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V. Endocrinological aspects of silk production

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It is usual to associate gland secretion of given protein(s) with the stimulation of a specific hormonal effector. This general picture is valid from some well known tissues such as the oviduct which produces ovalbumin and related white egg proteins under the action of progesterone, and the hen liver which secretes the yolk protein, phosvitin, under oestradiol action. Prolactin induces various lactalbumines in the mammary gland. In avians and mammals, this mode of action as well documented: nuclear receptors have been identified, kinetics investigated and antagonists studied. Nevertheless, the final molecular basis for the hormonal activity remains obscure.

What is the effect of hormonal stimulation on silk proteins' secretion by the silk gland cells in *Bombyx mori?* The key role of ecdysteroids and juvenile hormones in the general development and activity of the silk gland is obvious as is the correlation of its activity and the molting cycle. Despite their interest, endocrinological aspects of silk production are very little investigated and documented. A main question can be raised: are ecdysones and/or juvenile hormones really involved in silk secretion? Is there a specific 'silk secretion factor' responsible for the repeated turning on and off of fibroin and sericin genes?

Quantitative assays of the fibroin mRNA[1] showed that the cognate gene transcription is turned off during the larval apolysis after which the pre-existing fibroin mRNA molecules are completely degraded. Fibroin gene transcription turns on after re-feeding which follows ecdysis. A similar behavior is also true for the P25 mRNA, coding for a small silk protein[2].

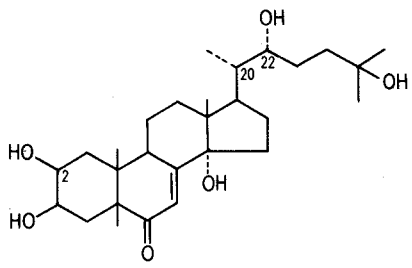
1. Ecdysteroids

Since the pioneer observations of S. Fukuda in 1940, who demonstrated by ligation and transplantation experiments that prothoracic glands release active substance(s) in the silkworm blood at critical periods, we know that larval and pupal molts are controlled by ecdysteroid hormones. They were purified for the first time by Butenandt and Karlson in 1954 using silkworm male pupae, formerly described as the α-ecdysone in 1965. A 2nd molting hormone, the β-ecdysone or ecdysterone (20-hydroxy ecdysone) was also isolated from pupae by Hoffmeister in 1966. Both are C-27 sterols with pronounced polar properties.

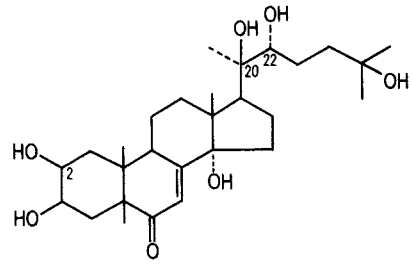
a) Quantitative assays

Several attempts at quantitative assays based either on bioassays using a *Calliphora* test or more recently radioimmunoassays (RIA) combined with further chromatographic fractionations[3] yield accurate data on free and conjugate ecdysteroids all along the larval cycle of *B. mori* for many tissues (ovaries, pupae, embryos)[4-8]; but surprisingly not yet for silk glands.

Ecdysone changes during larval and pupal development have been described by Calvez et al.[9] in the hemolymph of *B. mori* and *Philosamia cynthia,* of the tobacco hornworm *Manduca sexta* by Gilbert's group[10,11] and recently for *Galleria mellonella* by



Ecdysone



20-Hydroxyecdysone

Plantevin et al.[12]. These results for *B. mori* and *G. mellonella* are presented in figure 1. A recent refinement within the first days of the last larval instar of *B. mori* has been reported by Calvez[13].

Assuming that ecdysteroids easily go through the silk cell barrier and possibly parallel the blood content, we are able to ascertain the following pattern valid for 85–90% of total ecdysteroids – the free forms – and not for the non-migrating conjugates and some apolar derivatives such as 3-dehydro ecdysones:

Throughout most part the larval instars the ecdysone level is very low or not detectable, except for a small peak on the 3rd day of the last larval instar of *G. mellonella*. On the contrary, before ecdysis – at the 4th day of the 4th molt and day 8 for the 5th molt for *B. mori* – the ecdysone level sharply rises, the peak at the last larval instar being twice as high as that at the 4th instar. This peak is apparently preceded by secondary peaks; spinning starts during that period. The hormone activity is low, but never suppressed when larval and pupal ecdyses take place. The α/β ratio dramatically reverses in *B. mori* during the last larval instar. The peak at the end of the 4th instar is made up of 95% ecdysterone (β form). It gradually moves to the α form (35% at day 10, 88% at day 11 of the 5th instar). Finally, the α-ecdysone is the only one found in the pupae. Oenocytes could be involved in the α to β conversion. Thus tritiated ecdysone injected at the beginning of the 5th instar is partly converted into ecdysterone (see mini-review of Coulon[14]).

b) Biological significance

High levels of ecdysteroids are closely related to intensive morphogenetic and anabolic processes ordered in space and time (oocyte maturation, embryonic pre-diapausal development, postincubation). The exposure time of the cells to the hormone plays an important role in the differentiation of many target tissues, especially imaginal discs and cuticle. What about the silk gland cell? They apparently are not a target tissue for ecdysteroids, since these secretory cells are completely histolyzed during spinning, several metabolites being transiently stored in the fat body. Akai[15] reported the effects of massive injection of ecdysone in the middle stage of the 5th instar inducing precocious autolysis of the posterior silk gland part. After 6–12 h the granular endoplasmic reticula are concentrated in lamellar structures enclosed by a separate membrane with some mitochondria and ground matrix. Within 12–24 h they become lytic vacuoles through the autophagosomes. Similar ultrastructural changes in the gland cell occur during larval and pupal molting stages after an ecdysteroid stimulation. However, during larval-pupal metamorphosis the silk glands are completely histolyzed.

In the anterior part of the silk gland devoid of secretory function similar autolysis has been observed in vitro after administration of ecdysone to the culture

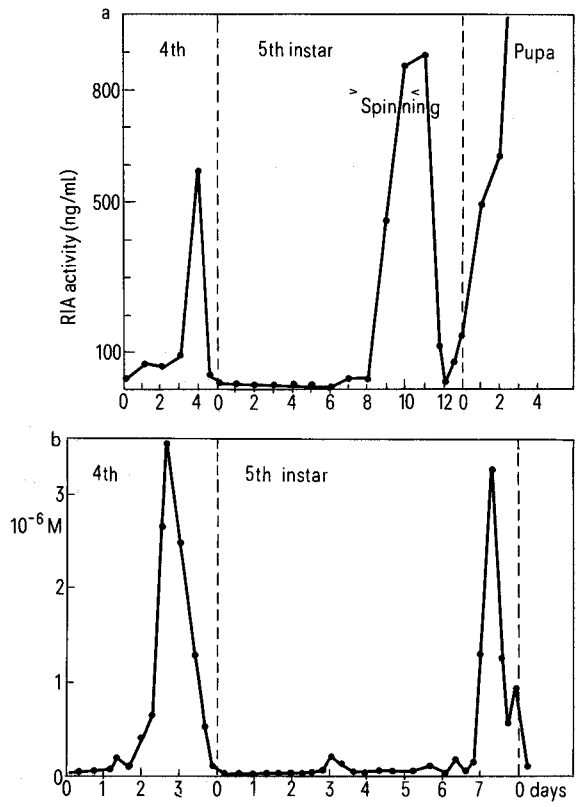


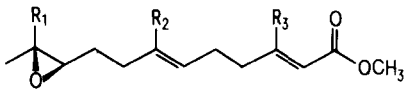
Figure 1. Ecdysone changes in the hemolymph of *Bombyx mori* and *Galleria mellonella*. Ecdysteroids titers have been determined by radioimmunoassay according to De Reggi et al.[3] from a sample (20–200 µl) of hemolymph immediately lyophilized. The anti-ecdysone serum used shows a reduced affinity for dehydroecdysone and inactive derivatives. Day 0 corresponds of the ecdysis. *B. mori* data are taken from Calvez et. al.[9], those from *G. mellonella* from Plantevin et al.[12] and Plantevin[32].

medium by Chinzei[16]. In both cases a critical period of development should be reached before the target tissue becomes competent. However, the cellular permeability seems to be modified according to the level of hemolymph ecdysteroids.

Finally, these first observations suggest a general effect of ecdysteroids on the metabolic pathways of the silk gland cells without any specificity on the silk protein production. The molting de-differentiation and the final histolysis involve either reversible disorder of the protein biosynthesis apparatus or its total damage leading in both cases to a halt in silk production and secretion. At any event, the relationship between ecdysteroid hormones and biochemical parameters of the silk gland remains a topic for active further investigation.

2. Juvenile hormones

This family of at least 4 closely related terpenoid compounds secreted by the corpora allata was first isolated from male imago abdomen of *Hyalophora cecropia* in 1965, their chemical structures elucidated a few years after for JH I, JH II and JH III[14] and more recently for JH 0[17]. Many synthetic structural analogues (methoprene or Manta, ZR 515,...) are commonly available drugs.



JH 0 C_{19} $R_1=R_2=R_3=C_2H_5$
JH I C_{18} $R_1=R_2=C_2H_5$, $R_3=CH_3$
JH II C_{17} $R_1=C_2H_5$, $R_2=R_3=CH_3$
JH III C_{16} $R_1=R_2=R_3=CH_3$

The main interest of JH in silk spinning is founded on the basic discovery of Akai and Kobayashi[18] that topical application of this hormone is able to prolong the feeding period of 5th instar larvae and consequently increase the cocoon shell weight. So far, JH is the only experimental effector capable of promoting higher silk production than is produced in native animals. Its effects on nucleic acid metabolism and protein synthesis in the silk gland have been actively investigated.

a) Quantitative assays

No quantitations are yet available for *B. mori*, but over the last few years some chromatographic quantitative assays based on thin-layer, high-pressure liquid chromatography and gaz-liquid chromatography for JH in *H. cecropia* and *M. sexta* have been done. Using recent radioimmunoassay techniques developed by Strambi et al.[19], Plantevin et al.[12] were able to quantitate JH in the hemolymph of *G. mellonella*. Similar RIA titrations are under progress for *B. mori* tissues, including silk glands at Lyons University.

Figure 2 describes total JH levels for free or circulating forms in *G. mellonella* hemolymph. If their intake into silk gland cells is not selective, whereas their turnovers differ from one tissue to another, we could consider general features of JH behavior to be as follows:

Absolute levels are about 100 times lower than those of ecdysteroids (around 10^{-8} M). - Changes are more or less regular. 2 peaks are visible during the 4th instar and possibly 4 during the last one.

Reduced levels occur during ecdysis. They immediately rise after re-feeding or spinning in *Galleria*. JH titers irregularly decrease throughout the last larval instar. - Measurements of native or biological diols from untreated samples show fluctuations ranging over 10–19% of total JH. - Internal distribution of the 3 major JH components during the last instar points out the predominancy of JH I, the gradual disappearing of JH II and the corresponding enhancement of JH III.

Thanks to the recent investigation of Shigematsu[20], the behavior of JH is being better understood within silk gland cells of *B. mori*. Exogeneous tritiated JH injected on the 3rd day of the 5th instar is quickly taken up, metabolized and excluded, mostly within a day's time, and possibly within a few hours. Most of the labeled JH is recovered in soluble cell fractions, but significant amounts are found in cell debris and nuclear fractions. Singular formation of acidic metabolites is seen in the posterior silk gland alone - not in fat body, gut or skin. In the posterior part, the ratio of intermediate metabolites was high in JH diol from the supernatant and in JH acid from the nuclear fraction. However, JH III was more rapidly metabolized than JH I. Acidic derivatives were detected in larger amounts in chromatin than in other cellular fractions.

b) Physiological effects on 5th instar duration and larva growth

As previously mentioned, the application of JH or analogues (which are more stable, since they are not subjected to an esterase activity) during the first days of the last larval instar leads to longer duration of the

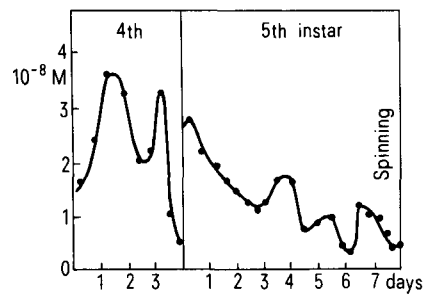


Figure 2. Juvenile hormones changes in the hemolymph of *Galleria mellonella*. Total circulating JH titers have been quantitated by radioimmunoassay according to Strambi et al.[19] and expressed in nmoles/JH I equivalent.

larval stage and up to a 30% increase of cocoon shell weight. Depending on the doses of JH and the time of application, larvae became permanent or molted to the 4th additional instar. Reversely, an allatectomy during the first days of the 4th instar induces precocious pupation whereas silk glands develop similarly to a 5th instar normal pattern. Electron microscope studies of the silk gland cells corroborate the functional pattern for the development of organelles involved in the silk protein synthesis and transport[21]. Detailed investigation of JH application to silkworm larvae have been reported by several groups[13,18,22-24] (for mini-reviews see Daillie[25] and Kurata[26]).

c) Physiological effects on silk gland growth and silk production

When the proper dose of JH analogues was administered in the early 5th instar, the growth rate of silk glands was lowered (fig. 3). However, this rate quickly recovered and then the glands grew bigger than in controls due to the longer duration of the instar. A single dose applied just before the 1st meal was still effective 3 days later[25]. It was only on day 5 that the posterior silk gland resumed growth. An application after 1 day of development prolonged the growth inhibition. In all cases, an accentuated relative decrease in weight was observed in the middle part; the inhibited silk protein synthesis failed of course to accumulate in that gland part.

It is well known that silk protein synthesis increases conspicuously after the mid-5th instar and reaches a maximum level in the late period interrupted by spinning. In larvae administered with JH in the early stage, the silk synthesis is delayed along with the domestic protein production, which is reflected in the tissue growth measurements. After a time of inhibition - without any JH application - the silk synthesis exceeds that of the native larvae, so that the total amount secreted becomes 30–50% higher. In larvae administered with JH in the late 5th instar, silk protein synthesis rapidly decreases.

It appears that JH is a potent inhibitor of protein synthesis. It is striking that during the early period of hormonal treatment, this growth retardation affects only the silk gland, but not other organs. It emphasizes therefore that the silk gland is a target tissue for JH, at least at this stage of development.

d) Biochemical effects on DNA and RNA synthesis

We know that fibroin and possibly sericin production directly depend upon the RNA accumulation (both rRNA and mRNA)[27,28] and RNA is mainly controled by nuclear activity.

In normal larvae, DNA accumulates within 2 replication cycles during the first half of the 5th instar and reaches a plateau after 4 days. When larvae were treated with JH in the early stage, the time needed to complete these 2 cycles was increased, based on thymidine incorporation as well as on thymidine kinase and DNA polymerase activities[24,25]. In JH-treated larvae the DNA content is not affected. Consequently, the 2 DNA synthesis cycles which overlapped in native larvae dissociate following an early JH application.

As reported by Shigematsu[20], JH is a potent inhibitor of DNA synthesis and requires to be metabolized in the target site or tissue - the silk gland in preference to the fat body. JH possibly binds to the active chromatin and therefore inhibitis DNA synthesis.

Concerning RNA accumulation, an early JH administration leads to a drastic inhibition of RNA transcription over 2–3 days depending on the dose and number of applications. Thus, RNA synthesis resumes so that the final amount of accumulated RNA is much higher than in controls. An almost similar behavior was seen in all RNA species (ribosomal, transfer and messenger). A typical curve in the posterior silk gland is given in figure 4.

Using a suitable mathematical model derived from Volterra-Kostitzin for studying the dynamics of macromolecular populations, Pave's theoretical analysis[29] of RNA data from treated and native larvae suggest that:

The rate of RNA synthesis remains remarkably constant.

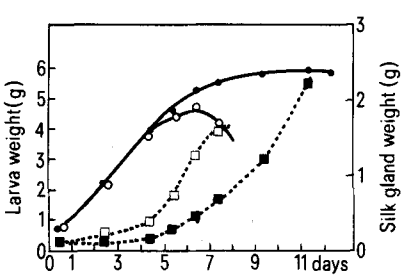


Figure 3. Effects of juvenile hormone analogue ZR 515 on silk gland growth and silk production from *Bombyx mori*. Data are taken from Daillie[25]. Circles for body weight, and squares for silk gland weight. Open signs for controls and closed signs for treated silkworms.

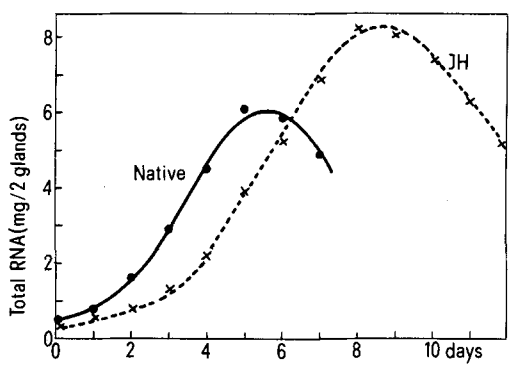


Figure 4. Effect of Juvenile hormones on the dynamics of total RNA in silk glands of *Bombyx mori*. RNA quantitative data have been collected from Prudhomme and Couble[28] and Kurata and Daillie[24]. Reproduced from Pave[29].

The degradation parameter differs significantly between 2 larvae populations. In other words, we reasonably assume that the enhancement of RNA directing the silk over-production in JH-treated larvae is related to a lower turnover of RNA, which in turn is due to a delayed activity of domestic nucleases. This last point, however, has to be checked experimentally.

e) Practical use of juvenile hormones in sericulture

As emphasized many times by Japanese[15,22,26,30,31] researchers, the methoprene or Manta was developed as a commercial drug for increasing silk production and was used until 1977. This JH analogue is usually spread out on mulberry leaves and silkworms fed with those treated leaves at the 3rd and 4th days of the 5th instar. The silk increase ranged from 5 to 15%. It is higher in races with low productivity and in the spring than in other rearing seasons. Let us note that the amount of mulberry leaves ingested by JH-treated larvae was larger than that by the control ones.

In addition, the raw silk thread was somewhat longer and thicker, but its reelability and neatness less than that of native silk. Of course, neither the fibroin/sericin ratio nor the amino acid composition of fibroin was altered.

3. General relationship between ecdysteroids and juvenile hormones: an introduction to a developmental program

The relatively poor 'harvest' of endocrinological information about respective effects of ecdysteroids and JH on silk production does not facilitate a ready understanding of their action on either larval or pupal development in the silk gland and in other silkworm tissues. A recent attempt at clarifying their interaction can be summarized in figure 5 according to Calvez[13].

From a nutritional point of view, the 5th instar can be divided in 2 feeding periods: period I or the obligatory feeding when the larvae starved during the first half of the last instar become incapable of spinning. Period II or the optional feeding occurs when the larvae starved after day 4 are capable of spinning a lighter cocoon. Larval and/or pupal developmental programs are not correlated with feeding periods, but they approximately overlap. Calvez[13] shows that JH I increases the duration of period I under larval expression and never prevents larvae from going through the period II. In addition, neither JH nor ecdysteroids control the transition from obligatory to optional feeding.

When ecdysterone was injected at day 5 - and even 2.5 days after JH application - into larvae previously treated with JH I, all underwent a complete larval molt. But when larvae treated with JH from day 5 to day 7.5 were injected with ecdysterone, no typical pupal molt occurred; only wing discs evaginated. In these 7.5-day-old larvae treated wtih JH, the developmental programm expressed larval properties, although injected ecdysterone revealed a pupal program in 5-day-old larvae. During that time lapse, the pupal program was not stabilized. Cellular self-reprogramming occurs at the onset of the last developmental period, probably under the control of ecdysteroids in the absence of JH. Unfortunately, direct effects of this cross-interaction of JH and ecdysteroids upon silk protein synthesis has not been yet investigated.

Conclusion

If we logically assume that the 'silk developmental program' is regulated as a sub-larval program, we must accept that silk glands are a specific target tissue for JH and not for ecdysteroids, regardless of the total absence of glands during pupation. However, binding sites and receptors as well as the mode of action of JH components at the chromatin level remain completely

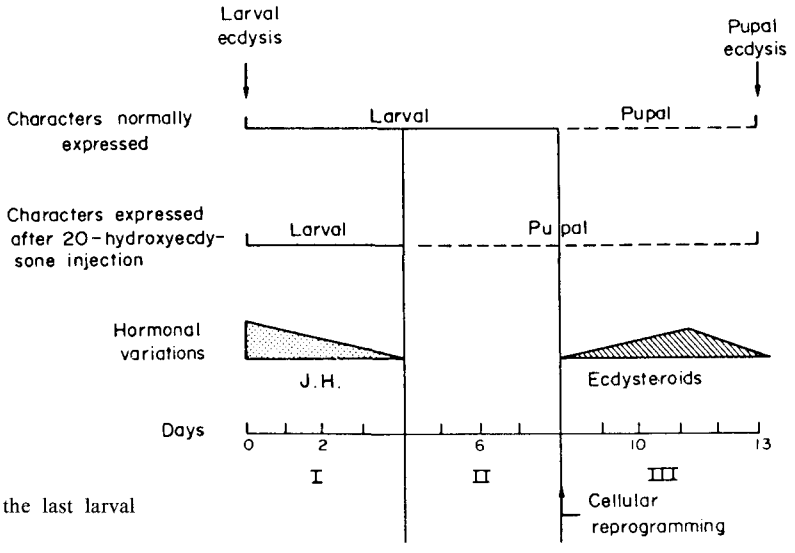


Figure 5. Progress of developmental program in the last larval instar of *Bombyx mori*. Reproduced from Calvez[13].

obscure - this is particularly true of their hypothetical specificity for silk genes and related genes.

In my opinion, there is no experimental evidence at present to suggest a direct relationship between JH activity and silk protein synthesis. Accurate quantitative assay of JH within the silk glands of *B. mori* should be available in order to confirm or refute the possible high level of JH I at the early stage of the 5th instar, which would not prevent the irreversible initiation of silk mRNA transcription. In addition, the protein synthesis inhibition directed by JH application - including inhibition of silk production - should be found at the transcription level, since this has been blocked, whereas the ribosomal apparatus is degraded at its normal turnover rate. Lastly, when JH dissociates from chromatin as a result of esterase activity and the larval developmental program resumes, silk protein synthesis is also resumed. Its accumulation is always proportional to the corresponding amount of mRNA available within the cell, which in turn depends upon the instar duration associated with a lower RNA turnover[28].

Finally, JH improves silk production because of the prolonged larval programming of the larvae. It is not necessary to postulate any affinity for the silk subprogram. This silk sub-program of larval character is controlled by other specific effector(s), possibly of a neurohumoral nature, independent of the major ecdysteroids and JH. This 'silk secretion factor', assuming it exists, has to be detected within the few hours which follow the 1st meal.

We hope that active and suitable research on ecdysteroids and JH action in the silkworm, as well as research on the silk program, will continue to progress. We are convinced that both are largely independent, but both are required for a better understanding of gene expression and developmental regulation.

* Acknowledgments. I thank G. Plantevin for communicating recent data, A. Bosquet and J. Cl. Prudhomme for their critical reading and Ch. Warren for her assistance in English style. We also are indebted to Professor K. Shimura for his invitation to join this review. Present address: C.A.R.T., 6ter, rue Rameau, F-63000 Clermont-Ferrand, France.

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VI. Sterol metabolism in insects and biosynthesis of ecdysone in the silkworm

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Insects lack the capacity for de novo sterol synthesis and require a dietary or exogenous source of sterol for their normal growth, development and reproduction. Thus they differ from vertebrates and most plants, which are capable of endogenous biosynthesis of sterols from acetate. This sterol requirement of the insect is in most cases satisfied by cholesterol (**1**); this is one of the principal sterols in insects, and serves as