Present Status of the Endocrine Regulation of Growth And Differentiation in the Silkworm, *Bombyx mori*

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In insects, pioneering works of morphological and physiological studies on endocrine organs were published about 40 years ago and studies on the internal secretion have made rapid progress in the field of experimental morphology in the past about 25 years.

Therefore, a systematic explanation of the mechanisms of larval molting, pupation and imaginal differentiation have gradually become possible.

In insects, studies of hormones are slower in progress than in higher animals. Studies of insect hormones, however, have made so much progress recently on the chemical properties, physiological activities and actions on various metabolic processes that the hormones have come to be tried as insecticides, growth-regulating agents for the silkworm and also medicines.

The author will explain in the following how endocrinological studies in the silkworm have been promoted in the course of these advances.

Molting hormone

In the silkworm, larval molting is induced by cooperative actions of the corpus allatum and the prothoracic gland, and pupation and imaginal differentiation are brought about by a secretion from the prothoracic gland activated under the influence of the brain.

The prothoracic gland in lepidopterous insects was first discovered by Lyonet in *Holcocerus vicarius* about 200 years ago. In the silkworm Verson found it in 1900, Toyama (1902) made an embryological study of it in details, and Tanaka (1925) named it "Zenkyō-sen" in Japanese.

Though the morphological study of this gland was almost completed by Japanese researchers in a period from 1940 to 1955, the character of the hormone secreted from the gland could not be made clear by them. The true form of the hormone had remained veiled for a long time but it was finally isolated by Butenandt and Karlson in Germany in 1954 and named ecdysone.

Ecdysone was proved to induce the pupation of *Caliphora erythrocephara* in a dose of 0.01 μg and its structural formula was determined in Germany in 1965. It may be said that 1966 was an epoch in the study of molting hormone-active substances, that is, in this year not only the synthesis of α-ecdysone was accomplished in Germany and the United States and ecdysterone was isolated from silkworm pupae but also Japanese chemists first demonstrated the presence of some substances with molting hormone activity in plants.

In the first place, Nakanishi et al. separated four kinds of new steroids from leaves of *Podocarpus nakaii* HAY of Formosa and named them ponasterones A, B, C and D. They reported that these substances have also molting hormone activity on the fly.

Takemoto et al. isolated independently two kinds of crystalline components (m.p. 242°C and 255°C, respectively) from roots of *Achyranthes*

fauriei, that is, Achyrantis Radix. They evidenced the molting hormone activity of these substances on the fly and, after various chemical examinations, identified the substance which melted at 242°C as ecdysterone.

Such being the case, the study of Takemoto et al. can be said to be the first discovery of molting hormones in plants. The other component which melted at 255°C was found to be a new steroid and named inokosterone. After that, Nakanishi et al. isolated also ponasterone E, but the chemical formulae of ponasterones D and E are unknown at present because of their low yields.

It is possible that various studies will be promoted in the future by the use of these phytoecdysones, because some of them are obtainable in large yields from plants.

Kobayashi et al. (1967, 1968), using "Dauer-pupae" and isolated abdomens of pupae, confirmed that all these substances had strong activity for the induction of imaginal differentiation in the silkworm though there were some differences in the effect among them.

Hasegawa (1967) obtained similar results. Morohoshi et al. (1967) found that silkworm larvae injected with ecdysterone just after the fourth molting were induced to molt supernumerarily to become sixth instras.

Kobayashi et al. (1968) carried out similar experiments and examined histologically the formation of new cuticle after the injection of ecdysterone. They also demonstrated the effect of ecdysterone on the formation of chitin in the cuticle by administering ^{14}C-glucose to silkworm larvae.

The molting active substances obtained from plants were also studied for their application to the induction of the simultaneous mounting of silkworm larvae at the end of the larval period.

It has been found that when the substances are administered orally with mulberry leaves to the larvae from about one day before the appearance of mature individuals, the mounting of these larvae is made considerably uniform as compared with that of controls, which shows that there is hope of their application as mounting-uniforming agents (Okauchi et al. 1968, Ito et al. 1968).

Studies on the metabolism-regulating effect of ecdysone have been mainly carried out in foreign countries in relation to puparium formation and nucleic acid and protein syntheses in the fly.

In Japan, the relation of this hormone to carbohydrate metabolism has been investigated (Kobayashi and Kimura 1967). That is, when "Dauer-pupae" of the silkworm were injected with a fixed amount of both ecdysone and 1-^{14}C-glucose or 6-^{14}C-glucose to see the transfer of the labeled compound into trehalose and glucose, the transfer of the compound to blood trehalose was promoted by the action of ecdysone to reach the maximum one hour after the injection and then decrease, while in controls which were not injected with ecdysone the transfer increases with time.

When the incorporation of the labeled compound into glycogen was examined in the fat body, the value in controls was the highest just after the injection, decreasing gradually, while in injected pupae the value was low, though it increased a little with time, and the ratio in radioactivity of glycogen to the fat body was kept almost constant.

From these results it has been understood that ecdysone has an influence on the synthesis of trehalose and glycogen in the fat body and especially promotes the trehalose synthesis.

Similar experiments were carried out in diapausing brainless pupae of *Samia cynthia pryeri*, giving a result that ecdysone influenced to increase the transfer of labeled glucose to expiratory CO_2. This is an interesting result which shows a physiological difference between the two insects because such a phenomenon has never been observed in "Dauer-pupae" of *Bombyx mori*.

Since the transfer of 1-^{14}C-glucose to CO_2 was always larger in amount than that of 6-^{14}C-glucose in both hormone-injected pupae and their controls, it is known that in lepidopterous insects the pentose phosphate cycle in glycolysis is more active than in the other

insects.

While ecdysone plays an important part in the puparium formation of the fly, in the mutant silkworm line of black pupa Hashiguchi (1964) has indicated that the manifestation of black color is under the control of a black pupa hormone secreted by the brain-thoracic ganglion complex. The separations of this hormone has been advanced recently.

An active substance has been obtained from a water-soluble fraction of a methanol extract by purifying through a cellulose column.

Possibel factors necessary for the manifestation of the black color of pupae are: molting hormone (prothoracic gland hormone); black pupa hormone; black pupa gene; phonol oxidase activity in the blood and skin; polyphenol oxidase-activating substance in the blood and low temperature at the maturing stage of larvae (Hashiguchi and Yoshitake 1966).

Molting hormones were examined for their effects on the mutation rate in the slkworm and ecdysterone was shown to increase the rate, while inokosterone had no such effect (Tazima 1971). It was reported that molting hormones had no effect on the induction by the cold treatment of nuclear polyhedrosis but decreased that of cytoplasmic polyhedrosis in the silkworm (Aruga 1971).

RNA synthesis proved to be promoted by the ecdysterone in the body wall of the silkworm larva but the effect was inhibited when it was used in combination with the juvenile hormone (Kobayashi 1971).

Inokosterone promotes the biosynthesis of protein the silkworm though the effect is variable according to the concentration of protein in a diet (Ito 1971).

Juvenile hormone

As mentioned above the corpus allatum is a gland tissue which induces larval molting in cooperation with the prothoracic gland and is thought to be a terminal organ to excrete the neurosecretion from the brain in the silkworm.

The corpus allatum in insects was reported for the first time by Nebert in 1913 and considered to be a ganglion in insects until the publication of Ito's paper.

According to Ito (1918), who made histological studies of the corpus allatum in the silkworm and other lepidopterous insects, this is an endocrine organ whose function is active in the moth.

Considering his conclusion together with the extraction of the juvenile hormone from the moth 40 years after that, we are deeply impressed with his penetration.

As for the function of the corpus allatum, pioneering experiments were mainly carried out in France (Bounhiol 1937) and Japan (Kim 1938, 1939) followed by Fukuda's well-known detailed studies. Studies on the nature of the juvenile hormone secreted from the corpus allatumn was mostly carried out in the United States and Germany and the isolation of the active substance was attained in 1967, making its structural formula clear.

In Japan during this period, experimental studies were chiefly promoted morphologically by Fukuda (1962) in the silkworm.

He experimented on the implantation of corpora allata from the pupae of various ages into the fourth instar larvae which had been deprived of their own corpora allata.

He observed that when corpora allata at the beginning of the pupal stage were implanted, the recipients became early-maturing larvae because the secretion of the implanted corpora allata had not yet reached the threshold.

In contrast to these, the larvae which received corpora allata from middle-aged or older pupae never showed early maturing but sometimes prothetelic characters. And the larvae implanted with corpolla allata from months or individuals at the end of the pupal stage molted normally.

The corpus allatum thus proved to be active in secretory function from the middle to the end of the pupal stage.

Fukuda also reported that the presence or absence of diapause factors had no important

influence on the activity of the corpus allatum.

When corpora allata of silkworm larvae were implanted into fourth instar larvae deprived of their own corpora allata in advance, the recipients were induced to molt normally, becoming moths through pupae, and if the implanted corpora allata of such moths were reimplanted into fourth instar larvae deprived of their own corpora allata, the implants showed to have activity to secrete the juvenile hormone.

The implantation of corpora allata from female moths into third instar larvae induced a supernumerary molting of the recipients to reach the sixth instar, while the recipients of corpora allata from male moths became normal fifth instar larvae, showing no supernumerary molting.

Decerebration at the beginning of the pupal stage had no influence on the sexual difference in the activity of corpora allata of resultant moths. That is, the corpora allata of brainless female moths had activity to secrete the juvenile hormone, while those of the brainless male moths did not have the activity. The sexual difference in activity of the corpora allata of silkworm moths is said to have relation to neither the gonad nor the brain (Fukuda 1963).

In the silkworm, the activity test of farnesol, which had been isolated in Germany as a juvenile hormone-active substance in hemimetabolous insects, showed that scale formation was not observed on the treated parts when the substance was applied to the abdominal skin of pupae (Fukuda 1964).

Hasegawa et al. (1968) reported that the development of embryos was inhibited by the application of juvenile hormone-active substances to the surface of silkworm eggs.

This result together with their known effects on the embryonic development of the other insects seems to suggest a hope of the insecticidal use of the active substances.

Kobayashi et al. (1968) have shown that the juvenile hormone, whose chemical structure was elucidated by Röller, of the *Cecropia*-silkworm and ecdysterone also induce larval molting in the *Bombyx*-silkworm.

Morohoshi, who reported previously (1959) that the corpus allatum had relation to the nondiapause of eggs in the silkworm, has experimented with silkworm varieties of variable voltinism. According to him, the implantation of corpora allata into fourth instar larvae causes a shortening of the pupal period in the recipients, and the resultant moths indicate a clear tendency to lay nondiapause eggs.

He says from these results that the corpus allatum affects the amount of hormone, which is secreted from the suboesophageal ganglion through the period of larval develoment and influences indirectly the voltinism (Morohoshi and Oshiki 1968).

He also demonstrated that an extract of corpora allata had an action to increase the pulse rate, promoting the decomposition of lipid and carbohydrate in the silkworm, and said that an extract of suboesophageal ganglia had an antagonistic action to these effects of the corpus allatum extract (Morohoshi 1968).

Brain hormone

Though an important role of the brain in the induction of insect metamorphosis was clearly shown by the work of Kopéc in *Lymantria dispar* in 1922, the brain was thought for a long time to be unnecessary for metamorphosis in the silkworm. Kobayashi has carried on his studies on the brain of the silkworm since 1955 and obtained the results outlined in the following:

When silkworm pupae (variety: N122 × C115) were divided into several groups according to hours after their pupation and deprived of their own brains, it was found that in a group of pupae which were decerebrated just after pupation many individuals remained in the form of pupae, never becoming adults, for more than 40 days (artificially diapausing brainless pupae="Dauer-pupae") at 25°C, while nonoperated normal pupae became adults about 12 days after pupation at 25°C.

Implantation of fresh brains from pupae

or moths into such "Dauer-pupae" induced the emergency of adults in all the recipients, while wounding along of "Dauer-pupae", accompanied with implantation of no brain, had not such an effect on the emergence of adults. The role of the brain in the internal secretion has been made clear by this experiment.

Then to examine the relation between the brain and the prothoracic gland which had been known to be the secretory organ of the molting hormone (prothoracic gland hormone), brains of fifth instar larvae were implanted into abdomens of other individuals of the same age and the recipients were divided into two groups: one for removing their own original brain just after pupation and the other for ligaturing them behind the thorax just after pupation to obtain isolated abdomens by cutting their head and thoracic parts off.

Both groups were kept at 25°C thereafter to see their development to moths and it was found that many moths emerged from the first group while almost no emergence of moths was observed in the second group.

From the facts that the presence of the brain alone was not able to induce imaginal differentiation and even an implanted brain could induce the emergence of moths if prothoracic glands were present in the recipients, if was concluded that a hormone from the brain acted as a prothoracic gland stimulant and as a result the hormone related to the induction of metamorphosis was secreted from the prothoracic gland.

To investigate the secretory center of the brain hormone, histological and histochemical studies of the neurosecretory cells were carried out in the brain of the silkworm and a secretion from the neurosecretory cells of the A and B types located in the brain was presumed to be the brain hormone or its precursor. Uwo (1961) observed electron microscopically the ultrastructure of the neurosecretory cells.

The finding of the hormonal action of the brain aroused our interest in the extraction of the brain hormone (Kobayashi and Kirimura 1958). A crude extract, which showed the brain hormone activity in a dose of 0.1 mg on "Dauer-pupae" of the silkworm, was used as a starting material for the purification of the hormone.

One of the two active fractions, which had been obtained by counter-current distribution and different in chemical property from each other, was purified further and identified from its chemical properties as cholesterol, showing the presence of steroid hormones in insects (Kirimura et al. 1962).

Thereafter, it was proved by Karlson and Hoffmeister (1963) and Nakanishi et al. (1970) that cholesterol was converted to ecdysone in *Calliphora* and *Bombyx*.

Thus, the author and his colleagues are inclined to believe that cholesterol mimics the action of the brain hormone on the bioassay, that is, cholesterol converts to ecdysones and the latter affects the prothoracic gland in the test-animal (Kobayashi and Yamazaki 1971).

On the other hand, Ichikawa and Ishizaki (1961, 1963) found that a water-soluble fraction of a methanol extract of silkworm brains had brain hormone activity, indicating the presence of proteinic brain hormones. They put its purification forward by column chromatography.

Kobayashi and Yamazaki (1966), to purify a water soluble active fraction obtained by counter-current distribution mentioned above, ground silkworm brains in 0.1 M phosphate buffer, centrifuged it, purified the supernatant liquid through a CM-cellulose column and obtained a highly purified substance with brain hormone activity.

Ishizaki and Ichikawa (1967) purified an extract of silkworm brains by gel filtration on Sephadex and chromatography on column of DEAE cellulose and obtained three active fractions, among which the most active fraction showed the hormone activity in a dose of 0.002 μg protein on *Samia cynthia pryeri* and was estimated to be 9,000~31,000 in molecular weight.

Using ^{14}C-glucose, Kobayashi (1967) com-

pared the effects of the proteinic brain hormone and ecdysone on the carbohydrate metabolism in the pupa of *Samia cynthia pryeri* and confirmed that both of them had relation to the synthesis of trehalose.

The proteinic brain hormone also promotes the synthesis of RNA in the prothoracic gland of the silkworm larva. But this is not a specific effect on the prothoracic gland because the hormone has the same effect on other tissues at the same time (Kobayashi et al. 1968).

Yamazaki and Kobayashi (1969) advanced the purification of this hormone further and obtained a highly purified substance which showed brain hormone activity in a dose of 0.02μg. This substance with an isoelectric point of 8.35~8.65 and a molecular weight of about 20,000 is inactivated by trypsin, pronase or nagarse.

Diapause hormone

The silkworm is an insect which passes diapause in the embryonic stage and the diapause is controlled by a hormone secreted from the suboesophageal ganglion in the pupal stage as found by Hasegawa and Fukuda, respectively, in 1951.

When suboesophageal ganglia taken out of diapause egg-producing pupae derived from eggs which had been treated so that the resultant moths would lay diapause eggs are implanted into nondiapause egg-producing pupae derived from eggs which had been treated so that the resultant moths would lay diapause eggs, the female recipients are changed so as to lay diapause eggs after emergence.

Neurosecretory cells found in the suboesophageal ganglion have been pointed out to be possible secretory parts of this hormone (Kobayashi 1957).

Fukuda and Takeuchi (1967), who experimented on the transplantation of neurosecretory cells at a certain part of the ganglion and made histological observation of the cells, have reported that the hormone is secreted from a pair of neurosecretory cells which are situated on the ventral side at the level about two-thirds from the anterior end of the ganglion.

On the other hand, this hormone was extracted by Hasegawa in 1957, and its purification has been continued by him since then. When this extract is injected into the nondiapause egg-producing pupae, the moths emerged from these pupae lay diapause eggs in which the embryos cease to develop within a few days, entering diapause. The mechanism of the action of diapause hormone has been studied by Yamashita and Hasegawa (1964).

When an extract containing the diapause hormone is injected into the nondiapause egg-producing pupae, in the first place blood trehalose, which is the main blood sugar in insects, is incorporated into the ovary to be synthesized into glycogen and the glycogen content of fat body decreases because of its coming out into the blood.

A series of changes as above are said to be due to feedback accompanying the sugar metabolism, which was triggered by the injection of the diapause hormone, in the ovary (Hasegawa and Yamashita 1965).

In addition, 3-hydroxykynurenine, a precursor of ommochrome (triptophane pigment), is increased in amount in the ovary and the egg of the silkworm by the injection of an extract containing the diapause hormone (Yamashita and Hasegawa 1964, 1966).

They also examined the relation between the trehalase activity and the diapause hormone by removing the suboesophageal ganglion and injecting the hormone extract in connection with glycogen synthesis in the ovary.

They found that trehalase activity in the ovary was increased by the hormone and came to the conclusion that this hormone probably mediated the synthesis of enzymatic protein to activate trehalase (Yamashita and Hasegawa 1967). The diapause hormone does not constantly act on the ovary but only at a certain stage of ovarian development (Yamashita and Hasegawa 1970).

Conclusion

In the field of silkworm endocrinology which started with techniques of experimental morphology, there still remain many problems to solve. Therefore, it is probable that biological studies of the unsettled questions will be promoted together with the physiological and biochemical studies on various physiologically vital active substances. On the basis of these results the application to agriculture and the introduction into agricultural techniques of insect hormones will be put forward in the future.

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