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Metabolism of [^{14}C]Cholesterol in *Manduca sexta* Pupae: Isolation and Identification of Sterol Sulfates, Free Ecdysteroids, and Ecdysteroid Acids

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[^{14}C]Cholesterol was injected into fifth-instar larvae of *Manduca sexta*, and the metabolites were isolated and identified from 8-day-old male and female pupae. A major portion of the metabolized cholesterol was esterified either with a sulfate group or with fatty acids. The predominant ecdysteroid metabolites were 20-hydroxyecdysone, 20,26-dihydroxyecdysone, 20-hydroxyecdysonoic acid, and 3-epi-20-hydroxyecdysonoic acid. Smaller amounts of ecdysteroids were identified as conjugates of 26-hydroxyecdysone, 3-epi-20-hydroxyecdysone, 20,26-dihydroxyecdysone, and its 3α-epimer. The metabolic profiles were similar for both male and female pupae. The two ecdysteroid acids were identified by nuclear magnetic resonance spectroscopy and chemical ionization mass spectrometry and by mass spectral analyses of their methyl esters. Detection of 3-epi-20-hydroxyecdysonoic acid as a major metabolite is significant, as its occurrence has been scarcely reported. 3-Epiecdysteroid acid formation is discussed as a possible ecdysteroid-inactivating pathway that may be operating specifically in lepidopterous insects or in particular developmental stages such as eggs or pupae.

Key words: tobacco hornworm, epiecdysteroids, ecdysteroid conjugates, 3-epi-20-hydroxyecdysonoic acid, mass spectrometry, NMR spectroscopy

INTRODUCTION

The regulation of molting hormone titer in insects is greatly influenced by the metabolism of ecdysteroids leading to their inactivation in the form of

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either end-products or temporary storage compounds. Metabolic inactivation is particularly critical during insect developmental stages that consist of closed systems such as eggs or pupae in which hormone clearance via active excretion cannot occur. Ecdysteroids are metabolized in insects primarily by one or more reactions (see [1,2] for review): 1) hydroxylation at C-20 and/or C-26; 2) epimerization at C-3; 3) oxidation to 26-oic acids; and 4) conjugation at C-2, C-22, or C-26 (esterification with phosphate, acetate, or glucose [3] moieties). Ecdysteroid conjugates are characterized by their susceptibility to enzymatic hydrolysis, whereas ecdysteroid acids are unhydrolyzable. Another characteristic of ecdysteroid acids is their ability to form methyl esters upon diazomethane treatment [1].

3-Epiecdysteroids and ecdysteroid acids (Fig. 1), resulting, respectively, from reactions 2 and 3 above, are generally thought to represent major inactivation products of the molting hormones, based on their relative inactivity in bioassays [4,5]. However, the combination of both reactions leading to the formation of 3-epiecdysteroid acids does not appear to be widespread in occurrence. Although 20-hydroxyecdysonoic acid, resulting from the action of processes 1 and 3 upon ecdysone (Fig. 1), has been found in many insect species [1], the presence of its corresponding 3α-epimer, 3-epi-20-hydroxyecdysonoic acid (Fig. 1), has been reported in only a few cases. There has also never been unequivocal structural identification of 3-epi-20-hydroxyecdysonoic acid by such means as NMR or mass spectrometry.

The extensively studied tobacco hornworm, *Manduca sexta* (L.), has served as a model animal in our analyses of ecdysteroid metabolism during different stages of insect development, particularly embryogenesis [3,6,7]. The current study investigates the administration of [^{14}C]cholesterol to last-instar *M. sexta* larvae and its subsequent incorporation and conversion into ecdysteroids and other metabolites in 8-day-old male and female pupae. This age was the reported time of peak molting hormone activity during pupal-adult development of *M. sexta* [8]. In particular, we report the isolation and characterization of 20-hydroxyecdysonoic acid and 3-epi-20-hydroxyecdysonoic acid as major metabolites and discuss the significance of the 3-epiecdysteroid acid formation as a possible inactivation pathway for ecdysteroid hormones in *M. sexta*.

MATERIALS AND METHODS

Biological Material

M. sexta larvae were reared on an artifical diet [9]. One microcurie of purified commercial [4-^{14}C]cholesterol (radiochemical purity >99% by TLC, specific activity 53.7 mCi/mmol) in 25 μl of a saline solution [10] containing 3% Tween 80 was injected with a microsyringe through the dorsal horn of each newly molted fifth-instar larva. Eight days after larval-pupal ecdysis, sexed pupae were collected, weighed, slit several times through the cuticle to enhance solvent penetration, and stored in MeOH* at −20°C until extraction.

*Abbreviations: BuOH = butanol; CI-MS = chemical ionization mass spectrometry; E = ecdysone; 20E = 20-hydroxyecdysone; 26E = 26-hydroxyecdysone; 2026E = 20,26-dihydroxyecdysone; EA = ecdysonoic acid; 20EA = 20-hydroxyecdysonoic acid; 20E22P = 20-hydroxyecdysone 22-phosphate; 26E26P = 26-hydroxyecdysone 26-phosphate; EtOH = ethanol; HPLC = high-performance liquid chromatography; HPTLC = high-performance thin-layer chromatography; LSIMS = liquid secondary ion mass spectrometry; ME = methyl ester; MeOH = methanol; PMR = proton magnetic resonance.

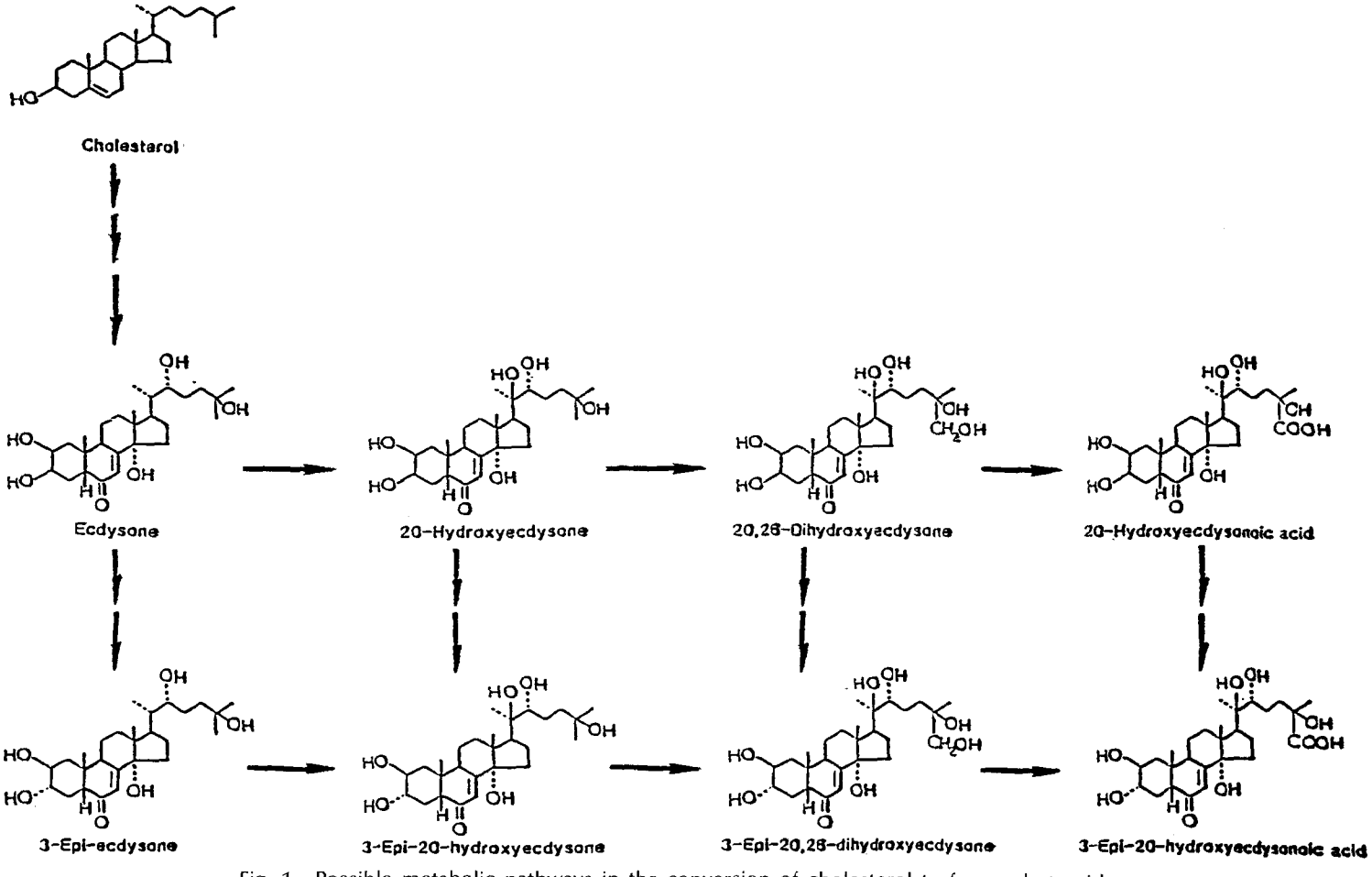

Fig. 1. Possible metabolic pathways in the conversion of cholesterol to free ecdysteroids and ecdysteroid acids in *M. sexta*.

Extraction and Separation of Free and "Polar" Ecdysteroids

Pupae were extracted as described previously [11]. The extract was dried under vacuum, and the residue was partitioned between hexane and 70% MeOH [12]. Apolar lipids from the hexane layer were separated on a column of silica gel 60 [13]. The 70% MeOH phase was dried under vacuum, and the residue was partitioned between BuOH and water [12]. Sterol sulfates and nearly all of the free ecdysteroids partitioned into the BuOH phase. Highly polar ecdysteroids, i.e., ecdysteroid acids and conjugated ecdysteroids (phosphate esters, etc.), partitioned mostly into the aqueous phase.

Purification of Free Ecdysteroids

Following evaporation, the BuOH phase was chromatographed on a Florisil column [3]; fractions were monitored by HPTLC. Fractions that contained both 2026E and cholesterol sulfate, which eluted closely together, were combined and partitioned (five transfers of the upper phase over three tubes) in a system of cyclohexane/BuOH/water (4:6:10) in order to isolate 2026E. Free ecdysteroid fractions were combined and purified on a C_{18} SEP-PAK before HPLC analysis.

Purification of Acidic and Conjugated Ecdysteroids

The aqueous phase from the BuOH/water partition was treated as described previously [11], primarily by XAD-16 and XAD-2 column purification, to yield partially purified polar ecdysteroids. These were applied to a column of DEAE-Sephadex A-50 (Pharmacia) and eluted with a discontinuous NaCl gradient system (0.01–0.20 M), which efficiently separated ecdysteroid acids from ecdysteroid conjugates [11]. Combined fractions from the DEAE-Sephadex column representing each ecdysteroid class were desalted on an XAD-2 column and purified on a C_{18} SEP-PAK before HPLC analysis.

High-Performance Thin-Layer Chromatography

Steroids were analyzed on high-performance silica gel 60 plates incorporated with a UV_{254} phosphor (Merck). Apolar lipid fractions (sterol ester, free sterol, etc.) were analyzed using the solvent systems hexane/diethyl ether/acetic acid (80:20:1) and $CHCl_3$/EtOH (96:4). Free ecdysteroids, sterol sulfates, and methyl ester derivatives of the ecdysteroid acids were analyzed in $CHCl_3$/EtOH (65:35). Migration of the ecdysteroid acids was obtained in $CHCl_3$/MeOH/10 N ammonium hydroxide (28:20:2). Plates were radioassayed as before [11].

HPLC/Radioassay

Reversed-phase HPLC was used to analyze free ecdysteroids on an ODS-Hypersil C_{18} column (Shandon, Sewickley, PA) by isocratic elution with 38% MeOH in water. Ecdysteroid acids and conjugates were analyzed by ion-suppression reversed-phase HPLC on an MOS I-Hypersil C_8 column (Shandon) by isocratic elution with 26% MeOH in 0.03 M aqueous NaH_2PO_4 solution (pH 5). Methyl ester derivatives of ecdysteroid acids were analyzed

by adsorption HPLC on an Ultrasphere silica column (Rainin, Woburn, MA) eluted isocratically with methylene chloride/isopropanol/water (120:35:2). The size of each column was 4.6 mm × 25 cm, 5-μm particles. Analyses were carried out at 33°C and a flow rate of 1.0 ml/min. Effluent absorbance was detected at 254 nm. Fractions (0.5 ml) collected for monitoring radioactivity were mixed directly with 4 ml Hydrofluor scintillation fluid (National Diagnostics, Somerville, NJ) and counted. Preliminary identification of metabolites was accomplished by comparison with ecdysteroid standards and previously characterized ecdysteroid metabolites.

Enzymatic Hydrolysis of Polar Ecdysteroids

Aliquots of the polar ecdysteroids were incubated with an enzyme mixture as described previously [11]. The reaction mixture was then partitioned between BuOH and water.

Methylation of Ecdysteroid Acids

Freshly distilled diazomethane in diethyl ether was added in excess to a methanolic solution of each ecdysteroid acid (20 μg in 100 μl). After 30 min at 22–25°C, the diazomethane and solvent were evaporated under nitrogen, and the methylated products were stored in MeOH at −20 °C before analysis.

Mass Spectrometry

Mass spectra were obtained with a model 4500 spectrometer (Finnigan, San Jose, CA). Ecdysteroids were analyzed by desorption CI-MS using ammonia, methane, and isobutane as reagent gases. Sterol sulfates were analyzed by LSIMS using cesium primary ions with energy 4 kV; samples were dissolved in methanol and applied to the probe in a glycerol matrix, and 1 μl of 1 N HCl was added.

NMR Spectroscopy

Fourier transform PMR spectra were recorded on a Bruker 400-MHz instrument. Samples were dissolved in D_2O, and spectra were referenced to 3-trimethylsilyl-2,2,3,3-tetradeuteropropionic acid.

RESULTS

Incorporation of [^{14}C]Cholesterol Into *M. sexta* Pupae

Larvae of *M. sexta* were injected with [^{14}C]cholesterol (1 μCi/larva) during the 1st day of the fifth (final) instar. About 70% of the injected radioactivity was subsequently incorporated into (i.e., recovered from) both male and female 8-day-old pupae (Table 1). Some of the sterol substrate was metabolized, as shown by the distribution of recovered radioactivity into the various solvent partitions and steroid metabolites (Table 1). Radioactivity from each sex was distributed similarly. The hexane partition contained over 97% of the recovered activity. Silica gel column chromatography and HPTLC analysis of the hexane phase associated 98% of its radioactivity with free sterols and less

TABLE 1. Incorporation and Metabolism of [^{14}C]Cholesterol in *M. sexta*

	Male	Female
[^{14}C]Cholesterol injected into fifth-instar larvae	65 μCi	36 μCi
Radioactivity recovered from 8-day-old pupae	47.0 μCi	23.2 μCi
Percentage distribution of recovered radioactivity		
Hexane partition (against 70% MeOH)		
Free sterol	95.1	95.6
Sterol ester	1.9	1.9
BuOH-water partition		
BuOH phase		
Free sterol	0.2	0.1
"Apolar" steroids	0.2	0.1
Sterol sulfate	0.7	0.8
Free ecdysteroids	0.7	0.6
"Polar" ecdysteroids	0.05	0.05
Aqueous phase		
Free ecdysteroids	0.05	0.05
Ecdysteroid acids	0.7	0.5
Ecdysteroid conjugates	0.4	0.3

than 2% with sterol esters. Although most of the recovered radioactivity was in the form of unmetabolized sterol, about 1% of the recovered activity was in the form of cholesterol sulfate, and nearly 2% was represented by free and polar ecdysteroids.

Fractionation of Metabolites From the BuOH Phase

Radiolabeled steroids from the BuOH phase (partitioned against water) were separated on a Florisil column into the following successive fractions (distributed as shown in Table 1): cholesterol (a relatively small amount that was not partitioned completely into the hexane phase), unidentified "apolar" steroids (intermediate in TLC migration between cholesterol and 3-epi-E), free ecdysteroids, sterol sulfates, and a small quantity of "polar" steroids (which appeared to be ecdysteroid acids or conjugates and which were later combined with the aqueous-phase polar ecdysteroids). Coelution of the sterol sulfates and 2026E, which also migrated closely together in HPTLC, occurred in some fractions. These were separated efficiently by the cyclohexane/BuOH/water partition: sterol sulfates into the cyclohexane/BuOH phase and 2026E into the aqueous phase.

Analysis of Sterol Sulfates

After being purified on an additional silicic acid column, the suspected sterol sulfate fraction was analyzed further. It gave an R_f value of 0.20 in the HPTLC solvent system for free ecdysteroids (reference R_f values: E = 0.51; 20E = 0.42; 2026E = 0.22), but exhibited no absorbance at 254 nm on the HPTLC plate. Cesium-LSIMS analysis of authentic cholesterol sulfate produced a negative-ion spectrum with three diagnostically significant ions. Two of the ions, m/z 80 (28%) and 97 (100%), originated from the sulfate moiety and correspond, respectively, to the anions SO_3^- and HSO_4^- [14]. The third ion occurred at m/z 465 (20%) and is equivalent to $(M-H)^-$. Examination of

the pupal sample by LSIMS revealed a negative-ion spectrum with five significant ions. Two of these ions, at m/z 80 (25%) and 97 (100%), confirmed the presence of a sulfate group. The remaining three ions occurred at m/z 465, 479, and 493 and indicated the presence of the respective sulfate conjugates of cholesterol, campesterol, and sitosterol in the approximate ratio 60:10:30. The latter two sterols originated from the dietary wheat germ [9].

Analysis of Free Ecdysteroids

Free ecdysteroid profiles for 8-day-old *M. sexta* pupae are presented as reversed-phase HPLC/radioassays in Figure 2. The male and female profiles, which were very similar, each showed a predominance of two radioactive peaks that coincided with the retention times for 2026E (peak 1) and 20E (peak 3). After peaks 1, 3, and 5 (corresponding to E) were isolated by HPLC collection, each component exhibited an absorbance maximum at 240 nm (in MeOH), characteristic of the α,β-unsaturated keto group of ecdysteroids.

Methane CI-MS analysis of peak 1 revealed major ions at m/z 525 (5%, $M+C_2H_5)^+$, 497 (20%, $M+H)^+$, 479 (15%, $M+H-H_2O)^+$, 461 (30%, $M+H-2H_2O)^+$, and 443 (30%, $M+H-3H_2O)^+$. Methane CI of peak 3 produced ions corresponding to the above fragments at m/z 509 (5%), 481 (15%), 463 (10%), 445 (20%), and 427 (20%). Methane CI of peak 5 produced corresponding ions at m/z 493 (1%), 465 (3%), 447 (4%), 429 (4%), and 411 (2%).

Ammonia CI produced the following major ions: for peak 1, at m/z 514 (85%, $M+NH_4)^+$ and 496 (50%, $M+NH_4-H_2O)^+$; for peak 3, at m/z 498 (100%, $M+NH_4)^+$ and 481 (55%, $M+H)^+$; and for peak 5, at m/z 482 (2%, $M+NH_4)^+$ and 464 (2%, $M+NH_4-H_2O)^+$.

The mass spectra indicated that the free ecdysteroids 1, 3, and 5 possess the respective molecular weights of 496, 480, and 464 (the molecular weight of E) and that peak 1 contains one more hydroxyl group than peak 3 and two more hydroxyl groups than peak 5. HPLC, HPTLC, and mass spectral analyses thus indicate that the two major free ecdysteroids from 8-day-old pupae are 2026E (peak 1) and 20E (peak 3), and that E (peak 5) is one of the minor free ecdysteroids. Male pupae yielded 0.14, 1.07, and 1.21 μg/g of E, 20E, and 2026E, respectively, while female pupae contained 0.09, 1.04, and 1.24 μg/g of those three respective ecdysteroids, as determined by HPLC quantitation. The HPLC/radioassays of both free ecdysteroid fractions (Fig. 2) also suggested the presence of small amounts of 3-epi-2026E (peak 2) and 3-epi-20E (peak 4).

Analysis of Polar Ecdysteroids

Ion-suppression reversed-phase HPLC/radioassays of the partially purified polar ecdysteroids from 8-day-old male and female pupae of *M. sexta* are shown in Figure 3. These represent samples purified before DEAE-Sephadex column chromatography and therefore are profiles of the total polar metabolites. Each profile exhibited two predominant radioactive peaks at 9.7 and 12.3 min.

A DEAE-Sephadex column [11] separated the polar ecdysteroid metabolites from the male pupae into the acid and conjugate fractions producing the

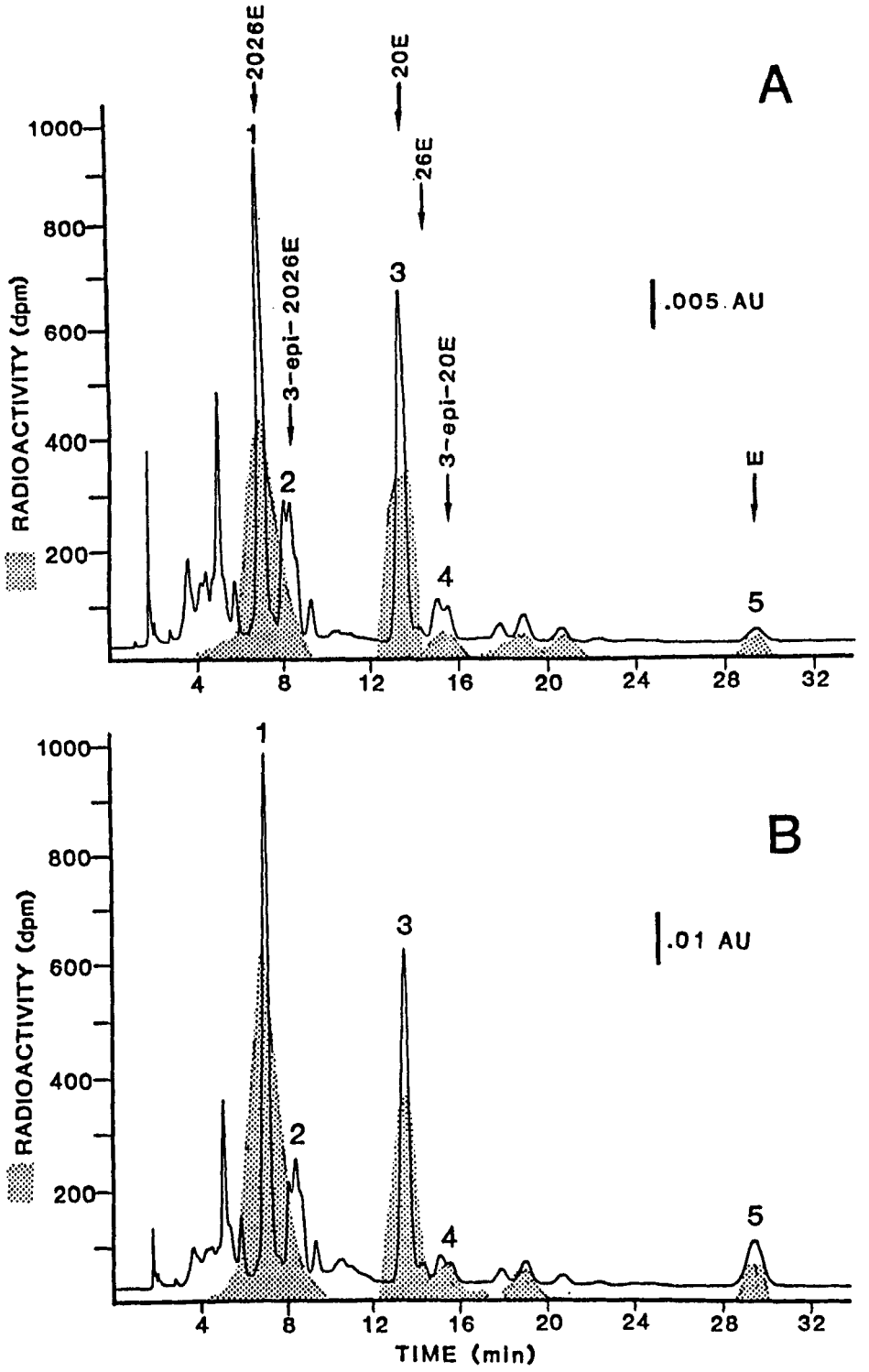

Fig. 2. Reversed-phase HPLC/radioassays of free ecdysteroids from (A) female and (B) male 8-day-old *M. sexta* pupae. Arrows denote elution positions of reference ecdysteroids (see "Abbreviations" footnote). Refer to "Results" for numbered peaks.

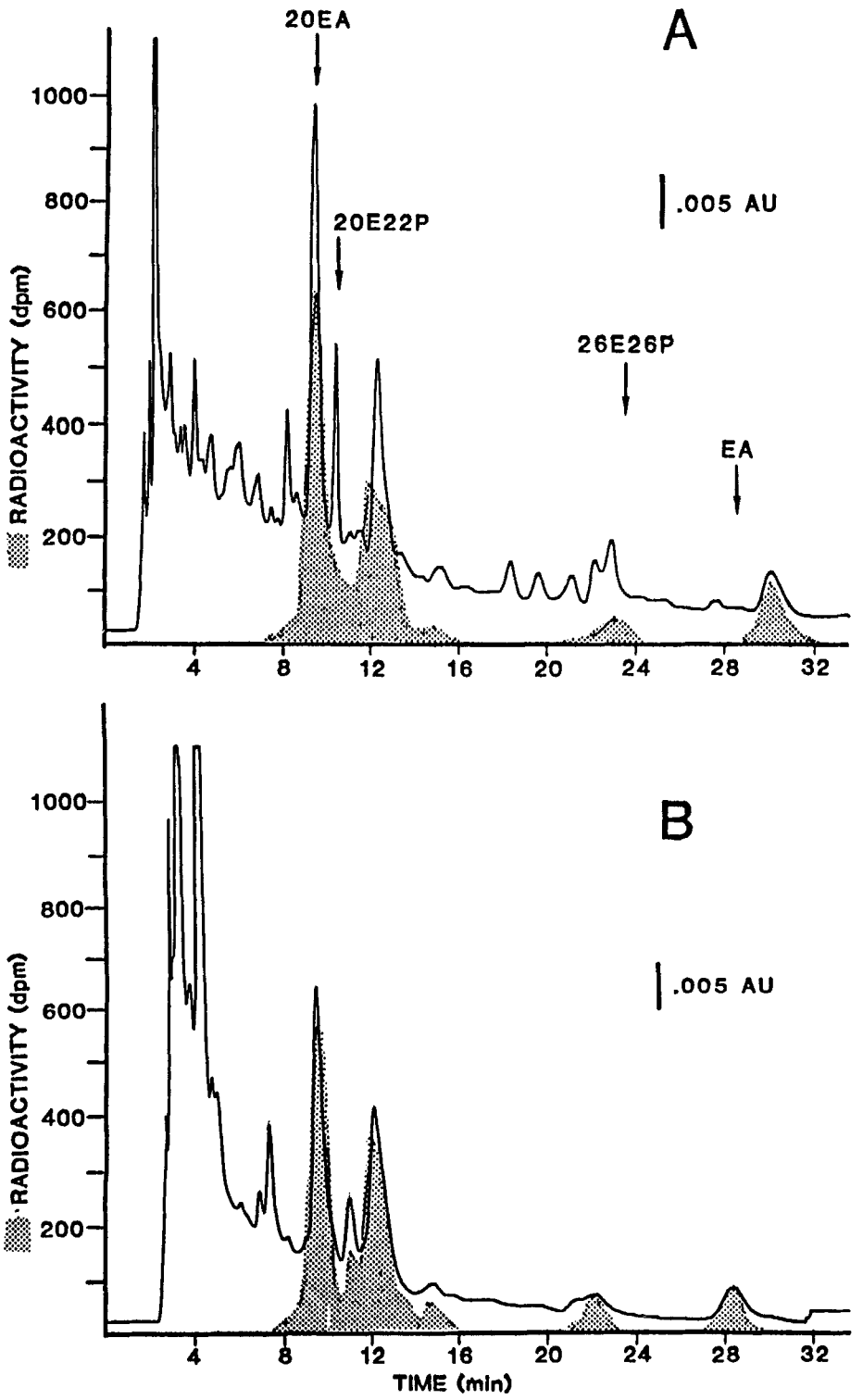

Fig. 3. Ion-suppression reversed-phase HPLC/radioassays of polar ecdysteroids from (A) female and (B) male 8-day-old *M. sexta* pupae. Arrows denote elution positions of reference ecdysteroids.

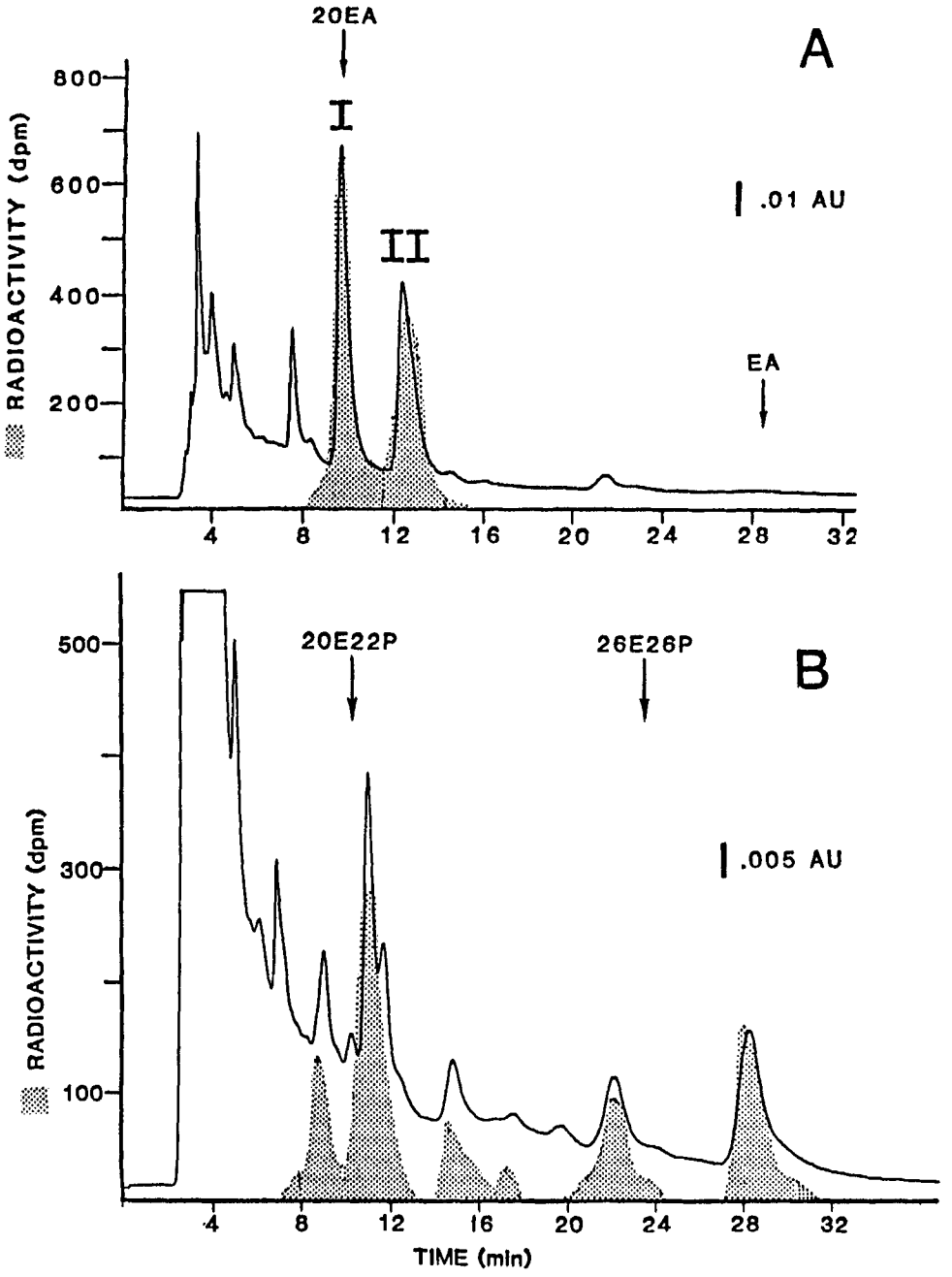

Fig. 4. Ion-suppression reversed-phase HPLC/radioassays of ecdysteroid acids (A) and conjugates (B) from 8-day-old male *M. sexta* pupae [11]. Arrows denote elution positions of reference ecdysteroids.

HPLC/radioassays shown in Figure 4. About 70% of the radioactivity from the total polar ecdysteroids was associated with the acid fraction, which revealed only two radioactive peaks (Fig. 4A), I and II, present in the approximate ratio 55:45 and with respective retention times of 9.7 and 12.3 min, the former time coinciding with that of authentic 20EA. Several radioactive peaks were present in the conjugate fraction (Fig. 4B); the largest component accounted for no more than 12% of the total polar ecdysteroid radioactivity. Thus, the major components of the polar ecdysteroid fraction from male pupae were two ecdysteroid acids, I and II. Analysis of the isolated metabolites from the female pupae revealed that their predominant polar ecdysteroids were identical with the two ecdysteroid acids I and II from the male pupae.

We verified the nature of the acid and conjugate fractions by subjecting each fraction to enzymatic hydrolysis. The conjugate fraction underwent complete hydrolysis and liberated free ecdysteroids; following incubation with the enzyme mixture, all of the radioactivity, which previously had partitioned into water (against BuOH), was now extracted into the BuOH phase. HPLC and HPTLC analyses of the purified BuOH phase revealed several radiolabeled free ecdysteroids, including 26E, 2026E, 3-epi-20E, and 3-epi-2026E. Complete identification of the ecdysteroid conjugates is currently being performed. Conversely, the acid fraction was resistant to hydrolysis: no radioactivity from the incubated mixture was liberated into the BuOH phase of a BuOH-water partition; HPLC and HPTLC chromatograms of the aqueous phase were unchanged from the original analyses.

Identification of Ecdysteroid Acids

The pupal ecdysteroid acids I and II (Fig. 4A) were separated by HPLC collection and purified in sufficient quantities for further analysis. Each of the two isolated compounds exhibited an absorbance maximum at 240 nm (in MeOH). In the HPTLC system for ecdysteroid acids, each compound gave an R_f value of 0.34. A molecular weight of 510 for compound I was indicated by the production of the following major CI-MS ions: by ammonia-CI, at m/z 527 $[M+(NH_3)_2H-H_2O]^+$, 510 $(M+NH_4-H_2O)^+$, and 492 $(M+NH_4-2H_2O)^+$; by methane-CI, at m/z 533 $(M+C_3H_5-H_2O)^+$, 521 $(M+C_2H_5-H_2O)^+$, 515 $(M+C_3H_5-2H_2O)^+$, 503 $(M+C_2H_5-2H_2O)^+$, 493 $(M+H-H_2O)^+$, and 475 $(M+H-2H_2O)^+$; by isobutane-CI, at m/z 549 $(M+C_4H_9-H_2O)^+$, 531 $(M+C_4H_9-2H_2O)^+$, 493, and 475. The corresponding mass spectra of compound II were essentially identical with those of compound I, signifying that they had the same molecular weight of 510, equivalent to 20EA.

Analysis of methylated derivatives of I and II. The presence of a carboxylic acid function in compounds I and II was demonstrated by their ability to be methylated via diazomethane treatment. The resulting methyl ester derivatives, ME-I and ME-II, were then characterized. In the HPTLC solvent system for free ecdysteroids, acids I and II remained at the origin, while ME-I (R_f 0.48) and ME-II (R_f 0.52) were resolvable and migrated close to E (R_f 0.51). In silica HPLC (CH_2Cl_2/isopropanol/water, 120:35:2), ME-I (retention time = 11.7 min) and ME-II (11.1 min) again migrated close to E (11.8 min; 20E =

16.1 min). A less-polar solvent ratio (125:30:2) achieved greater resolution: ME-I = 20.6 min; ME-II = 19.0 min; E = 17.0 min. When analyzed by reversed-phase HPLC in the free ecdysteroid solvent system, acid I was unretained (eluting at the void-volume time of about 2 min), while ME-I (11.8 min) eluted closer to 20E (13.7 min) than to E (29.0 min). This chromatographic behavior of ME-I concurred with previous results [5,15] which demonstrated that the methyl ester of 20EA migrated like E in silica HPLC but migrated closer to 20E in reversed-phase HPLC. The chromatographic behavior of the methyl esters, as well as that of the parent ecdysteroid acids, suggested that compound II was less polar than I.

ME-I and ME-II were purified by collection from silica HPLC. Each ME retained the UV absorbance maximum at 240 nm (in MeOH) possessed by their parent acids. A molecular weight of 524 for ME-I was indicated by the production of the following major CI-MS ions: by ammonia-CI, at m/z 542 $(M+NH_4)^+$, 524 $(M+NH_4-H_2O)^+$, 510 $(M+NH_4-MeOH)^+$, 506 $(M+NH_4-2H_2O)^+$, 493 $(M+H-MeOH)^+$, and 488 $(M+NH_4-3H_2O)^+$; by methane-CI, at m/z 553 $(M+C_2H_5)^+$, 535 $(M+C_2H_5-H_2O)^+$, 525 $(M+H)^+$, 507 $(M+H-H_2O)^+$, 489 $(M+H-2H_2O)^+$, and 471 $(M+H-3H_2O)^+$. The corresponding mass spectra of ME-II were essentially identical with those of ME-I, indicating a molecular weight of 524 for each methyl ester, equivalent to the methylated derivative of 20EA.

Thus, characterization of their methylated derivatives confirmed the identities of I and II as ecdysteroid acids with identical molecular weights of 510. Compound I cochromatographed (in reversed-phase HPLC) and agreed in molecular weight with authentic 20EA and was more polar than compound II.

PMR spectroscopy. Because 20EA was the suspected identity of ecdysteroid acid I, we compared the PMR spectra of 20E and compound I taken in D_2O (Fig. 5). The significant differences in the spectrum of compound I, relative to the spectrum of 20E, were the disappearance of the C-26 methyl signal (occurring at 1.238 ppm for 20E), the large downfield shift of the remaining C-27 methyl signal from 1.238 ppm to 1.354 ppm, and the small upfield shift of the C-18 methyl peak from 0.870 to 0.856 ppm. The spectral comparison indicated that the carboxylic acid group of compound I is at C-26 and that compound I is 20-hydroxyecdyson-26-oic acid (20EA). Our results concurred with a previous PMR analysis of 20E and 20EA taken in CD_3OD [16].

We next compared the PMR spectra of ecdysteroid acids I and II (Fig. 5). The major differences were related to the proton signals originating from the A ring of the steroid nucleus: large shifts of both the C-2 and C-3 proton signals and a smaller shift of the C-19 methyl peak. No differences in the proton signals from the ecdysteroid side-chain were observed. Therefore, compound II is also a 26-oic acid. Given that I and II have the same molecular weight and that the structure of compound I is 20EA, the chemical shifts suggested that I and II were epimeric with respect to one of their hydroxyl groups at C-2 or C-3. For confirmation, we compared the PMR spectra of authentic 20E and 3-epi-20E (Fig. 5). Indeed, the far upfield chemical shifts for the H-2 and H-3 signals of 3-epi-20E from those of 20E were analogous to the upfield chemical shifts for the H-2 and H-3 signals of compound II from

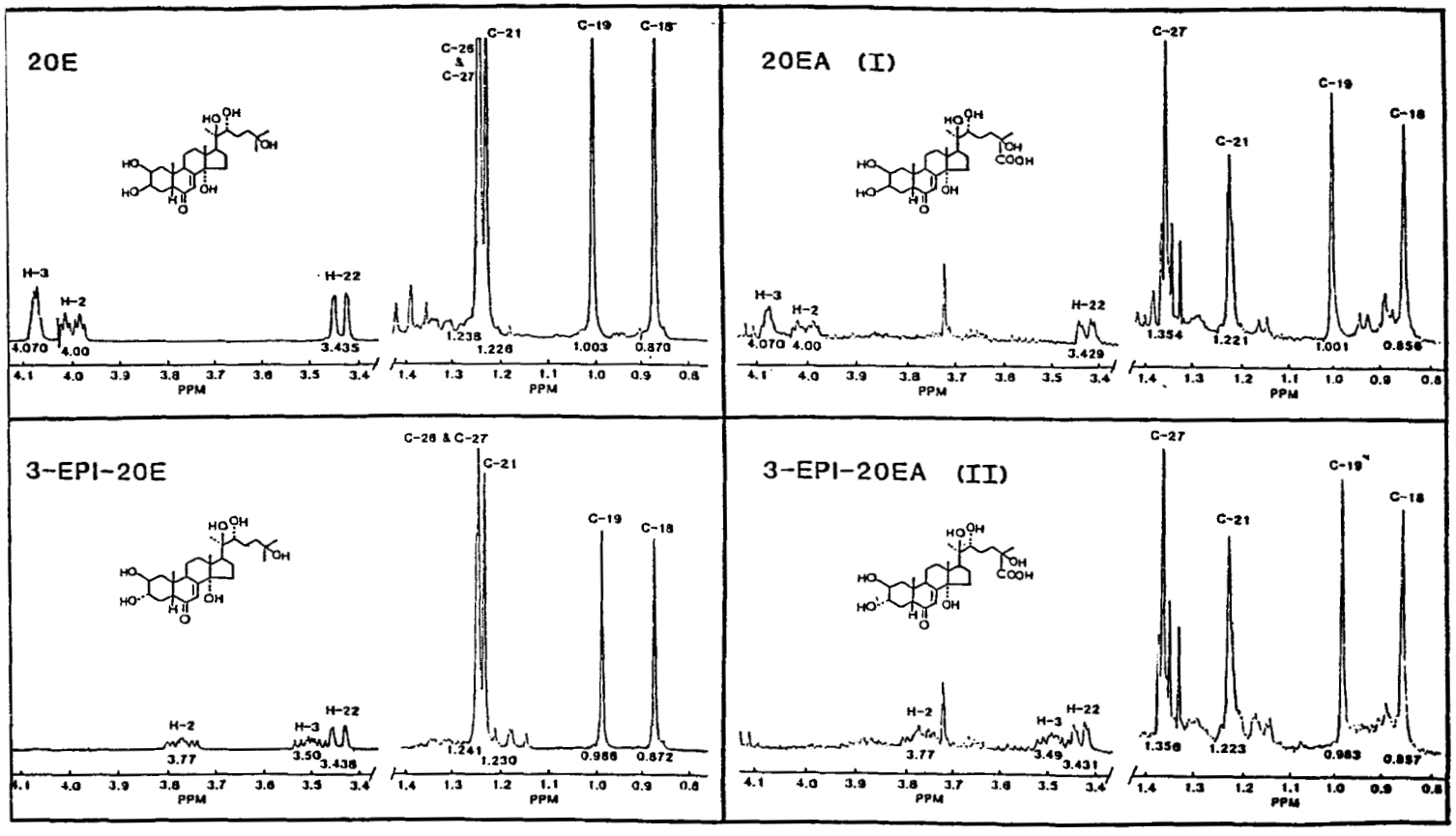

Fig. 5. Partial PMR spectra of 20E, 3-epi-20E, 20EA (I), and 3-epi-20EA (II) taken in D_2O.

those of compound I. The H-2 and H-3 signal positions for 20E and compound I were assigned by their peak width at half height [17]. 3-Epi-20E and 3-epi-20EA, however, gave wide signals at 3.49–3.50 and 3.77 ppm for both H-3 and H-2 protons, respectively. These signal positions were based on assignments made by 2D-COSY experiment of 3-epi-20EA [18]. As shown in Figure 5, the H-3, H-2, and C-19 signals of 3-epi-20E were shifted upfield by 0.57, 0.22, and 0.02 ppm, respectively, from their positions in the spectrum of 20E. The H-3, H-2, and C-19 signals of compound II were shifted upfield by 0.58, 0.23, and 0.02 ppm, respectively, as compared to the spectrum of compound I (20EA). Therefore, compound II is 3-epi-20EA.

Male pupae contained 1.25 and 1.10 μg/g of 20EA and 3-epi-20EA respectively, while female pupae contained 1.47 and 1.23 μg/g of those two respective ecdysteroid acids, as quantitated by HPLC.

DISCUSSION

Long-term [^{14}C]cholesterol labeling for the investigation of in vivo ecdysteroid metabolism during insect development has proven to be a successful and invaluable tool, as demonstrated in studies of *Pieris brassicae* [19,20] and *Schistocerca gregaria* [21,22]. Coupled with improved analytical techniques, this method has enabled us to elucidate ecdysteroid metabolic pathways during embryogenesis of *M. sexta* through the isolation and unequivocal identification of labeled ecdysteroid metabolites from [^{14}C]cholesterol [7]. We are using the same methodology to elucidate steroid metabolic pathways during the pupal-adult development of *M. sexta*. Warren and Gilbert [23] have previously performed excellent studies of ecdysteroid metabolism during this stage of *M. sexta* in which they employed elegant HPLC/RIA methodology. However, while their system has certain advantages over our experimental methods, the RIA antisera had limited affinity toward specific ecdysteroids (C-26 oxidized C-3 epimers), which we have been able to isolate and identify in our present study.

[^{14}C]Cholesterol injected into male and female last-instar larvae of *M. sexta* was converted to various labeled steroid metabolites. The radiolabeling of these steroids has allowed for their collection and isolation in minute quantities from 8-day-old pupae and has permitted us to follow their metabolic fate in the developing pupae. We chose this pupal age for initial study because it was reported as the approximate time of peak titer of molting hormone activity during pupal-adult development [8], as determined by the house fly bioassay. Eight days also represents approximately 40% of *M. sexta* pupal-adult development.

Injection of cholesterol during early fifth-instar results in a long interval of about 17 days before collection of 8-day-old pupae, allowing the labeled sterol to equilibrate within the endogenous sterol pool prior to its conversion to ecdysteroids. Long-term cholesterol labeling of ecdysteroids provides a more accurate physiological reflection of steroid metabolism than does the method of short-term ecdysone labeling, as pointed out by Beydon and Lafont [20].

We observed a relatively high percentage of incorporation of the administered radioactivity, even though a 9-day larval period existed before *M. sexta*

entered the closed pupal stage. Owing to the method of administration (i.e., injection as opposed to dietary ingestion) and because the administered sterol represented such a minute portion of the endogenous pool into which it equilibrated, it was not unexpected that most of the labeled sterol was sequestered and not excreted during fifth-instar.

The metabolic reactions of [^{14}C]cholesterol occurring in up to 8-day-old pupae were identified as esterification with fatty acids, conjugation to sulfate esters, and conversion to free ecdysteroids, ecdysteroid acids, and ecdysteroid conjugates. The sterol sulfate fraction from the pupae contained three conjugated sterols: 60% cholesterol, 10% campesterol, and 30% sitosterol, the two phytosterols being of dietary origin. Although cholesterol sulfate is commonly found in the excreta of higher vertebrates, its presence in invertebrates was not established until its isolation and identification from the pupal meconium of *M. sexta* [24], which contained the same three sterol sulfates as did our 8-day-old pupae. Sulfates may be significant metabolites of cholesterol throughout pupal-adult development. The most likely function of sterol sulfates in insects, as well as in mammals, is sterol excretion via a more water-soluble form. But sulfates might also function as sterol-storage forms or as metabolic intermediates whose relatively high water-solubility enhances their cellular transport and distribution to other tissues where hydrolysis to free sterol or further metabolism to ecdysteroids could occur [25]. Fatty acid esters of sterols are believed to function similarly as a reserve for free sterols, but rather in the form of an apolar molecule, in contrast to the polar sulfate. Two esterification mechanisms perhaps operate to reserve cholesterol either in a water-soluble form or in a relatively apolar form, depending upon the eventual location and function of the reserved sterol.

The free ecdysteroid profiles of this pupal age group exhibited no significant sexual differences, either qualitatively or quantitatively. 20E and 2026E were the major free ecdysteroids, signifying high activities of ecdysone 20-monooxygenase and the 26-hydroxylation enzyme system up to this stage of pupal-adult development, as previously demonstrated in *M. sexta* pupae by Warren and Gilbert [23]. High activities of the C-26 alcohol oxidation and C-3 epimerization, which are probably ecdysteroid-inactivating reactions, resulted in the predominance of two ecdysteroid acids, 20EA and 3-epi-20EA, as polar metabolites of cholesterol in both male and female pupae. Sexual differentiation of the ecdysteroid conjugate profiles remains a possibility to be examined. Indeed, sexual dimorphism may not be reflected in the ecdysteroid composition, if at all, until much later pupal ages approaching adult eclosion.

Ecdysteroid acids occur generally in insects. 20EA has been found in specific developmental stages of many diverse species, including Diptera (*Calliphora vicina* larvae [5] and *Sarcophaga peregrina* pharate pupae [26]); Orthoptera (*Schistocerca gregaria* embryos [16,22] and larvae [27], *Locusta migratoria* larvae [5] and feces from larvae and adults [28]); and Lepidoptera (*Pieris brassicae* larvae [18] and pupae [5], *Spodoptera littoralis* pupae [16], and *M. sexta* eggs [7] and pupae [23]).

In contrast, only a few insects have been reported to contain the corresponding 3α-epimer, 3-epi-20EA: pharate pupae of *S. peregrina* [26] and *P.*

brassicae [15] and late-stage pupae of *M. sexta* [23]. This study establishes the first unequivocal identification of a 3-epiecdysteroid acid (3-epi-20EA) isolated from a biological source based on NMR and mass spectral characterization. We have also detected 3-epi-20EA as a metabolite of [^{14}C]cholesterol in other developmental stages of *M. sexta*: in both early and late ages of pupae (unpublished data), in late phases of embryogenesis, and in newly emerged first-instar larvae [7]. In fact, 3-epi-20EA is the second major ecdysteroid metabolite in these newly emerged larvae. The pathway in *M. sexta* leading from the free ecdysteroids to 3-epi-20EA follows either (or both) of two routes (Fig. 1): 1) 26-oxidation of 2026E followed by 3-epimerization; 2) initial 3-epimerization of E, 20E, or 2026E followed by hydroxylation/oxidation reactions at C-20 and C-26. Epimerization at C-3 is an irreversible process, as demonstrated in *M. sexta* [29], *P. brassicae* [30], and *S. littoralis* [31], and so 3-epi-20EA is presumably an end-product. However,the possibility exists that 20EA or 3-epi-20EA can be metabolized further by conjugation at C-2, C-3, C-22, or C-26, though such conjugated acids have not yet been detected.

Ecdysteroid acid formation has been suggested as a stage-specific mechanism for the regulation of molting hormone activity in the fleshfly, *S. peregrina*, when EA, 20EA, and their 3-epimers were found in pharate pupae but were scarcely detected in mature larvae [26]. Based on the occurrence data, this stage-specific appearance of ecdysteroid acids may be characteristic only of certain species. Ecdysteroid acid formation could very well occur in most insects during every developmental stage to serve as an inactivation pathway, though the titer of ecdysteroid acids may vary throughout each stage.

On the other hand, the apparently rare occurrence of 3-epiecdysteroid acids in certain insects suggests that their production occupies a specific niche in insect ecdysteroid metabolism. Their rarity could, of course, be due to a failure to detect these metabolites in previous investigations. Nevertheless, our results, combined with previous findings, show that 3-epiecdysteroid acids are significant metabolites in eggs and pupae of *M. sexta* and in pupae of *P. brassicae* [15], two species belonging to the order Lepidoptera. Epimerization of 3β-ecdysteroids has been proposed as a process that specifically operates in closed insect systems [15]; eggs and pupae generally contain significant quantities of 3-epiecdysteroids, whereas mature larvae do not. The formation of 3-epiecdysteroid acids may thus be an inactivation mechanism that specifically predominates in closed stages (i.e., eggs and pupae) of lepidopterous insects.

The two reactions principally involved in epiecdysteroid acid production are 3-epimerization and 26-oxidation. From the levels detected in *M. sexta* pupae, the ratio of 3α- to 3β-acids (3-epi-20EA/20EA) is 0.86, which is several times greater than the ratio of the corresponding precursor free ecdysteroids (3-epi-2026E/2026E = 0.29). Interpretation of these ratios can be expressed in either of two ways: the 26-oxidation enzyme system converts 3α(epi)-ecdysteroids to acids more readily than it converts 3β-ecdysteroids; ecdysteroid acids are more favorable substrates for the 3-epimerase than are the free ecdysteroids. Therefore, 3-epimerization and 26-oxidation are linked together as an enzymatic mechanism that modifies and presumably inactivates possible binding sites at opposite ends of the ecdysteroid molecule, thereby creating a more efficient inactivation process.

Ecdysteroid conjugation and 3-epimerization in *M. sexta* have been linked together similarly as an inactivation mechanism; midgut cytosolic enzymes converted 3-epiecdysone to conjugates more readily than they converted ecdysone [32]. Two insects in the order Orthoptera possess an ecdysteroid-inactivation pathway that also involves a double-reaction modification. The related locust species, *L. migratoria* [28] and *S. gregaria* [27,33], produce C-2 and C-22 phosphate conjugates of ecdysone-3-acetate and 20-hydroxyecdysone-3-acetate. Acetylecdysteroid phosphates are perhaps characteristic of Orthoptera in the same manner that epiecdysteroid acids are associated with Lepidoptera.

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