.

Summary

1. The periodicity in the amount of ice formed in an insect body was calorimetrically studied, employing mainly the prepupa and partly the pupa of the "slug moth", *Monema flavescens*.

2. The theoretical foundation of the equation, which was used for estimating the amount of ice formed at the graded subfreezing temperatures, was considered from the thermodynamic standpoint.

3. Specific heat of the dry substance in the prepupa is estimated to be 0.32 ± 0.03 cal./gm./deg. as the average of fifteen measurements.

4. The amount of ice formed in the insect is a function of the freezing temperature, and the relation of the former to the latter is expressed by the following equation of hyperbola,

$$X' = a + \frac{b}{t}\ , \qquad (1)$$

where X' is the amount of ice in per cent of total body water, "t" the freezing temperature, and "a" and "b" constants. The values of "a" and "b", are different in every month. The amount of ice at any freezing temperature can be calculated from this equation.

5. From the theoretical consideration, the following relations are obtained,

$$a = 100\left(1-\frac{u}{M_w}\right), \qquad (2)$$

and

$$b = -a\,t_f, \qquad (3)$$

where u is mass of unfreezable water, M_w total mass of water contained in the insect, and t_f the freezing point of the blood. Then, "a" means the amount of freezable water in per cent of total water in the insect and $-b/a$ corresponds with the freezing point (t_f) of the blood.

6. It is clear from the statistical test that there is a periodicity in the amount of ice formed in the insect body. The amount of ice formed at a given freezing temperature begins to decrease in fall, it reaches the minimum in winter and then it begins to increase again in spring.

7. There is an obvious parallelism between the periodicity in the adjusted monthly means of the amount of ice and the periodicity in the freezing points of the blood. There exists similarly an intimate correlation between the latter and the seasonal variation of the monthly means of atmospheric temperatures (Fig. 22). The correlation coefficients in both cases are statistically significant, being 0.954 in the latter case and 0.983 in the former.

8. Combining equations (1) and (3), one gets

$$X' = a\left(1-\frac{t_f}{t}\right). \qquad (4)$$

This equation expresses the fact that, at a definite freezing temperature (t), the amount of ice formed in the insect body is directly proportional to the freezing point (t_f). This equation is satisfied by the adjusted monthly means of the amount of ice and the corresponding values of the freezing point of the blood each month; in this case, the values of "a" and "t" are estimated by the method of least squares to be 98.1 and -6.50, respectively.

9. The amount of ice formed in the prepupa, in the coelom of which glycerol has been injected, is considerably smaller than that in the normal one, and can be approximately evaluated on the basis of additional depression of the freezing point of the blood caused by the injection of glycerol.

10. From these facts, it may be clearly said that the freezing point of the blood is the dominant factor determining the amount of ice formed in the insect, and that the periodicity of the latter is brought about by the influence of the fluctuations of atmospheric temperature.

11. Since the water content of the prepupa is almost unchanged throughout the overwintering period, it has no direct correlation with the periodicity of the amount of ice formed in the insect.

12. Judging from the various results of the previous studies, the ice formation occurs only in the blood within the body cavity. As the ice crystal grows extracel-

lularly, the blood is concentrated and accordingly more and more water is drawn from the tissue cells by osmosis. Thus the water drawn from the cell interior may also take part in the extracellular freezing. That this is true was indicated by the quantitative relationship among the amount of water contained in the blood, that of total body water, and that of ice formed in the body at graded subfreezing temperatures.

13. Discussion was offered regarding the causes, by which errors may be introduced into the determination of the amount of ice by the calorimetric method used in the present study.

14. Since the undercooling point of extracted oil is as low as −26.0°C and the oil is contained in the form of minute globules within the fat cell, the oil may hardly congeal at the time of the body freezing.

15. In relation to the ice formation in the insect body, the problem of unfreezable or bound water was discussed.

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References

"L. T. S." in the following is the abbreviation for "Low Temperature Science", a scientific publication written in Japanese with English summary, issued by the Institute of Low Temperature Science, Hokkaido University, Sapporo, Japan.

1. ASAHINA, E. and AOKI, K. 1958a A method by which frost-hardy caterpillars survive freezing at a super-low temperature.* L. T. S., Ser. B, **16**, 55–63.
2. ASAHINA, E., AOKI, K. and SHINOZAKI, J. 1954 The freezing process of frost-hardy caterpillars. Bull. Ent. Res., **45**, 329–339.
3. AOKI, K. and SHINOZAKI, J. 1953 On the undercooling of the prepupa of slug moth.* L. T. S., **10**, 103–108.
4. SHINOZAKI, J. 1954 b On the freezing of the prepupa of slug moth.* L. T. S., Ser. B, **12**, 71–86.
5. ASAHINA, E. 1959 a Diapause and frost-resistance in a slug caterpillar. Kontyû, **27**, 47–55.
6. DITMAN, L. P., WEILAND, G. S. and GUILL, J. H. 1940 The metabolism in the corn earworm. III. Weight, water, and diapause. J. Econ. Ent., **33**, 282–295.
7. PAYNE, N. M. 1927. Freezing and survival of insects at low temperatures. J. Morph., **43**, 521–546.
8. PAYNE, N. M. 1928 Cold hardiness in the Japanese beetle, *Popillia japonica* Newman. Biol. Bull., **55**, 163–179.
9. SALT, R. W. 1961 Principles of insect cold-hardiness. Ann. Rev. Ent., **6**, 55–74.

10. PAYNE, N. M. 1926 The effect of environmental temperatures upon insect freezing points. Ecology, **7**, 99–106.
11. SALT, R. W. 1936 Studies on the freezing process in insects. Univ. Minn. Agr. Exp. Sta. Tech. Bull., **116**, 3-41.
12. ASAHINA, E., AOKI, K. and SHINOZAKI, J. 1953 Resistance mechanism to frost injury of overwintering slug caterpillar.* Kontyû, **20**, 11-17.
13. SALT, R. W. 1959 a Role of glycerol in the cold-hardiness of *Bracon cephi* (Gahan). Can. J. Zool., **37**, 59–69.
14. TAKEHARA, I. and ASAHINA, E. 1959 Glycerol content in some frost-hardy insects, a preliminary report.** L. T. S., Ser. B, **17** 159-163.
15. TAKEHARA, I. and ASAHINA, E. 1960 a Glycerol in overwintering prepupa of slug moth, a preliminary note.* L. T. S., Ser. B, **18**, 51–56.
16. TAKEHARA, I. and ASAHINA, E. 1960 b Frost-resistance and glycerol content in overwintering insects.* L. T. S., Ser. B, **18**, 57–65.
17. TAKEHARA, I. and ASAHINA, E. 1961 Glycerol in a slug caterpillar I. Glycerol formation, diapause and frostresistance in insect reared at various graded temperatures.* L. T. S., Ser. B, **19**, 29–36.
18. ROBINSON, W. 1928 Relation of hydrophilic colloids to winter hardiness of insects. Colloid Symp. Monog., **5**, 199–218.
19. ROBINSON, W. 1931 Free and bound water determinations by the heat of fusion of ice method. J. Biol. Chem., **92**, 699–709.
20. SACHAROV, N. L. 1930 Studies in cold resistance of insects. Ecology, **11**, 505–517.
21. SIEGLER, E. H. 1946 Susceptibility of hibernating codling moth larvae to low temperatures, and the bound-water content. J. Agr. Res., **72**, 329–340.
22. DITMAN, L. P., VOGT, G. B. and SMITH, D. R. 1942 The relation of unfreezable water to cold-hardiness of insects. J. Econ. Ent., **35**, 265–272.
23. SALT, R. W. 1955 Extent of ice formation in frozen tissues, and a new method for its measurement. Can. J. Zool., **33**, 391–403.
24. SCHOLANDER, P. F., FLAGG, W., HOCK, R. J. and IRVING, L. 1953 Studies on the physiology of frozen plants and animals in the Arctic. J. Cell. and Comp. Physiol., **42**, Suppl. **1**, 1–56.
25. SAYRE, J. D. 1932 Methods of determining bound water in plant tissue. J. Agr. Res., **44**, 669–688.
26. GREATHOUSE, G. A. 1935 Unfreezable and freezable water equilibrium in plant tissues as influenced by sub-zero temperatures. Plant Physiol., **10**, 781–788.
27. KANWISHER, J. W. 1955 Freezing in intertidal animals. Biol. Bull., **109**, 56–63.
28. KANWISHER, J. W. 1957 Freezing and drying in intertidal algae. Biol. Bull., **113**, 275-285.
29. THOENES, F. 1925 Untersuchungen zur Frage der Wasserbindung in Kolloiden und tierischen Geweben. Biochem. Zeitschr., **157**, 174–186.
30. ROTH, W. A. 1938 Die spezifischen Wärmen des Wassers (H_2O) zwischen 0° und 100°C. Zeitschr. phys. Chem., A, **183**, 38–42.
31. DORSEY, N. E. 1940 Properties of ordinary water-substance. In all its phases: water-vapor, water, and all the ices. Reinhold Publ. Corp., New York.
32. SHINOZAKI, J. 1957 The specific heat of insects. J. Fac. Sci., Hokkaido Univ., VI, Zool., **13**, 470–474.

33. ASAHINA, E. 1955 Freezing and supercooling as a method of storage of mobile animal, a preliminary experiment.* Zool. Mag. (Tokyo), **64**, 280–285.
34. AOKI, K. 1955 The initiation of the freezing in insect (preliminary note).* L. T. S., Ser. B, **13**, 51–57.
35. ST. JOHN, J. L. 1931 The temperature at which unbound water is completely frozen in a biocolloid. J. Amer. Chem. Soc., **53**, 4014–4019.
36. BĚLEHRÁDEK, J. 1935 Temperature and living matter. Borntraeger, Berlin.
37. SALT, R. W. 1959 b Survival of frozen fat body cells in an insect. Nature, **184**, 1426.
38. SALT, R. W. 1956 a Freezing and melting points of insect tissues. Can. J. Zool., **34**, 1–5.
39. MORAN, T. 1926 The freezing of gelatin gel. Proc. Roy. Soc., London, Ser. A, **112**, 30–46.
40. ASAHINA, E. 1959 c Prefreezing as a method enabling animals to survive freezing at an extremely low temperature. Nature, **184**, 1003–1004.
41. ASAHINA, E. and AOKI, K. 1958 b Survival of intact insects immersed in liquid oxygen without any antifreeze agent. Nature, **182**, 327–328.
42. KANWISHER, J. W. 1959 Histology and metabolism of frozen intertidal animals. Biol. Bull., **116**, 258–264.
43. CHAMBERS, R. and HALE, H. P. 1932 The formation of ice in protoplasm. Proc. Roy. Soc., London, Ser. B, **110**, 336–352.
44. ASAHINA, E. 1953 a Analysis of the freezing process of living organisms. X. Freezing process of egg cell of sea-urchin.* L. T. S., **10**, 81–92.
45. ASAHINA, E. 1956 The freezing process of plant cell. Contr. Inst. Low Temp. Sci., **10**, 83-126.
46. KISTLER, S. S. 1936 The measurement of "bound" water by the freezing method. J. Amer. Chem. Soc., **58**, 901–907.
47. SNEDECOR, G. W. 1946 Statistical methods. Applied to experiments in agriculture and biology. Iowa State Coll. Press, Iowa, U. S. A.
48. ASAHINA, E. 1953 b Freezing process of blood of a frost hardy caterpillar, *Cnidocampa flavescens*.* L. T. S., **10**, 117–126.
49. SHINOZAKI, J. 1954 a The velocity of crystallization of ice from the blood of prepupa of slug moth.* L. T. S., Ser. B, **11**, 1–11.
50. SALT, R. W. 1956 b Influence of moisture content and temperature on cold-hardiness of hibernating insects. Can. J. Zool., **34**, 283–294.
51. DUBACH, P., SMITH, F., PRATT, D. and STEWART, C. M. 1959 Possible role of glycerol in the winter-hardiness of insects. Nature, **184**, 288–289.
52. SALT, R. W. 1957 Natural occurrence of glycerol in insects and its relation to their ability to survive freezing. Can. Ent., **89**, 491–494.
53. SALT, R. W. 1958 Role of glycerol in producing abnormally low supercooling and freezing points in an insect, *Bracon cephi* (Gahan). Nature, **181**, 1281.
54. ASAHINA, E. 1962 Some notes on the mechanism of frost resistance in living animal and plant at climatic low temperatures. Bull. Marine Biol. Sta. Asamushi, **10**, 31–36.
55. LUYET, B. J. 1939 Water and the ultra-structure of protoplasm. Archiv exp. Zellforsch., **22**, 487–491.

56. ASAHINA, E. 1959 b Frost-resistance in a nematode *Aphelenchoides ritzema-bosi*.* L. T. S., Ser. B, **17**, 51–62.
57. SAKAI, A. 1956 Survival of plant tissue at super-low temperatures.* L. T. S., Ser. B, **14**, 17–23.
58. SAKAI, A. 1960 Survival of the twig of woody plants at −196°C. Nature, **185**, 393–394.

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* In Japanese with English summary.
** In Japanese.