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# Ecdysteroid Synthesis in Dissociated Cells of the Prothoracic Gland of the Silkworm, *Bombyx mori*

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**ABSTRACT**—A method for the preparation of viable cells of prothoracic glands of *Bombyx mori* was developed. Dissected prothoracic glands (PGs) from larvae at the spinning stage were incubated in the Grace's insect culture medium containing dispase (6,000 PU/ml of medium at pH 6.5) at 37°C for 15 min. After centrifugation for 2 min at 250 rpm, resultant cells were incubated with Grace's insect medium for designated times. After this treatment 87±7% of the cells remained viable as judged by exclusion of trypan blue. The amounts of ecdysteroid secreted into medium by these cells were approximately 70 to 80% of the amounts secreted by intact PGs. The rates of ecdysteroid secretion over 6 hr in organ culture and in cell culture were quite similar, although the amounts of ecdysteroid detected in the latter medium were somewhat lower.

## INTRODUCTION

The synthesis of radioactive ecdysone made possible the development of the radioimmunoassay (RIA) which provided an extremely sensitive, simple and cheap method of detecting ecdysteroid [2, 5, 17]. By using these techinques, it is now well established that ecdysone is the product of the PGs and that its production and release is controlled by the prothoracicotropic hormone (PTTH) [1, 3, 8, 13, 18].

In *Bombyx* the studies on the mode of action of PTTH on the PGs and biochemical processes on the regulation of ecdysteroid synthesis by PTTH are quite few, although identification and isolation of PTTH have been developed [6, 7, 9]. In the present study, we demonstrate an improved method for preparing the dispersed cells of PG for the investigation of mode of action of PTTH.

## MATERIALS AND METHODS

*Insects*

Larvae of the silkworm, *Bombyx mori* (J122×C115), were reared with mulberry leaves or artificial diet (Nihon Nosan Kogyo, Yokohama) in a rearing room of our laboratory at 25±1°C in a 16 hr light: 8 hr dark photoperiod. Larvae were staged on the day of 4th ecdysis, and this day was designed as Day 0 of 5th instar. Only female animals were used throughout the experiments.

*Preparation of larval prothoracic gland*

After immersing the larvae into 75% of ethanol for 2 to 3 min, the prothoracic glands (PGs) of the staged larvae were removed. The PGs were rinsed with physiological saline (0.85% NaCl) for *Bombyx* and then with Grace's medium (GIBCO, USA) two to three times to avoid contamination by haemolymph. Thereafter, PGs were incubated in Grace's medium or were treated with a medium containing dispase as described below.

*Preparation of incubation medium containing dispase*

To dissociate the cells of the PG, we used a proteolytic enzyme, dispase, (Godo Shusei, Tokyo). The optimal pH for this enzyme is between 7.5 and 8.0 and the optimal temperature is 25 to 37°C. The pH of the silkworm haemolymph is about 6.5. Accordingly, the pH of the medium was adjusted with 1 N NaOH to either pH 6.5 or pH 8.0 before use. The dispase was then dissolved in this medium. Activity of this enzyme was represented as protease units per ml of medium. Incubation temperature was 25 or 37°C.

*Incubation of prothoracic gland or its cells*

PGs were dissected from the staged animals and incubated immediately. A pair of glands was incubated in 300 μl of Grace's medium at 25°C. Thirty microliter aliquots of the medium were taken for RIA at various times and were replaced by 30 μl of fresh medium.

In the case of dissociated cells, larval PGs were incubated in various concentrations of dispase in Grace's medium for 15–60 min at either 25 or 37°C. After several rinses with Grace's medium, one pair glands equivalent of dissociated cells was incubated in 300 μl of medium. During the incubation, the reaction mixture was shaken gently with a micromixer (TAITEC: EM 33, Japan) and cells were dissociated by drawing the tissue in and out of a siliconized Pasteur pipette about 80 times at 15 min intervals for up to 1 hr.

*Viability of the dissociated cells of prothoracic gland*

After dispase treatment, the viability of the dissociated cells was checked using 0.1% trypan blue. Viable cells excluded this dye, whereas dead cells became blue.

*Estimation of ecdysteroids in haemolymph and in culture medium*

Haemolymph (10 μl) or medium (30 μl) was subjected to an ecdysteroid radioimmunoassay (RIA) [15]. Ecdysteroids were extracted with 300 μl of absolute methanol, then aliquots of the

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supernatant were assayed for ecdysteroid by RIA. Since 20-hydroxyecdysone was used as a standard, the average values of the amount of ecdysteroids are expressed as ng of 20-hydroxy-ecdysone equivalents ± standard deviation. The tritiated ligand[23, 24-$^3$H(N)] ecdysone (82.8 Ci/mmol) was purchased from New England Nuclear.

## RESULTS

*Dissociation of prothoracic gland cells by a proteolytic enzyme, dispase*

For studies of ecdysteroid synthesis in dissociated cells of the PG *in vitro,* the following prerequisites must be fulfilled by the experimental procedure: low variability among replicate samples, rapid and easy methods for handling of large numbers of cells, homogeneity among the dispersed cells, and maintenance of viability for at least 6 to 8 hr during the incubation.

The PG cells in *Bombyx* are compact and surrounded by a thick basal lamina (Fig. 1 [16]). Therefore, we tried various enzymes to obtain viable preparations of single PG cells. Preliminary incubations with either trypsin or collagenase were not satisfactory since most cells were not viable (data not shown). By contrast, with dispase treatment, PG cells partially dissociated and remained viable. In order to obtain viable cells of PG consistently, we sought to establish procedures for preparing single cells of PG.

Firstly, PGs from day 9, 5th instar *Bombyx* larvae, were incubated for various times in Grace's medium containing several concentrations of dispase at different pHs and temperatures. Five pairs of PG were removed and rinsed first with physiological saline for *Bombyx*, then with Grace's medium. These organs were incubated in a disposable plastic dish (Corning, 35×10 mm) with 2 ml of dispase solution (0 to 1,500 PU/ml Grace's medium).

At 25°C the cells of PG showed little dissociation even at the highest enzyme concentration used (1,500 PU/ml for 60 min) irrespective of pH (Table 1). Under these conditions, the cells were dispersed gradually with time and concentration of enzyme but some clusters of cells remained, and the dissociated cells were significantly damaged. Accordingly, these conditions were not sufficient for preparation of viable cells from PG. On the other hand, when the PGs were incubated with dispase at 37°C, quantities of dispersed cells could be obtained both at pH 6.5 (Fig. 2) and pH 8.0 (Table 1). However some precipitates of cellular debris were observed at higher enzyme concentrations at pH 8.0. Thus, it seemed that the treatment with dispase at pH 8.0 was unsatisfactory for preparing viable cells of PG.

Secondly, the effects of dispase concentration on the dissociation of PG cells were investigated. A pair of PG from a larva in the spinning stage (day-9) was treated with various concentration of dispase in Grace's medium (pH 6.5) at 37°C. As shown in Figure 3, there was no correlation between the enzyme concentration and the percentage of viable cells. The yield of viable cells was 86.8±7.2% for all concentrations from 1.5 to $10\times10^3$ PU/ml.

Lastly, we separated a pair of PGs and treated each individual PG with dispase (pH 6.5, 6000 PU/ml, 15 min, 37°C) to determine the cell number in the PG. There was no difference between the cell number in the right and the left PG throughout development in the 5th instar with the average number of cells between 160 to 230 in both right and left PG (data not shown).

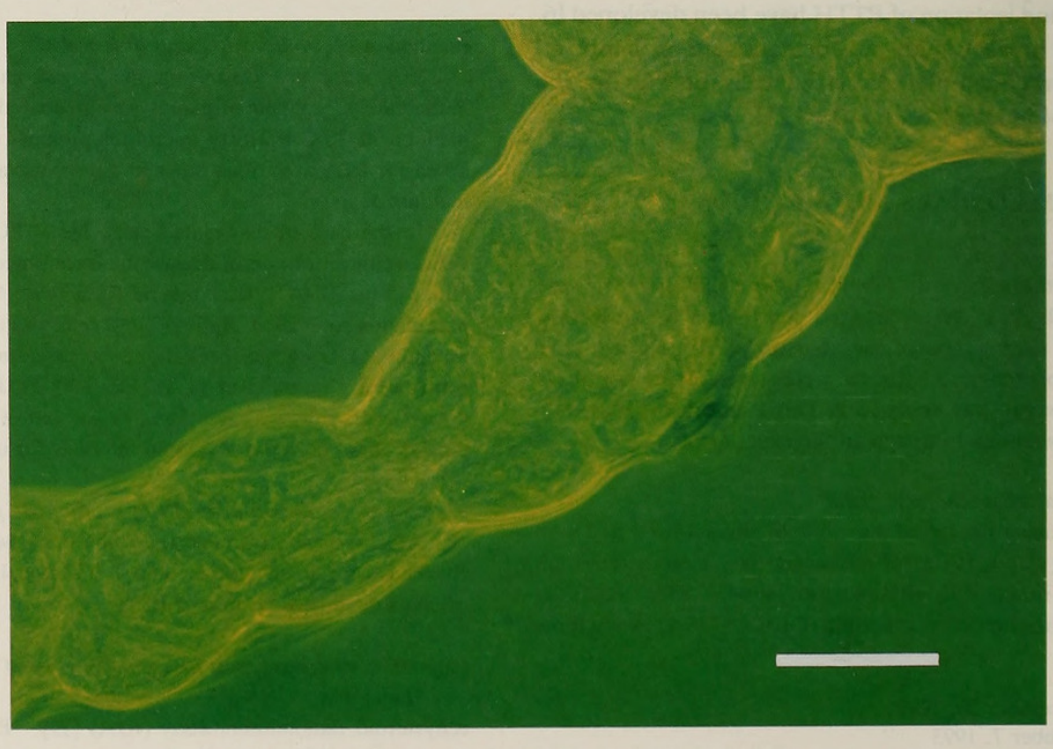

FIG. 1. Prothoracic gland in *Bombyx mori.* Bar, 100 μm.

TABLE 1. Dissociation of prothoracic glands by dispase under different conditions

| Concentration of dispase (PU/ml) | Incubation time (min) 15 | 30 | 45 | 60 | Incubation time (min) 15 | 30 | 45 | 60 |
|---|---|---|---|---|---|---|---|---|
| | [pH 6.5 at 25°C] | | | | [pH 6.5 at 37°C] | | | |
| 0 | − | − | − | − | − | − | − | − |
| 500 | − | − | ± | ± | ± | + | ++ | + |
| 1,000 | ± | ± | ± | ++ | ± | + | ++ | ++ |
| 1,500 | ± | + | + | ++ | ++ | +++ | +++ | +++ |
| | [pH 8.0 at 25°C] | | | | [pH 8.0 at 37°C] | | | |
| 0 | ND | ND | ND | ND | − | − | − | − |
| 500 | ND | ND | ND | ND | ND | ND | + | + |
| 1,000 | ND | ND | ND | ND | + | + | ++ | ++ |
| 1,500 | ND | ND | + | ++ | + | +++ | ++ | +++ |

The grades of the dissociation of prothoracic glands were as follows; −: not dissociated, ±: basal lamina was removed but cells were not dissociated, +: some clusters of prothoracic gland cells seen, ++: partial dissociation of prothoracic glands with a mixture of single cells and groups of 2 to 5 cells, +++: complete dissociation of the prothoracic gland yielding dispersed cells as shown in Fig. 2. ND: not determined.

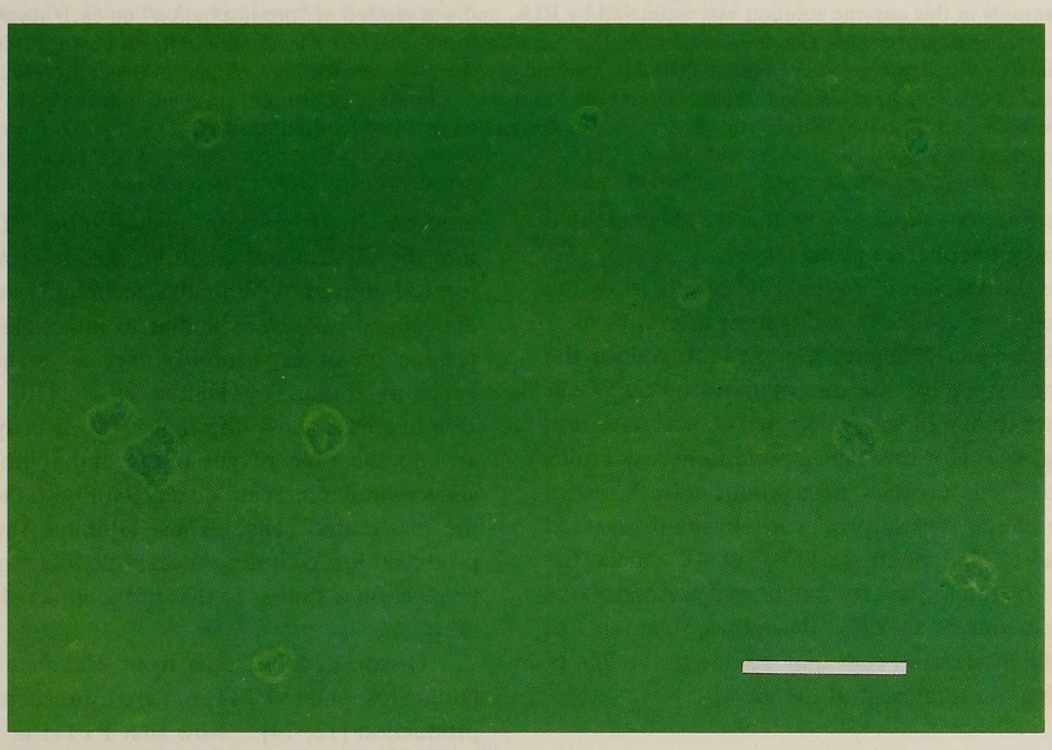

FIG. 2. Dissociated cells of prothoracic glands. The prothoracic glands were treated with 1,500 PU of dispase/ml Grace's medium (pH 6.5) at 37°C for 30 min. Bar, 350 $\mu$m.

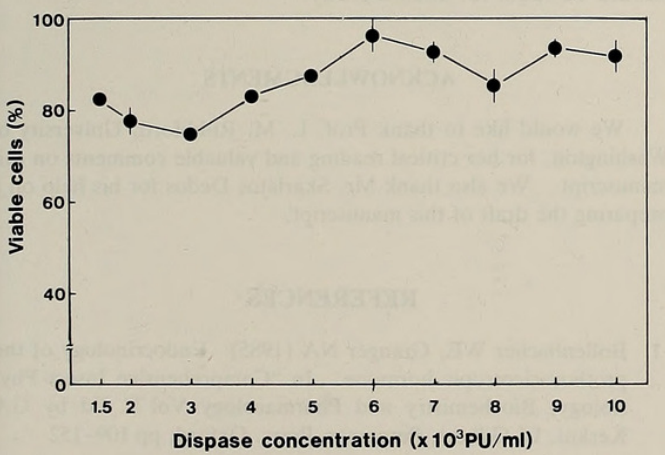


FIG. 3. Relationship between the concentration of dispase in Grace's medium (pH 6.5) and the percentage of viable cells as judged by exclusion of the trypan blue dye.

*Secretory activity of ecdysteroid by intact PGs and dissociated PG cells*

Intact PGs from larvae in the spinning stage (day-8 to day-10 of 5th instar) were incubated in Grace's medium for 6 hr to determine their secretory ability in comparison to those of dispersed cells. As shown in Figure 4, the secretory activity of PGs on day 8 (1 day after the onset of spinning) was relatively low (10.9±1.8 ng per 6 hr). By day 9, secretory activity increased about 3-fold to 28.6±4.1 ng per 6 hr

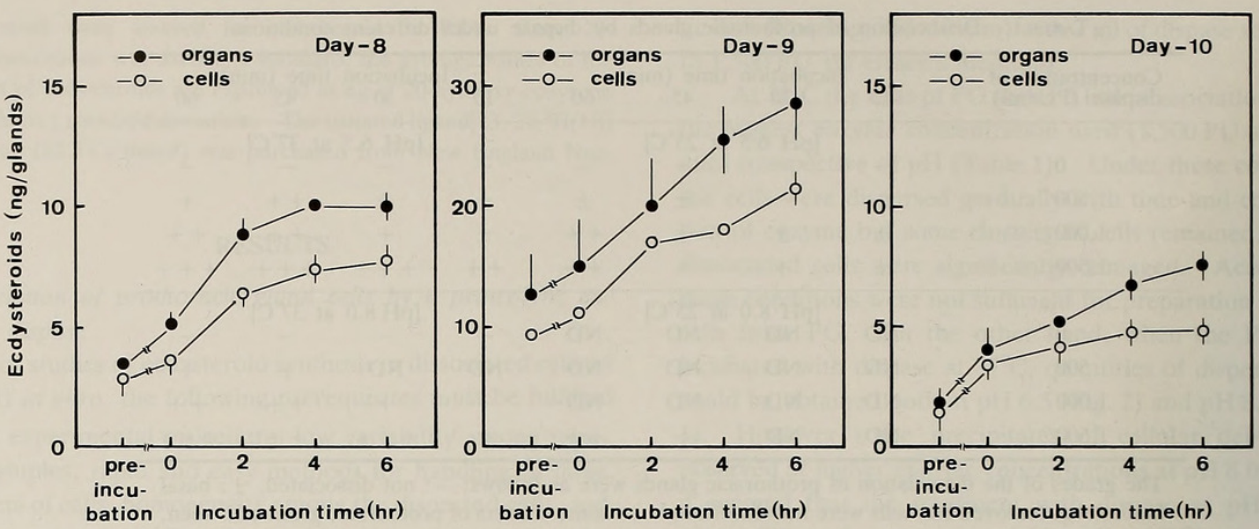


FIG. 4. Ecdysteroid synthesis following incubation of the dissociated cells and intact prothoracic glands of *Bombyx mori* on different days of the final instar. The cells of the prothoracic gland were dispersed with dispase (6,000 PU/ml of Grace's medium, pH 6.5) at 37°C for 15 min. The enzyme was removed by centrifugation at 250 rpm for 2 min. The amount of ecdysteroids in this enzyme solution was estimated by RIA, and was plotted as "pre-incubation" on each abscissa. The resultant cells of prothoracic glands were immediately rinsed with 300 $\mu$l Grace's medium. After centrifugation (250 rpm for 2 min), the cells were resuspended in 300 $\mu$l of medium at time "0" of incubation. A pair of intact prothoracic glands or a pair of one gland's equivalent of dissociated cells was used. The stage of prothoracic gland is indicated in the upper right side of each figure. The day of 4th ecdysis was designated as "day 0" of 5th instar.

incubation. Ecdysteroid produced by the glands decreased significantly by day 10 (pharate pupal stage).

To measure the amount of ecdysteroid produced by the dispersed PG cells, we used a centrifugation tube with filter (Ultrafree, C3SV, 5 $\mu$m Millipore Co. Ltd) to reduce the handling time necessary for the dissociation of PG. Cells from PGs of day 8 to day 10 larvae secreted ecdysteroids into the medium (Fig. 4). However, the amounts of ecdysteroids were about 30 to 35% lower than those produced by the intact PGs. The rate of ecdysteroid production in the dissociated cells was similar to that of intact PG (Fig. 4). Since the mortality of cells by the dispase treatment as described above ranged between about 14 to 25%, the reduced amounts of ecdysteroid in the dissociated cells may be due to the loss of viable cells during the treatment of the glands.

## DISCUSSION

The PG cells in *Bombyx* are surrounded by a thick basal lamina (Fig. 1), while the cells in *Hyalophora cecropia* are connected only by a thin strand [4] and the morphology of the glands of *Manduca sexta* is intermediate between these two extremes [3, 11]. *Manduca* PG cells have been successfully dissociated by either 0.4% trypsin/chymotrypsin/elastase [14] or 0.4% elastase [12]. In these experiments, the yield of viable cells was $\geq$95% and the cells remained viable for at least 4 hr [12, 14]. In the present experiment, a technique was developed whereby the intact PG of *Bombyx* could be dispersed into viable cells by using dispase in Grace's medium. The yield of viable cells ranged between 80 to 94% and these cells remained viable at least 6 hr.

To minimize the handling of the dissociated cells, we used an Ultrafree centrifugation tube. Thus, after incubation the supernatant could be readily removed and assayed for ecdysteroids. With this technique we showed that the dissociated cells were similar to intact glands in their linear production of ecdysteroids over a 2–6 hr period *in vitro*. Previous studies by Okuda *et al.* [10] have shown that *Bombyx* PGs show different rates of ecdysteroids synthesis around the time of gut purge and spinning. Our studies show a similar increase on day 9 for both the intact glands and the dissociated cells. Thus, although the dissociated cells produced less ecdysteroid than the intact gland, their rate of production is similar to that of the intact gland at a particular time.

Dissociated PG cells from *Manduca* lacking the basal lamina respond to PTTH *in vitro* by an increase in ecdysteroid production [12, 14]. Now that PTTH in *Bombyx* has been purified and sequenced [6, 7, 9], studies of its mode of action on the PG are needed. Our dispersed PG cell preparation should be ideal for such a study.

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