2-Deoxy-α-ecdysone from Ovaries and Eggs of the Silkworm, *Bombyx mori*

Abstract. *From ovaries dissected from developing and emerged adults of the silkworm,* Bombyx mori, *two substances having high molting hormone activity were isolated. One of these was identified as 2-deoxy-α-ecdysone by means of high-pressure liquid chromatography and mass spectrometry. Although this compound had previously been isolated from the fern* Blechnum minus *and postulated to be the precursor of α-ecdysone, it had not been obtained from insect material. The compound is also contained in the form of a conjugate in ovaries as well as in diapausing silkworm embryos.*

High molting hormone activity has been detected in the ovaries and eggs of several species of insects: from eggs of *Melanoplus differentialis* (*1*), *Bombyx mori* (*2*), *Manduca sexta* (*3*), and *Oncopeltus fasciatus* (*4*), and from ovaries of *Macrotermis bellicosus* (*5*) and *Locusta migratoria* (*6*). In the silkworm *Bombyx mori* α-ecdysone has been found in developing embryos (*7*), whereas most of the molting hormone–active substances in diapausing embryos are present in the form of conjugates (*8*). We have now isolated and identified 2-deoxy-α-ecdysone. The compound is also contained in diapausing embryos in a conjugated form. Although this compound had been obtained by Chong *et al.* from the fern *Blechnum minus*, it had not been obtained from an insect material (*9*).

Our starting materials were ovarian tissues (270 g from pharate adults, 6 days after larval-pupal ecdysis, and 498 g from adults, within 3 hours after emergence) or diapausing embryos (700 g, maintained for 6 months at room temperature after egg-laying). These materials were crushed and ground to powder in liquid nitrogen with a pestle in a mortar. The frozen powder was then dried without thawing. The dried powder thus obtained was extracted with 80 percent ethanol (1 liter for ovarian tissues from pharate adults, 1.5 liters for ovaries of emerged adults and for diapausing embryos) at 60°C for 20 minutes, and the residue was reextracted twice in the same way. The combined extracts were concentrated in a rotary evaporator. The concentrate was dissolved in 100 to 200 ml of 70 percent methanol, and the insoluble matter was washed twice with 50 to 100 ml of 70 percent methanol. The methanolic solution was shaken with an equal volume of petroleum ether to remove lipids. The methanolic phase was then concentrated to a syrup under reduced pressure. The concentrate was dissolved in a small volume of ethanol and stored at −80°C overnight. Gummy precipitates, composed mostly of carbohydrates, were removed by filtration through a sintered-glass filter, and the residue was washed with a small volume of ethanol. The ethanolic filtrates were combined and concentrated under reduced pressure. This concentrate was dissolved in a small volume of water and applied on a column of Sephadex G-15 (3.5 by 92.5 cm). Molting hormone activity at every purification step was estimated by bioassay with the use of isolated abdomens of fleshfly larvae according to Ohtaki *et al.* (*10*). The biologically active fraction was then subjected to silicic acid chromatography (*10*). The column consisting of 40 g of silicic acid (Mallinkrodt AR 100 mesh; 4.5 by 5.0 cm) was first eluted with 1.5 liters of a mixture of benzene and methanol at 90:10, then 500 ml of mixtures of benzene and methanol at 75:25, and 50:50, and finally with 2.5 liters of methanol. The bulk of free ecdysone came off the column with the solvent system of benzene-methanol at 90:10; whereas the conjugated forms appeared mostly in the methanol eluate.

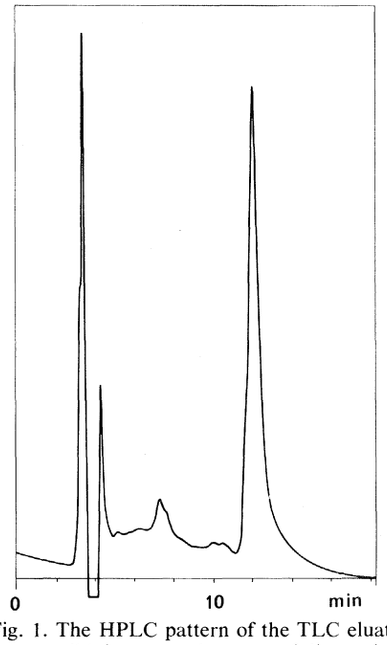

Fig. 1. The HPLC pattern of the TLC eluate from snail juice–treated extract of diapausing embryos. The fraction eluting at 12.0 minutes showed potent molting hormone activity. The TLC eluates from ovarian tissues of developing and emerged adults gave essentially the same chromatograms. The HPLC was performed (DuPont 830; column, Zorbax SIL 2.1 by 250 mm) in a solvent system of 8 percent methanol in methylene chloride, at a flow rate of 0.26 ml/min.

The fraction containing conjugates was treated with 400 mg of acetone powder of snail juice (*11*) in acetate buffer at *p*H 5.3, at 30°C for 5 to 6 hours. After the incubation, the mixture was treated with four volumes of ethanol, and the insoluble materials were removed by centrifugation. The preparation was then subjected to a second silicic acid chromatography (20 g, 4.5 by 2.2 cm). Elution with benzene-methanol (90:10) recovered most of the activity; subsequent elution with methanol yielded only minor activity, indicating that most of the conjugates had been hydrolyzed. The benzene-methanol eluate from the first silicic acid chromatography as well as that from the second chromatography were then subjected to preparative thin-layer chromatography (TLC) on precoated silica gel plates (Merck; 60 F_{254}; 20 by 20 cm, 2.0 mm thick) with a solvent system of chloroform and 96 percent ethanol (4:1). Ultraviolet-absorbing materials were visualized under an ultraviolet lamp. Major ultraviolet absorbing bands were scraped off and eluted with freshly distilled tetrahydrofuran. In cases of extracts from ovaries, materials from the bands having R_F values of 0.45 and 0.35 exhibited considerable biological activity. High-pressure liquid chromatography (HPLC) constituted the final step of purification; the material with an R_F value of 0.45 was eluted as a sharp peak at 12.0 minutes, which corresponded with the retention time of 2-deoxy-α-ecdysone (Fig. 1). Cochromatography of the material with the authentic 2-deoxy-α-ecdysone (isolated from *Blechnum minus* and supplied by D. H. S. Horn) yielded a single sharp peak at 12.0 minutes. The ultraviolet spectrum of the material collected from the fraction showed maximum absorbancy (ethanol) at 243 nm. Identity of the material with 2-deoxy-α-ecdysone was confirmed by mass spectrometry; it exhibited prominent peaks at 448 (M^+), 430 (M^+-18), 412 (M^+-18X2), 394 (M^+-18X3), 361 ($M^+-C_5H_{11}O$), 314, 284 (M^+−side chain 1). The material having an R_F value of 0.35 seems to be an ecdysone analog, since it has molting hormone activity (*Sarcophaga* test) and has an absorption maximum at 240 to 250 nm in ethanol.

From the snail juice digest of diapausing embryos, materials having R_F values of 0.45, 0.35, and 0.27 on TLC showed considerable biological activity. The materials with R_F values of 0.45 and 0.35 seem to be the same as the components

described above. The material having the R_F value of 0.27 was identified as α-ecdysone, by means of HPLC and gas chromatography of trimethylsilyl derivative (*12*). Similar conjugates of 2-deoxy-α-ecdysone and of the compound having an R_F value of 0.35 were also found in extracts of ovaries in a comparable amount to the free forms.

Assuming that the molecular extinction coefficients of 2-deoxy-α-ecdysone and of α-ecdysone are the same as that of β-ecdysone, and neglecting the loss during purification, the content of the compounds in ovaries and in embryos was calculated from the peak areas in the chromatograms of HPLC by comparison with a standard curve constructed with authentic β-ecdysone (*7*). The content of free 2-deoxy-α-ecdysone in pharate adult ovaries is 0.31 μg/g, while that of the conjugated form is 0.46 μg/g. The content of free 2-deoxy-α-ecdysone in emerged adult ovaries is 0.22 μg/g, while that of the conjugated form is 0.26 μg/g. The content of 2-deoxy-α-ecdysone in diapausing embryos is 0.36 μg/g in a conjugated form.

The discovery of 2-deoxy-α-ecdysone in insect material is significant, since it lends support to the hypothesis that 2-deoxy-α-ecdysone is the immediate precursor of α-ecdysone (*9*).

Reevaluation of the role of conjugates of ecdysones seems necessary. Conjugates have been postulated to be inactivation products (*13*), but the presence of the conjugates of 2-deoxy-α-ecdysone and of α-ecdysone suggests that they are normal metabolic intermediates or storage forms, part of which may eventually be transformed into free α-ecdysone during the course of embryonic development (*7*).

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11. Japanese common landsnails, *Euhadra* sp., were collected on the campus of Nagoya University and starved for 1 week; digestive fluid was then squeezed from the intestines. An acetone powder of the snail juice was prepared by mixing ten volumes of cooled (−80°C) acetone, by filtering the resultant precipitate with a small Buchner funnel, and by drying it in an evacuated desiccator.
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14. Part of our work was presented at the Annual Meeting of the Japanese Comparative Endocrinology, March 1976, and at the 15th International Congress of Entomology, August 1976. Supported by grant 30404 from the Ministry of Education, Japan. We thank Professor T. Takemoto of Tokushima University for the α- and β-ecdysone and the Katakura Sericultural Experiment Station and the Kanebo Silk Company for silkworm materials.

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Transmitter Release During Repetitive Stimulation: Selective Changes Produced by Sr^{2+} and Ba^{2+}

Abstract. *The addition of Sr^{2+} or Ba^{2+} to the solution bathing the frog neuromuscular junction leads to an increased release of transmitter by each nerve impulse during and following repetitive stimulation. The mechanisms by which Sr^{2+} and Ba^{2+} increase release are not the same. Each ion appears to act selectively on a different process involved in transmitter release.*

The amount of transmitter released from a synapse by each nerve impulse varies as a function of previous synaptic activity (*1*). The factors which give rise to this variation in transmitter release have traditionally been distinguished from one another by differences in the duration of their effects. On this basis at least four processes (*2*) have been shown to increase transmitter release at the synapse of the vertebrate neuromuscular junction: a first component of facilitation which decays with a time constant of about 50 msec (*3*); a second component of facilitation which decays with a time constant of about 300 msec (*3*); augmentation which decays with a time constant of about 7 seconds (*4*); and potentiation (post-tetanic potentiation) which decays with a time constant of tens of seconds to minutes (*5*). Some of the factors involved in facilitation, augmentation, and potentiation of transmitter release appear to be different (*6*), but until now it has been difficult to distinguish among these processes except on the basis of their time constants of decay. We now report that Sr^{2+} and Ba^{2+}, two ions that can substitute for Ca^{2+} in evoked transmitter release (*7, 8*), have differential effects on the processes involved in transmitter release. While Sr^{2+} selectively increases the magnitude and time course of the second component of facilitation, Ba^{2+} selectively increases the magnitude of augmentation. These differential effects of Sr^{2+} and Ba^{2+} provide a new way to distinguish among the processes which increase transmitter release during repetitive stimulation and suggest that these processes can act relatively independently of one another.

End-plate potentials (EPP's) were recorded from the sartorius nerve-muscle preparation of the frog *Rana pipiens* by means of intracellular and surface recording techniques (*4*). Surface recording sums the responses from many endplates giving a good measure of average intracellular activity (*3, 9*). Standard frog Ringer (115 m*M* NaCl, 2 m*M* KCl, 1.8 m*M* $CaCl_2$, 2.16 m*M* Na_2HPO_4, 0.85 m*M* NaH_2PO_4, 5 m*M* glucose, 0.03 m*M* choline) was modified by reducing Ca^{2+} to 0.35 to 0.6 m*M* and adding 5 m*M* Mg to decrease transmitter release and thus prevent muscle twitch. The effect of Ba^{2+} on transmitter release was examined by adding 0.05 to 1.0 m*M* Ba^{2+} to this control bathing solution (*10*). The effect of Sr^{2+} was examined by adding 0.2 to 0.6 m*M* Sr^{2+} to the control solution or by replacing the Ca^{2+} in the solution with 0.6 to 1.5 m*M* Sr^{2+}.

At the low levels of transmitter release in these experiments transmitter release is approximately proportional to EPP amplitudes (*4*). Facilitation, augmentation, and potentiation are all defined by the fractional increase in EPP amplitudes that they produce (*4, 11*). The contribution that each makes to increasing transmitter release was determined by successively peeling off the multiple exponentials used to describe the decay of the EPP amplitudes following the conditioning trains (*4*) or by using stimulation patterns which can test for each process during a conditioning train (*6*).

Figure 1 shows that replacement of the Ca^{2+} in the bathing solution with Sr^{2+} leads to an increase in the magnitude and time course of the second component of facilitation. The motor nerve was stimu-