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Review

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Biochemical Changes during Embryonic Diapause in Domestic Silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae)

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Ecophysiologically diapause represents a syndrome of physiological and biochemical characteristics, all of which ensure survival during a long period of dormancy. Since, silkworm enters diapause as embryo at the early embryonic stage, the duration of egg life depends on the duration of embryonic diapause. The nature of diapause in silkworm, *Bombyx mori*, is primarily determined by genetic characters and endocrinological mechanisms, mediated by environmental factors such as temperature and photoperiod. Hibernating potency value besides nucleic acid and carbohydrate metabolism, production and utilization of sorbitol are also equally responsible for induction, initiation, determination, maintenance and termination of diapause. Embryonic diapause in *Bombyx mori*, induced by active secretion of sub-oesophageal ganglion is attributed to hormonal system and metabolic adjustment, which serves to bring about a new physiological state. Metabolic conversion of trehalose to glycogen at induction, glycogen to sorbitol at initiation and sorbitol to glycogen at termination of diapause is correlated and in each metabolic shift a key enzyme becomes active in response to hormonal and environmental stimulation. An attempt has been made in this review article to discuss briefly the nature of embryonic diapause, influence of various factors on diapause nature, hormonal mechanism of diapause besides biochemical composition of egg, nucleic acid and carbohydrate metabolism, production and utilization of sorbitol in relation to induction, determination, maintenance, initiation and termination of diapause in the silkworm, *Bombyx mori*.

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Introduction

The silkworms reared for commercial silk production includes *Bombyx mori, Antheraea pernyi, A. yamamai, A. mylitta, A. assama, A. proyeli* and *Philosamia cynthia ricini.* Among these species, the domesticated silkworm, *Bombyx mori* (L), is most widely used and the techniques for its rearing, seed production, maintenance, preservation and reeling etc. have been very well defined. Rapid development in sericulture industry has been achieved by the development of silkworm breeds using hybrid breeding and the expansion of rearing throughout the year by controlling embryogenesis (Yokoyama, 1973).

Biochemical diapause in insects is characterized by low metabolic rate and turnover of metabolites (Harvey, 1962), and the induction and termination of diapause are under the control of endocrinological mechanisms mediated by environmental stimuli (Wigglesworth, 1970).

In the silkworm, intense efforts have been made on the studies of diapause, cold storage and artificial hatching of eggs and appropriate techniques have been developed for obtaining hatchable eggs at any time of the year (Yamashita and Yaginuma, 1991).

Diapause, an arrested state of overt development, is a sophisticated biological phenomenon (Singh, 1989) and is characterized by diapause development physiogenesis analogous to morphogenesis which is a pre-requisite for resumption of development.

Various environmental, physiological, biochemical and metabolic changes are associated with the induction, initiation, maintenance, determination and termination of diapause (Yamashita *et al.*, 1975; Yaginuma and Yamashita, 1978; Irie and Yamashita, 1980; Yaginuma *et al.*, 1990) in the silkworm.

In this review article, an attempt has been made to highlight briefly the advances achieved in relation to diapause, physiological and biochemical studies on induction, determination, maintenance, initiation and termination of embryonic diapause in the silkworm.

I. Diapause

Pigmentation or colouration in insects is mainly due to melanin, pterine, pigments, ommochrome and carotenoid. In some insects colouration is closely correlated with diapause and colour pattern is sometimes used as an index to distinguish diapause from non-diapause. Among these pigments, ommochrome is well documented in relation to embryonic diapause (Yamashita and Hasegawa, 1985). Diapause development is under the control of endocrinological system mediated by environmental factors such as temperature, photoperiod, humidity etc. Hence, diapause is characterised by an active mechanism for adaptation to the adverse seasons.

Diapause can occur at embryonic (*Bombyx mori, Aedes aegypti*), larval (*Cydia pomonella, Gilpinia polytoma*), pupal (*Antheraea mylitta, Hyalophora cecropia*) or adult (*Eurydema* sp., *Leptinotarsa decemlineata)* reproductive stages of life cycle and the stages are characteristically fixed in each species (Table 1).

Nature of diapause

The nature of diapause in the silkworm is primarily determined by genetic character (Morohoshi, 1979). The insects having only one generation in a year (univoltine), embryonic diapause occurs in all generation regardless of environmental conditions, is known as "obligatory diapause", while those having two or more generations in a year (bivoltine or polyvoltine), diapause is expressed in the next generation when the mother receives specific stimuli from environment is termed "facultative diapause" (Thiele, 1973).

The univoltine forms of *B. mori* lay only hibernating (diapausing) eggs, which are also called "Kurodane eggs", and multivoltine forms lay only non-hibernating eggs, which are known as "Nemadane eggs", while the behaviour of bivoltine eggs are intermediate (Tazima, 1978). Bivoltine silkworm breeds lay non-hibernating eggs during first generation and hibernating eggs in the next generation, which hatches out in the following spring and thus producing only two generations in a year. Practically, univoltine and bivoltine silkworm breeds produce superior cocoons to those of multivoltines in both quality and quantity. Generally silkworms programmed to lay diapause eggs are superior to those that lay non-diapause eggs.

The diapause eggs of silkworms are dark brown (pigmented) due to presence of ommochrome formed in serosal cells, while non-diapause eggs are yellow / white due to lack of this pigment. Ommochrome is synthesized in developing embryos and other tissues during tryptophane metabolism (Inagami, 1955).

Tryptophane metabolism in insects has been studied extensively and the pathway from tryptophane to ommochrome has been reviewed in detail by Linzen (1974). In general, three major metabolites directly involved in the biosynthesis of the pigments are formyl-kynurenine, kynurenine and 3-hydroxy kynurenine. The corresponding enzymes responsible for their production are kynurenine-formamidase, kynureninase and kynurenine-3 hydroxylase. Tryptophane responsible for the formation of ommochrome has been reported to accumulate in eggs of *B. mori (*Koga and Osanai, 1967). The steps involved in the formation of ommochrome in the silkworm eggs from tryptophane is indicated below:

Hibernating potency value

Nagatomo (1953) assumed a series of three sex-linked alleles, Hs, Hs2 and hs and three pairs of autosomal genes

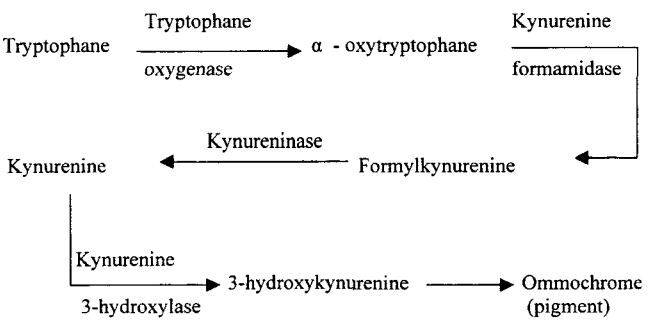

Table 1. Diapause nature in different insect species (modified after Singh, 1989)

Nature of diapause	Example
Embryonic diapause	*Bombyx mori, Melanoplus differentialis, Aedes aegypti, Aeschna mixta*
Larval diapause	*Cydia pomonella, Anax sp., Epistrophe bifasiata, Gilpinia polytoma, Cephus cinctus, Lucilia caesar*
Pupal diapause	*Antheraea pernyi, Hyalophora cecropia, Antheraea mylitta, Philosamia cynthia, Saturnia pavonia, Mimas tiliae*
Adult diapause	*Eurydema sp., Pterostichus sp., Leptinotarsa decemlineata, Musca autumnalis*

Table 2. Relationship between hibernating potency value and voltinism in silkworm (*Bombyx mori*) (source: Nagatomo, 1953)

Hibernating Potency value	Voltinism	Characteristics
0-1	Multivoltine	Always lay non-hibernating eggs.
2-4	Intermediate between multi and bivoltine	Lay some non-hibernating eggs at high incubation temperature.
5-7	Bivoltine	Lay non-hibernating eggs at low temperature and hibernating eggs at high temperature of incubation.
8-10	Intermediate between bivoltine and univoltine	Lay both hibernating and non-hibernating eggs at low incubation temperature.
11-12	Univoltine	Always lay hibernating eggs.

H1h1, H2h2 and H3h3 responsible for the hibernation and reported different kinds of voltinism in relation to "Hibernating potency value" (H. V.) (Table 2). He stated that eggs with 0 – 1 H. V. value always lay non-hibernating eggs (multivoltine) while those with 11 – 12 H. V. value will lay only hibernating eggs (univoltine). Bivoltine silkworm races have HV value in between 5 – 7. The silkworm eggs with HV value of 2 – 4 are intermediate between multivoltine and bivoltine and lays some non-hibernating eggs at high incubation temperature. Contrary to this, the silkworm eggs with HV value of 8 – 10 are intermediate between bivoltine and univoltine and lay both hibernating and non-hibernating eggs at low incubation temperature.

Change of diapause nature

In the silkworm diapause determination is almost maternal. That is, temperature and photoperiod are most efficient at the embryonic stage of previous generation (Table 3) and only supplemental in the post-embryonic stages.

The sensitive embryonic stages begin just after blastokinesis. Incubation of eggs of bivoltine strain at a high temperature results in the induction of diapause eggs in the next generation, and a low temperature incubation results in the production of non-diapause eggs (*c. f.*, Yokoyama, 1973). Incubation as low as 15°C causes production of non-diapause eggs in next generation, whereas diapause eggs are induced by incubation at 25°C. When eggs are incubated at intermediate temperature of 20°C, the developmental fate remains undetermined in the embryos. High temperature at younger larval stages and low temperature at later larval stages acts to induce diapause eggs (Tazima, 1978).

Photoperiod is one of the principal physical factors regulating diapause. In *B. mori*, egg diapause is regulated by photoperiod as well as temperature during the embryonic stage of the female and is completely independent of photoperiod during post-embryonic development. Thus, pho-

Table 3. Effects of temperature and photoperiod on egg diapause in bivoltine breeds of mulberry silkworm, *Bombyx mori*

Temperature and Photoperiod during incubation	Temperature°C/developmental stage		Resulting moths laying diapause and non-diapause eggs
	I-II	IV-pupal	
25°C			
Light	25 or 20	25	Diapause
Dark	25 or 20	25	Diapause
20°C	25 or 20	25 or 20	Diapause
Light	20	25	Diapause<<Nondiapause
Dark	25	25	Diapause < Nondiapause
	25	20	Diapause>>Nondiapause
15°C			
Light	20	25	Diapause < Nondiapause
	25	25	Diapause > Nondiapause
	25	20	Diapause>>Nondiapause
Dark	25 or 20	25 or 20	Non-diapause

Note: Light, 16 hrs light and 8 hrs dark; Dark, 16 hrs dark and 16 hrs light.

Table 4. Relationship between the conditions of incubation, rearing, mounting and diapausing character of the resultant eggs in bivoltine silkworm races (source: Tazima, 1978)

Development stage	Environmental conditions	Effect	Remarks
	Temperature		
	High	Diapausing	Whole eggs at 25°C or above
	Low	Non-diapausing	Whole eggs at 15°C or below
	Humidity		
	Humid	Diapausing	Humidity affects the voltinism only when the incuba-
During Incubation	Dry	Non-diapausing	tion temperature are at 15 – 25°C
	Light	Diapausing	Light (18 hours or more a day)
			Photoperiod affects only when incubated at 15 – 25°C
	Dark	Non-diapausing	In case of 12 hrs darkness a day.
Early larva	Temperature		
	High	Diapausing	Only when incubation temperature were at 15 – 25°C
	Low	Non-diapausing	
Late larva	Temperature		
	High	Non-diapausing	Only when incubation temperature were at 15 – 25°C
	Low	Diapausing	
Mounting and later	Temperature		
	High	Non-diapausing	Only when incubation temperature were at 15 – 25°C
	Low	Diapausing	

toperiod becomes effective in regulation of development only when eggs are incubated at an intermediate temperature. In these eggs, long photoperiod causes induction of diapause and short photoperiod non-arrested state of development (Table 4).

In silkworm, injection of uranyl nitrate (Hasagawa, 1957), quabain (Takeda and Hasegawa, 1975) and 5'-AMP (Suzuki *et al.*, 1978) into females destined to lay diapause eggs caused them to lay non-diapausing eggs. Converse was demonstrated by injection of KCl (Yoshitake, 1954) and quabain (Takeda and Hasegawa, 1976) into pupae of non-diapause type. But despite extensive studies, the mechanism of action of these chemicals remains unknown.

Termination of diapause

Exposure of silkworm diapause eggs to oxygen is one of the artificial methods (Okada, 1971) of termination of diapause. The exposure of diapause destine eggs to oxygen gas can prevent the expression of diapause (Sonobe *et al.*, 1979) and eggs resumed embryonic development while under an oxygen-deficient environment non-diapause eggs ceased their development. Oxygen is also indispensable for diapause development during chilling of diapause eggs (Ando, 1974). Diapause in silkworm eggs can be terminated to obtain effective hatchability by means of cold storage - chilling (hibernation schedule), hydrochlorisation (hot or cold acid treatment), and cold storage and hydrochlorisation (chilling and treatment).

The application of these methods depends on the programme of hatching desired. Chilling is one of the effective method of terminating diapause. Exposure of diapausing silkworm eggs to a temperature as low as 5°C over 60 days completely terminates diapause and embryogenesis resumes when these eggs are transferred at 25°C (Takami, 1969). Optimum temperature to break the embryonic diapause is in between 5 to 7.5°C. The required chilling duration to break the diapause depends on the time gap the eggs have been kept for aestivation at 25°C after oviposition. Various comprehensive hibernation schedules for preservation of bivoltine eggs for different durations to get the hatching at appropriate time have already been recommended and some of them are in-vogue (Tazima, 1978; Reddy and Samson, 1995; Manjula and Hurkadli, 1995, 1996; Dandin *et al.*, 2000) to meet the demand of seed supply throughout the year.

"HCl" treatment (hydrochlorisation) of silkworm diapause eggs has been the method of choice for blocking the diapause both at commercial and basic research laboratory level. There are two methods of HCl treatment. In the first method 20 – 24 hrs old oviposited eggs are soaked in HCl solution (specific gravity, 1.075 at 15°C) at 46.1°C for 5 minutes. This avoids the eggs to enter into diapause and hence when incubated at 25°C, larvae hatches in 10 – 11 days after treatment. In the second method, 20 – 24 hrs old oviposited eggs are soaked in HCl solution (specific

gravity, 1.10 at 10°C) at room temperature for 60 to 90 minutes. Sometimes, to block the diapause for longer period in order to get hatching at desired time, chilling followed by acid treatment is also used. In this method 48 hrs old eggs are first chilled at 5°C for more than 30 days and then soaked in HCl solution (specific gravity, 1.10 at 15°C) at 48°C for 5 minutes (Takami, 1969). This treatment causes diapause eggs to hatch at any desired time within a period of two months time after oviposition.

However, it is still an open question how hydrochlorisation blocks diapause. Park and Yoshitake (1970) suggested that HCl infiltrated into eggs impedes the embryonic protein synthesis system in yolk cells, which results in resumption of embryogenesis. Further, HCl treatment is reported to stimulate the activity of specific esterase isozyme (Kai and Nishi, 1976) and RNA synthetic activity (Kurata *et al.*, 1979) in treated diapause eggs. Activity of 'esterase A' increased dramatically within 30 minutes of HCl treatment. The esterase attains maximum activity before the eggs are competent to develop, and Kai *et al.* (1987) suggested that activation of 'esterase A' is a pre-requisite for resumption of development.

Table 5. Amino acid composition of diapause hormone -A (DH-A) and diapause hormone -B (DH-B) (after Yamashita and Hasegawa, 1985)

Amino acid	Diapause hormone-A	Diapause hormone-B
Lysine	0.3	1.2
Arginine	0.8	1.0
Aspartic acid	1.3	1.3
Threonine	1.0	0.9
Serine	1.0	1.5
Glutamic acid	2.1	2.2
Proline	3.0	2.4
Glycine	2.1	2.1
Alanine	2.2	2.1
Valine	1.7	1.0
Isoleucine	1.9	1.2
Leucine	3.0	3.0
Tyrosine	0.9	0.7
Phenylalanine	0.9	0.8
Tryptophane	–	1.0
Glucosamine	0.9	0.0
Galactosamine	0.9	0.0

Values represent mole ratio.

Hormonal control of diapause

In diapause, development is arrested at a specific stage of embryogenesis. Cell division is arrested and embryogenesis ceases immediately after formation of cephalic lobe and telson and sequential segmentation of mesoderm (Kitazawa *et al.*, 1963; Sonobe *et al.*, 1986). Yolk cells and yolk granule changes shape and their physical properties as a part of diapause process (Miya *et al.*, 1972) besides fine structure of some cellular organelles is adaptively modified (Okada, 1970a). The most stricking feature of the diapausing eggs is the dark colouration of the serosal cells due to the formation of ommochrome pigment (Koga and Osanai, 1967).

Of the endocrine system, sub-oesophageal ganglion (SG) has been reported to play very vital role in the induction of diapause. It is generally accepted that egg diapause of *B. mori* is induced by an active principle secreted from a pair of large neurosecretory cells located in SG (Fukuda and Takeuchi, 1967) and are under the control of four other neurosecretory cells (Park, 1973). This active principle is known as 'Diapause Hormone' (DH) (Yamashita and Hasegawa, 1985). Two forms of DH (DH-A and DH-B) have been identified, which are neuropeptides having molecular weight of 2,000 – 3,000 and consist of 12 – 14 amino acids and are responsible for induction of embryonic diapause in the silkworm (Isobe and Goto, 1980; Yamashita and Hasegawa, 1985; Yamashita, 1996). The DH-A molecules contain 14 amino acids and two amino sugars, while DH-B contains the same amino acids but no amino sugars (Table 5). This reveals that amino sugar component of DH-A is apparently not essential for hormonal activity (Kubota *et al.*, 1979). Katagiri *et al.* (2001) reported that DH plays multiple functions in directing the diapause-associated metabolism in the silkworm.

DH is actively syntesised and released from the SG of female pupae programmed to lay diapause eggs, but only trace secretion occurs in SG of pupae programmed to lay non-diapause eggs (Yamashita and Yaginuma, 1991). Substances that are DH-active appear to be produced in brain, corpora allata, corpora cardiaca and thoracic ganglion of silkworms, as well as in several other insects, because implantation of these organs into non-diapause type pupae induces diapause eggs just like injection of DH (Takeda, 1977a, 1977b). Sonobe *et al.* (1986) using a mutant strain (pnd) of the silkworm indicated that the parental genotype is required for expression of the embryonic diapause programmed by DH.

There are several differences between diapause and non-diapause eggs after oviposition. Diapause eggs showed high accumulation of 3-hydroxykynurenine, glycogen and ecdysteroids (Coulon, 1988) with reduced accumulation of cyclic guanosine monophosphate (GMP). Among these, 3-hydroxykynurenine and glycogen exhibit dramatic changes on the commencement of diapause,

where 3-hydroxykynurenine is oxidised to ommochrome, resulting in the dark colouration of the diapause eggs and glycogen is mainly converted into sorbitol which was synthesized from haemolymph trehalose in developing embryos of pharate adult and acts as an anti-freeze for the diapause embryo (Chino, 1958). DH also exerts an inhibitory effect on 'esterase A', which is an enzyme essential for mobilising the yolk needed for completion of embryogenesis (Kai and Hasegawa, 1973; Kai and Haga, 1978). By blocking esterase A, DH enforces the diapause by denying the embryo access of yolk. Molecular analysis of DH action has demonstrated that DH directly induces trehalase gene expression in developing embryos, which eventually brings about hyperglycogenism in mature eggs, leading to sorbitol production at the onset of diapause (Su *et al.*, 1994). DH function is conceived to be the initial and essential reaction leading to the diapause-associated metabolism in silkworm eggs (Yamashita, 1996).

II. Biochemical changes during embryogenesis

Since the first detailed study on the development of silkworm eggs, embryological studies were confined especially in solving the practical problems such as identification of suitable stages for refrigeration of early embryos and the initiation, continuation and termination of diapause in order to develop an effective system for long-term cold storage of silkworm eggs (Takami and Kitazawa, 1960). In these studies morphological changes of embryos were used to assess their long-term survival. Moreover, the tolerance of eggs to cold storage varies with the stage of embryonic development besides genotypes because of adaptation phenomenon, metabolic changes and also genetic variations etc (Sander *et al.*, 1985; Sonobe *et al.*, 1986) and hence relatively recent studies on silkworm eggs becomes more concern with physiology, biochemistry and metabolic activity associated with termination of embryonic diapause (Yaginuma *et al.*, 1990; Yamashita and Yaginuma, 1991) for effective handling.

Biochemical composition of silkworm egg

Newly laid eggs of silkworm are composed of proteins (~10%), lipids (~8.5%), glycogen (~2.5%), chorion (~18 %) and water (~60%) (Yamashita and Irie, 1980). More than 95% of the total protein is yolk protein: vitellin (~40%), 30 kDa protein (~35%) and egg-specific protein (ESP) (~20%). These proteins are quite different from each other in their physiochemical and biological properties (Zhu *et al.*, 1986). Each protein exhibits unique profile of degradation during embryogenesis, *viz.*,

i) Egg-specific protein is utilized early and completely disappeared by the time of hatching. ESP is degraded during embryogenesis by a process called hydrolysis catalysed by trypsin like seryl protease and alters the carboxyl site of Lys^{114} and $Argenine^{210}$ of ESP (Indrasith *et al.*, 1988a). The developmental increase in activity is due to increase at transcription of mRNA for this enzyme protein (Indrasith *et al.*, 1988b). Therefore, the utilization of ESP is a programmed event correlated with embryogenesis.

ii) Vitellin degradation occurred only at the later stage of embryogenesis and about 40% of the initial amounts remained unutilized in hatched larvae (Irie and Yamashita, 1980). Thus, vitellin metabolism appears to be independent of the diapause phenomenon in *B. mori* eggs. Yamashita and Irie (1980) concluded that vitellin in silkworm is not essential for egg maturation, embryogenesis and diapause while conducting experiment on vitellin deficient eggs.

iii) 30 kDa proteins are less utilized during embryogenesis (Zhu *et al.*, 1986).

The second major component of egg is lipid (~8.5%), which is composed of triacylglycerols (~80%) and phospholipids (~20%). Other constituents such as free fatty acids, mono and di-glycerides are usually present in small quantities (Chen, 1971). Most of the metabolic energy (approximately 70% of the total energy) is derived from the oxidation of triacylglycerol. The oxidation of lipids is advantageous for embryogenesis of terrestrial cleidoic eggs because large amount of metabolic water (1.07 g/g lipid) is released. Phospholipids are distributed as major component of yolk and used for formation of embryonic cells. Lipid is the major component consumed during diapause (Chino, 1958) and the concentration of lipid is higher in diapausing silkworm eggs than in non-diapausing eggs (Kim *et al.*, 1981). Developing ovaries accumulate more triglyceride in the presence of SG and the accumulated lipid decreases at the end of pupal adult life. The lipid concentration of silkworm diapause eggs decreases as diapause proceeds and considered to be the major substrate consumed during diapause. Nakasone (1979) reported that in *B. mori* embryos, triglyceride accounts for about 60% of the total CO_2 expired, while phospholipids and sterol remain almost constant level throughout embryogenesis. This indicates that lipid is used as an energy source during both diapause and post-diapause development.

Diapausing insects accumulate high level of glycerol and sorbitol led to the recognition that diapause is achieved by unique metabolic route that is not needed for non-diapausing eggs (Yamashita and Hasegawa, 1985). In silkworm eggs glycogen undergoes dramatic changes during diapause. In the diapause eggs, the following reversible reaction occurs with the initiation and termination of diapause:

Glycogen ⟷ Sorbitol + Glycerine

These metabolic shifts are induced by a particular set of hormones, which is responsible for diapause initiation. With the initiation of diapause, the glycogen is converted into sorbitol while during termination the reaction is in reversed direction (Yaginuma and Yamashita, 1977, 1978). The experiment with ^{14}C glycine showed that sorbitol is totally derived from glycogen, while glycerine is produced only when glycogen content reached the lowest level. About the mechanism of reversible reaction, following steps exists:

glucose + NADPH ⟷ sorbitol + $NADP^+$

glucose-6-p/fructose-6-p + NADPH ↔ sorbitol-6-p + $NADP^+$

glyceraldehyde + NADPH ⟷ glycerine + $NADP^+$

2-OH acetone-p + NADH ⟷ 2-p glycerine + NAD^+

Yamashita and Hasegawa (1985) stated that NAD^+ sorbitol dehydrogenase is the key enzyme in the utilization of sorbitol during termination of diapause in *B. mori* eggs. One of the remarkable differences between diapause and non-diapause eggs within a few days of oviposition is that glycogen rapidly disappears in diapaused eggs but remains at its initial level in non-diapaused eggs. $NADP^+$ sorbitol dehydrogenase is relatively high in both newly laid diapaused and non-diapaused eggs but remains high during embryogenesis of non-diapaused eggs as well as during the long period of diapause (Yaginuma and Yamashita, 1979). On the other hand, the activity of NAD^+ sorbitol dehydrogenase is almost absent in newly laid non-diapause eggs but appears 2 days after oviposition in non-diapause eggs.

Table 6. Relationship between diapause and metabolism in mature eggs of the silkworm, *Bombyx mori*

Characters	Nature of eggs	
	Non-diapause	Diapause
DNA (μg/mg)	0.87(100)	0.65(74.71)
RNA (μg/mg)	18.84(100)	14.04(74.52)
Glycogen (%)	1.60(100)	1.87(116.88)
Lipids (%)	26.5(100)	27.5(103.77)
Oxygen consumption (mm^3/g/h)	26.82(100)	13.64(50.19)

Table 7. Nucleic acid metabolism during early embryonic development of diapause and non-diapause eggs in *Bombyx mori* (*c.f.*, Kurata *et al.*, 1980)

Embryo development	*24	*48	**72	**120
DNA (ig/mg)				
Non-diapause	1.37	1.67	1.86	2.25
Diapause	1.00	1.49	1.54	1.58
RNA (ig/mg)				
Non-diapause	15.84	15.98	15.54	17.76
Diapause	14.27	14.33	14.00	13.58
DNA / RNA				
Non-diapause	0.086	0.104	0.120	0.127
Diapause	0.071	0.103	0.110	0.117

*Pre-diapaused period; **Diapaused period.

Diapause and nucleic acid metabolism

In the silkworm eggs, diapause is decided during the maturation process of egg in the ovary of pupal body. Therefore, there is a close relationship between diapause occurrence and metabolism of egg cells. In insects, nucleic acid is not only related to the expression of genes but also influence protein synthesis, cell division, growth and development. In univoltine genotypes, if sub-oesophageal ganglion is removed at early pupal stage, female silkworm will lay non-diapausing eggs, while normal female lay diapause eggs. If the mature eggs inside the ovariole of above two groups are taken out, it is found that DNA and RNA content of diapause eggs is 25.29% and 25.48% respectively lower than that of non-diapause eggs but the DNA / RNA ratio of these two groups were the same (Table 6). Hence, it is inferred that DNA content of mitochondria of diapause eggs is probably lower than non-diapause eggs. DNA content increases logarithmically in both non-diapause and diapause embryos at 1st day after oviposition. In non-diapause eggs, DNA increase continues for a few more days but in diapause eggs no further increase is observed after 2 days of oviposition when diapause is established (Kurata *et al.*, 1980) (Table 7). RNA plays a major role in protein metabolism and embryo-morphogenesis. It is reported that mRNA carrying out the early morphogenesis information is synthesised during the egg formation process and is deposited in the cytoplasm of egg before fertilization. The entrance of sperm activates the egg and the mRNA in latent condition is activated first. During the pre-diapause stage, the DNA content in both non-diapause and diapause eggs are used very rapidly. But 48 hrs after oviposition, the DNA metabolism of these two types of eggs is quite different (Table 7). The fact that DNA content of diapause eggs keeps constant after 48 hrs of oviposition is perhaps the biochemical pre-condition for stopage of embryo morphogenesis and initiation of embryonic diapause. On contrary, the DNA content of non-diapause eggs continues to increase rapidly. In non-diapause eggs within 24 hrs of oviposition, with the increase of DNA content, RNA content decreases. After 72 hrs of oviposition large amount of

RNA is synthesised and accumulated in the non-diapausing eggs. This is closely related to the synthesis of protein during embryonic development. On the contrary, in diapause eggs within three days of oviposition, the RNA content keeps constant though large quantity of DNA is synthesised and accumulated. Within 24 hrs of oviposition the DNA / RNA ratio in both the types of eggs (diapause and non-diapause) increases rapidly but after 48 hrs they reach the same level. Later the DNA / RNA ratio of non-diapause eggs increased rapidly.

Diapause and carbohydrate metabolism

Carbohydrate metabolism is the main pathway of biochemical regulation of diapause in the silkworm. It is proved that induction, initiation, maintenance and termination of diapause are related to carbohydrate metabolism. Amount of glycogen accumulated in diapause eggs is 1.7 times higher than in non-diapause eggs (*c.f.*, Furasawa *et al.*, 1992; Yamashita *et al.*, 1981). More than 90% of carbohydrate accumulated in the silkworm diapause eggs is glycogen. Further, the metabolic fate of glycogen in eggs is completely decided by diapause conditions. Thus, glycogen in eggs serves not only as a stored reserve for metabolic energy, but also as the resource for diapause-associated metabolism. The concentration of glycerin varies with genotype besides seasons from 32 – 40 mg/g egg. Glycogen accumulated in the silkworm egg is from the glycogen stored in the fat body during pupal stage, which is converted into trehalose and is released into haemolymph and then absorbed by developing oocyte. The trehalase localises in plasma membrane of vitellogenic follicles (Azuma and Yamashita, 1985), where haemolymph trehalose hydrolises into glucose and are to be taken up by oocytes. The glucose is immediately used to synthesise glycogen as a storage reserve, by which hyperglycogenia is induced in diapause eggs. Consequently, DH provides the metabolic state leading to diapause-associated carbohydrate metabolism in silkworm eggs (Yamashita, 1996). It has been demonstrated that enzymes involved in trehalose synthesis include trehalase, hexokinase, phosphoglucomutase, UDP glucose-pyrophosphatase and glycogen synthetase. Of these trehalase is a membrane-bound restriction enzyme existing on the surface of oocytes and follicle cells. The amount of trehalose in the pupal body fluid entering the ovary and the amount of glycogen synthesised in the egg is related to the trehalase activity. Thus, the increase of glycogen content in diapause eggs depends on the increase of trehalase activity.

At the initiation of diapause, the stored glycogen is converted into sorbitol by the activation of phosphorylase 'b' to 'a' under anaerobic condition (Yamashita *et al.*, 1975) and thus during the whole diapause stage, the glycogen content in the silkworm eggs keeps at a very low level but the sorbitol content keeps at a very high level. The pathway of glycogen to sorbitol is coupled to pentose phosphate pathway (Chino, 1960; Suzuki and Miya, 1975; Kageyama, 1976). In these eggs, activity of glucose-6-phoaphate (G-6-P) dehydrogenase is about twice higher than that of phosphofructokinase (Suzuki and Miya, 1975) and G-6-P amounts equivalent to about 10% of the accumulated sorbitol are oxidised through the pentose-phosphate pathway. The other fraction of G-6-P is eventually metabolised to glycerol (Yaginuma *et al.*, 1990). After the termination of diapause, sorbitol is again converted into glucose and the key enzyme involved in the activity is NAD^+ sorbitol dehydrogenase, which plays significant regulatory role in the metabolic cycle.

Accumulation of huge quantity of sorbitol in the diapausing eggs is generally regarded as a protecting agent of eggs from cold injury in winter. Yamashita *et al.* (1975) reported that about 80% of glycogen is being converted into sorbitol during the first 10 days of oviposition, whereas glycerol is accumulated during the later stage of diapause. It is now very well established that the conversion of glycogen to sorbitol is a specific feature related to the initiation of diapause in the silkworm eggs (Yaginuma and Yamashita, 1979). Gycogen rapidly disappears in the diapausing eggs within a few days of oviposition but remains at its initial level in the non-diapause eggs.

The rate-limiting enzymes in each pathway have been identified: trehalase in glycogen synthesis in the ovary at the induction of diapause, glycogen phosphorylase in sorbitol synthesis at the initiation of diapause and NAD dependent sorbitol dehydrogenase in glycogen synthesis at the termination of diapause. All these enzymes catalize the initial step in each pathway to determine flux, and activating of these key enzymes is strictly regulated by the treatments evoking each phase of diapause; trehalase activation by DH for diapause induction, glycogen phosphorylase activation by anerobiosis for diapause initiation, NAD-sorbitol dehydrogenase activation by chilling or HCl treatment for diapause termination.

The utilisation of sorbitol is controlled by 'nicotinamide adenine dinucleotide - sorbitol dehydrogenase' (NAD-SDH) (Yaginuma and Yamashita, 1979). Yaginuma *et al* (1990) reported that incubation of diapause eggs at 25°C does not induce the activity of NAD-SDH until 90 days. Moreover, the activity appears at very low level between 90 – 160 days. Similarly, chilling at 5°C for less than 30 days does not induce activity but 40 days period of chilling effectively stimulates activity. In contrast, 0.5°C never induces activity even with an exposure exceeding 250 days. Utilization of sorbitol in diapause eggs thus appears

to be controlled by NAD-SDH activity through mediation of temperature (Yamashita and Yaginuma, 1991). In non-diapause eggs, the sorbitol content increased on 2nd day of oviposition and then decreased rapidly (Furasawa and Yang, 1987). Polyol content (sorbitol and glycerol) can be used as the biological indices to monitor the diapause status of the silkworm eggs (Furasawa *et al.*, 1992).

Initiation of diapause and sorbitol production

There is a significant biochemical difference at the time of oviposition between eggs destined to become diapause and non-diapause. The eggs developed in the presence of diapause hormone accumulate large quantity of glycogen (Yamashita. *et al*, 1981). When oogenesis proceeded in the absence of diapause hormone, the laid eggs becomes non-diapause with less accumulation of glycogen and during embryogenesis, conversion of glycogen to sorbitol does not occur.

Sorbitol is an ideal metabolite to assess the diapausing state of silkworm eggs (Yamashita *et al.*, 1988). In diapause eggs, the initiation of sorbitol synthesis is correlated with the time when embryogenesis progressively slowed down. Conversion of glycogen to sorbitol at this stage is monitered by the enzyme 'glycogen phosphorylase-a activity. Conversion of glycogen phosphorylase-'b' to 'a' is being reported absent in non-diapause eggs under normal conditions (Yamashita *et al.*, 1975).

Glycogen phosphorylase-b kinase responsible for conversion of 'b' to 'a' form is identified in silkworm eggs and is reported to have some similarities in kinetic properties to that in other insect tissues (Ziegler *et al.*, 1979). There is no difference in their activity between diapause and non-diapause eggs.

When newly laid non-diapause eggs are exposed to low temperatures, sorbitol is accumulated and the extent of accumulation is closely dependent on the temperatures of exposure. In this case, glycogen is also used as an initial substrate for the formation of sorbitol. Thus, in non-diapause eggs, sorbitol production seems to be a biochemical adaptation against low temperature stress (Furasawa and Shikata, 1982).

In diapause eggs, sorbitol began to decrease after continuous chilling for atleast two months or by treatment with HCl on one month chilled eggs. The more advanced fall in sorbitol took place in eggs chilled for longer period. In all cases, sorbitol is stoichimometrically reconverted to glycogen at the time of termination of diapause (Yamashita *et al.*, 1988) via fructose by NAD-SDH enzyme (Yaginuma and Yamashita, 1979, 1986). Thus, the conversion of sorbitol to glycogen is specific sign signally the breakdown of diapause in silkworm eggs. In the metabolic pathway from sorbitol to glycogen, NAD-sorbitol dehydrogenase, which catalyses the reaction from sorbitol to fructose is noticed to be the key enzyme. This activity is not being detected in eggs unchilled or chilled for less than two months but abruptly appears in eggs if chilled for more than two months.

The duration of diapause may be determined by analysing the amount of oxygen required to prevent the decrease of sorbitol content in the eggs (Furasawa *et al.*, 1987) which indicates that conversion of sorbitol to glycogen upon termination of diapause is closely related to the respiratory system via - GP cycle. Diapause termination is also closely related to the oxygen consumption during diapause period (Furasawa *et al.*, 1987) and that the activity of NAD-SDH enzyme is associated with the oxygen required for the termination of diapause. They demonstrated that supply of oxygen to diapause eggs affect the maintenance of diapause period and when eggs are kept under anaerobic condition, the conversion of sorbitol to glycogen is delayed leading to delayed diapause termination.

Sorbitol utilization in relation to diapause termination

Diapausing silkworm eggs chilled at 5°C for at least three months when transferred to 25°C, the eggs resumed embryogenesis and hatches within two weeks (Yamashita *et al.*, 1988). Hatching can also be brought about by soaking the pre-chilled diapausing eggs into hot HCl solution (Yamashita, 1984). In these eggs, sorbitol content began to decrease after continuous chilling at least for two months or by treatment with HCl on one month chilled eggs. Greater fall in sorbitol content took place in eggs chilled for longer periods. Conversion of sorbitol to glycogen does not take place if eggs are kept continuously at 25°C (Yamashita *et al.*, 1988) even for more than six months.

After diapause, all sorbitol in the eggs is converted into glycogen. During embryogenesis, the main carbohydrate consumed is glycogen. The glycogen-sorbitol-glycogen metabolic process is roughly the same as the process of diapause onset, maintenance and termination. In other words, the change of carbohydrate metabolism of the silkworm eggs is a close relative to the phenomenon of diapause.

The sequence of major physiological events occurring in *B. mori* eggs till the establishment of diapause at 25°C is as follows:

- **One day after oviposition**
  - Optimum age for HCl treatment to block diapause initiation, beginning of ommochrome formation in serosal cells.
  - Beginning of glycogen decrease and maximum glycogen consumption (Chino, 1957, 1958).

- **Two days after oviposition**
  - Steep decline in oxygen consumption (Chino, 1958).
  - Abrupt increase of glycogen phosphorylase - a activity (Yamashita *et al.*, 1975).
  - Decline in affinity of lysosomes in embryonic cells to acridine orange with supra vital staining. The affinity is lost during diapause (Okada, 1970b).
  - Arrest of increase in DNA content (Kurata *et al.*, 1980).
- **Three days after oviposition**
  - Arrest of nucleic acid synthetic activity in nuclei of embryonic cells and gradual decrease in yolk cells in-vitro (Perk and Yoshitake, 1970).
- **Four days after oviposition**
  - Arrest of mitotic activity in embryo (Kitazawa *et al.*, 1963; Okada, 1970a).
  - Continued steep decline in glycogen content and gradual accumulation of sorbitol and glycerol respectively (Yaginuma and Yamashita, 1977).

Changes in amino acid pool

Significant changes in some free amino acids occurred during the initiation and termination of diapause. In particular, a sudden large increase in alanine content (about 50 mol µmol/g eggs) occurred at the initiation of diapause. (Suzuki *et al.*, 1984; Osanai and Yonezawa, 1986). Then alanine declines gradually with the increase of glutamate and especially proline. Content of proline, which is always low during the initiation and maintenance of diapause, increased suddenly during the termination period indicating the conversion of alanine to glutamate and proline in diapausing eggs. In diapausing insects, a high concentration of free amino acids as well as of polyols and sugers serves to decrease the super cooling point (Somme, 1982). The super cooling point of *B. mori eggs* is lower during diapause and hibernation (Suzuki *et al.*, 1983), and the increase of total amino acids and the accumulation of alanine or proline might be responsible for this effect. Proline serves as an energy source for later stages of embryonic life (Osanai and Yonezawa, 1986).

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