Natural Occurrence of Glycerol in the Slug Caterpillar, *Monema flavescens**

by

Ichiro TAKEHARA
竹 原 一 郎
Biological Section, The Institute of Low Temperature Science

Received July 1966

Abstract

The behavior of glycerol in the overwintering prepupae of the slug caterpillar, *Monema flavescens*, is described in detail.

From the results obtained it seems apparent that: 1) glycerol is formed from glycogen and reconverted to glycogen; 2) a temperature of 10°C is optimum for causing glycerol formation in the prepupae; 3) at 20°C and 0°C practically no glycerol is formed; 4) the entry into diapause alone is not sufficient to cause glycerol formation in the prepupae, although diapause seems to provide a physiological precondition favourable for the formation of glycerol; 5) at the same time the termination of diapause is not necessarily required for the disappearance of glycerol in the prepupae.

The behavior of glycerol in the prepupae is tentatively interpreted to be a result of a certain process which may probably occur in the diapausing prepuae placed at 10°C.

I. Introduction

The accumulation of glycerol in insects was independently reported by CHINO in the diapausing egg of the silkworm, *Bombyx mori*[1], and by WYATT and KALF in the pupa of the giant silkworm, *Hyalophora cecropia*[2]. According to CHINO, glycogen is converted completely into sorbitol and glycerol within about thirty days from oviposition in the diapausing egg of the silkworm[3]. Sorbitol and glycerol remain at their highest level throughout the diapause

* Contribution No. 776 from the Institute of Low Temperature Science.
Doctor thesis submitted to the Tohoku University.

Table 1. Occurrence of glycerol in insects

Species and stage	Glycerol (approx. maximum % of fresh wt.)	Reference
Hemiptera		
Pterocomma smithia, eggs	15.5*	36
Lepidoptera		
Loxostege sticticalis, larvae	4.3	5
Pyrausta nubilalis, larvae	4.0	11
Monema flavescens, prepupae	5.0	24
Alsophila pometaria, eggs	15.1*	36
Hyponomeuta evonymellus, larvae	11.5*	37
Laspeyresia strobilella, larvae	17.3*	37
Acrolita naevana, eggs	8.3	37
Philudoria albomaculata, larvae	0.5	46
Spilosoma niveus, larvae	1.8	46
Bombyx mori, eggs	1.1	3
Hyalophora cecropia, pupal blood	2.8	4
Telea polyphemus, pupal blood	0.5	4
Papilio maacki, pupae	3.5	46
Papilio xuthus, pupae	4.1	45
Papilio machaon, pupae	3.4	9
Papilio alcinous, pupae	0.1	46
Pieris rapae crucivora, pupae	0.5	46
Araschnia levana, pupae	3.3	46
Coleoptera		
Cetonia roetotsi, larvae	3.8	46
Dendroctonus monticolae, larvae	23.4*	36
Hymenoptera		
Camponotus pennsylvanicus, adults	10.0	35
Camponotus obscuripes, adults	5.3	12
Camponotus herculeanus, adults	5.8*	36
Hoplismenus obscurus, adults	3.0	46
Pterocormus molitorius, adults	4.5	46
Bracon cephi, larvae	25.0	6
Eurytoma gigantea, larvae	23.4*	36
Megachile rotundata, larvae	2.2*	36
Diptera		
Eurosta solidaginis, larvae	2.0	5
Diplolepis radicum, prepupae	6.4*	37
Diplolepis sp., prepupae	17.6*	37
Rhabdophaga globosa, larvae	32.4*	37

* Per cent of the sum of the water content plus glycerol

period. When diapause has been artificially broken by cold treatment, these two polyhydric alcohols are almost completely reconverted into glycogen. CHINO believes that the resynthesis of glycogen in silkworm eggs is associated with the termination of diapause *per se* and not with the subsequent process of post-diapause development[3]. In the *cecropia* silkworm, on the other hand, glycerol appears in the blood about the time of pupation, and then gradually accumulates during diapause to reach a level of about 0.3 M[4]. With the initiation of adult development, glycerol disappears very quickly. No glycerol is found in the blood of larva or developing pupa just before emergence[4].

Insects have long been known to be the largest and highest whole animals so far proven to tolerate freezing without fatal injury. The remarkable effect of glycerol in protecting living cells against freezing injury has also been well known for more than ten years. In this connection some cryobiologists supposed that the discovery of glycerol in insects might open the way to solving the mechanisms of frost resistance in insects[5-7]. In fact the possession of glycerol has recently been observed in a variety of overwintering insects (Table 1). Detailed reviews of the relation of glycerol to insect frost hardiness have been presented by SALT[8] and more recently by ASAHINA[9].

A series of works concerning the problem mentioned above has also been carried on in our laboratory for some years. The slug caterpillar, *Monema flavescens*, has been used as the main experimental material in our work. In this insect the presence of glycerol was first detected in 1958 by AOKI and SHIKAMA[10]. It has been demonstrated that mere possession of glycerol by an insect is not a prerequisite for exhibiting the ability to survive freezing[11-13]. However, a previous paper has also revealed that the increase in glycerol content in the diapausing prepupa of the slug caterpillar clearly increases its frost resistance[13]. In the course of the studies on the mechanisms of frost resistance in insects, it became, therefore, desirable to know various properties in the natural occurrence of glycerol and factors related to controlling the glycerol metabolism in the insect. In the present paper, the behavior of glycerol as it naturally occurred in the overwintering prepupae of *M. flavescens*, will be described in detail. A remarkable dependency of glycerol metabolism upon temperature will also be shown with relation to the physiological state of the insects.

II. Materials and Methods

Material

The overwintering prepupae of the slug caterpillar, *Monema flavescens* WALKER, were employed as materials throughout the present experiments.

The adults of this moth usually emerge in July. Most of the larvae from eggs laid by them become full-grown by the beginning of September, when they construct hard oval cocoons. The period of their spinning cocoons continues generally from the end of August to the end of September. After spinning cocoons, the larvae soon transform to smooth-skinned prepupae in cocoons (Fig. 1, a, b). In this state, they overwinter on the twigs or trunks of trees, such as maple, chestnut, plum, pear and so on. Their pupation begins usually in June of the next summer, though the prepual diapause of the prepupae seems to terminate by the biginning of January[14].

The larvae of this insect were collected usually near the end of August in Sapporo and reared on maple trees in our laboratory, till they spun their cocoons. During the period from the end of September to the beginning of October, these cocoons were collected from the maple trees.

Treatment of insects

Prepupae were kept in a cage at outdoor temperature or in a thermostat at a constant temperature of 20°C, till they were employed in experiments. In these prepupae futher morphological development can scarcely take place as long as they are kept at 20°C[14] *.

Glycerol or other reagents were injected into the body cavity of prepupae through the anus with a micro-injection device furnished with a No. 26 gauge needle. The wound made by the injection was not artificially sealed. The injected prepupae were placed in petri dishes and kept separately at temperatures of 0°C, 10°C and 20°C. Such treatment frequently results in the death of the insects kept at 0°C, but not of those at 10°C and 20°C.

Ligation experiments were made of prepupae tightly ligated around the body between the thorax and abdomen with thread. The softness of their body was convenient for this purpose. Since some of the ligated prepupae were apt to die while being kept at 10°C, only those prepupae that could actively move their abdominal muscle were employed for glycerol analysis.

The intensity of prepupal diapause was examined as follows: Each group of 10 prepupae in intact cocoons was put in a petri dish and kept in a thermostat at a constant temperature of 20°C. After the desired period of time (usually 70 days), all the cocoons in a group were opened and the morphological development of the prepupae was examined (Fig. 1, b, c). The intensity of diapause was expressed as the percentage of diapausing prepupae in the group, which had not yet been able to begin developing. This is, therefore,

* An exception was observed only in 1962, when some of prepupae began to develop even at 20°C.

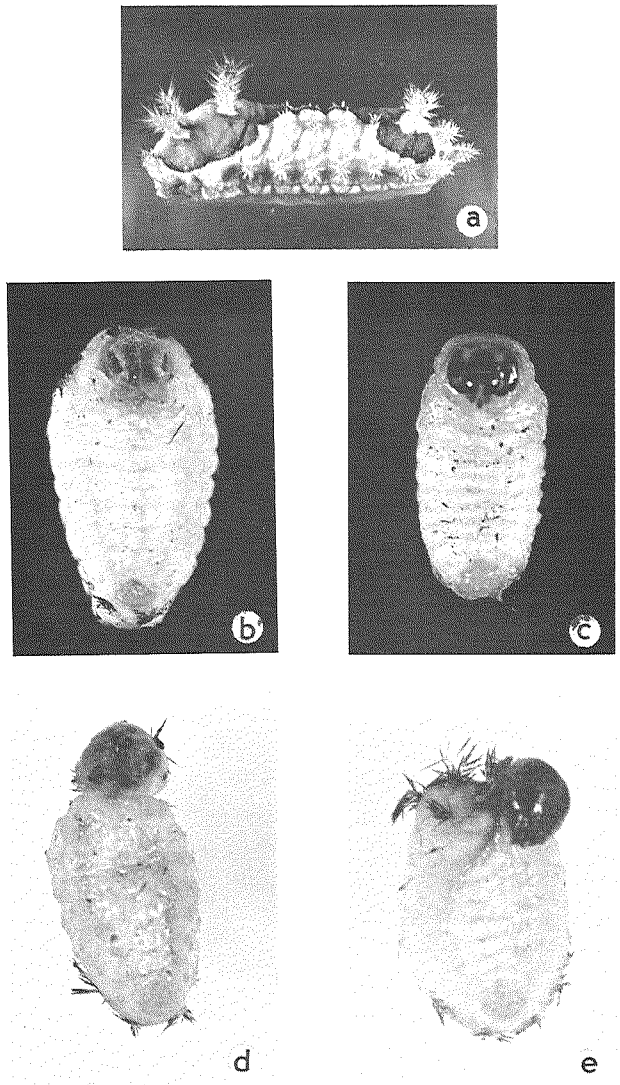

Fig. 1. A larva and prepupae of the slug caterpillar, *Monema flavescens*. a: A full-grown larva, side-view. b: A diapausing prepupa, ventral side. c: A prepupa shortly before pupation. Morphological changes in both head and abdomen are clearly observed. d: A ligated prepupa kept at 10°C for a long time. No change in appearance. e: A ligated prepupa transferred to 20°C after keeping at 10°C for several months. Morphological change is observed only in head, but not in abdomen

not the intensity of diapause in an individual prepupa, but the relative intensity in each group of prepupae.

Estimation of glycerol

A single prepupa, or two to three prepupae were ground in 80 per cent ethanol and an extract was obtained by centrifuging. The remaining residue was washed twice with 80 per cent ethanol. The ethanol extracts obtained in this way were combined and dried with a warm air stream. The resulting residue was suspended in a small amount of anhydrous pyridine and insoluble matter was removed by centrifuging. The supernatant obtained was used for paper chromatographic analysis. Aliquots of the supernatant were applied to a strip of Whatman No. 1 paper and developed by the ascending method with *n*-butanol-acetic acid-water (4 : 1 : 2) as the solvent[15]. The spot of glycerol was detected by TREVELYAN'S alkaline silver nitrate method[16]. The appropriate area was cut from the unsprayed paper chromatogram strip and the glycerol on the strip was eluted with 20 ml of deionized water. When the amount of glycerol was small, the eluate was concentrated under reduced pressure. The glycerol content was determined colorimetrically by the periodic acid oxidation and subsequent chromotropic acid-formaldehyde reaction[17].

Estimation of carbohydrate

A prepupa was ground in 80 per cent methanol, and an extract was obtained by centrifuging. The remaining residue was washed twice with 80 per cent methanol. These methanol extracts were combined and a small amount of powdered charcoal was added to remove organic substances which would interfere with color reaction. The extract containing the charcoal was dried with a warm air stream. The dried residue was suspended in an appropriate amount of 5 per cent trichloroacetic acid solution and the suspension was filtered. An aliquot of the filtrate containing the soluble carbohydrate to 80 per cent methanol was analyzed colorimetrically with anthrone reagent[18]. The carbohydrate analyzed in this way was the mixture of some sugars, but each of them had not yet been identified.

Since glycogen is insoluble in 80 per cent methanol, the glycogen present in prepupae can be detected from the precipitated residue remaining after extracting sugars with methanol. The residue was suspended in 5 ml of 5 per cent trichloroacetic acid and the glycogen was extrated by heating at 100°C for 15 min. The amount of glycogen was colorimetrically determined as sugar with anthrone reagent. Glycerol does not interfere with the determination of carbohydrate.

III. Results

1 *Seasonal changes of glycerol and carbohydrate content*

It has been known that the slug caterpillar shows a remarkable seasonal variation in some characters: Undercooling point of the insect body[19,20], velocity of ice formation in the blood from the prepupa[21], resistance to body freezing in the insect[14], and amount of ice formed in the prepupa[22]. SALT suggested a close correlation between glycerol and the frost-hardiness in insects[5]. In this connection it is interesting to know the seasonal change of the glycerol content in the prepupa of this insect. Amounts of glycerol, glycogen and sugars were estimated in the insect at intervals after a few days from the spinning of cocoon under the outdoor temperature conditions. Even after 10 days from the spinning, the larva did not completely transform to a smooth-skinned

Table 2. Water content of prepupa after spinning cocoon

Days after spinning	Water content %
1—2	63.2
	58.1
	60.8
10	59.6
	63.2
	63.1
25	65.3
	60.6
	63.7
38	59.0
	56.6
	63.5
55	65.5
	64.3
	65.6
69	65.6
	65.2
	60.5
94	66.6
	63.5
	68.9
124	68.4
	62.1
	62.1

prepupa. However, the water content was almost constant in the insect after the spinning of cocoon (Table 2). Therefore, the amount of these compounds was expressed as milligrams per gram of fresh body weight.

In Fig. 2 the changes of glycerol and carbohydrates (glycogen and sugars) content in prepupae during the period from September 1959 to March 1960 are shown. The total sugar content decreased to a minimum (about 3 mg/g) during the first 10 days after the spinning of cocoons, and remained at almost a constant level, at least till the middle of March. On the other hand, the glycogen content increased during the first 25 days and thereafter promptly decrased from the beginning of October. It reached a minimum in the middle of November, which amounted to less than 0.5 mg/g. Glycogen remained at the minimum level till March. For about three weeks after the spinning of cocoons practically no glycerol was produced in the insects. Glycerol began to increase in the middle of October and reached a maximum after 30 to 40 days. The high glycerol level did not change at least until the end of March. Such a maintenance in the amount of glycerol and carbohydrates may assumably

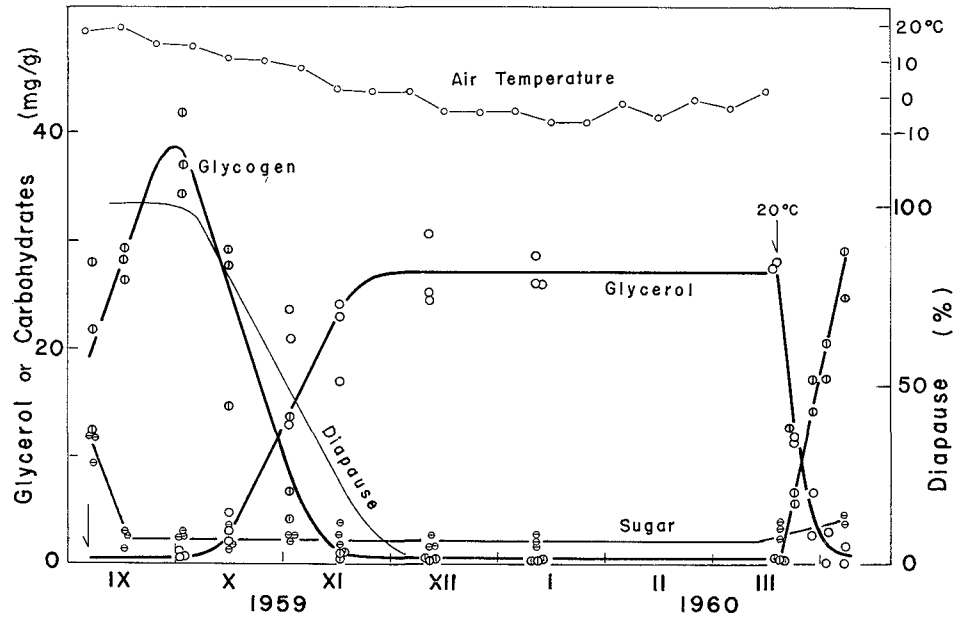

Fig. 2. Seasonal changes in the content of glycogen, glycerol and total sugar, and also in percentage diapause in the overwintering prepupae of the slug caterpillar, *M. flavescens*. An arrow on the abscissa shows the time of the spinning of cocoons. Another arrow on the curve for glycerol content shows the time of transference of the prepupae to 20°C from outdoors

result from the fact that these prepupae were exposed to a low outdoor temperature during their overwintering period (Fig. 2). When the prepupae were transferred at the end of March to a constant temperature of 20°C from outdoors the glycerol content rapidly decreased while the glycogen content simultaneously increased. In such a case, pupal development was obseved to occur in the prepupae transferred from outdoors to 20°C and some of them transformed to pupae within 20 days. In the developing prepupae glycerol completely disappeared before their pupation. The same changes were also observed in prepupae reared at outdoor temperature in 1960 to 1961 (Fig. 3). During the cold five months, the maximum level of glycerol was about 37 mg/g on an average. On the other hand, the glycogen content in the end of September averaged about 41 mg/g. This suggests a greater conversion of glycogen to glycerol in this insect group than in the 1959–1960 group, in which the glycerol maximum was about 27 mg/g and the glycogen maximum was about 37 mg/g, although the reasons for these variations are not clear. The high level of glycerol was maintained until the middle of April, but by the middle of May most glycerol had already disappeared, when the morphological change

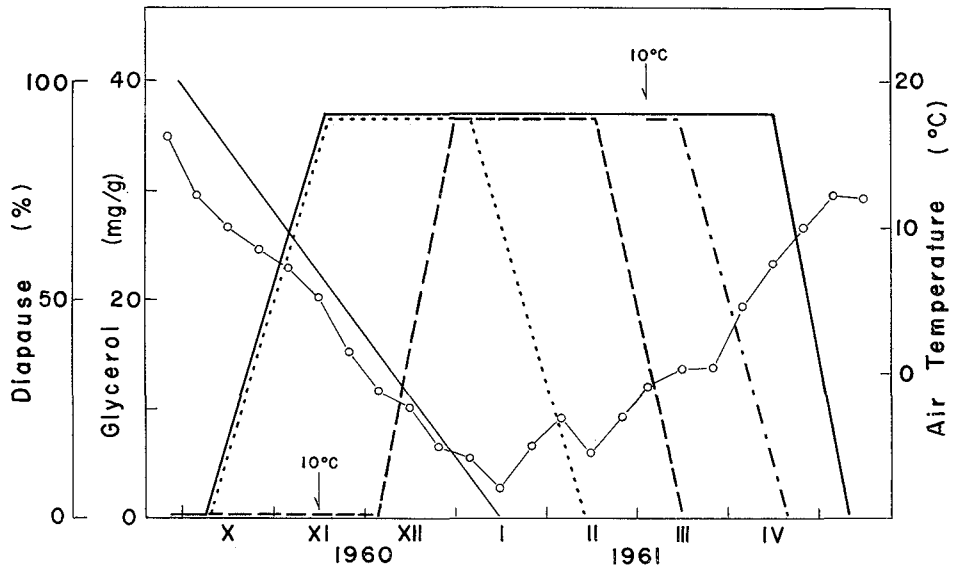

Fig. 3. Schematic presentation of changes in glycerol content in the prepupae reared outdoors, at 10° and 20°C. ——, glycerol in prepupae reared outdoors; — — —, transferred to 10°C from 20°C, arrow shows the time of transference; — – —, transferred to 10°C from outdoors, arrow shows the time of transference; – – – –, reared at 10°C; ——, intensity of diapause; —○—, mean atmospheric temperature

for pupation was observed in the prepupae.

2 *Effect of temperature on glycerol accumulation and disappearance*

In a preliminary report, it was shown that there was no accumulation of glycerol in the prepupae kept at 20°C, at which temperature the insects were unable to develop futher to pupae[23]. The data presented in Fig. 2 also suggests that the accumulation of glycerol does not occur in prepupae kept outdoors

Table 3. Changes of glycogen, glycerol and sugar content at 10°C

Date	Glycogen mg/g	Glycerol mg/g	Sugar mg/g
28/ 9 /60	42.0	0.1	4.0
	46.4	—	2.3
	34.3	—	2.6
11/10/60	24.4	4.6	2.4
	23.5	1.2	2.7
	26.6	3.7	4.0
17/10/60	26.5	12.8	2.4
	17.6	12.1	3.2
	25.3	8.0	1.5
28/10/60	7.8	16.5	4.0
	13.1	21.2	5.6
	5.7	23.6	2.9
11/11/60	5.1	37.0	3.6
	7.7	38.8	3.6
	—	33.6	2.3
25/11/60	1.5	41.1	2.6
	2.3	28.5	5.6
	—	31.8	—
10/12/60	1.6	40.8	2.0
	3.6	44.9	1.9
26/12/60	1.1	33.5	3.1
	—	37.0	4.4
9/ 1 /61	1.2	33.7	2.9
	—	32.3	4.6
25/ 1 /61	4.3	15.1	3.8
	17.2	20.8	5.0
9/ 2 /61	9.1	0.7	4.4
	18.3	0.8	3.9
20/ 2 /61	32.2	0.5	7.0
	18.5	9.6	4.1
7/ 3 /61	22.2	0.1	3.9
	—	11.2	—

before the atmospheric temperature falls to about 10°C. Glycerol disappeared rapidly in naturally overwintering prepupae which were transferred to a temperature of 20°C from outdoors in March, while in prepupae which remained outdoors at nearzero or subzero atmospheric temperatures, no decrease of glycerol was observed. It appeared, therefore, worthy to investigate the effect of temperature on glycerol accumulation and disappearance.

Prepupae kept outdoors until the 28th of September, were separately placed in three thermostats set at 0°, 10° and 20°C. The contents of glycerol, glycogen, and sugars in these insect groups were determined at intervals.

At a constant temperature of 10°C, glycerol first appeared in the prepupae in small amounts in the beginning of October; it continued to increase thereafter and reached a maximum level within about 40 days. This maximum level did not change during the period of 40 to 50 days, and then the glycerol content began to decrease, it almost disappeared after 40 days or so (Fig. 3, Table 3). In the prepupae transferred to a constant temperature of 10°C from outdoors at the beginning of March, when the average outdoor temperature was yet below zero, the glycerol content began to decrease after two weeks and then almost disappeared after 40 days or so (Fig. 3). In such cases, no morphological change was observed in the prepupae in which glycerol once formed had already disappeared. In the prepupae transferred to 20°C from outdoors (Fig. 2) or kept outdoors until May (Fig. 3), on the other hand, morphogenesis apparently proceeded.

At a constant temperature of 20°C the accumulation of glycerol was hardly observed, at least for about 50 days. After the 50 days' storage at 20°C, these prepupae were divided into two groups. Each of them was transferred to the constant temperatures of 10° and 0°C respectively, and then was analyzed for glycerol, glycogen, and sugars. As shown in Fig. 3 and Table 4, the change of the glycerol content in the 10°C group was similar to that in prepupae originally placed at 10°C. On the other hand, scarcely any accumulation of glycerol was observed at 0°C. This was also the case in the prepupae originally kept at 0°C (Table 5).

As already observed under outdoor conditions (Fig. 2), accumulation and disappearance of glycerol at a constant temperature of 10°C were also associated with a marked decrease and increase of the glycogen content (Table 3). This reverse relationship between the content of glycerol and glycogen was not necessarily a quantitative one, since their content considerably varied among individuals and also seasonally. It appears, however, that glycogen is presumably the source of glycerol in this insect. Unlike the marked changes of glycerol and glycogen, the content of sugar was not appreciably changed in

Table 4. Changes of glycogen, glycerol and sugar content at 20°C and these changes after transferring to 10° and 0°C from 20°C

Changes at 20°C, mg/g			
Date	Glycogen	Glycerol	Sugar
28/ 9 /60	42.0	0.1	4.0
	46.4	—	2.3
	34.3	—	2.6
10/10/60	39.3	0.1	2.9
	31.6	—	2.4
	35.0	—	2.3
18/10/60	41.1	0.1	1.8
	40.2	—	3.2
	39.0	—	2.9
1/11/60	53.7	0.1	2.7
	42.7	—	2.8
	43.1	—	3.7
15/11/60	34.3	0.1	1.9
	32.3	—	2.8

Changes after transferring to 10°C from 20°C on 15/11/60, mg/g				Changes after transferring to 0°C from 20°C on 15/11/60, mg/g			
Date	Glycogen	Glycerol	Sugar	Date	Glycogen	Glycerol	Sugar
29/11/60	28.1	0.1	4.9	30/11/60	31.5	0.1	5.1
	25.4	—	4.6		27.9	—	3.3
	37.3	—	4.8		30.1	—	5.1
14/12/60	12.4	35.4	4.2	15/12/60	41.0	0.8	5.1
	19.8	11.2	4.9		44.1	0.5	4.8
27/12/60	5.6	35.0	3.1				
	12.6	41.9	2.4				
18/ 1 /61	5.0	28.7	1.9				
	6.0	37.9	1.7				
1/ 2 /61	2.7	30.4	2.8	2/ 2 /61	39.0	0.1	5.8
	2.8	32.6	3.1		42.3	0.1	6.3
17/ 2 /61	11.7	38.7	3.3				
	3.0	39.6	2.0				
6/ 3 /61	12.0	21.0	2.2	1/ 3 /61	—	0.1	—
	6.2	5.5	3.3		—	0.1	—

prepupae reared at any of the different temperatures. Therefore, sugar may not be important for the metabolism of glycerol in this insect.

Table 5. Changes of glycogen, glycerol and sugar content at 0°C

Date	Glycogen mg/g	Glycerol mg/g	Sugar mg/g
28/ 9 /60	42.0	0.1	4.0
	46.4	—	2.3
	34.3	—	2.6
19/10/60	29.3	0.2	4.0
	26.3	—	4.2
	27.2	—	5.0
2/11/60	33.6	0.6	5.6
	35.4	2.3	4.6
	35.8	0.8	7.3
12/11/60	35.4	0.4	4.2
	41.2	0.6	7.1
	25.1	1.0	4.5
28/11/60	34.6	0.2	6.0
	31.4	0.3	6.6
	31.4	2.2	1.3
19/12/60	30.0	1.6	4.0
	37.0	1.8	6.0
10/ 1 /61	37.9	1.1	9.5
	35.6	—	4.3
1/ 2 /61	37.8	0.2	3.2
	38.1	0.1	6.6
2/ 3 /61	28.5	3.8	6.6
	25.8	0.1	6.7

3 *Changes in glycerol content with environmental temperature*

From the data presented before, the temperature of 10°C seems probably to be optimum for glycerol accumulation, for at 20° and 0°C glycerol scarcely accumulated in the insect. To obtain further evidence for the temperature dependency of glycerol accumulation and disappearance, the behavior of glycerol in prepupae artificially subjected to a two or three-step temperature treatment was observed (Fig. 4). Prepupae used in this experiment had been kept at 20°C for 30 to 50 days after they were collected in September.

In Fig. 4 the changes of glycerol content in the prepupae, which were first kept at 10°C, then transferred to 20°C and finally again to 10°C, are presented as Curves A and B. After two weeks' keeping at 10°C, when the glycerol had accumulated in some amounts, the prepupae were transferred to 20°C, where the glycerol content decreased. One group of these prepupae was returned to 10°C after three weeks at 20°C (Curve A) and another group

after 7 weeks (Curve B). In the former, the glycerol content began to increase after 15 days of transference and in the latter, after 25 days. In the case of Curve A, glycerol once got its maximum level and then disappeared after about 130 days from returning to 10°C. In Curve B, the changes of the glycerol content seem to behave similarly, although it may differ in its maximum level. Curve C in Fig. 4 shows the behavior of glycerol in the prepupae which were first kept at 10°C for 5 weeks, then at 20°C for 5 weeks, and finally

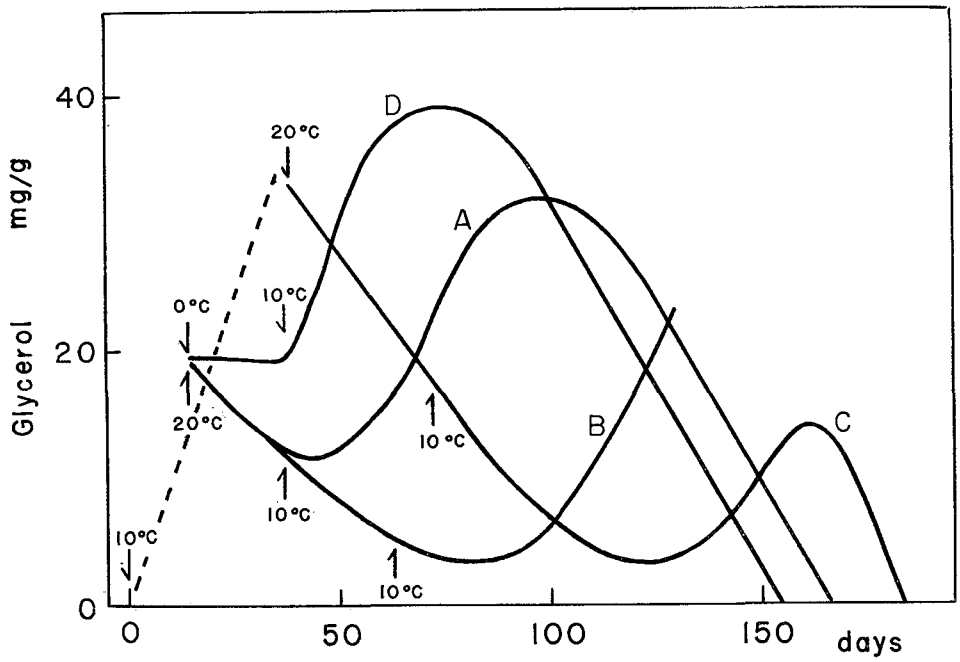

Fig. 4. Changes in glycerol content in the prepupae with changes in environmental temperature. Each curve denotes glycerol behavior in the prepupae subjected to the following temperature treatment; A: at 10°C for 2 weeks, then at 20°C for 3 weeks, finally at 10°C; B: at 10°C for 2 weeks, then at 20°C for 7 weeks, finally at 10°C; C: at 10°C for 5 weeks, then at 20°C for 5 weeks, finally at 10°C; D: at 10°C for 2 weeks, then at 0 °C for 3 weeks, finally at 10°C. Arrows show the time of transference to the indicated temperature. Prepupae employed were collected in September, 1963 and kept at 20°C until they were used for experiments in November

were returned to 10°C where the glycerol content began to increase after about 60 days; soon after it had reached a small maximum amount, however, it disappeared rapidly. Curve D in Fig. 4 shows the change of the glycerol content in the insects which had been kept first at 10°C, transferred to 0°C, then returned to 10°C. After two weeks' keeping at 10°C, the glycerol content

reached about 20 mg/g, then the prepupae were transferred to 0°C, at which temperature the amount of glycerol ceased to increase. After three weeks at 0°C, they were returned to 10°C, and the glycerol immediately began to increase again. In this case, the behavior of glycerol was almost the same as that of control insects which were kept at 10°C throughout the period of the experiment (Fig. 4). It seems, therefore, that in the insects which are producing glycerol at 10°C, a short transfer to 0°C probably has no effect upon later glycerol formation at 10°C.

In Fig. 5 some temperature induced behaviors of glycerol exhibited in prepupae collected in 1964 are shown. This is almost the same experiment at those shown by Curve A and B in Fig. 4. By keeping prepupae at 10°C,

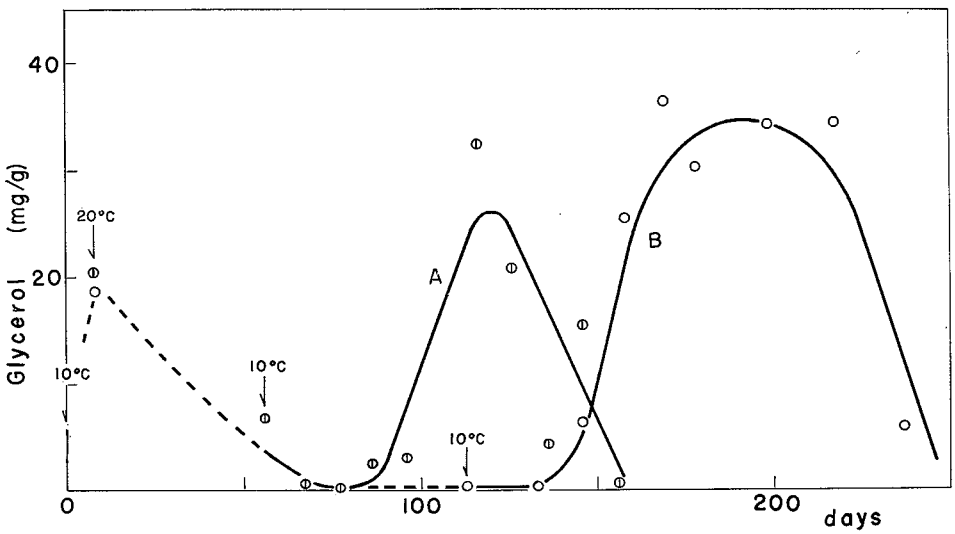

Fig. 5. Changes in glycerol content in the prepupae transferred to 10°C from 20°C. Curve A: glycerol in prepupae returned to 10°C after 7 weeks keeping at 20°C; Curve B: returned to 10°C after 15 weeks keeping at 20°C. Arrows show the time of transference to the indicated temperature. Prepupae employed were collected in September, 1964 and kept at 20°C until November, then reared at 10°C to allow for some accumulation of glycerol

glycerol first accumulated in the insects to a level of about 20 mg/g, then they were transferred to 20°C. After 7 weeks at 20°C (Curve A), one group of them was returned to 10°C and another one after 15 weeks (Curve B). Thereafter, they were analyzed at intervals for glycerol. In the prepupae kept for 7 weeks at 20°C, the content of glycerol reached nearly the same maximum as was reached in the control insects at 10°C, but soon decreased rapidly even at the same temperature. In the prepupae previously kept for 15 weeks at

20°C, on the other hand, the change of glycerol content at 10°C was almost the same as that in controls which were kept at 10°C throughout the period of experiment. These results seem to show that a short previous treatment at 10°C has no effect on the increase and decrease of glycerol during final treatment at 10°C, provided that a long stay at 20°C precedes the final temperature treatment.

The effect of alternative changes in the keeping temperature of the insects on the change of glycerol content is shown in Fig. 6. The temperature was repeatedly alternated in the following manner: for a week at 10°C and

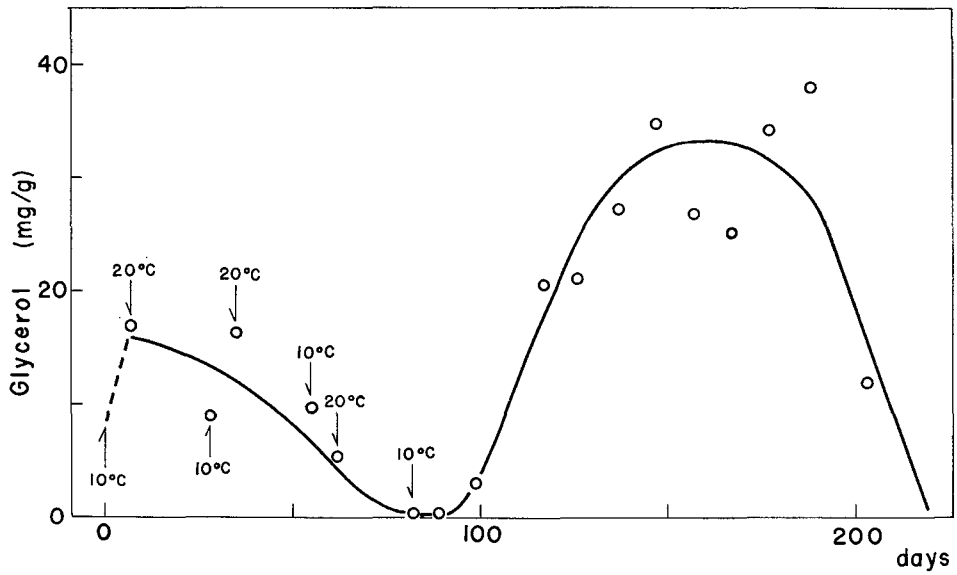

Fig. 6. Effect of alternative changes in temperature on the glycerol content. After the temperature was alternated 3 times in the following manner, at 10°C for a week and then 20°C for 3 weeks, the prepupae were then transferred to the final constant temperature of 10°C. Arrows show the time of transference to the indicated temperature. Prepupae employed were collected in September, 1964 and kept at 20°C until November, when they were used for the experiment

then for three weeks at 20°C. It appeared that the alternative temperature change had hardly any effect on the increase and decrease of glycerol in the prepupae during the final treatment at 10°C, except that the maximum level of glycerol was slightly lower than in the control. Moreover, it is to be noted in Fig. 6 that the glycerol once formed gradually disappears during the alternation of these temperatures. It seems, therefore, that the increase and decrease of glycerol in the prepupae does not result directly from a shift of relative

rate in the reaction of glycerol metabolism by temperature.

The prepupae reared at 0°C for a long period of time were studied to reveal the fate of glycerol once produced in the insects (Fig. 7). After three weeks' keeping at 10°C, when the glycerol content had reached a certain level, the prepupae were transferred to 0°C (Fig. 7, Curve A). Glycerol in the insects decreased very slowly, though each of their amounts ranged widely. On the other hand, when the prepupae were transferred to 0°C after 9 weeks' keeping at 10°C, where the glycerol content had already been at maximum level, the rate of decrease in glycerol content was greater than in the case mentioned

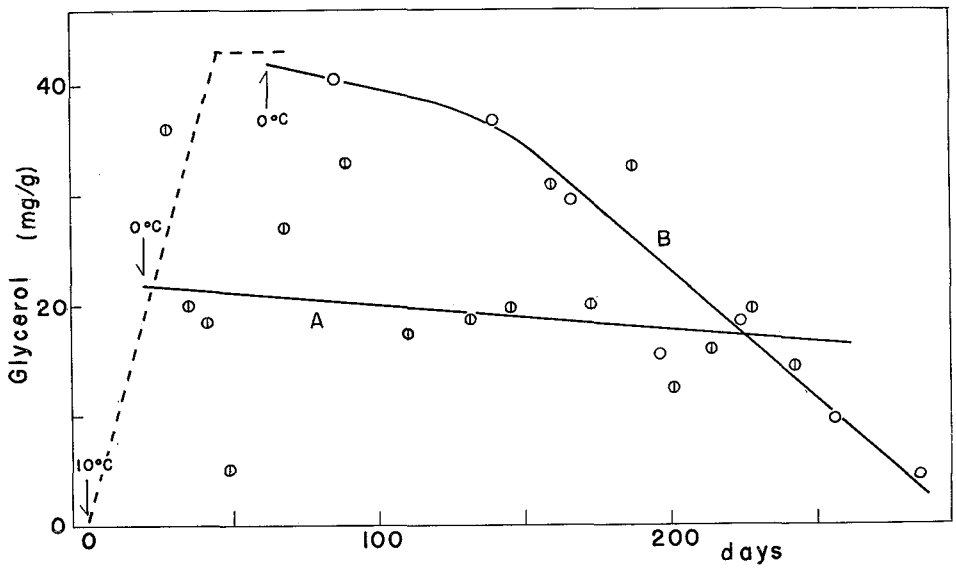

Fig. 7. Changes in glycerol content at 0°C in the prepupae transferred from 10°C. A: prepupae transferred to 0°C after 3 weeks keeping at 10°C; B: prepupae transferred to 0°C after 9 weeks keeping at 10°C. Arrows show the time of transference. Prepupae employed were collected in September, 1963 and kept at 20°C until November, when they were used

above (Fig. 7, Curve B). Such difference in glycerol behavior presented by Curve A and Curve B seems to be concerned with some changes which have perhaps occurred as a result of a physiological process developed during the 9 weeks at 10°C in the insect.

4 *Effect of injected glycerol*

In order to know the regulative factor in the accumulation of glycerol in the insects, RINGER's solution containing glycerol was injected into diapausing prepupae, which had been kept at 20°C for about one month in mid-autumn.

The prepupae were then kept at 10°C and 20°C to examine their glycerol content. Fig. 8 shows the effect of injected glycerol on the changes of the glycerol content in prepupae at 10°C. The amount of glycerol injected was sufficient to raise the glycerol content to about 50 mg/g as an average, which was higher than the maximum value of glycerol found in normal prepupae. The behavior of glycerol in the glycerol injected group and uninjected control was compared. As clearly shown in Fig. 8, the injection of glycerol inhibited the glycerol accumulation; the rate of the glycerol accumulation was about

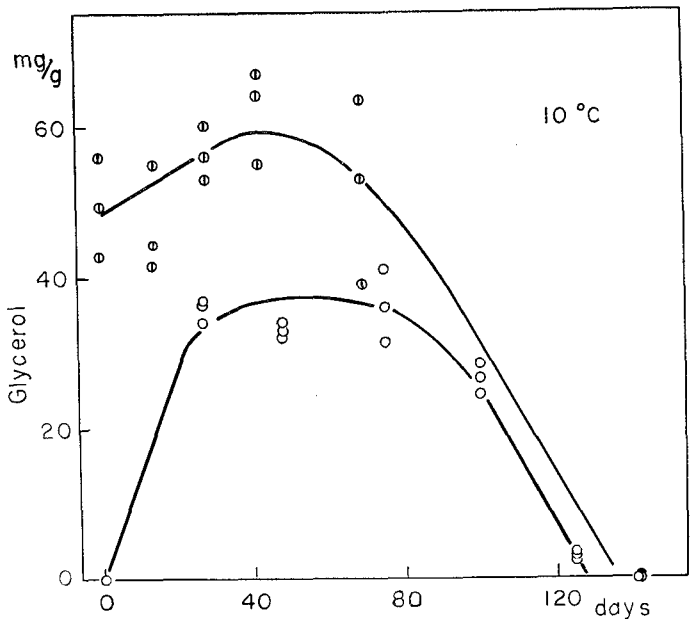

Fig. 8. Effect of injected glycerol on the change of glycerol content at 10°C. ⓘ: glycerol content in injected prepupae; ○: normal control. Prepupae employed were collected in September, 1962 and kept at 20°C until November, when they were used

one fifth of that in the normal prepupae and the highest content of glycerol produced in the insects was about one fourth of the normal maximum. However, it appeared that the rate and the time of glycerol disappearance were almost the same in both groups of prepupae.

The behavior of glycerol injected into diapausing prepupae was also observed at 20°C, at which temperature practically no insects were released from diapause for a long time. As a result of this experiment, about 40 mg/g of glycerol injected into the prepupae mostly disappeared within about 70 days

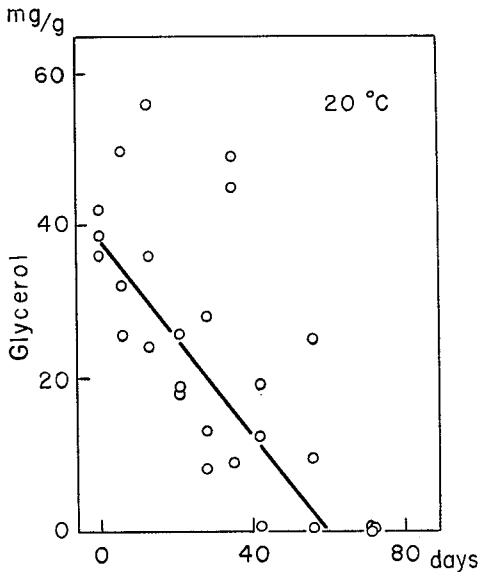

Fig. 9. Change in the amount of glycerol injected into prepupae at 20°C. Prepupae employed were collected in September, 1962 and kept at 20°C until November, when they were used

after injection (Fig. 9). However, the rate of disappearance of glycerol in diapausing prepupae was considerably slower than that in post-diapausing prepupae. In the latter about 30 mg/g of glycerol rapidly disappeared within about 20 days at 20°C (Fig. 2). This suggests that a reaction system for glycerol disappearance exists also in a diapausing prepupa and thus the termination of diapause does not seem to be a necessary condition for disappearance of glycerol in the insect.

Observation of injected glycerol was also done using prepupae kept at 0°C. Although almost all the prepupae died soon after the glycerol injection at this temperature, the injected glycerol seemed to be scarcely if at all decreased for a long time at 0°C. The results presented in Fig. 7 also support this view.

5 *Effect of some reagents*

In order to get some clue to explain the mechanism of glycerol metabolism, RINGER'S solution containing one of the reagents which may inhibit some reactions in the insect was injected into prepupae, which had been kept at 20°C. After the injection of these reagents the prepupae were kept at 10°C to observe the glycerol behavior in the insects. The concentration of the reagents was 1/50 M, 1/5 M and 1/50 M for potassium cyanide, sodium fluoride

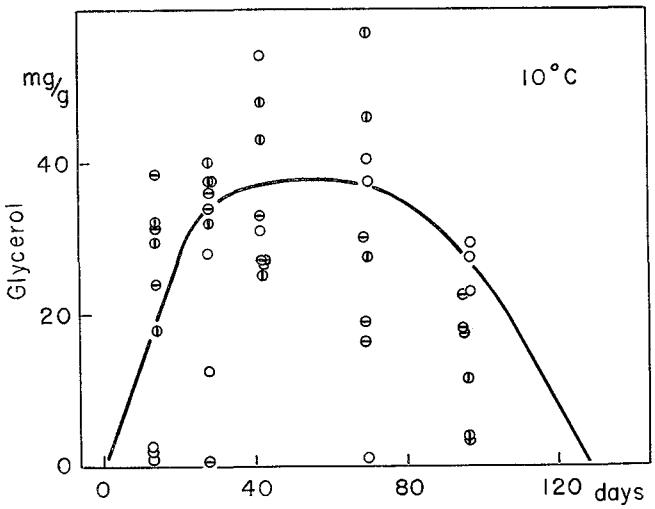

Fig. 10. Effect of some reagents on the change of glycerol content at 10°C. ⊕: KCN; ○: NaF; ⊖: Iodoacetate. Solid curve denotes glycerol behavior in uninjected control insects. Prepupae employed were collected in September, 1962 and kept at 20°C until November, when they were used

and monoiodoacetic acid respectively. The amount of injected solution was 0.02 to 0.03 ml per gram of fresh body weight. The changes in the glycerol content within these prepupae are given in Fig. 10. No clear results were obtained from these experiments concerning the variation of glycerol content. However, it was to be noted that the maximum level of the glycerol content in the prepupae in which these reagents were injected was almost the same as in normal uninjected ones. Therefore, these reagents seem to have no effect on the glycerol formation in the insect.

Some other reagents were also administrated to the prepupae in the same manner. As a result of this experiment 2,4-dinitrophenol, methylene blue, and potassium ferricyanide were observed to have no effect on the accumulation of glycerol. In addition fructose-1,6-diphosphate, a possible intermediate for glycerol metabolism, was injected into prepupae but that also produced no effect on the glycerol formation in the insects.

Although the data obtained in the above-mentioned experiments are too little to get any clue as to the mechanism of glycerol metabolism it seems notable that many injected reagents exhibit scarecely any effect on glycerol metabolism in the insect. Probably, the inhibition or other effects of the reagents may not be apparent under the present experimental conditions. The

explanation of the results will remain uncertain until further experiments are performed.

6 *Diapause and glycerol formation*

In Fig. 2 and Fig. 3 the seasonal variation in the intensity of prepupal diapause is shown. The intensity of prepupal diapause appeared to be closely related with the glycerol increase. In the 1959–1960 group of prepupae, the release from diapause proceeded as the glycerol content increased. In the middle of October, when the glycerol content began to increase rapidly, only 20 per cent of prepupae had been released from diapause; by the middle of November, 80 per cent of prepupae had been released, and then in the middle of December, when the glycerol content came up to maximum level, the prepupal diapause had already terminated (Fig. 2). However, it is to be noted that until the end of March at least, no decrease of glycerol content was observed in these prepupae.

In the 1960–1961 group, too, almost the same results as in the 1959–1960 group were obtained, although the termination of diapause in the former group was somewhat prolonged (Fig. 3). All of the prepupae, kept outdoors as well as at 10°C, were released from diapause within about 4 months from the spinning of cocoons. In the prepupae kept at 10°C the glycerol content began to decrease from the beginning of January, when the prepupal diapause had msotly terminated. In this case, however, no morphological development to pupa was observed in the prepupae even after their glycerol had already disappeared. The prepupae transferred to 10°C after being kept at 20°C for 50 days, were also completely released from diapause with in 75 days. In this case too, the glycerol content began to decrease by this time[24].

From the results mentioned above, it appears that glycerol accumulation in the prepupae may be accompanied by the termination of their diapause. However, as previously mentioned, it is impossible in the present experiment to know both intensity of diapause and the amounts of glycerol in the same individual insect. Therefore, the fact that the termination of diapause and the incerase of glycerol proceed simultaneously in a group of prepupae does not necessarily mean that every insect in which glycerol has reached a certain amount has terminated its diapause. To make the relation between diapause and glycerol metabolism clear, the behavior of glycerol in ligated prepupae was examined.

Since the hormone which causes the metamorphosis for pupation has been known to be secreted from the prothoracic gland by the stimulation of the brain hormone in some lepidopterous insects[25-27], a ligated abdomen separated

from a thorax is reasonably supposed to remain in the state of diapause regardless of the environmental temperature. The isolated abdomen without head and thorax was used for glycerol analysis in ligated prepupae. As a result of this experiment, the glycerol content in ligated prepupae at 10°C increased and decreased in the same manner as in the normal control (Fig. 11). It appears, therefore, that the termination of diapause is not required for the accumulation and disappearance of glycerol at 10°C, as far as diapause in an

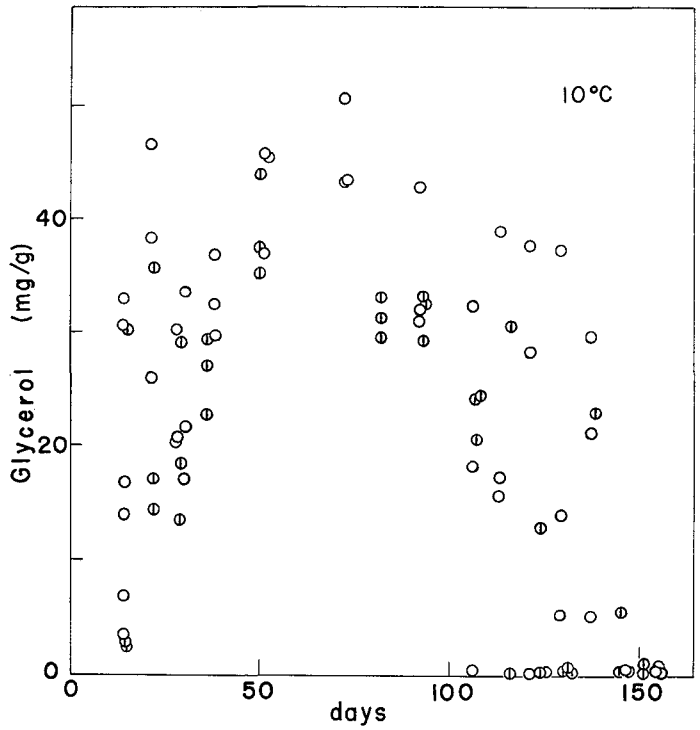

Fig. 11. Change in glycerol content in the ligated prepupae at 10°C. ⓘ: ligated prepupae; ○: normal control. Prepupae employed were collected in September, 1963 and kept at 20°C until November, when they were used

isolated abdomen is considered to be due simply to the absence of the growth factor. In this connection, whether or not pupal development did really take place in the isolated abdomen of ligated prepupae was examined. The ligated prepupae, after being kept at 10°C for about 6 months, were transferred to 20°C to observe further development. Even after two months or more at 20°C, morphological changes did not occur at all in the abdomen of the ligated insects,

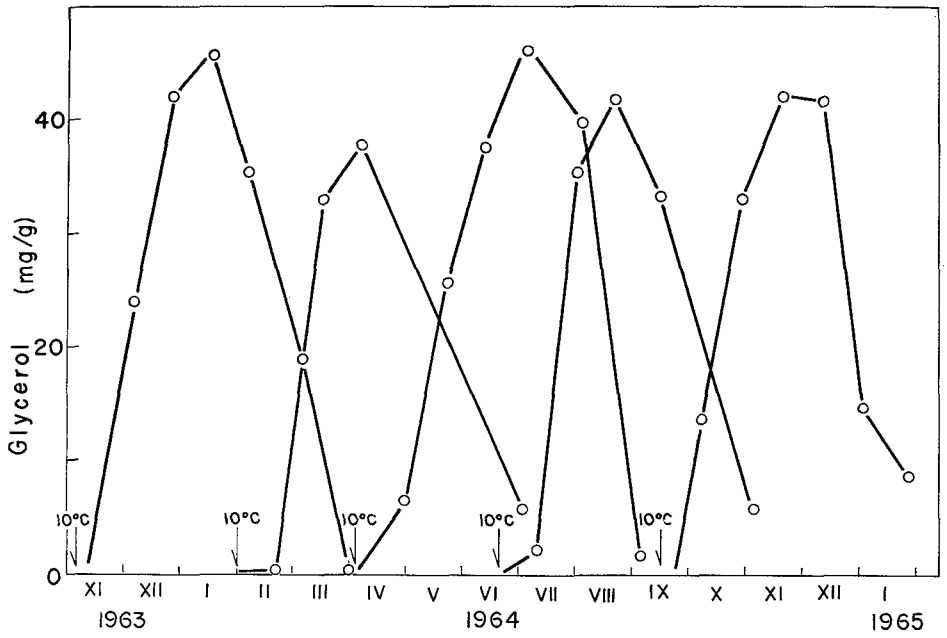

Fig. 12. Changes in glycerol content in the prepupae transferred to 10°C after a long period of storage at 20°C. Arrows show the time of transference to 10°C from 20°C. Prepupae were collected in September, 1963 and kept at 20°C until they were transferred to 10°C

but it did in the head and the thorax of the same individual insects (Fig. 1, d, e).

Whether diapausing prepupae have the capacity for glycerol formation whenever they are transferred to 10°C was the next problem to be examined. As mentioned before, prepupal diapause in the insect scarcely terminated for long period of time, so far as they were kept at 20°C. Using these prepupae kept at 20°C for a long time the glycerol content was determined after they were transferred to 10°C. It was revealed that whenever these prepupae were transferred to 10°C from 20°C, glycerol increased and decreased in almost the same manner, even after being kept at 20°C for a full year (Fig. 12). This suggests that the as long as they remain in diapause, they maintain the capacity for glycerol formation.

At a constant temperature of 0°C, on the other hand, the prepupal diapause completely terminated in the prepupae after 120 days[28,29]. When such prepupae were transferred to 10°C from 0°C, there occurred no appreciable glycerol increase[29]. In the 1960–1961 group of prepupae, however, the termination of prepupal diapause proceeded very slowly at 0°C. Only about 30 per cent of the diapausing prepupae were set free from diapause after they had

been kept at 0°C for 100 days[24]. The reason underlying the difference is not yet clear.

IV. Discussion

CHINO demonstrated in eggs of *Bombyx mori* that sorbitol and glycerol were formed from glycogen, and that the latter was later resynthesized from these polyhydric alcohols[3]. WYATT and MEYER also suggested that glycogen was presumably the chief source of glycerol in pupae of *Hyalophora cecropia*, although glycogen had not been determined in their experiment[4]. In the prepupae of *Monema flavescens*, too, glycerol is assumably formed from glycogen during the autumn and it is reconverted to glycogen in the following spring (Fig. 2 and 3). However the increase of glycerol is slightly less than the decrease of glycogen. This slight difference may be explained as a result of formation of fat during autumn. TAKEDA and HUKUSIMA reported that when glycogen decreased during the overwintering period, fat increased in the prepupae of *M. flavescens*[30].

CHINO indicated a tentative scheme for the formation of sorbitol and glycerol from glycogen[3], and reported the presence of polyol dehydrogenases responsible for the formation of these polyols in the eggs of the silkworm[31]. Later he found phosphatase (s) which might split both sorbitol-6-phosphate and α-glycerol phosphate[32]. It was also found that the prepupae of the slug caterpillar contained phosphorylase[33], aldolase[33] and phosphatase[34]. Although only a few enzymes which may possibly be involved in the glycerol metabolism have been found in this prepupae, it appears that the change in the glycerol content presumably occurs along the same possible pathway presented by CHINO.

In non-diapausing insects, so far found to have natural glycerol, repeated increase and decrease in the glycerol content may occur at any time throughout a year depending on the changes in environmental temperature. This was shown first by DUBACH *et. al.* in the black carpenter ant, *Camponotus pensylvanicus*[35]. By keeping the ants for about 6 days at 0°C to 5°C, glycerol was found to be produced in their tissue. After about three days rearing at 20°C to 25°C, glycerol could no longer be detected in them. Similar changes were observed in detail by TANNO in the carpenter ant, *Camponotus obscuripes*[12]. By keeping the ants at 0°C, glycerol increased in the insect at the rate of about 1.1 mg/g/day for more than 40 days. These ants completely lost their glycerol after being reared at 25°C for 10 days. Moreover, at temperatures above 5°C, glycerol was scarcely observed to increase in this insect[12]. SØMME also observed in non-diapausing larvae of the mountain pine beetle, *Dendroctonus monticolae*, that an increase and a decrease in glycerol content

resulted from storage at −5°C and 0°C to 5°C respectively[36]. According to these results, the changes in glycerol content seem to be exclusively controlled by environmental temperature in some non-diapausing insects, although there are differences among them in the effective temperature required for the control of glycerol behavior.

In diapausing insects, on the other hand, the increase and decrease in glycerol content does not seem to depend only on the effect of environmental temperatures. The accumulation of glycerol in these insects was observed to begin usually during the autumn in a rather wide range of temperature, though the rate of increase in the glycerol content varied with temperatures to which they were exposed. In the eggs of *Bombyx mori*, the increase of glycerol content was observed at 5° and 25°C[3]; in the eggs of *Pterocomma smithia*, at −5°, 0°, 5° and 20°C[36]; in the eggs of *Acrolita naevana*, at 0° and 20°C[37]; in the pupae of *Hyalophora cecropia*, at 6° and 25°C[4]; in the pupae of *Papilio machaon*, at −5°, 0°, 10° and 20°C[9]; in the larvae of *Eurosta solidaginis*, at 5°C but no change at −5° and 20°C[36]. Unlike these insects, however, in the prepupae of *Monema flavescens* a remarkable temperature-induced increase and decrease of glycerol content were observed at 10°C and 20°C respectively, unless they remained too long at 10°C. Glycerol accumulated at 10°C in the prepupae; transferring them to 20°C, its content decreased; returning to 10°C, it again accumulated (Figs. 4, 5 and 6). SØMME also reported similar changes in the glycerol content in diapausing larvae of the pea moth, *Laspeyresia strobilella*[37]. In the larvae of this insect placed at 20°C, all glycerol disappeared and if these larve were transferred to 0°C or −4°C, glycerol again accumulated.

The decrease of glycerol content in diapausing eggs of insects appears to be associated directly with the termination of diapause. On the other hand, in diapausing larvae or pupae the disappearance of glycerol has been observed to be associated with the initiation of post-diapause development. In the case of the slug caterpillar, *Monema flavescens*, too, normally overwintered prepupae in spring rapidly lost their glycerol as soon as they were exposed to 20°C with the simultaneous initiation of post-diapause development (Fig. 2). Besides, the prepupae of this species were also able to decrease their glycerol content even in the diapause stage if they were transferred to 20°C (Fig. 4). When glycerol was injected into the diapausing prepupae at 20°C, at which temperature diapause never terminated in most of these insects, it disappeared completely within about 70 days after injection (Fig. 9), although the rate of their decrease was very slow as compared with that in the post-diapausing insects transferred to 20°C (Fig. 2). Moreover, in the prepupae transferred to 10°C from outdoors

at the beginning of March, when the glycerol level is still at a maximum, the glycerol content begins to decrease only after about two weeks, in spite of the fact that their diapause has already terminated (Fig. 3). These results suggest that the termination of diapause is not necessarily required for the disappearance of glycerol in the prepupa of the slug caterpillar, although there is no question that glycerol easily decreases in post-diapausing prepupae of this insect unless the environmental temperature is too low.

CHINO postulated that the metabolism of sugar alcohols in the eggs of *Bombyx mori* might be related to their electron transport system and he tested this assumption, but his expected results have not yet been obtained[38]. When they first discovered glycerol in the pupa of *Hyalophora cecropia*, WYATT and MEYER also supposed that the decline in the cytochromes, by which respiration in the diapausing insect was limited, could lead to an increased ratio of DPNH to DPN and to reduced effective activity of the particulate α-glycerophosphate oxidase. Both of these effects would favour accumulation of α-glycerophosphate, which could presumably be hydrolyzed to glycerol[4]. But he suggested later that the accumulation of glycerol in diapausing pupae of *H. cecropia* might not be due to impeded electron transfer, since α-glycerophosphate was more abundant in actively developing stages of the insect than during diapause[39]. In overwintering prepupae of *M. flavescens* a remarkable drop in oxygen uptake was observed by SHINOZAKI even at 20°C[40]. Such a drop in oxygen uptake is generally regarded to be associated with diapause[41,42]. However, glycerol accumulates significantly in this insect only at about 10°C but not at 20°C. It appears, therefore, that the formation of glycerol in this prepupa may not be directly attributable to the blocking of the electron transport system.

SALT suggested that the relation of glycerol formation to diapause appeared to be a coincidence arising from concurrent timing[8]. However, except the case of the carpenter ants and a pine beetle mentioned before, all the insects which have so far been found to have glycerol are invariably in diapause or post-diapause. The physiological state of the diapausing insect seems, therefore, to provide some physicochemical conditions favourable for the production of glycerol. However, under natural atmospheric conditions no glycerol accumulates in the slug caterpillar as soon as it enters diapause, only when the atmospheric temperature has dropped to around 10°C does glycerol rapidly increase (Fig. 2). Besides, even at 10°C, the most favourable temperature condition for glycerol formation, glycerol dose not begin to increase usually until more than two weeks have passed after the diapausing insect has been transferred from 20°C to 10°C. These results suggest that some process which

proceeds at about 10°C may precede glycerol formation. Since glycerol behavior is entirely similar in ligated insects to normal diapausing ones, the temperature dependent process just suggested should occur in diapausing insect tissue without any supply of hormone-like substances from the prothoracic gland or brain. On the other hand, such an assumed process is supposed to occur under a certain physiological condition favourable to the termination of diapause, since at 10°C prepupae of the slug caterpillar can be most readily set free from their diapause.

An interesting fact which may be involved in the above-mentioned process has been found in the pupae of *Hyalophora cecropia* by GILBERT. He states "the detection of hormone in newly pupated animals but not in pupae chilled for 6 months indicates that juvenile hormone originally present is inactivated during prolonged chilling, and that the corpora allata are not activated during the animal's storage at low temperature. This agrees with the previous finding that the pupa can inactivate injected hormone even at low temperatures"[43].

Let us consider now two reaction systems for glycerol formation and disappearance, both assumably exist in a diapausing prepupae of the slug caterpillar. These systems are assumed to be different from each other in some reactions, in other words, the production and disappearance of glycerol in the insect are not conducted by the same reaction system though some of the enzymes are shared by both the opposing processes. Therefore, the reaction rates in the two opposite directions may be different even under the same physiological conditions. An application of the effect of juvenile hormone (or any other factor induced by juvenile hormone) tentatively supposed to interfere in these two reaction system may provide a possible explanation for the glycerol behavior in the slug caterpillar, although such an effect of juvenile hormone on glycerol metabolism in the insect has not yet been studied. FUKAYA and MITSUHASHI observed that corpora allata in overwintering larvae of the rice stem borer was continuously active in producing juvenile hormone until the termination of diapause[44]. The same will perhaps be true in the case of the prepupae of the slug caterpillar kept at 20°C from autumn, since at this temperature they can hardly be set free from diapause. If juvenile hormone existing in these prepupae may inhibit the assumed reaction systems for glycerol metabolism, the results in the present experiments will possibly be explained. The relation of activity of these reaction systems to the intensity of the inhibitory factor, or concentration of juvenile hormone, tentatively proposed in the diapausing prepupae is presented in Fig. 13. In this figure, the activities of the reaction systems for glycerol formation and disappearance are expressed by a broken and a solid line respectively. When the inhibitory

factor is present in sufficient amounts, glycerol disappearance somewhat exceeds glycerol formation in activity. With the gradual decrease of the inhibitory factor in the insect the two lines in Fig. 13 cross each other resulting in the excess of glycerol formation over glycerol disappearance. However, as the inhibitory factor decreases, these two lines come nearly in contact with each other. Thus the two reaction systems gradually become equal in activity. Finally, these two lines cross again, since the original activity without any

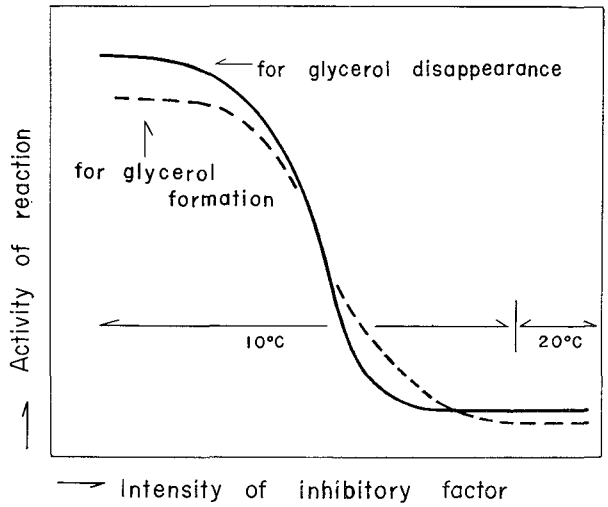

Fig. 13. Relation of activity of glycerol metabolism to intensity of inhibitory factor tentatively proposed in the slug caterpillar. Solid and broken curves denote the change of rate of the raction systems for glycerol disappearance and formation respectively. These curves indicate that accumulation and disappearance of glycerol depend upon the relative rate of both reaction ststems. Since the inhibitory factor is sufficiently present, glycerol can never be formed but only decreases at 20°C. With the gradual decrease of inhibitory factor at 10°C, glycerol accumulates at first and then keeps a maximum level for several weeks and finally disappears completely in the insect. For the details see text

inhibitory factor is assumed to be less in the system for glycerol formation than in that for glycerol disappearance.

As long as diapause persists at 20°C, juvenile hormone remains in the insect at a sufficient level to inhibit both reactions for glycerol formation and disappearance to some extent. Even under such conditions, the activity of the reaction system for glycerol disappearance exceeds that for glycerol formation. This results in difficulty for the glycerol accumulation process. In

the case of prepupae possessing some amounts of glycerol which have already been produced during a previous treatment, they gradually lose glycerol by being transferred to 20°C. The observation at 10°C where glycerol in the insect remarkably increases first, then remains at a maximum level for about seven weeks, and finally disappears completely, will be also interpreted as follows: At this temperature juvenile hormone only gradually decreased to a certain level, its inhibitory effect becomes so small that the activity of the reaction system for glycerol formation exceeds that for glycerol disappearance (see Fig. 13). Thus glycerol accumulates in the insect. In the case of the insect transferred from 20°C to 10°C, it will be some time until juvenile hormone decreases to the level just mentioned. This is perhaps the reason why glycerol never increases just after the transfer of the insect. As juvenile hormone decreases, these two reaction systems gradually become equal in activity (see Fig. 13). This results in the maintenance of a maximum level in the glycerol content for some time. Such a relation in activity between two reaction systems continues until juvenile hormone has almost disappeared in the insect when the relation again reverses (Fig. 13) and glycerol begins to decrease in the insect. In such a case, the inhibitory factor remains insignificant in the insect; the rate of glycerol disappearance is, therefore, expected to be high. This results in an easier decrease of glycerol content in the insect at 10°C than in the diapausing insect at 20°C (see Figs. 3 and 9).

Glycerol behavior in the prepupae transferred from 10°C to 0°C and kept at 0°C for a long time (Fig. 7) may possibly be interpreted by the same view as described above. If a prepupa, which is producing glycerol at 10°C, is transferred to 0°C during the process of reaching a maximum glycerol level, it still has a certain amount of inhibitory factor or juvenile hormone. The content of glycerol in such an insect will remain nearly constant since the disappearance of the inhibitory factor will reasonably take a long time. On the other hand, when the insect is transferred from 10°C to 0°C after its glycerol content has reached a maximum level, it will retain nearly the same level of glycerol content for some time until the inhibitory factor has slowly reduced to so small an amount that the glycerol decrease in the insect becomes appreciable (see Fig. 13). The apparent slowdown in the rate of glycerol decrease in the insect kept at 0°C will be reasonably explained by a simple temperature effect.

Although some of the glycerol behavior in the slug caterpillar can be explained by the assumed character of the reaction systems in the insect, detailed interpretation of glycerol metabolism seems to be very difficult, since the explanation applied deals with only two reaction systems in a whole insect which

is supposed to be an extremely complicated balanced system including a number of processes. At present the balance in enzyme activities, level of coenzymes, distribution of substrates in the insect tissue and other properties, which may be involved in glycerol metabolism, still remain uncertain. When the knowledge about these problem has been accumulated, a better explanation for glycerol metabolism in the insect will be possible.

Summary and Conclusion

In order to clarify various properties in the natural occurrence of glycerol and factors concerning the control of glycerol metabolism in the insect, the behavior of glycerol contained in the overwintering prepupae of the slug caterpillar, *Monema flavescens*, was examined.

Seasonal changes of glycerol and glycogen was observed in the prepupae. No glycerol was produced in the insects for about three weeks after the spinning of their cocoons. Glycerol began to increase in the middle of October and reached almost a maximum after 30 to 40 days. The maximum amount of glycerol was usually about 40 milligrams per gram of fresh body weight. The maximum level of glycerol was held until the middle of April in the following spring, but by the middle of May most glycerol had disappeared. On the other hand, the glycogen content increased during the 25 days just after the spinning of cocoons and thereafter decreased from the beginning of October. It reached a minimum in the middle of November. The minimum level of glycogen was maintained until the middle of April in the following spring, but thereafter increased.

The effect of temperature on glycerol metabolism was examined at 0°C, 10°C and 20°C. At a constant temperature of 0°C and 20°C glycerol scarcely accumulated in the insect. On the other hand, at a constant temperature of 10°C glycerol first appeared in the prepupae in small amounts after about two weeks and reached a maximum within 40 days. The maximum level was held during the period of 40 to 50 days, and then glycerol almost disappeared after about 40 days. In this case the accumulation and disappearance of glycerol coincided with the decrease and increase of glycogen content.

To obtain further evidence for the temperature dependency of glycerol metabolism, the behavior of glycerol with environmental temperature was observed in the prepupae. In the prepupae transferred to 10°C and 20°C from outdoors in early spring, when the average atmospheric temperature was yet below zero, glycerol almost disappeared after about 40 days and 20 days respectively.

By using prepupae reared at 20°C from the beginning of autumn, the following series of experiments was done. After two weeks' storage at 10°C, when glycerol had been produced, the prepupae were transferred to 20°C, where the glycerol content slowly decreased. The prepupae were then returned to 10°C, where an increase followed by a decrease in the glycerol content was observed, although the maximum level and the time of disappearance of glycerol were different from each other according to the previous treatments.

After two weeks' storage at 10°C, the prepupae were then transferred to 0°C, at which temperature glycerol ceased to increase. When the prepupae were returned to 10°C after three weeks at 0°C, glycerol began to increase immediately. However, if the prepupae had been kept at 0°C for a long period of time, the glycerol content slowly decreased. In this case, the rate of decrease was greater in the prepupae previously kept for 9 weeks at 10°C than in the prepupae previously kept at 10°C for three weeks.

When the environmental temperature was repeatedly alternated (for a week at 10°C and then for three weeks at 20°C), clear temperature dependency of glycerol behavior was not observed. Moreover, previous alternation in temperature had hardly any effect on the behavior of glycerol in the prepupae during final treatment at 10°C.

At a constant temperature of 10°C, the injection of glycerol (ca. 50 mg/g) into the diapausing prepupae inhibited both the rate of accumulation and the maximum amount of glycerol. At a constant temperature of 20°C, glycerol (ca. 40 mg/g) injected into diapausing prepupae mostly disappeared within about 70 days after injection. The injection of reagents, such as potassium cyanide, sodium fluoride, iodoacetate and others produced no effect on glycerol formation and disappearance in the insects at 10°C.

To make the relation between the termination of diapause and glycerol metabolism clear, the behavior of glycerol in ligated prepupae was examined. As a result of this experiment, the glycerol behavior in ligated prepupae at 10°C was observed to occur in the same manner as in the normal control. The result seems to suggest that the termination of diapause is not required for the increase and decrease of glycerol at 10°C, as far as diapause in an ligated abdomen is considered to be due simply to the absence of the growth factor. It was observed that as long as the prepupae remained in diapause, they maintained the capacity for glycerol formation for a full year at least.

These results presented above seem to indicate that glycerol is assumably formed from glycogen and it is reconverted to glycogen. One of the most important factors is controlling the glycerol metabolism is temperature. A temperature of 10°C is probably optimum for causing glycerol formation in

the prepupae; practically no glycerol is produced at 20°C and 0°C. The formation of glycerol in the prepupae may not be directly attributable to diapause, although diapause seems to provide some physiological conditions favourable for the formation of glycerol. The termination of diapause is not necessarily required for the disappearance of glycerol in the prepupae.

It may be concluded from these results that the behavior of glycerol observed in the overwintering prepupae of the slug caterpillar is not directly associated with the steady physicochemical state characteristic of diapausing insect, but with a certain process which may easily occur probably in the diapausing prepupae placed at 10°C. On the basis of the above mentioned assumption, a tentative explanation for the behavior of glycerol in the insect was made.

Acknowledgements

The author is much indebted to Prof. K. AOKI of Tohoku University and Prof. E. ASAHINA of the Institute of Low Temperature Science for their interest and encouragement in this work and their valuable criticism of the manuscript. His thanks are also due to colleagues of the Biological Section in the Institute of Low Temperature Science for their invaluable help in various ways.

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"*Low Temp. Sci.*" in the following is the abbreviation for "Low Temperature Science", a scientific publication written in Japanese with English summary, issued by the Institue of Low Temperature Science, Hokkaido University, Sapporo, Japan.

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