Öocyte Age and Glycogen Synthesis in Pupal Ovaries
of the Silkworm, *Bombyx mori* L.
(Lepidoptera : Bombycidae)¹

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The ovarioles of silkworm pupae comprise many öocytes showing successive development in sequence with the largest one at the posterior end of each ovariole. The approximate weight of an öocyte can be indicated as an index to öocyte age on which glycogen synthesis, as well as protein accumulation, is closely dependent. The glycogen synthesizing activities are most striking during the few days when pupal öocytes start to grow. Such activity was observed 4 days after pupation in about 20 öocytes lying at the posterior end of each ovariole. The accumulation of these two substances aforementioned was completed within 2 or 3 days, starting with the posterior öocytes and going on towards the terminal of each ovariole.

INTRODUCTION

From previous studies on the mode of action of the diapause hormone, it has been proved that the physiological age of pupal ovaries has a close relation to the hormone action (HASEGAWA, 1963; YAMASHITA and HASEGAWA, 1966 a). When Cu^{++} or UO_2^{++} was injected into silkworm pupae, the öocytes degenerated by the action of the former one and the “diapause” öocytes were converted into “non-diapause” by the latter. These reactions occurred at different positions of each ovariole according to the age of the treated pupae (HASEGAWA, 1947 b, c).

From these observations, it is likely that the öocytes in silkworm pupae respond to the diapause hormone and to various chemicals during a special stage in the course of pupal-adult development. We tried without success to determine this stage histochemically. Ovarioles of *Bombyx* pupae consist of many öocytes showing successive development in sequence with the fastest at the posterior end of each ovariole and without clear-cut differences between neighbouring öocytes (HASEGAWA, 1943; OZAWA, 1959), the same as *Hyalophora cecropia* (TELFER and RUTBERG, 1960). The development of the öocytes at the posterior, middle and terminal parts of each ovariole is very different according to the pupal age. It is, therefore, necessary to pay attention to the developmental stages of the öocytes while physiological and biochemical studies are undertaken on the pupal ovaries of these insects.

Glycogen synthesis and protein accumulation in pupal ovaries differ according

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to the developmental age of each oöcyte. In this paper we have taken the approximate weight of the oöcyte as an index to its age and examined glycogen synthesis as well as protein accumulation from this standpoint.

MATERIALS AND METHODS

The silkworm used was a hybrid between Kinshu and Showa. The female pupae transformed uniformly into adults on the thirteenth day after pupation at 25°C.

In order to analyse biochemical events of oöcytes, it is desirable to collect a sufficient number of them at the same physiological stage. In silkworm ovaries of the polytrophic type, the descendants of each oogonium ultimately form complexes, consisting of an oöcyte and seven nurse cells. The oöcyte-nurse cell complex is surrounded by follicular epithelium, and oöcytes mature by nutrients supplied from nurse cells and follicular epithelia (MACHIDA, 1941; OTSUKI, 1965). In pharate adults where almost all oöcytes have matured and nurse cells have degenerated, many oöcytes locate in ovarian stalks after stripping off follicular epithelia. In this case we can easily collect mature oöcytes by disrupting ovarian stalks, but it is impossible to separate and collect developing oöcytes from nurse and follicle cells. As a matter of convenience, therefore, we call the oöcyte-nurse cell complex with follicular epithelium "oöcyte" in this paper, although recently POLLACK and TELFER (1969) named it "follicle".

The most convenient index to an oöcyte developmental stage may be its weight. As the weighing of individual, fragile, young oöcytes without chorion was difficult, the oöcyte weight was approximately calculated from averages of ovariole weight containing definite number of oöcytes. The paired ovaries of 10 pupae each consisting of 8 ovarioles were dissected and freed from other tissues in cold saline solution (0.75 per cent NaCl). Each ovariole of adults of Kinshu×Showa contains about 80 mature eggs. Every 20 oöcytes of successive development were collected together in one group starting from the posterior end of each pupal ovariole. Pupae were dissected at different ages and many groups were selected. The oöcytes in each group were considered to be belonging to almost the same developmental stage². For example, each ovariole of 8-day old pupae was divided into four groups; the first group consisted of 20 oöcytes from the first to 20th oöcyte in numerical position in the ovariole, the second group from 21st to 40th, the third group from 41st to 60th and the fourth group from 61st to the terminal of the ovariole. In the last group, therefore, we could not estimate the number and weight of the oöcytes. Pupal ovarioles younger than 2 days old contained too young oöcytes to be divided into groups so that the whole ovarioles were considered as one group. These procedures for one series of experiments were generally completed within one hour after dissection. Neither swelling nor shrinking of the oöcytes was visible until the homogenation of pooled samples. It happened that some ovarioles were 'injured during the dissection of 10 pupae. In such cases we could not recognize the order of the oöcytes, and the ovarioles

² Although, less the number of oöcytes in each group the more real oöcyte weight could be achieved, we selected 20 oöcytes in each group for convenience.

were discarded. The other intact ovarioles were grouped, and pooled samples were weighed gravimetrically and used for subsequent analyses.

Glycogen concentration in each group was determined by the sulphuric acid method (KEMP and KITS VAN HEIJNINGEN, 1954) as described previously (YAMASHITA and HASEGAWA, 1966 a, b). In addition, we performed the following two experiments to see the glycogenetic activities in the öocytes at different ages; (1) on the day of pupation female pupae were injected with glucose- ^{14}C (U) ($0.2 \mu\text{Ci}/0.01 \text{ ml}/\text{pupa}$) and the pupae were sacrificed every other day starting 4 days after the injections, (2) the same amount of the isotope was injected into each female pupa every two days after pupation and the pupae were killed after 6 hr pulse labelling. Ten pupae injected with the isotope were dissected in each experiment. Ovarioles were divided into groups as stated above, blotted on filter paper, weighed quickly and then homogenized in an all-glass homogenizer with 5 vol. of cold distilled water. Ten vol. of 80 per cent ethanol were added to the homogenate while stirring, the mixture being centrifuged at 3,000 rev./min for 5 min to separate glycogen from the ethanol-soluble sugars. The ethanol sediment was extracted twice with 5 per cent trichloroacetic acid in a refrigerator (5°C). The extract was added with 6 vol. of ethanol in order to reprecipitate glycogen. This precipitate was washed twice with 80 per cent ethanol, followed with successive washings of ethanol-ether (2:1) and ether. Glycogen precipitate was then dried and redissolved into 5 ml of distilled water. One ml of this solution was used for determination of the glycogen concentration by the sulphuric acid method using glucose as a standard. The remainder of the solution was dried onto a stainless planchet, and the radioactivity of this sample was determined by a windowless, gass-flow proportional counter.

Protein content in each group of ovarioles was determined by the method of LOWRY et al. (1951) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

It was assumed that the öocytes in one group were nearly identical in developmental age. Making an admission of this assumption, we calculated approximate weights of öocytes by dividing the total weight of each group by the number of öocytes in the group. The results are summarized in Table 1. As shown in this table, the ratios of the weight of group I to the sum of each group weight are more than 60 per cent at middle pupal ages, followed by successive decrement to 25 per cent. But the ratios of group II and III 6- and 8- day old are 20.9 per cent and 13.2 per cent, respectively. Then, the ratios in both groups increase to about 25 per cent at the end of pupal life. Such is the case with the ratio of group IV.

Approximate weight of one single 4-day old öocyte in group I is $129 \mu\text{g}$ and increases about 6-folds in the successive 2 days³. The increasing pattern of öocyte

³ As stated in MATERIALS AND METHODS, "öocyte" in this paper is tentatively called the öocyte-nurse cell complex surrounded with follicular epithelia. In young pupae, therefore, öocyte weight does not indicate the weight of developing öocyte alone. Öocyte weight of pharate adult, however, means matured öocyte weight with ovarian sheath which is negligible in weight.

Table 1. CHANGES IN APPROXIMATE WEIGHT OF OOCYTES AT EACH POSITION OF OVARIOLES DURING ADULT DEVELOPMENT

Pupal age in days	Grouping of oöcytes from the posterior end of ovarioles							
	1st to 20th (I)		21st to 40th (II)		41st to 60th (III)		Above 61st (IV)	
	Group wt./sum of each group wt. (%)	Weight per oöcyte (μ g)*	Group wt./sum of each group wt. (%)	Weight per oöcyte (μ g)*	Group wt./sum of each group wt. (%)	Weight per oöcyte (μ g)*	Group wt./sum of each group wt. (%)	
0	100.0†							
2	100.0†							
4	61.1	129	38.9†					
6	65.9	741	20.9	290	13.2†			
8	49.8	888	28.0	750	13.2	200	9.0†	
10	31.3	773	31.3	773	27.9	780	9.5†	
12	25.7	764	25.5	755	25.2	745	23.6†	

Silkworms used are Kinshu $\times$ Showa; each series of experiment consists of 10 pupae at same age; the order of oöcytes is in sequence with the oldest at the posterior end of ovarioles; grouping of ovarioles in this table applies to all other figures in this paper.

* Calculated approximately from dividing each group weight by the number of oöcytes included in the group; † measured with whole ovarioles of young pupae or with terminal parts of ovarioles consisting of numbers of small oöcytes.

Fig. 1. Protein concentration in each group of ovarioles (A) and per oöcyte in each group (B) during adult development. Roman numerals : each group of ovarioles; * and broken line : surveyed together without grouping (The same applies to all other figures in this paper).

weight observed in group I is also recognized in the second and third groups. These facts tell us that the development of young oöcytes is striking only during 2 days when they are about to develop and that the weight of oöcytes is approximately 740 to 760 μ g at the end of pupal life, early developing oöcytes in group I being a little heavier than the late ones in the other groups.

Protein concentration in each group (mg/g wet weight) and estimated protein content in oöcytes (μ g/oöcyte) in each group during pupal adult development are illustrated in Fig. 1. Accordingly, protein concentration per wet weight of each group (Fig. 1 A) is not markedly variable among the groups irrespective of pupal age. However, as shown in Fig. 1 B indicating protein content per oöcyte, oöcyte protein was accumulated in the second group 2 days later than in the first

one and the third group 2 more days later, and the öocyte protein content in each group increased abruptly only during 2 days. This is agreeable with the increasing pattern of öocyte weight shown in Table 1. These results demonstrate clearly that the öocytes mature rapidly through consecutive development from the posterior öocytes to the terminal of ovarioles.

Glycogen accumulates rapidly in pupal ovaries during pupal-adult development (YAMASHITA and HASEGAWA, 1964). To see when glycogen accumulation occurs actively in öoplasm⁴, glycogen content in each group was surveyed. The results are indicated in Fig. 2A. The first group containing early developing öocytes are

Fig. 2. Glycogen concentration in each group of ovarioles (A) and per öocyte in each group (B) during adult development.

actively synthesizing glycogen and reach the maximum level 6 days after pupation. The second and third groups follow the same pattern of glycogen accumulation as observed in group I, but the glycogen accumulation of each group is delayed by 2 days successively. When the pupae transform into adults, glycogen concentration in each group is nearly the same.

The state of this affair is documented more clearly by glycogen content per öocyte. The content per öocyte was calculated approximately by dividing the glycogen amount in each group by the number of öocytes included in the group (Fig. 2B). The glycogen content per öocyte in group I is about 2 µg at 4 days old pupal age, and during successive 2 days it reaches to maximum level of glycogen per öocyte about 15 µg. In groups II and III the glycogen amount per öocyte is less than 5 µg at the beginning but increases abruptly during the successive 2 days. When the pupae emerge 12 days after pupation each öocyte contains nearly the same amount of glycogen irrespective of its numerical position in the ovariole. From these results it is clear that the glycogen accumulation in öoplasm occurs rapidly during the short time of active development and the glycogen content at the end of the pupal life is the same in each öocyte.

To get further information on glycogen accumulation pattern in the öocytes of each group, 0.2 µCi of glucose-¹⁴C (U) was injected into each pupa on the day of pupation and the incorporation of the isotope into öocyte glycogen was surveyed every other day, from 4 days after the injection onwards (Fig. 3). As shown in Fig. 3A illustrating cpm/mg glycogen in each group, groups I and II

⁴ Ovary glycogen was detected histochemically in öoplasm but not in nurse and follicle cells (HASEGAWA, unpublished observation).

Fig. 3. Incorporation of glucose-¹⁴C into glycogen of each group of ovarioles (A) and per oöcyte in each group (B). Injections of the isotope, 0.2 μ Ci per pupa on the day of pupation.

took up much quantity of the labelled glucose into the glycogen fraction after 4-day labelling, especially the latter group. From 6-day pupal age onwards, the isotope incorporated into each group was low and not significantly different among each group.

High glycogenesis in groups I and II of 4-day old ovarioles would be interpreted as follows; on the day of pupation almost all the ovarioles are enveloped with ovarian sheath and the oöcytes in the ovarioles are very young, but a very small amount of glycogen is already present in a few posterior oöcytes. One or two days after pupation ovaries develop, the ovarioles are liberated from ovarian sheath and oöcytes grow at the expense of histolyzed materials derived from larval tissues. Labelled glucose injected on the day of pupation is firstly taken up into various tissues, such as blood trehalose, fat-body glycogen, ovary glycogen and so on one or two hr after the injections, besides some amount are expired as ¹⁴CO₂ (unpublished observations). Therefore, an aliquot of the isotope injected into fresh pupae is directly taken up into developing oöcytes in group I. Prospective oöcytes of group II are too young to synthesize glycogen at this stage, and a few days later they begin to synthesize it at the expense of materials originated from labelled larval tissues. As already indicated in Fig. 2, the glycogen contents in the 4-day old ovarioles, especially group II, are very low. This would be the reason for the extremely high specific activities per mg glycogen in groups I and II, particularly in the latter at that stage.

When labelled glucose is injected into fresh pupae, the isotope is at first distributed equally among tissues such as blood, midgut, and fat-body. Two and four days later, fat-body glycogen has highest radioactivity among tissues, and the activity becomes low rapidly at middle pupal ages when ovarian development proceeds vigorously (unpublished observations). This would interpret that specific activities in each group of ovarioles at 6-day old age are not markedly different among groups and are low in each group.

Total radioactivity of glycogen per oöcyte in each group, however, increases markedly during 2 days of development (Fig. 3B). At 8-day old stage the oöcytes of group I cease to accumulate glycogen and they may have accomplished

maturity, while the other groups are on their way to glycogen synthesis.

To test what stage of öocyte is the most active to synthesize glycogen, the following experiments were undertaken; 0.2 μ Ci of glucose- 14 C (U) was injected into each pupa every second day after pupation, and the pupae were sacrificed 6 hr after injections. The results are illustrated in Fig. 4. As shown in Fig. 4A

Fig. 4. Incorporation of glucose- 14 C into glycogen of each group of ovarioles (A) and per öocyte in each group (B) during 6 hr pulse labelling. Injections of the isotope, 0.2 μ Ci per pupa every second day after pupation; the pupae, sacrificed 6 hr after the injections.

indicating specific activity of each group, the maximum capabilities of glycogen synthesis in groups I, II, and III are 4, 6, and 8 days after pupation, respectively. Eight days after pupation, the glycogenesis in groups I and II is scarcely observed, but that in group III is very high. The fourth group, including terminal öocytes in the ovarioles, shows decreasing curve without any increase until adult emergence.

When pupal öocytes become mature, they strip off their follicle epithelia, which begins 3 or 4 days before adult emergence. The matured öocytes, as such, come down into ovarian stalk. These öocytes increase in number day by day, and almost all the matured ones exist in the ovarian stalks at adult emergence (HASEGAWA, 1943). On the other hand, the immature öocytes in pupal ovaries are more numerous than prospective mature ones and the surplus öocytes degenerate sooner or later. The degeneration also begins 3 or 4 days before adult emergence (HASEGAWA, 1943, 1947a). Groups I and II, from 8-day old ovarioles or 4 days before adult emergence, contain almost completed öocytes, while group III includes those under development. Some öocytes in group IV are on their way to maturity and some fatal to degeneration. Matured öocytes and degenerating ones cannot synthesize glycogen. The sequence of öocyte development in silkworm pupae, therefore, is reflected from the pattern of the glycogen synthesis. The state of this affair is also demonstrated by the incorporation of labelled glucose into each öocyte glycogen during 6 hr pulse (Fig. 4B). The 4-day old öocytes in group I take up a high amount of the isotope or synthesize glycogen actively and, 4 days later, take up no isotope or stop to synthesize glycogen. In the other groups the synthetic activity is postponed by 2 days in the order of groups II and III. In addition, we can see from this experiment the glycogenesis

in oöcytes is restricted by the stage peculiar to their physiological age.

From the present series of experiments it is disclosed that each oöcyte in pupal ovaries matures day by day from the posterior to the terminal of each ovariole passing through a special stage when glycogen synthesis takes place actively during a few days. But whether glycogen synthesis takes place mainly in oöplasm, nurse cells or in follicle cells remains to be proven in *Bombyx*, although in scorpion fly *Panorpa* with polytrophic ovarioles as well a probable site of glycogen synthesis is reported as periplasm (RAMAMURTY, 1968).

REFERENCES

- HASEGAWA, K. (1943) Studies on the development of oöcytes in the silkworm, *Bombyx mori*.
I. Ovarian development during pupal stages. *Bull. Seric. Exp. Sta.* **11** : 359-377.
- HASEGAWA, K. (1947 a) Studies on the development of oöcytes in the silkworm, *Bombyx mori*.
II. On the degenerated oöcytes. *Bull. Seric. Exp. Sta.* **12** : 481-492.
- HASEGAWA, K. (1947 b) Studies on the development of oöcytes in the silkworm, *Bombyx mori*.
III. The influence of some metallic ions, with special reference to Fe- and Cu- ion on the development of the oöcytes. *Bull. Seric. Exp. Sta.* **12** : 493-512.
- HASEGAWA, K. (1947 c) Studies on the voltinism in the silkworm, *Bombyx mori* L. I. *Bull. Seric. Exp. Sta.* **12** : 513-530.
- HASEGAWA, K. (1963) Studies on the mode of action of the diapause hormone in the silkworm, *Bombyx mori* L. I. The action of diapause hormone injected into pupae of different ages. *J. Exp. Biol.* **40** : 517-529.
- HASEGAWA, K. and O. YAMASHITA (1967) Control of metabolism in the silkworm pupal ovary by the diapause hormone. *J. Seric. Sci., Tokyo.* **36** : 297-300.
- KEMP, A. and A. J. M. KITS VAN HEIJNINGEN (1954) A colorimetric micromethod for the determination of glycogen in tissues. *Biochem. J.* **56** : 646-648.
- LOWRY, D. H., N. J. ROSEBROUGH, A. L. FARR, and R. L. RANDALL (1951) Protein measurement by the Folin phenol reagent. *J. Biol. Chem.* **176** : 367-388.
- MACHIDA, J. (1941) On the formation of yolk and the egg membrane in the domesticated silkworm, *Bombyx mori* L. *J. Col. Agri. Tokyo. Imp. Univ.* **15** : 83-131.
- OTSUKI, Y. (1965) Studies on the yolk formation in the silkworm, *Bombyx mori* L. An analysis of its mechanism revealed by the comparison between normal and abnormally small egg. *Bull. Fac. Tex. Fib. Kyoto Univ. Indust. Arts Tex. Fib.* **4** : 314-344.
- OZAWA, T. (1959) Studies on the development of the egg cells of the silkworm, with special concern to the stage variation by their arranged order in an ovariole. *J. Seric. Sci. Tokyo.* **28** : 211-219.
- POLLACK, S. B. and W. H. TELFER, (1969) RNA in Cecropia moth ovaries : site of synthesis, transport, and storage. *J. Exp. Zool.* **170** : 1-24.
- RAMAMURTY, P. S. (1968) Origin and distribution of glycogen during vitellogenesis of the scorpion fly, *Panorpa communis*. *J. Insect Physiol.* **14** : 1325-1330.
- TELFER, W. H. and L. D. RUTBERG (1960) The effect of blood protein depletion on the growth of the oöcytes in the Cecropia moth. *Biol. Bull.* **118** : 352-366.
- YAMASHITA, O. and K. HASEGAWA (1964) Studies on the mode of action of the diapause hormone in the silkworm, *Bombyx mori* L. IV. Effect of diapause hormone on the glycogen content in ovaries and the blood sugar level of silkworm pupae. *J. Seric. Sci. Tokyo* **33** : 407-416.
- YAMASHITA, O. and K. HASEGAWA (1966 a) The response of the pupal ovaries of the silkworm to the diapause hormone with special reference to their physiological age. *J. Insect Physiol.* **12** : 325-330.
- YAMASHITA, O. and K. HASEGAWA (1966 b) Further studies on the mode of action of the diapause hormone in the silkworm, *Bombyx mori* L. *J. Insect Physiol.* **12** : 957-962.