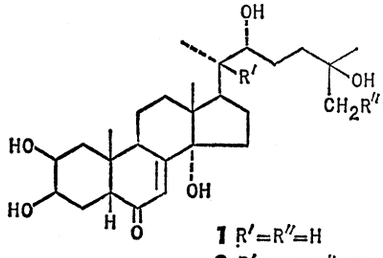
26-Hydroxyecdysone: New Insect Molting Hormone from the Egg of the Tobacco Hornworm

Abstract. *Five kilograms of tobacco hornworm eggs (48 to 64 hours old) afforded 26.5 milligrams of a new crystalline insect molting hormone identified as 26-hydroxyecdysone. The three known insect ecdysones—α-ecdysone, 20-hydroxyecdysone, and 20,26-dihydroxyecdysone—were also present but in much smaller quantities. The new hormone is the predominant molting hormone in the hornworm during this stage of embryonic development. These results taken in context with the current knowledge of the chemistry and biochemistry of the molting hormones during postembryonic development in the hornworm indicate quantitative and qualitative differences in the biosynthetic-metabolic pathways as well as in the ecdysones in different developmental stages of this insect.*

The known insect molting hormones, α-ecdysone (**1**), 20-hydroxyecdysone (**2**), and 20,26-dihydroxyecdysone (**3**) have been isolated and identified from insects (*1*) during postembryonic development. Although high levels of molting hormone activity have been reported during embryonic development (*2*), the qualitative nature of the ecdysones in this stage of insects has not yet been determined. In our continuing studies with the molting hormones of the tobacco hornworm, *Manduca sexta* (L.), we observed that the extracts from 48- to 64-hour-old eggs exhibited biological activity in the housefly assay (*3*) greater than that found during pupal-adult development (*4*). By contrast, the extracts of 1- to 4-hour-old eggs had negligible biological activity, an indication that the hormones are produced by the developing embryo. The observations prompted us to accumulate a sufficient amount of 48- to 64-hour-old eggs for analyses, and we now report that although the known insect ecdysones (**1**, **2**, and **3**)



1 R′=R″=H
2 R′=OH; R″=H
3 R′=R″=OH
4 R′=H; R″=OH

were isolated in very small quantities from the developing embryo, the predominant ecdysone present is a new hexahydroxy steroid, 26-hydroxyecdysone—2β,3β,14α,22*R*,25,26-hexahydroxy-5β-cholest-7-en-6-one (**4**).

Collections were made over a period of several years until we accumulated 5 kg or approximately 3.5×10^6 eggs. The eggs set aside for these studies were held at ambient temperature (28° ± 3°C) until 48 to 64 hours old, and after removal of 100 eggs from each collection to determine hatch (average hatch 85 percent), the material was frozen until extracted. The eggs were blended for 20 minutes with methanol at 0.5 ml/g and then three times again with 0.5 ml/g of 75 percent methanol. Examination under the dissecting microscope of the pulp after decantation of the final methanolic supernatant did not reveal intact eggs. Our methods of extracting, fractionating, and isolating the molting hormones have been described (*4–6*). However, when we fractionated the biologically active extract on silicic acid (*7*) according to our previous method, the new hormone was eluted in both the α-ecdysone and 20-hydroxyecdysone (benzene and methanol system, 9 : 1) and in the 20,26-dihydroxyecdysone (benzene and methanol system, 75 : 25) fractions. Furthermore, in the countercurrent distribution (CCD) system of cyclohexane, butanol, and water (5 : 5 : 10), the new hormone [*K* (distribution coefficient) = 0.39] overlapped the tubes for 20-hydroxyecdysone ($K = 0.52$) (*4*). However, thin-layer chromatography (TLC) on preparative silica gel G plates in the solvent system of chloroform and ethanol (8 : 2) with wick, readily separated the new hormone (R_F 0.08) from 20-hydroxyecdysone (R_F 0.15). The new steroid (R_F 0.31) in the solvent system of chloroform and ethanol (65 : 35) (*8*) with wick, could be separated from 20,26-dihydroxyecdysone (R_F 0.22) by TLC on preparative silica gel G plates and by countercurrent in the solvent system mentioned previously. On recrystallization of the new steroid from ethyl acetate and methanol, we obtained 26.5 mg of needlelike crystals, melting at 252° to 256°C with slight decomposition, an ultraviolet absorption maximum at 245 nm (methanol), ε 11,600, ν_{max} in Nujol 3380 cm^{-1} (associated hydroxyls), 1650 cm^{-1} (α,β-unsaturated ketone), mass spectrum (*m/e*, relative intensity) M^+ 480(< 1), 462(9), 444(33), 426(17), 413(12), 395(8), 330(9), 300(100), 291(45), 285(26), 267(29), 211(40), 181(60), 142(48), 115(45), 93(38). The prominent peaks at *m/e* 462, 444, and 426 are due to ions formed from the molecular ion by successive elimination of one, two, and three molecules of water respectively, while the peak at *m/e* 413 (M-36-31) results from loss of two molecules of water plus CH_2OH (from scission of the bond between C-25 and C-26). The base peak at *m/e* 300 resulting from cleavage of the bond between C-17 and C-20 accompanied by a hydrogen atom transfer and loss of one molecule of water indicate that the ecdysone side chain has three hydroxyl groups. The peak at *m/e* 115 is the side chain fragment resulting from scission of the bond between C-20 and C-22 followed by a loss of one molecule of water.

The nuclear magnetic resonance (NMR) spectrum recorded at 60 megacycles in deuterated pyridine with methyl resonances at δ 0.74(18-H), 1.08(19-H), 1.23, 1.32(21-H), 1.47 (27-H) and the mass spectral data indicate that this compound is 26-hydroxyecdysone (**4**).

The 26-hydroxyecdysone in the housefly assay is about one-tenth to one-fifteenth as active as α-ecdysone or is approximately equal to the activity of 20,26-dihydroxyecdysone (*8*). In addition to 26-hydroxyecdysone we also isolated 1.2 mg of 20-hydroxyecdysone and could account for about 0.5 mg of α-ecdysone and 1.5 mg of 20,26-dihydroxyecdysone. These hormones were identified by comparison of their *K* (CCD) and R_F (TLC) values, and their mass and NMR spectra with those of the authentic steroids.

Since insects possess the biochemical mechanism for forming ecdysone conjugates as glucosides (*9–11*) and as sulfates (*10, 11*), portions of the highly polar fractions from the egg were subjected to enzymic hydrolysis and our results with sulfatase yielded small but identifiable amounts of 26-hydroxyecdysone. Ecdysone moieties from glucosidase hydrolysis if present were below the range of our method of detection. Apparently conjugation does not occur to an appreciable extent dur-

ing this period of development. From the foregoing results, the biosynthetic–metabolic pathways for the molting hormones during this stage of embryonic development can best be expressed as follows:

α-ecdysone (**1**) → 26-hydroxyecdysone (**4**)
↓
20-hydroxyecdysone (**2**) →
20,26-dihydroxyecdysone (**3**)

In the egg, α-ecdysone serves as the common precursor for both routes; but clearly the pathway to 26-hydroxyecdysone is the principal one as indicated by the strikingly large quantities of this hormone, whereas the conversion of α-ecdysone to 20-hydroxyecdysone is a minor pathway. However, during pupal-adult development α-ecdysone serves as a precursor for 20-hydroxyecdysone, which at this stage is the predominant molting hormone (*4, 8*). This has been substantiated in studies with the labeled ecdysone precursor 22,25-dideoxyecdysone which is efficiently converted to the three insect ecdysones (**1, 2, 3**) during both prepupal and pupal-adult development (*5, 11*). However, during larval development in the hornworm, this same ecdysone precursor is principally metabolized to a number of ecdysone analogs which lack the hydroxyl group at C-22 (*6*).

On the basis of the chemical and biochemical information on the molting hormones in the tobacco hornworm, then, different biosynthetic-metabolic pathways as well as quantitative and qualitative differences in the ecdysones occur in different developmental stages of this insect. Consequently, different ecdysones could function at different stages of insect development, and the qualitative nature of the molting hormones could well dictate the type of molt. If this is true, certain of the current concepts concerning the hormonal control of molting and metamorphosis in insects may require a reevaluation and revision.

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Octopamine-Sensitive Adenylate Cyclase: Evidence for a Biological Role of Octopamine in Nervous Tissue

Abstract. *An adenylate cyclase that is activated specifically by very low concentrations of octopamine has been identified both in homogenates and in intact cells of the thoracic ganglia of an insect nervous system. This enzyme appears to be distinct from two other adenylate cyclases present in the same tissue, which are activated by dopamine and by 5-hydroxytryptamine, respectively. The data raise the possibility of a role of octopamine-sensitive adenylate cyclase in the physiology of synaptic transmission.*

Recent studies have indicated that the actions of some neurotransmitters may be mediated through adenosine 3′,5′-monophosphate (cyclic AMP). For example, evidence now suggests that cyclic AMP mediates dopaminergic transmission, thereby modulating cholinergic transmission, in mammalian sympathetic ganglia (*1*). Included in this evidence is the observation that low concentrations of dopamine increase ganglionic cyclic AMP through the activation of a dopamine-sensitive adenylate cyclase (*2*). Evidence has also been presented that cyclic AMP may mediate the inhibitory effect of norepinephrine on Purkinje cells of the cerebellum (*3*), a tissue known to contain a norepinephrine-sensitive adenylate cyclase (*4*).

It has been suggested that octopamine (*5*), which is found in both vertebrate and invertebrate nervous systems, may, in some species, function as a

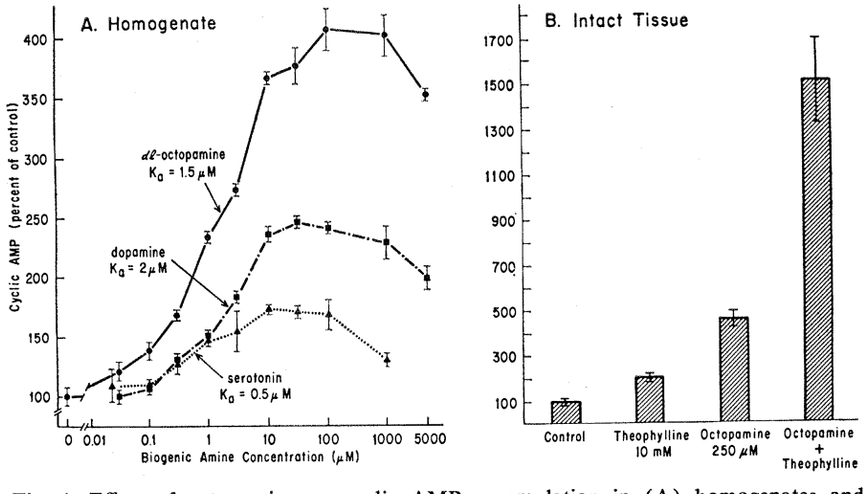

Fig. 1. Effect of octopamine on cyclic AMP accumulation in (A) homogenates and (B) intact tissue preparations of cockroach thoracic ganglia. (A) Effect of various concentrations of *dl*-octopamine, dopamine, and serotonin on adenylate cyclase activity in homogenates. The control activity, per milligram of protein, in the absence of added biogenic amine was 10.0 ± 1.5 pmole/min. (B) Effect of 250 μ*M* *dl*-octopamine and 10 m*M* theophylline, alone and in combination, on the accumulation of cyclic AMP in intact hemiganglia. The control was 17.7 ± 2.7 pmole per milligram of protein. The values shown in both (A) and (B) are the means and ranges for two to three replicate samples, each assayed in duplicate.