Both α-ecdysone and 20-hydroxyecdysone have been isolated and identified from several species of insects during pupal-adult development (1, 2), and in certain specific cases, the molting hormones have also either been detected in or isolated from adult insects (3, 4, 5, 6, 7). However, the larvae or nymphs that have been examined to date show very low levels of molting hormone activity (5, 7, 8, 9), which makes it extremely difficult to obtain information as to the qualitative nature of the ecdysones in these early stages of insect development. On the other hand, the feces from immature insects have been shown to contain appreciable amounts of molting hormone activity (10). The isolation and conclusive identification of the ecdysones from larval excreta could provide some insight into the nature of the molting hormones produced during these earlier stages and thus allow for a comparison to be made with the ecdysones of the later developmental stages.

In present studies, only frass from 5th-instar larvae of the tobacco hornworm, Manduca sexta (L.), was used since nearly 90\% of the total frass excreted during larval development is eliminated at this instar. The molting hormone activity of extracts from several samples of frass using the house fly assay (11) indicated the presence
of about 0.10 to 0.20 μg-equivalents/g (wet wt.) of ecdysones based on the biological activity of α-ecdysone. These levels are about 1/10 to 1/20 the levels of the free ecdysones detected in tobacco hornworm pupae at peak ecdysone titer (12). Although direct comparisons cannot be made with the values reported for other species of insects, the levels present in frass from the tobacco hornworm based on dry weight of the frass are comparable to those values for feces from 4th-to 5th-instar nymphs of the locust *Schistocerca gregaria* (Forskal) but are greater than those values found for feces from larvae of the silkworm, *Bombyx mori* (L.) (10).

After we processed and separated the active components from 40 kg of frass, comparative TLC, NMR, and mass spectral analyses showed that 20-hydroxyecdysone was the predominant hormone. Similar analyses of the other major active compound present confirmed its identity as α-ecdysone. The relative distribution of the biological activity for 20-hydroxyecdysone and α-ecdysone was approximately 70 and 30%, respectively. Although α-ecdysone but not 20-hydroxyecdysone is present in feces of certain other insects examined (10, 13), our studies showed that α-ecdysone and 20-hydroxyecdysone do co-exist in frass from 5th-instar tobacco hornworm larvae. This report is the first conclusive demonstration of the presence of 20-hydroxyecdysone in insect excreta. As in the case of tobacco hornworm pupae (12), these studies indicate that α-ecdysone and 20-hydroxyecdysone are both present in 5th-instar larvae as reflected by their occurrence in the frass and suggest that 20-hydroxyecdysone may also serve as the major molting hormone in the earlier stages of the tobacco hornworm. Whether tobacco hornworm
larvae also produce 20,26-dihydroxyecdysone, as found during pupal-adult development (14) or possess, in addition, other ecdysones or ecdysone-like compounds in the free or conjugated form remains to be determined.

That tobacco hornworm larvae are capable of hydroxylating the C-26 position and of conjugating an ecdyson analog and its metabolites has been demonstrated in studies with the labeled ecdyson analog 22,25-dideoxyecdysone (15).

**EXPERIMENTAL**

A total of 40 kg (wet wt.) of frass from 5th-instar tobacco hornworm larvae was homogenized for 5 minutes with 1 ml of methanol/g. The homogenate was transferred to glass cups and centrifuged at 2500 rpm for 5 minutes. The supernatants were decanted off, and the residues in the tubes were pooled and rehomogenized twice with 75% methanol (1 ml/g). The supernatants were combined, and the crude extractives containing the ecdysones were isolated as previously described (12, 14). The crude extractives (84 g) were chromatographed on Woelm alumina (neutral grade I + 20% water) (15) with methanol as the eluting solvent. The methanol eluate (21 g) from the alumina columns was further purified by silicic acid chromatography with increasing percentages of benzene-methanol (15). The fractions from the silicic acid columns that eluted α-ecdysone and 20-hydroxyecdysone (4.5 g) were subjected to 50 transfers in a countercurrent distribution system of cyclohexane, butanol, water (5:5:10) with 100 ml each of the upper and lower phases. The residue (234 mg) from tubes 13-22 was further purified by column chromatography on silicic acid, Woelm alumina (neutral grade I + 20% water) and by thin-layer chromatography (15) to a final mass of 4.6 mg. Crystallization from ethyl acetate-methanol provided 1.02 mg of 20-hydroxyecdysone, m.p. 226-230 °C with dec.; λ max 245 nm, (methanol) ε 11,521. (M+ 480), NMR, δ 1.21 (18-H), 1.08 (19-H) 1.58 (21-H) 1.38 (26- and 27-H).

The residue (724 mg) from countercurrent tubes 29-44 was further purified by column chromatography on silicic acid and Woelm alumina (neutral grade I + 20% water) and by thin-layer chromatography to give a mass of 0.6 mg. Crystallization from ethyl acetate-methanol gave about 0.2 mg of α-ecdysone m.p. 221-225 °C, λ max 245 nm (methanol). (M+ 464), NMR, δ 0.73 (18-H), 1.06 (19-H) 1.25, 1.32 (21-H) 1.39 (26- and 27-H). Due to the small amounts of material available for recrystallization, the melting points are somewhat lower than those reported in the literature (1). However, TLC analyses, NMR, and mass spectra of the α-ecdysone and 20-hydroxyecdysone isolated from frass were identical to the authentic standards of these two steroids.
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REFERENCES


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