Biosynthesis of α-Ecdysone by Prothoracic Glands in vitro

Abstract. *An in vitro study in which isolated prothoracic glands of the* Bombyx *silkworm were cultured has provided definite evidence that the prothoracic gland is the site where molting hormone is synthesized. The hormone behaved very similarly to free ecdysone on thin-layer chromatography. Analysis by liquid chromatography and mass fragmentography revealed that the hormone is identical with α-ecdysone.*

Endocrinological (*1*) and chemical studies (*2*) on the insect molting hormone were first achieved about 30 years ago. It has been generally believed that the prothoracic glands are responsible for ecdysone synthesis. However, there has been no direct evidence to establish this as a fact. Instead, recent endocrinological and chemical studies (*3*) on the insect molting hormone have been becoming more and more confused with respect to the elementary question of whether the prothoracic gland is really the site of ecdysone synthesis. Some investigators (*4*) have shown that isolated prothoracic glands produce the molting hormone in vitro. However, the amounts of secreted hormone have been always too small to provide proof that this gland is the site of molting hormone synthesis. We believe that the crucial experiment to answer this question should be conducted by means of organ culture of isolated prothoracic glands, extraction of hormones from the culture system, and both bioassay and chemical analysis. This approach, along with a fundamental improvement of the culture system, has led to success in demonstrating that the prothoracic glands synthesize and secrete a large amount of α-ecdysone.

The prothoracic glands were dissected from last instar *Bombyx* silkworm larvae 1 day after spinning and subjected to organ culture, in which one pair of glands was cultivated in 0.04 to 0.06 ml of culture medium for 5 to 7 days at 25°C in a hanging drop. Usually, more than 20 cultures were conducted at the same time. After cultivation, the hormone was extracted (*5*) from the glands or the culture medium and subjected to bioassay, with the use of *Sarcophaga* test abdomens (*6*) to determine the hormonal activity. The activity was calculated from the standard curve and puparium index for β-ecdysone and expressed in nanograms of β-ecdysone equivalent.

We cultivated the glands in synthetic media such as Grace's or Wyatt's (*7*). The amount of hormone produced was very small (less than 5 ng per pair of glands), thus confirming the very poor yield of the hormone reported by others. We then tried culturing in insect hemolymph, since it was assumed that hemolymph would be the most physiologically satisfactory medium and that the cholesterol associated with hemolymph lipoproteins (*8*) may be utilized as the substrate of ecdysone. We used hemolymph from diapausing pupae of the Cynthia silkworm, after removing the hemocytes. However, on exposure to air an expected difficulty arose, the hemolymph darkened, because of the tyrosine-tyrosinase reaction. It may theoretically be possible to use some reagents such as phenylthiourea to prevent such darkening, but such chemicals are poisonous or physiologically deleterious. Therefore, we applied the hemolymph to a Sephadex column to separate the protein fraction from the low-molecular substances, including tyrosine. The protein fraction was collected through a Sephadex G-25 column equilibrated with saline and diluted with an equal volume of Wyatt's insect culture medium (omitting tyrosine), filtered with a Millipore filter, and then used as the culture medium.

The medium, referred to as "hemolymph medium," contained about 40 percent of the original hemolymph on the basis of the amount of protein, and did not blacken even after prolonged standing in air. We made another change in the culture system: an excess of oxygen (0.5 unit partial pressure) was continuously supplied to the small incubator in which the organ culture was performed, since the anatomical fact that the prothoracic glands are associated with a highly developed tracheal system appears to show that their function requires an ample supply of oxygen.

This improved culture system, in which the prothoracic glands were cultivated in hemolymph medium under a continuous supply of excess oxygen, led to the first successful result. A typical set of data (Table 1) reveals that, before cultivation, the amount of hormonal activity detected is extremely low in the glands and completely lacking in the culture medium, whereas, after cultivation, a considerable amount of activity is found only in the medium and none in the glands themselves. This result indicates that as soon as the hormone is synthesized by the glands it is secreted into the medium, which may explain why no investigator has succeeded in detecting an appreciable amount of molting hormone in extracts from the prothoracic gland itself.

Table 2 shows the efficiency of various culture media; in comparison with glands cultured in hemolymph medium, those in synthetic media produce only a small amount of hormone. The reason why hemolymph medium is so effective in supporting hormone synthesis by the glands while synthetic media lack such capacity may at least partly be explained by the following assumption. Since cholesterol is a precursor of ecdysone (*9*), the cholesterol present in the hemolymph, which is known to be associated with two major lipoproteins, lipoproteins I and II (*8*), may be utilized as the substrate, whereas the synthetic media contain no such available cholesterol. To test this assumption, we purified lipoproteins I and II from hemolymph (*8*), and prepared culture media consisting of lipoprotein

Table 1. Amounts of molting hormone found in glands and media before and after cultivation (expressed in nanograms of β-ecdysone equivalent per pair of glands). Culturing conditions are described in the text.

Medium or organ	Gland cultures (No.)	Amount of hormone
Before cultivation		
Prothoracic gland	40	2.5
Hemolymph medium (1 ml)		0.0
After cultivation		
Prothoracic gland	25	0.0
Hemolymph medium	25	120.0

Table 2. Effect of culture medium on the synthesis of molting hormone by prothoracic glands. The amount of hormone found in culture medium after cultivation is expressed in nanograms of β-ecdysone equivalent per pair of glands. The amounts of proteins contained in one culture medium were 1.3 mg for hemolymph, 0.54 mg for lipoprotein I, and 0.50 mg for lipoprotein II.

Medium	Gland cultures (No.)	Amount of hormone
Grace's	14	4.8
Wyatt's	24	17.7
Hemolymph	25	120.0
Lipoprotein I	21	148.0
Lipoprotein II	16	108.0

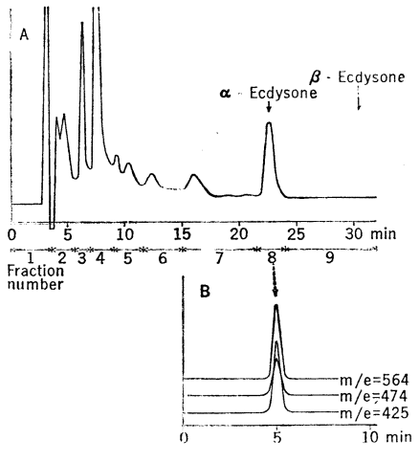

Fig. 1. (A) Liquid chromatogram of the fraction eluted from TLC plate. High-pressure liquid chromatography: instrument, DuPont 830 liquid chromatograph; column, Zorbax SIL (25 cm by 2.1 mm inside diameter); mobile phase, CH_2Cl_2 and CH_3OH (9 : 1); flow rate, 0.3 ml/min; column pressure, 1000 pounds per square inch; column temperature, 20°C; and detector, ultraviolet photometer at 254 nm. (B) Mass fragmentogram of TMS derivative of fraction 8. Instrument, LKB 9000s MID PM 9060S; column, 1.5 percent OV-101 on Chromosorb W HP 80 to 100 mesh (1 m by 4 mm inside diameter); column temperature, 270°C; ionization current, 60 μa; ionization voltage, 20 ev; accelerating voltage, 3.5 kv; and ion source temperature, 290°C.

I or II and Wyatt's medium. Both the two lipoproteins as well as hemolymph are very satisfactory in culture media (Table 2) and appear to be more efficient than hemolymph on the basis of the protein amount, suggesting that the above assumption is correct.

A continuous supply of excess oxygen was also beneficial. Glands cultured in air (0.2 oxygen partial pressure) produced only 25 ng (per pair of glands) of the hormone equivalent to β-ecdysone, while the production in 0.5 oxygen partial pressure (97 ng) was approximately four times greater.

In order to determine the chemical nature of the molting hormone formed in our culture system, we first used thin-layer chromatography (TLC). For this experiment, the hormonal fraction was extracted from the culture medium after cultivating 40 pairs of glands. One half of the fraction was directly subjected to bioassay to determine the hormonal activity, and the other half was applied to a TLC plate (ready-made silica gel plate, Merck) which was developed with a mixture of chloroform and methanol (2 : 1). Pure β-ecdysone was run at the same time as a marker. After the plate was developed, the hormone was eluted with tetrahydrofuran (*10*) from several regions with different R_F's, including the solvent front and the origin, and was then bioassayed. The results revealed that all the hormonal activity was located in a distinct region corresponding to β-ecdysone ($R_F = 0.53$). Since it is well known that α- and β-ecdysone show very close and sometimes overlapping R_F's on TLC, this observation suggests that the hormone in question represents either α- or β-ecdysone, or both.

Further evidence on the chemical nature of the hormone was obtained from high-pressure liquid chromatography. An amount of hormonal fraction extracted from the culture medium equivalent to 200 pairs of glands was first applied to TLC. A portion of the fraction eluted from the region corresponding to ecdysone on TLC was subjected to bioassay to determine the hormonal activity, and the remaining portion was submitted to liquid chromatography. The new fractions detected were collected individually and bioassayed. A typical chromatogram (Fig. 1A) shows the presence of eight or more fraction peaks, indicating that the extract from TLC still contains many different substances. Fraction 8 was identical with α-ecdysone but not with β-ecdysone with respect to retention time. Furthermore, the chromatography in which fraction 8 was run with standard α-ecdysone showed that this fraction was not separable from α-ecdysone but gave a distinct single peak. On the other hand, the results of the bioassay revealed that eight fractions (1st to 7th and 9th) had no hormonal activity at all, and that only fraction 8 had such activity, which was equal to the total activity of the material applied to the chromatography. In order to obtain the crucial data on the structure of the hormone, we subjected this fraction to mass fragmentography. The fraction 8 material collected from another run of liquid chromatography was applied to a gas chromatography–mass spectrometry system, with a trimethylsilyl (TMS) derivative of the fraction (*10*). The ions m/e 425, 474, and 564 which are most characteristic of α-ecdysone TMS derivative (*10*) were monitored. As shown in Fig. 1B, the retention time of each of these three peaks exactly coincided with that (5.0 minutes) of the α-ecdysone TMS derivative, and the relative intensities of these three peaks were satisfactorily consistent with those of the standard spectrum of the α-ecdysone TMS derivative (*10*).

Our experimental results, together with our present knowledge on the insect molting hormone, lead to the conclusion that the prothoracic gland is indeed the site of α-ecdysone synthesis. Another research team working independently has provided chemical evidence, including complete mass spectrometry, that the isolated prothoracic glands of the tobacco hornworm, *Manduca sexta*, produce α-ecdysone (*11*); this result is consistent with our conclusion.

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