26-Hydroxyecdysone, which is the major free recoverable ecdysteroid of older age groups of embryonated eggs of the tobacco hornworm was also the major component in 4- to 18-hour-old embryonated eggs. The other 3β-ecdysteroids, ecdysone, 20-hydroxyecdysone, and 20,26-dihydroxyecdysone, were also present and accounted for all the molting hormone activity; 26-hydroxyecdysone was devoid of molting hormone activity in the house fly assay. 20-Hydroxyecdysone was a minor component, which confirms the earlier observations that the main metabolic route for ecdysteroids during embryonic development is that leading to 26-hydroxyecdysone, whereas formation of 20-hydroxyecdysone is a minor pathway. A new 3α-ecdysteroid, 3-epi-26-hydroxyecdysone, also devoid of molting hormone activity, was the second major ecdysteroid isolated from the eggs. 3-Epi-20,26-dihydroxyecdysone was detected in very minute amounts. In addition to the six 3β- and 3α-ecdysteroids there were at least an equivalent number of unknown ecdysteroids all of which lacked molting hormone activity. Their physical properties including chromatographic behavior are discussed.

INTRODUCTION

The naturally occurring ecdysteroids from two different age groups of embryonated eggs of the tobacco hornworm, Manduca sexta (L.), 48- to 64-hour and 24- to 44-hour-old were reported in previous communications (1,2,3). In both of these age groups, 26-hydroxyecdysone accounted for 80 percent of the free recoverable ecdysteroids. The three other commonly known 3β-ecdysteroids of insects, ecdysone, 20-hydroxyecdysone and 20,26-dihydroxyecdysone, accounted for most of the remaining 20%. Because of the preponderance of 26-hydroxyecdysone, it was proposed that during embryonic development the major biosynthetic-metabolic pathway for these ecdysteroids during embryonic development involves the formation of

* Retired, August 1979
26-hydroxyecdysone, and pathways leading to 20-hydroxyecdysone and 20,26-dihydroxyecdysone are only minor ones.

This report is concerned with the profile of the naturally occurring ecdysteroids isolated from young embryonated hornworm eggs (4- to 18-hour-old).

EXPERIMENTAL METHODS AND RESULTS

Instrumentation: A Waring Blender, 3.8 liter capacity (4), served to homogenize the eggs. An International Model PR-2 portable refrigerated centrifuge was used to centrifuge the extracts. A manually operated Craig-Post 60 tube counter-current distribution (CCD) unit with 10 ml volumes each of the upper and lower phase was used to separate or purify the ecdysteroids. Precoated thin-layer chromatography (TLC) Silica Gel 60F-254 plates (E. Merck, Darmstadt, Germany) were employed for the separation, purification, and analyses of the compounds. A Chromato-vue instrument box permitted direct visualization of the spots or zones under short wavelength ultraviolet light. Spots were also detected by spraying the plate with 50% sulfuric acid and heating at 100°C. Ultraviolet spectra were taken in methanol with a Bausch and Lomb Spectronic 505. Mass spectra were secured with an LKB Model 9000 mass spectrometer equipped with a Varian Spectra System 100 MS data system. The samples were introduced directly into the ionization chamber (ionization energy was 70 ev). NMR spectra were recorded at 60 MHz with a Varian A-60A NMR Spectrometer equipped with a Varian C-1024 time averaging computer, and with a JEOL FX-60-Q Fourier Transform NMR equipped with a 1.7 mm capillary probe. Deuterated pyridine was used as the solvent and TMS as the internal standard. IR spectra of samples coated on a AgCl plate were obtained with a Nicolet model 7199 Fourier Transform IR.

Biological Material: Tobacco hornworm eggs from 4- to 18-hour-old were
collected daily from leaves of the tobacco plant (5). A sample of 100 eggs from each collection was set aside to determine hatch (average hatch 87.5%). At ambient temperature (28 ± 3°C) hatch occurs in 3 days. The other eggs were weighed, transferred into screw cap glass containers and held frozen (-20°C) until 3000 g were accumulated for workup.

Bioassay: The house fly assay (6) was used to detect molting hormone (MH) activity during extraction and fractionation of the ecdysteroids. The pure ecdysteroids, depending on their polarity, were assayed either as aqueous or as methanolic (5-10%) solutions. For those ecdysteroids that possess MH activity, a house fly unit is equivalent to that quantity of the pure hormone that results in 50-60% puparium formation of the treated insects.

Column chromatography: Both macro-and micro-adsorption column chromatography was used to purify and/or separate the ecdysteroids. The first macrocolumn consisted of 50 g [4.4 (I.D.) x 2.5 cm] of Woelm neutral alumina (activity Grade 1 ± 20% water). The adsorbent was packed in a minimum amount of methanol without allowing any of the solvent to pass through the column. After introduction of the crude extract in a minimum amount of methanol directly on the adsorbent, the column was eluted first with 800 ml of methanol and then with 800 ml of 75% aqueous methanol. The second macrocolumn was 18 g (4.4 x 2.5 cm) of benzene-washed silicic acid (7) capped with a layer of sand. After introduction of the methanol eluate from the alumina column, the silicic acid column was eluted as follows: [1] 1200 ml of benzene-methanol (95:5); [2] 400 ml of benzene-methanol (95:5); [3] 800 ml of benzene-methanol (90:10); [4] 400 ml of benzene-methanol (75:25); and [5] 400 ml of methanol. A microcolumn of silicic acid was used as the final step in the purification of the individual ecdysteroids. It consisted of 1.13 g (1.1 x 2.5 cm) of benzene-washed silicic acid capped off with sand. After introduction of the ecdysteroid, the column was eluted as follows: [1] 75 ml of benzene-methanol (95:5); [2] 25 ml of benzene-methanol (95:5); [3] 50 ml of benzene-methanol (90:10); [4] 15 ml of benzene-methanol (75:25); and lastly [5] 15 ml of methanol.

Thin-layer Chromatography (TLC): Precoated TLC Silica Gel 60F-254 plates in conjunction with column chromatography and CCD were also used to purify and separate the ecdysteroids. The three solvent systems used to develop the plates in chamber with wick were: chloroform-ethanol 4:1 (8); chloroform ethanol 65:35 (9); and chloroform ethanol 9:1 (10). Once the upper and lower limits of the zones were demarked under UV light, they were scraped off the plate into a 50-ml glass stoppered centrifuge tube equipped with a glass funnel. Methanol (15 - 20 ml) was used to rinse the funnel and the glass rod that was used to break up clumps and for extracting the slurry. After vigorous shaking and centrifugation, the initial extract was removed and the silica gel was extracted twice again with methanol. Recoveries by this procedure were not quantitative, nevertheless; TLC was necessary for separating and purifying the large number of ecdysteroids encountered in this study.

Ecdysteroid Derivatives: The acetonides were prepared by adding 25 μl of acetone that contained 10 μg of p-toluenesulfonic acid to 10 μg of the ecdysteroid and allowing the reaction mixture to stand for 2 hr at 30°.
All or part of the reaction mixture was spotted on a TLC plate and the plate was developed in the solvent system of chloroform-ethanol (12:1).

The acetates were obtained by allowing the ecdysteroid to react with pyridine-acetic anhydride (3:1) for 24 hr at 25°C. The excess acetic anhydride was allowed to react with methanol, the solvent was removed with a stream of nitrogen gas and the residue was redissolved into methanol and spotted on a TLC plate. The plate was developed twice in the solvent system of benzene-ethyl acetate (1:3).

Analysis of Crude Sulfatase Solution for Ecdysteroids: Sigma sulfatase (crude solution, Type H-2, Lot No. 880-9004) from Helix pomatia was used to determine both the presence of any biological activity inherent in the enzymatic preparation and as the enzyme source for hydrolysis of sulfate conjugates of insect extracts. One ml of the crude sulfatase solution was transferred to a 50-ml centrifuge tube and diluted to 10 ml with distilled water saturated with l-butanol. The diluted solution was extracted three times with equal volumes of butanol saturated with water and then further processed and worked up in the same manner as the butanol extractive of hornworm eggs. The residues from column fractions 3 and 4 were analyzed by TLC.

No UV absorbing material was detected under UV light in the ecdysteroid region for either fraction though a single microgram of an ecdysteroid standard following development is readily detected under UV light. Since ec dysone and 20-hydroxyecdysone are the most active molting hormones in the house fly assay, the areas of silica gel corresponding to these ecdysteroids were extracted and bioassayed. Biological activity was associated with the area of 20-hydroxyecdysone. The quantity of "20-hydroxyecdysone" in this lot number of Helix sulfatase solution was on the order of 300 ng/ml.

Extraction of Hornworm Eggs and Column Fractionation of Egg Extracts: After the frozen eggs were transferred to a water jacketed blender and allowed to thaw overnight in 0.5 ml methanol per gram of tissue, they were homogenized alternately at low and high speed for 20 min. The homogenate was transferred to 250-ml glass cups and centrifuged at 2200 RPM for 10 min. The supernatants were decanted and filtered under vacuum through medium pore, fritted disc glass funnels. The pulp in the tubes as well as the residue in the funnels were combined and rehomogenized three times with 75% methanol at 0.5 ml/g and treated as before. The procedure for homogenizing the eggs was complete since no intact eggs were observed when several samples of the final pulp were examined under the dissecting microscope.

Further purification of the extract via partitioning between butanol-water, followed by partitioning of the residue from the butanol phase between 70% methanol-hexane are described elsewhere (11).

From the workup of the 3 kg of hornworm eggs, the 70% methanol extract yielded 3.0 g of residue, (total activity 1.2 x 10^6 HFU). The residue was incubated with 1 ml of the crude Helix sulfatase in 100 ml of sodium acetate-acetic acid buffer solution (pH 6.2) for 24 hours at 37°C (12). To recover the ecdysteroids, the buffer solution was extracted three times with 1/2 volumes of butanol and the extracts were combined and the solvent removed under vacuum. Bioassay of the residue (2.3 g) did not result in an increase in the total biological activity (1.0 x 10^6 HFU). The butanol extractive was dissolved in a minimum amount of
methanol and fractionated on a macrocolumn of Woelm neutral alumina. The methanol eluate (0.97 g) possessed 90% of the biological activity. The eluate was dissolved in 2.0 ml of methanol, adjusted with benzene to a final concentration of benzene-methanol (95:5), and subjected to a macrocolumn of benzene-washed silicic acid. The flask containing the eluate was rinsed twice with 10 ml volumes of methanol-benzene as described above and added to the column. Bioassay, and TLC analyses of column fractions 2 through 5 revealed that fractions 3 and 4 possessed activity.

Isolation and Identification of the Ecdysteroids Eluted in Column Fraction 3: Fraction 3 (143.7 mg), which is known to elute ecdysone, 20-hydroxyecdysone, and a considerable quantity of 26-hydroxyecdysone (1), was subjected to 50 transfers in a CCD system of cyclohexane, butanol, water (5:5:10). The upper and lower phases of each tube were then collected, reduced to dryness, weighed, dissolved in a known volume of methanol for UV and/or TLC analyses. For TLC, a 15-μg sample from each tube was spotted on a 20 x 20 cm plate along with ecdysteroid side markers, and the plate was developed in chloroform-ethanol (65:35). The material from CCD tubes 8 through 23 gave UV absorbing spots with an $R_f$ value for 26-hydroxyecdysone (0.27) and an ecdysteroid with an $R_f$ value of 0.31. The contents of tubes 8 through 21 were pooled (24.0 mg); analysis by UV showed $λ_{max}$ at 245 nm, which suggests an α,β-unsaturated ketone system (7-ën-6-one) that is typical of ecdysteroids. The material was streaked equally on six 20 x 20 cm plates (4 mg/plate) and the plates were developed in chloroform-ethanol (65:35). When the plates were viewed under UV light, three zones were detected, a lower zone with an $R_f$ value of 26-hydroxyecdysone (0.27) a middle zone with an $R_f$ of 0.31, and an upper barely discernible zone with an $R_f$ value of 0.38, which is similar to that of 20-hydroxyecdysone. The ecdysteroids from the lower (16.3 mg) and middle (1.75 mg) zones were set aside until preparative TLC purification was obtained of the ecdysteroids eluted in column fraction 4. The material representing the upper zone (possibly 20-hydroxyecdysone) was lost when the UV analysis was completed. Based on its UV spectrum the total mass of this ecdysteroid was in the order of 50 μg.

TLC analyses of samples from CCD tubes 23 through 39 each showed three major UV absorbing spots with $R_f$ values of 0.34, 0.43 and 0.48 that differed in $R_f$ from any previously isolated tobacco hornworm ecdysteroids except for ecdysone ($R_f$ 0.45). The contents of tubes 23 through 39 were pooled (13.5 mg), analyzed by UV spectrometry and the material was streaked equally on three 20 x 20 cm plates and developed once in the solvent system of chloroform-ethanol (65:35). Only two major UV absorbing zones were now detected, a lower zone with an $R_f$ value of 0.34, and a broad upper zone with an $R_f$ value of 0.46 (zones coinciding with $R_f$ values of the spots of 0.43 and 0.48 were not resolved). The NMR spectrum of the material (1.0 mg) from this lower zone ($R_f$ 0.34) indicated a mixture of compounds. Further purification by TLC on a 10 x 20 cm plate developed 9 times in the solvent system of chloroform-ethanol (4:1) yielded 300 μg of an ecdysteroid of structure yet to be determined (unknown 1). The NMR spectrum of its acetate indicates that the compound formed a triacetate. The comparative $R_f$'s of this acetate and acetates of the other ecdysteroids that were isolated and of the standards are presented in Table 1. The new ecdysteroid was inactive.
<table>
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<tr>
<th>Ecdysteroid</th>
<th>Acetates</th>
<th>$R_f$ values*</th>
<th>Acetonides</th>
<th>$R_f$ values**</th>
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<td>diacetone</td>
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<td>Unknown 6 ---</td>
<td>0.36</td>
<td>---</td>
<td>11111</td>
<td></td>
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</table>

* TLC plate developed twice in chamber with wick in the solvent system of benzene-ethyl acetate(1:3)

** TLC plate developed in chamber with wick in the solvent system of chloroform ethanol(12:1)
in the house fly assay at 0.5 μg, the highest level tested.

The upper zone (R<sub>f</sub> value of 0.46) yielded 5.0 mg of material. NMR analyses indicated it to be a mixture. The mixture was streaked on a single 20 x 20 cm TLC plate and developed 4 times in the slower moving solvent system of chloroform-ethanol (4:1). Under UV light, three UV absorbing zones were detected. The material recovered from each of the zones gave λ<sub>max</sub> at 245 nm (methanol). The upper zone (215 μg) on the basis of analyses by NMR, was impure and was purified further by TLC using a 5 x 20 cm plate and developed in the solvent system of chloroform-ethanol (9:1). The UV absorbing zone yielded 110 μg of an unknown ecdysteroid(s) (unknown 2). The new ecdysteroid was inactive in the house fly at 1.0 μg, the highest dose tested.

The middle zone gave 1.4 mg of material. This ecdysteroid was identified by TLC, NMR, and mass spectral data as ecdysone (Fig. 1). Crystallization from methanol-ethyl acetate yielded 220 μg of crystals, m.p. 231-234° with decomp., λ<sub>max</sub> 245 nm, ε 11,450. Its biological activity in the house fly assay was the same as that of authentic ecdysone.

The lower zone yielded 1.2 mg of material. Analysis both by CCLY and NMR, however, indicated it to be a mixture of compounds. The mixture was streaked on a 10 x 20 cm TLC plate and developed six times in the solvent system of chloroform-ethanol (4:1), resulting in three UV absorbing zones. The material recovered from each zone gave sharp λ<sub>max</sub> at 245 nm. The upper, middle, and lower zones gave 125, 110, and 65 μg, respectively of new ecdysteroids (unknowns 3, 4, 5). All three steroids were inactive in the house fly assay at 0.24 μg, which was the highest dose tested.

Each of these ecdysteroids gave distinctly different NMR spectrum. The NMR spectrum of the acetate of the ecdysteroid (unknown 3) from the upper zone indicated a triacetate; that of the middle zone (unknown 4) indicated a diacetate and the presence of other minor acetoxy groups which suggested the presence of other compounds that were also acetylated. The TLC of these acetates also confirmed the presence of small amounts of acetoxy compounds other than those that could result from partial acetylation of an hydroxyl group at C-25, which suggested the presence of other impurities. Unknown 5 was not derivatized or further analyzed.

Isolation and Identification of the Ecdysteroids from Column Fraction 4:

Fraction 4 (104.6 mg), which is known to elute 26-hydroxyecdysone and 20,26-dihydroxyecdysone (1), was also subjected to 50 transfers by CCD and worked up as described for column fraction 3. The contents from tubes 0 through 6 gave two UV absorbing spots when analyzed by TLC in the solvent system of chloroform-ethanol (65:35), a lower spot with an R<sub>f</sub> value of 0.08 (unknown 6) and an upper spot with an R<sub>f</sub> value of 0.19 which coincides with that of 20,26-dihydroxyecdysone. The material (3.14 mg) from tubes 0 through 6 was streaked on a 10 x 20 cm plate and developed in the solvent system of chloroform-ethanol (65:35). Two major and a minor UV absorbing zones were detected, a lower major zone with an R<sub>f</sub> value of 0.00 (unknown 6), a middle major zone with an R<sub>f</sub> value of 0.19 (20,26-dihydroxyecdysone), and a minor upper zone, not detected in the preliminary TLC analysis, with an R<sub>f</sub> value of 0.22, which is similar to that for 3-epi-20,26-dihydroxyecdysone. The material from both the lower and middle zone gave sharp λ<sub>max</sub> at 245 nm. However,
the material from the upper zone yielded a UV spectrum with considerable
end absorption and a barely discernible shoulder at 245 nm.

The very polar lower zone (unknown 6, Rf 0.08) yielded 355 μg of
material that was not amenable to mass spectral analysis. Additional
analyses will be required before the structure of this ecdysteroid is
determined. This steroid was inactive in the house fly assay at levels
up to 0.5 μg, which was the highest dose tested.

The middle zone gave 260 μg of an ecdysteroid which, by TLC, NMR and
mass spectral analyses, was identified as 20,26-dihydroxyecdysone
(Fig. 1). The ecdysteroid was 1/10 to 1/15th as active as ecdysone in
the house fly assay. The upper zone yielded about 15 μg, of material
(based on its UV spectrum) and by TLC analyses behaved as 3-epi-20,26-di-
hydroxyecdysone. However, because of the insufficient quantity, the
ecdysteroid was not subjected to bioassay or additional physical
analyses.

The contents of CCD tubes 8 - 23 yielded two UV absorbing spots by
TLC, one with an Rf value similar to that of 26-hydroxyecdysone (0.27)
and another with an Rf value of 0.31. The combined material from tubes
8 through 23 (31.6 mg) was streaked on 8 full size plates and developed
in the solvent system of chloroform-ethanol (65:35). The lower zone (Rf
value 0.27) yielded 23.7 mg of material that was combined with the 16.3
mg of ecdysteroid (Rf value 0.27) recovered from the TLC plates in
fraction 3. The combined material (40.0 mg) recrystallized from ethyl
acetate-methanol yielded 35.7 mg of 26-hydroxyecdysone, m.p. 251-255°
with decomp., λmax 245 nm, ε 11,632 [Lit (1) m.p. 252-256°,
λmax 245 nm, ε 11,600]. The TLC, NMR, and mass spectral data
were identical with those of 26-hydroxyecdysone previously isolated
(1,2). The material was inactive in the house fly assay at levels up to
1 μg, which was the highest dose tested.

The material (1.5 mg) from the upper zone was combined with the 1.75
mg of ecdysteroid with a similar Rf value recovered from the TLC plates
from fraction 3 and rechromatographed on a 20 x 20 cm plate. The UV
absorbing zone yielded 2.1 mg of the ecdysteroid with an Rf value of
0.31, λmax 245 nm, ε 9,700 (ca. 80% purity). The NMR and mass
spectral data of this ecdysteroid were almost indistinguishable from
those of 26-hydroxyecdysone. This information and the fact that the
compound by TLC analyses was only slightly less polar than 26-hydroxy-
ecdysone and forms only a monoacetonide (13,14,15) identified the ecdy-
steroid as 3-epi-26-hydroxyecdysone (Fig. 1). The new ecdysteroid was
inactive in the house fly assay up to the 1.0 μg level, which was the
highest dose tested.

DISCUSSION

In our first communication we stated that MH activity in 48- to
64-hour-old eggs exceeded that found during pupal-adult development (11),
whereas extracts from 1- to 4-hour-old eggs possessed negligible MH
activity. The low biological activity of the free ecdysteroids in
extracts from 1- to 4-hour-old eggs as opposed to the high biological activity of the free ecdysteroids in extracts from the older eggs suggested that in the older eggs the hormones were produced by the developing embryo (1), or that the developing embryo was capable of converting the inactive ecdysteroids or conjugates already present in eggs to MH active ecdysteroids. The view that the hormones were produced by the developing embryo was reinforced when highly polar column fractions from the crude extracts of the older eggs were subjected to enzymic hydrolyses yielded small but identifiable quantities of 26-hydroxyecdysone, which indicated that the ecdysteroid conjugates in this period of embryonic development were present only in small amounts (1). In a subsequent study (2) with 24- to 44-hour-old embryonated eggs, MH activity of the free ecdysteroids was approximately two thirds that found in the older eggs (1). This study also confirmed the high levels of free 26-hydroxyecdysone relative to the other ecdysteroids (2) and supported the view that the developing embryo produced its own hormone(s).

Subsequent reports by other researchers who have worked with ovaries and young eggs from other species of insects (16,17,18) have indicated, however, that both adult ovaries and newly laid eggs possess high levels of ecdysteroids as conjugates and that the ecdysteroids present in the egg are in part the result of direct transfer of the hormones by the mother to her eggs. These latter reports prompted us to subject the extracts from young embryonated eggs (4- to 18-hour-old) to enzymic hydrolysis before any further processing. However, bioassays of extracts obtained prior to \((1.2 \times 10^6 \text{ HFU})\) and after hydrolysis \((1.0 \times 10^6 \text{ HFU})\) did not show a difference in the total MH activity, indicating that the Helix enzyme did not hydrolyze conjugates of active ecdysteroids.
The predominant ecdysteroid in young eggs (4- to 18-hour-old) is 26-hydroxyecdysone (Fig. 1). Moreover, these eggs possess higher levels of 26-hydroxyecdysone, about 37 mg (12 µg/g wet wt); of the 24- to 44-hour-old and 48- to 64-hour-old embryonated eggs possess 15.3 (3.0 µg/g wet wt.) and 26.5 mg (5.3 µg/g wet wt.), respectively. However, on the basis of recovery of both known and unknown ecdysteroids, the relative percentage of 26-hydroxyecdysone is about 90% and thus is in the magnitude of that found for the older groups.

The higher levels of 26-hydroxyecdysone could be interpreted to indicate that the adult female hornworm contributes by transmitting certain ecdysteroids in the free and/or conjugated form to the eggs. However, this conclusion would be premature since embryogenesis has already begun in the 4- to 18-hour-old eggs. A final decision of this issue must await further studies on adults, their ovaries, and eggs under 4-hour-old. In relation to the latter, we have already begun to collect 1- to 4-hour-old eggs for future studies. Analyses of these eggs for both free and conjugated ecdysteroids, and subsequent enzymic hydrolysis of the conjugates followed by the isolation and identification of the ecdysteroids should resolve whether ecdysteroids in Manduca eggs are a result of direct transfer from the female hornworm or are produced by the developing embryo.

A surprising development during biological evaluation of the ecdysteroids was the lack of MH activity in the house fly assay for 26-hydroxyecdysone isolated both in this study and an earlier study (24- to 44-hour-old). Concurrent assays of both lots of 26-hydroxyecdysone gave negative results at levels as high as 1 µg. Previously (1), this ecdysteroid from embryonated eggs was thought to exhibit molting hormone
activity of about 1/10th to 1/15th that of ecdysone (for ecdysone 3.0 ng = 1 HFU). The discrepancy can most likely be explained by a contamination on the earlier samples with 20-hydroxyecdysone.

Three other 3β-ecdysteroids, ecdysone, 20-hydroxyecdysone (identified on the basis of TLC) and 20,26-dihydroxyecdysone, besides 26-hydroxyecdysone were also present. These accounted for about 1.5% of the total recovered ecdysteroids and for all the MH activity. 20-Hydroxyecdysone, which is the major hormone at peak titer of MH activity during pupal-adult development (11), was present in minute amounts (ca. 50 μg). Such low titers of 20-hydroxyecdysone are consistent in hornworm eggs throughout embryonic development, and similar observations have been reported for eggs and ovaries of certain other insect species as well (18). Although nanogram quantities of ecdysone and 20-hydroxyecdysone have been detected in the Helix pomatia enzymes (16,19), the molting hormone activity (equivalent to 300 ng of 20-hydroxyecdysone) that was detected in the amount of snail juice used in the enzymic hydrolysis, can be discounted as the principal source of this hormone.

In addition to the 3β-ecdysteroids, 3α-ecdysteroids were also isolated from this group of hornworm eggs. Of the three known 3α-ecdysteroids (3-epi-ecdysone, 3-epi-20-hydroxyecdysone, and 3-epi-20,26-dihydroxyecdysone) which were first isolated and identified from the tobacco hornworm during post-embryonic development in vitro (14) and/or in vivo (13), only 3-epi-20,26-dihydroxyecdysone, detected in minute amounts (ca. 15 μg), was tentatively identified. Both 3-epiecdysone and 3-epi-20-hydroxyecdysone have previously been isolated and tentatively identified from eggs (17) of the wax moth, Galleria mellonella (L.).

The new 3α-ecdysteroid, 3-epi-26-hydroxyecdysone (Fig. 1) that
comprised 3% of the total recovered ecdysteroids was the second major component isolated from this group of hornworm eggs. The ecdysteroid exhibited a Rf value of 0.31 (26-hydroxyecdysone, Rf 0.27) and its NMR spectra recorded at 60 MHz in deuterated pyridine showed methyl resonances that were identical to those of 26-hydroxyecdysone and suggested that the environment of the methyl groups were similar. Its mass spectrum was almost indistinguishable from that of 26-hydroxyecdysone. The previously identified 3α-ecdysteroids also exhibited similar features (13,14,15). Like 3α-ecdysteroids, the reaction of the compound with acetone yielded one less acetonide group than the corresponding 3β-epimer (26-hydroxyecdysone). For these reasons and arguments and conclusions presented previously (13,14,15), we conclude that this ecdysteroid differs from 26-hydroxyecdysone only in the orientation of the hydroxyl group at the C-3 position and that it has the assigned 2β,3α-diol structure, and is 3-epi-26-hydroxyecdysone.

The discovery of 3-epi-26-hydroxyecdysone completes the series of 3α-ecdysteroids for each of the known 3β-ecdysteroids of the tobacco hornworm (ecdysone, 20-hydroxyecdysone, 26-hydroxyecdysone and 20,26-di-hydroxyecdysone). Just what specific roles the 3α-ecdysteroids play in insect development is not clear. Recently, it has been proposed that conversion of the active 3β-ecdysteroids to the corresponding and considerably less active 3α-ecdysteroids serves as a means of inactivation (13,15,20).

The exceptionally high levels of MH activity in eggs in comparison to levels of MH activity in other developmental stages of the tobacco hornworm (3) makes for an interesting discussion, especially when one considers that 26-hydroxyecdysone is obtained at a higher level (per g of
hornworm egg) than any other ecdysteroid of the hornworm and that it is devoid of MH activity, even at the one microgram level. One can speculate that 26-hydroxyecdysone possesses biological activity related specifically to embryogenesis rather than to MH activity, or it may simply be an inactivation product of ecdysone. All this further points to the need for the development of assays for determining function(s) of ecdysteroids other than that of molting.

Six ecdysteroids (Fig. 1) thus were conclusively or tentatively identified from young hornworm eggs, but there is at least an equivalent number of unknown ecdysteroids, all lacking MH activity at the levels tested. However, when these compounds were acetylated, their analyses by TLC and NMR spectroscopy indicated that certain of the compounds were mixtures (Table 1). These acetates, at this point, provide a promising alternative method for the complete separation, purification, and identification of these ecdysteroids.

Ecdysteroids of undetermined structure have likewise been reported from ovaries of Locusta migratoria and from eggs of the wax moth (16,17, 18). All of the unknowns from hornworm eggs possess an α,β-unsaturated ketone as indicated both by UV and IR analyses. The IR spectra do not show the presence of an isolated keto group, which means that they are not dehydro ecdysteroids. The NMR spectra exhibited methyl resonance in the δ 0.75 region for the C-18 methyl, indicating that the unknowns lack a hydroxyl group at C-20. This latter observation supports our earlier conclusion that hydroxylation at C-20 is a minor pathway for ecdysteroid biosynthesis in this developmental stage of the hornworm.

There are two biosynthetic pathways for ecdysteroids during embryonic development of the hornworms: the pathway to 26-hydroxyecdysone is
the principal route, whereas the conversion of ecdysone to 20-hydroxy-
edysone and 20,26-dihydroxyecdysone is a minor pathway. The latter two 
edcysteroles exhibit MH activity in the house fly assay but 26-hydroxy-
edysone does not. This is especially interesting since 20,26-dihydroxy-
edysone, though only 1/10 to 1/15 as active as ecdysone or 20-hydroxy-
edysone in the house fly assay, differs structurally, as far as we know, 
from 26-hydroxyecdysone only in having an additional hydroxyl group at 
C-20. Apparently hydroxylation of ecdysone at C-26 renders it inactive 
in the house fly assay, and further hydroxylation at C-20 reintroduces MH 
activity. It is also possible that the hydroxyl group at C-20 in 
20,26-dihydroxyecdysone makes it more favorable for the receptors for MH 
activity in the house fly than the ecdysteroid which lacks the hydroxyl 
group at C-20. Another alternative is that 26-hydroxyecdysone and 
20,26-dihydroxyecdysone differ in stereoechemistry at C-25 and C-26.

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REFERENCES

4. Mention of a commercial or proprietary product in this paper does not constitute an endorsement of this product by the U. S. Department of Agriculture.

7. Unisil, 100 to 200 mesh, Clarkson Chemical Co., Williamsport, PA.


**TRIVIAL AND IUPAC EQUIVALENT NAMES**

Ecdysone = $\beta_3,3\beta,14\alpha,22R,25$-Pentahydroxy-$5\beta$-cholest-7-en-6-one

20-Hydroxyecdysone = $\beta_3,3\beta,14\alpha,20R,22R,25$-Hexahydroxy-$5\beta$-cholest-7-en-6-one

26-Hydroxyecdysone = $\beta_3,3\beta,14\alpha,22R,25,26$-Hexahydroxy-$5\beta$-cholest-7-en-6-one

20,26-Dihydroxyecdysone = $\beta_3,3\beta,14\alpha,20R,22R,25,26$-Heptahydroxy-$5\beta$-cholest-7-en-6-one

3-Epi-ecdysone = $\beta_3,3\alpha,14\alpha,22R,25$-Pentahydroxy-$5\beta$-cholest-7-en-6-one

3-Epi-20-hydroxyecdysone = $\beta_3,3\alpha,14\alpha,20R,22R,25$-Hexahydroxy-$5\beta$-cholest-7-en-6-one

3-Epi-20,26-dihydroxyecdysone = $\beta_3,3\alpha,14\alpha,20R,22R,25,26$-Heptahydroxy-$5\beta$-cholest-7-en-6-one

3-Epi-26-hydroxyecdysone = $\beta_3,3\alpha,14\alpha,22R,25,26$-Hexahydroxy-$5\beta$-cholest-7-en-6-one