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In Vitro Diapause Substance in the Silkworm, *Bombyx Mori*

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Summary

By means of in vitro studies, in which isolated suboesophageal ganglions of the *Bombyx* silkworm were cultured, it was shown that at least two kinds of substances are biosynthesized and exert independent effects on determination of diapause or non-diapause in silkworm eggs. They are referred to as the diapause and non-diapause substance, respectively. Whether diapause or non-diapause eggs are laid may depend upon the different quality of these substances.

I. Introduction

ALTHOUGH it is commonly recognized that the suboesophageal ganglion (SG) of the silkworm (*Bombyx mori*) releases a hormone into the maternal circulation, which causes the newly laid eggs to enter diapause (FUKUDA, 1951, 1952; HASEGAWA, 1951, 1952; KOBAYASHI, 1957; MOROHOSHI and OSHIKI, 1969 a,b), this diapause substance has not been verified at the level of molecular structure.

The extraction of diapause hormone from the complex of brain and SG of the silkworm pharate adults was first conducted by HASEGAWA (1957). During the course of diapause hormone purification, ISOBE et al. (1973) demonstrated the molecular weight of diapause hormone using millions of male adult heads, but problems of the molecular structure and identification still remain. PARK and YOSHITAKE (1971) reported that in the diapause factor cells located in the SG, there was a difference in the rough-surfaced edoplasmic reticulum in the process of cytoplasmic granule formation between producers of diapause and non-diapause eggs. In addition, PARK (1973) found that diapause regulator producing cells in the SG may give information to the diapause factor cells.

This finding led us to suggest a hypothesis as to whether the diapause or non-diapause of silkworm eggs may be dependent on different qualities of a substance already synthesized, presumably under mutual interaction of diapause factor- and regulator-producing cells.

In some insects the prothoracic glands (PG) can biosynthesize a substance in vitro which participates in the moulting process. This material has been identified as α-ecdysone by chromatographic analysis (CHINO et al. 1974; ROMER et al. 1974). To date, there has been little success in tissue culture studies with the suboesophageal ganglion to extract or identify the diapause substance responsible for induction of diapause in eggs.

Accordingly, the authors performed experiments to demonstrate the synthesizing ability of the SG in vitro and to resolve the difference of the substances secreted from both diapausing and non-diapausing SG in *B. mori*

II. Materials and Methods

Animals

In order to obtain silkworms destined to lay diapause and non-diapause eggs, a bivoltine Daizo strain was incubated at 25°C under illumination and 15°C in the dark, respectively. Two or 3 days after pupation

the pupae were used for tissue culture of the suboesophageal ganglion.

Tissue culture

The suboesophageal ganglions were removed under a dissecting microscope and cultured for 3 days in 0.04 to 0.06ml of tissue culture medium as described by GRACE (1962). In this experiment insect haemolymph from silkwom pupae was not added in tissue culture media. The pH was adjusted to 6.5. All cultures were incubated at 25±1°C (TAKAMI, 1966, modified hanging drop method).

Bioassay

The medium (0.1ml) after the culture of diapause-SG was injected into the abdomen of female larvae destined to lay non-diapause eggs on the day before pupation. At the same time an equal amount of medium of the non-diapause SG was also given to diapause egg-producing larvae. Both diapause and non-diapause egg producing larvae were also injected with pure culture medium at the same time injections were made with cultured media. The experimental and control insects were kept at 25°C until they became adults and laid eggs.

III. Results

Among the 10 experimental insects, both in diapause and non-diapause, several survived, became adults, and laid eggs. The balance died during the pupal stage. It is uncertain whether death of pupae was caused by physical damage of injection, or by reaction of the cultured media injected in the body.

Diapause substance

As seen in Fig. 1, the few eggs laid by experimental non-diapause egg producers began to form pigment on the serosa for the onset of diapause; whereas the majority of the eggs in the same batch did not show any pigmentation, remaining yellowish-white in colour until hatching about 10 days after oviposition. All eggs laid by the controls of non-diapause egg producing larvae did not form any pigmentation of the serosa, and hatching was similar to non-diapause eggs.

The above-mentioned results suggest that when the isolated suboesophageal ganglion (of diapause) was cultured in vitro, a neurosecretory substance can be releassed and stored in the culture medium. This substance has the ability to produce diapause eggs, even partially, if applied as late as the pupal stage

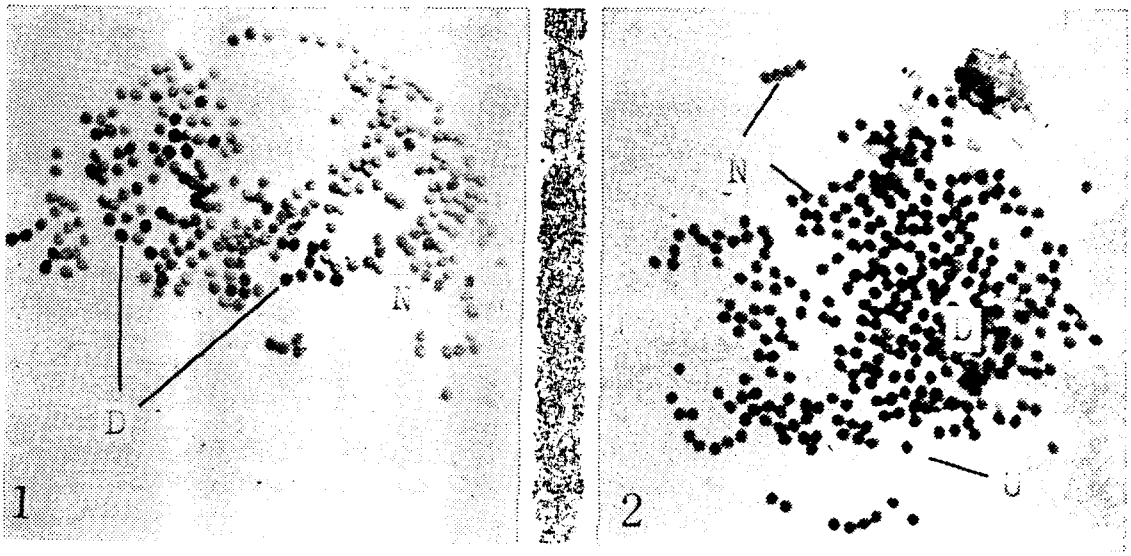

Fig. 1. Diapause eggs from the experimental non-diapause egg producer injected by "diapause substance". D, diapause egg; N, non-diapause egg.

Fig. 2. Non-diapause eggs from the experimental diapause egg producer injected by "non-diapause substance". Non-diapause eggs are darker than unfertilized eggs. D, diapause egg; N, non-diapause egg; U, unfertilized egg.

and affects the ovaries of non-diapause egg producers. The authors term the neurosecretory substance responsible for the induction of diapause eggs, in short, 'diapause substance'.

Table 1 is a summary of the results of experiments.

Table 1. Effect of diapause substance on the induction of diapause eggs from non-diapause egg producers, *Bombyx mori*

Series		Total No.of Eggs*	No. of Diapause Eggs	Remarks
Experimental	1	284	13	
non-diapause	2	301	6	
egg producers	3	—	—	Died during pupal stage
	4	192	7	
	5	—	—	Died during pupal stage
	6	—	—	Moth didn't lay eggs
	7	255	21	
	8	271	0	
	9	312	5	
	10	436	22	
Controls	1	203	0	
	2	414	0	
	3	386	0	
	4	332	0	
	5	275	0	

* Data for unfertilized eggs omitted from calculations.

Table 2. Effect of non-diapause substance on induction of non-diapause eggs from diapause egg producers, *Bombyx mori*

Series		Total No. of Eggs	No. of Non-diapause Eggs	Remarks
Experimental	1	—	—	Died during pupal stage
diapause egg	2	288	21	
producers	3	189	8	
	4	264	19	
	5	—	—	Died during pupal stage
	6	47	—	Unfertilized eggs
	7	316	0	
	8	—	—	Died during pupal stage
	9	—	—	Died during pupal stage
	10	327	23	
Controls	1	172	0	
	2	254	0	
	3	206	0	
	4	418	0	
	5	299	0	

* Data for unfertilized eggs omitted from calculations.

Non-diapause substance

The majority of eggs laid by experimental diapause egg producers showed serosal pigmentation, and became darker after several days. Some of the eggs in the same batch retained their slight brownish-yellow colour (Fig. 2).

All eggs hatched about 10 days after oviposition. The conrols entered diapause, presenting the proper color of diapause eggs of the race.

This finding may explain how the neurosecretory substance released from the isolated non-diapause-SG can take part in the determination of voltinism as a non-diapause factor; it is called, in short, "non-diapause substance" by the authors.

Table 2 presents the occurrence of non-diapause eggs by injecting the non-diapause subtance into female larvae destined to produce diapause eggs.

IV. Discussion

The experiments described here demonstrate the ability of the SG from *B. mori.* to synthesize the diapause substance in vitro.

CHINO et al. (1974) succeeded in the biosynthesis of a large amount of α-ecdysone by means of the organ culture of isolated prothoracic glands from larval *B. mori.*

ROMER et al. (1974) showed in *Tenebrio molito* that when prothoracic glands and oenocytes are cultured together, the α-ecdysone derived from the PG is oxidized by the oenocytes to β-ecdysone.

Thus what relationship exists between these substances synthesized in the SG and the voltinism of silkworm eggs? In this experiment, if the synthesized substance from the isolated SG (of diapause) was injected into the female of non-diapause egg producers, the experimental insects laid mainly non-diapause eggs, including a few of diapause eggs.

On the contrary, when females of diapause egg producers were treated with the substance (of non-diapause), in the main diapause eggs were laid together with a small number of non-diapause eggs. This illustrates that in the SG of *B. mori* at least two kinds of substances are synthesized, and independently exert direct effects on the determination of diapause or non-diapause in silkworm eggs. One material is a diapause-inducing substance (diapause substance), and the other is a non-diapause inducing substance (non-diapause substance).

It is conceivable that there may be differences in quality between these diapause and non-diapause substances, which in turn regulates eggs in pupal ovaries to become diapause or non-diapause types.

During experiments on diapause of the silkworm, PARK and YOSHITAKE (1971) revealed a difference in the rough-surfaced endoplasmic reticulum of diapause factor cells located in the SG. This occurred in the process of cytoplasmic granule formation, and was dependent on the amount of cytoplasmic granules between the diapause and non-diapause egg producers.

Furthermore, PARK (1973) found that diapause regulator producing cells were partially surrounded by diapause factor cells. He observed a highly electron dense material of lysosomes in the diapause egg producers in the DR cells.

These lysosomes were not present in the non-diapause egg producers. At this juncture, the present experiment corresponds well with the opinions of PARK who demonstrated that the silkworm eggs entering diapause or non-diapause are closely related to the quality of the secretory hormone(from the DF cells), which may depend upon a certain material from DR cells.

The authors can not calculate the quantity of diapause or non-diapause substances produced by the cultured SG in vitro. It must be pointed out, however, that the activity of cultured SG is markedly low compared with that of the intact SG in vivo. Therefore, the occurrence of diapause eggs from the experimental non-diapause egg producers was comparatively small, and the same results occurred vice versa.

Furthermore, it can also be considered that in the ovaries of injected female pupa there exists a competition between the substance injected out and the pre-existing substance of the recipient's own SG. Such competition brought about some pigmentation of serosa even in the non-diapause eggs laid by the experimental diapause egg producers. It is apparent that the activity of the non-diapause substance injected out is predominant over that of the diapause substance of the recipient's own SG.

On the other hand, HASEGAWA (1957) conducted trials to isolate the diapause hormone by treating the brain-SG complex of the silkworm pupae with methanol, ether, and finally chloroform. There was a high tendency to produce diapause eggs when the extract was injected into an insect destined to lay non-diapause eggs. But the authors do not agree with his assertion, because the voltinism of *B. mori* would be determined only after the SG biosynthisizes the diapause or non-diapause substance and released it into the maternal circulation during the pupal stage. Perhaps HASEGAWA might have placed greater importance on the ganglion itself prior to the neurosecretory function of the SG. In this connexion the diapause substance (or non-diapause substance) may be different from the diapause hormorne, a chemical extract of the SG itself, as pointed out by HASEGAWA.

Consequently, the authors arrived at the conclusion that the production of diapause and non-diapause silkworm eggs may be dependent on the different quality of a substance already synthesized in the suboesophageal ganglion. The authors cannot prove the chemical properties, including the molecular structure of the diapause substance (or non-diapause substance). A more detailed account of related experiments will be reported in a subsequent paper.

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家蠶의 休眠物質에 관한 硏究

成 洙 一·朴 光 義

家蠶의 休眠物質을 究明하기 위하여 休眠性蠶 및 非休眠性蠶의 食導下神經球(SG)를 各各 摘出, Grace氏 培養液을 使用하여 體外培養實驗하였다.

培養完了後, 培養產物液에 對한 生物檢定實驗(Bioassay)結果, 休眠性蠶의 SG및 非休眠性蠶의 SG는 化性에 各各 獨立的으로 影響하는 質的으로 相異한 物質, 즉 休眠物質(Diapause Substance) 및 非休眠物質(Non-diapause Substance)을 分泌하고 있음이 밝혀졌다.

蠶卵의 休眠性및 非休眠性의 決定은 이 兩物質間의 質的인 差異에 基因하는 것으로 생각된다.