6-position of the purine nucleus and of the steric requirements for binding to adenosine deaminase is continuing.

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Sterol Metabolism in Larvae of the Confused Flour Beetle, *Tribolium confusum*

By EDWARD E. SMISSMAN, NEIL A. JENNY, and STANLEY D. BECK

Dehydroepiandrosterone, pregnenolone, and progesterone were isolated as metabolic products during normal insect metabolism of dietary sterols in the confused flour beetle, *Tribolium confusum*. Their identity was established by elemental analysis, thin-layer chromatography, gas chromatography, ultraviolet absorption, optical rotation, and 2,4-dinitrophenylhydrazone derivatives compared to authentic samples. A proposed metabolic pathway of dietary sterols in *T. confusum* larvae is outlined.

The indispensability of sterols for the growth of insects has been amply established by numerous nutritional studies on a variety of species (1–3). Higher animals are able to synthesize sterols from simple compounds such as acetate, while the lack of sterol synthesis seems to be a metabolic defect common to all insects (4).

The requirement for specific sterols varies widely among the insecta, and most larvae exhibit only moderate specificity with respect to their need for dietary sterols. Several dietary sterols of the larvae of *Tribolium confusum* are converted to cholesterol and 7-dehydrocholesterol which are the principal tissue sterols of the insect (5). The present and related metabolic studies (5–7) indicate the ability of insects to metabolize dietary sterols to the extent of altering the side chain and the degree of saturation in the B ring of the sterol nucleus. The objective of this study is to demonstrate the molecular alteration of side-chain substituted sterols during normal insect metabolism by isolation and characterization of the ketosteroid metabolic products.

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EXPERIMENTAL AND RESULTS

Since the results obtained in a previous study indicated that dietary sterols are converted into cholesterol and 7-dehydrocholesterol (6), it was decided to grow the *T. confusum* larvae on a natural diet supplemented with a dietary sterol that could be converted to cholesterol. The beetle larvae were reared from egg to larval maturity using a basic diet of 4 parts white flour, 4 parts graham wheat flour, and 1 part yeast with the additional dietary sterol, ergosterol, present in the yeast. Before isolation of lipids, the larvae were held for 24 hours on a sterol-free diet to minimize interference from sterols contained in the gut contents.

Extraction.—In previous studies of steroid isolation, many types of solvent systems have been used. Weakly polar solvents are used most commonly. Since it was difficult to determine which procedure would be best for this study, several extraction procedures were used in an attempt to separate the steroids from the numerous other constituents of the insect body. In all cases the larvae were first washed with diethyl ether and skellysolve B to remove any diet material on the surface of the insects and any steroids present in the insect epicuticle.

Skellysolve B Extraction.—After washing the larvae, 250 Gm. of larvae in 100 ml. of skellysolve B was homogenized in a Waring Blendor for 5 seconds. This homogenate of larvae was extracted with 1.5 L. of skellysolve B in a Soxhlet apparatus for 48 hours. The skellysolve B extract was concentrated, redissolved in 100 ml. of 5% alcoholic potassium hydroxide solution, and refluxed for 1 hour. The hydrolyzate was poured into 300 ml. of distilled water, and the nonsaponifiable material was extracted three times with 400 ml. of skellysolve B. The organic layer was washed several times with water and dried over anhydrous magnesium sulfate.

Skellysolve B Followed by Continuous Ether Extraction.—The skellysolve B extract of nonsaponifiable material (see above) was concentrated and mixed with 200 ml. of aqueous 5% sodium carbonate. This mixture was extracted with 1 L. of diethyl ether in a liquid-liquid continuous extractor for 4 days. The diethyl ether extract was washed several times with water and dried over anhydrous magnesium sulfate.

Butyl Alcohol Extraction.—After washing the larvae, 100 Gm. of larvae was homogenized in a Waring Blendor for 5 seconds with 50 ml. of butyl alcohol. To a solution of 150 ml. of butyl alcohol, 20 Gm. of sodium hydroxide, and 100 ml. of water was added the larval homogenate, and the mixture was refluxed for 2 hours. Ten grams of celite filter aid was added and the mixture filtered. The filter cake was extracted with 50 ml. of hot butyl alcohol and the fractions combined. The solvent was removed by distillation, and the residue was extracted with 250 ml. of skellysolve B. Finally, the skellysolve B extract was washed several times with water and dried over anhydrous magnesium sulfate.

Although these three different extraction procedures were effective, the skellysolve B and butyl alcohol methods were more efficient.

Chromatography.—Heilbron (8) has succeeded in separating steroidal material from nonsaponifiable matter by aluminum oxide chromatography. A modification of his procedure, using deactivated neutral aluminum oxide, grade III (9), and a similar solvent sequence, proved useful for chromatographic separation of the metabolic products.

Adsorption Chromatography.—The residue from several skellysolve B extractions was placed on a chromatographic column (3 × 50 cm.) containing 5 Gm. of deactivated neutral aluminum oxide, grade III (Woelm, suitable for chromatography) for every 50 mg. of nonsaponifiable materials (see Table I). Since only the ketosteroid metabolic products would be isolated and characterized in this study, infrared spectra of all the fractions were performed to determine which fractions contained the desired material. Fraction *7* contained most of the carbonyl material, although the infrared spectra of fractions *1*, *5*, and *9* also indicated their presence in small amounts.

Before work was initiated on characterization of these carbonyl containing fractions, attempts were made to improve the separation of various components of the nonsaponifiable material. One solution of this problem was to reduce the tailing of bands during elution. This can be accomplished by using gradient elution technique (10).

Gradient Elution Adsorption Chromatography.—The residue from five skellysolve B extractions was placed on a chromatographic column (2 × 40 cm.) containing 5 Gm. of acid-washed aluminum oxide (Merck, suitable for chromatography) for every 200 mg. of nonsaponifiable material. The following eluent sequence was used: 500 ml. of skellysolve B, 500 ml. of benzene, 1000 ml. of diethyl ether, and 500 ml. of absolute ethanol. Ten-milliliter fractions were collected using an automatic fraction collector, and a total of 218 fractions were collected (see Table II). Again infrared spectra of the fractions were used to determine the location of the desired carbonyl material. Ultraviolet absorption spectra of tubes 81 to 145 were determined to give an indication of the ketosteroid products present. These ultraviolet maxima correspond favorably to the known maxima of progesterone and androstenedione at 240 and 241 μ. Fractions 81 to 126 contained most of the carbonyl material, although fractions 127 to 145 also contained a small amount.

Table I.—Adsorption Chromatography of Nonsaponifiable Material (1.06 Gm.)

Fraction	Amt., ml.	Eluent	Product
1	150	Skellysolve B	655 mg. white, waxy solid
2	200	Skellysolve B–benzene, 10:1	31 mg. yellow oil
3	250	Skellysolve B–benzene, 8:1	Nothing
4	150	Skellysolve B–benzene, 6:1	Nothing
5	150	Skellysolve B–benzene, 2:1	120 mg. yellow semisolid
6	150	Benzene	Nothing
7	150	Benzene–diethyl ether, 1:1	279 mg. tan solid
8	150	Diethyl ether	Nothing
9	200	Diethyl ether–ethanol, 3:1	29 mg. white solid
10	100	Diethyl ether–ethanol, 1:1	Nothing
11	100	Ethanol	Nothing

Table II.—Gradient Elution Adsorption Chromatography of Nonsaponifiable Material (3.8 Gm.)

Chromatographic Tubes	Product, mg.
1–4	Nothing
5–9	White, waxy solid, 100
10–18	White crystals, 220
19–24	Nothing
25–29	Yellow oil, 60
30–52	Colorless oil, 360
53–59	Colorless oil, 230
60–80	Colorless oil, 340
81–92	Colorless oil, 250
93–105	Light yellow oil, 310
106–126	Yellow oil, 520
127–130	Orange oil, 60
131–145	Yellow oil, 280
146–152	Colorless oil, 100
153–154	Orange oil, 30
155–157	Colorless oil, 40
158–160	Orange oil, 20
161–167	Colorless oil, 80
168–173	Yellow oil, 60
174–218	Colorless oil, 100

Thin-Layer Chromatography.—The analytical separation of slightly polar steroids has been realized by paper partition chromatography on acetylated or impregnated paper (11). These methods have some drawbacks, such as the long time of development. Van Dam (12) described a modification using adsorbent-coated glass plates which allow microadsorption chromatography on a flat surface. This procedure was used to separate further and identify partially the metabolic products in this study.

The chromatoplates were prepared according to the general procedure in the Desaga thin-layer apparatus manual (13). An amount of 10–100 mcg. of sample was placed on the Desaga silicic acid

TABLE III.—APPROXIMATE R_f VALUES FROM THIN-LAYER CHROMATOGRAPHY

			Solvent					
Sample	Benzene		Benzene:2 Ethyl Acetate:1		Benzene:3 Ethyl Acetate:2		Toluene:9 Ethyl Acetate:1	
7-Dehydroepiandrosterone	...		0.65		0.69		0.45	
Cholesterol	0.11		0.53		0.59		0.28	
Ergosterol	0.09		0.65		0.69		0.43	
β-Sitosterol	0.11		0.44		...		0.33	
Testosterone	0		0.28		...		0.12	
Androsterone	0		0		...		0	
Dehydroepiandrosterone	0.02		0.38		0.42		0.14	
Pregnenolone	...		0.42		0.46		0.16	
Progesterone	...		0.50		0.53		0.25	
Androstenedione	...		0.53		0.56		0.28	
Fraction 7	0.02	0.20	0.09	0.62	...		0.15	0.49
	0.14	0.62	0.38	0.78			0.24	0.74
			0.48					
Tubes 81–92	...		0.29	0.52	0.24	0.42	0.06	0.2
			0.43		0.51		0.17	
Tubes 93–105	...		0.02	0.33	0.02	0.36	0.07	
			0.19	0.41	0.18	0.51	0.14	
			0.25	0.52	0.22			
Tubes 106–126	...		0.02	0.39	0.02	0.37	0.03	0.22
			0.16	0.51	0.16	0.49	0.06	0.37
			0.24		0.21	0.57	0.14	

chromatoplates and then were developed. After thorough drying at 105°, they were sprayed with a saturated solution of antimony trichloride in chloroform and heated for 5 minutes at 105°. The reading of the spots was aided using long-wave ultraviolet light. An outline of the R_f values found in the different experiments is given in Table III. Known steroids were included in each experiment as internal standards. Fraction 7 had R_f values corresponding to pregnenolone and/or dehydroepiandrosterone, androstenedione and/or progesterone, and ergosterol. Tubes 81 to 92 had R_f values corresponding to androstenedione and/or progesterone and pregnenolone. Tubes 93 to 105 and tubes 106 to 126 had R_f values corresponding to pregnenolone and/or dehydroepiandrosterone and androstenedione and/or progesterone.

TABLE IV.—RELATIVE RETENTION TIMES FROM GAS CHROMATOGRAPHY

	Relative Retention Times			
Sample	50–300°[a]		100–300°[a]	
Cholestane	1.00[b]		1.00[c]	
Progesterone	0.818		0.801	
Pregnenolone	0.788		0.764	
Dehydroepiandrosterone	0.754		0.733	
Androstenedione	0.732		0.705	
Fraction 7	0.141	0.685	...	
	0.301	0.775		
	0.517			
Tubes 81–92	0.219	0.631	0.240	0.713
	0.560	0.726	0.555	0.774
			0.616	
Tubes 93–105	...		0.555	0.780
			0.616	0.818
			0.700	
Tubes 106–126	0.685	0.772	0.700	
	0.732	0.819	0.780	

[a] Column, 6 ft. × 1/4 in. O.D., 3% SE-30 on Chromosorb W. 80/100 mesh; programmer switch, 11°/min.; block temperature, 280°; injection port temperature, 240°; bridge current, 150; helium flow, 100 ml./minute; reference flow, 20 ml./minute; and helium pressure, 20 p.s.i. on the F and M model 500 programmed temperature gas chromatograph. [b] Time, 32.5 minutes. [c] Time, 29.2 minutes.

Gas Chromatography.—Numerous studies on the separation and identification of steroids by gas chromatography have been performed, and a recent review article (14) summarizes most of the work in this field. Although it has been reported that steroids decompose at temperatures over 260° to give broad peaks, gas chromatography studies were performed using programmed temperatures to 300°. Reproducible results were obtained for a number of known steroids, including the suspected metabolic steroids; therefore, these conditions were used for partial identification of the isolated metabolic products. A summary of the relative retention times is given in Table IV. Fraction 7 and tubes 81 to 92 had relative retention times comparable to androstenedione and pregnenolone. Tubes 93 to 105 had relative retention times comparable to progesterone, pregnenolone, and androstenedione. Tubes 106 to 126 had relative retention times comparable to progesterone, androstenedione, and dehydroepiandrosterone.

2,4-Dinitrophenylhydrazones.—Along with the small amounts of ketosteroids, large amounts of hydrocarbons are present in the nonsaponifiable material (15). All compounds with double bond systems absorb strongly in the far ultraviolet region but this is the region of decreased sensitivity in laboratory spectrophotometers and where interference from solvent occurs. To solve these problems, a study was initiated that dealt with the formation and isolation of steroidal 2,4-dinitrophenylhydrazones, (2,4-DNPs), as well as a determination of their absorption characteristics in the visible range. The 2,4-DNPs of the ketosteroids in the fractions containing ethanol-soluble components were prepared according to the method of Reich (16). The colored solid which resulted was purified by chromatography on an acid-washed aluminum oxide column (1 × 20 cm.). (See Table V.)

Another method of 2,4-DNP preparation was employed for fractions not completely soluble in ethanol (17). After drying, the 2,4-DNP derivative was purified by chromatography on an acid-washed aluminum oxide column. (See Table V.)

TABLE V.—ADSORPTION CHROMATOGRAPHY OF 2,4-DINITROPHENYLHYDRAZONES

Fraction or Tubes	Eluent	2,4-DNP Product
81–92	Benzene	Pregnenolone
81–92	Benzene–chloroform, 5:1	Progesterone (bis 2,4-DNP)
93–105	Benzene	Dehydroepiandrosterone
93–105	Benzene–chloroform, 3:1	Progesterone (20-mono-DNP)
106–126	Benzene	Dehydroepiandrosterone
106–126	Benzene	Progesterone (3-mono-DNP)
106–126	Benzene–chloroform, 3:1	Progesterone (20-mono-DNP)
7	Skellysolve B–benzene-diethyl ether, 80:10:5	Progesterone (bis-2,4-DNP)
7	Benzene	Pregnenolone
7	Benzene	Dehydroepiandrosterone
7	Benzene	Progesterone (3-mono-DNP)

All of the pure 2,4-DNP products in Table V were identified by ultraviolet spectral studies, melting point, mixed melting point with authentic samples, and analysis.

The bis-2,4-DNP of progesterone was recrystallized from a mixture of chloroform and absolute ethanol to give dark red plates, m.p. 270° dec. [lit. m.p. 282–283° (16)], which did not depress the melting point when mixed with an authentic sample of bis-2,4-DNP of progesterone. $\lambda_{max.}^{CHCl_3}$ 380 mμ, ϵ = 51,800 (lit. $\lambda_{max.}^{CHCl_3}$ 380 mμ) (16).

Anal.—Calcd. for $C_{33}H_{38}N_8O_8$: C, 58.74; H, 5.68; N, 16.60. Found: C, 58.52; H, 5.73; N, 16.60.

The 2,4-DNP of pregnenolone was recrystallized from a mixture of chloroform and absolute ethanol to give bright yellow plates, m.p. 253–254° [lit. m.p. 253–254° (16)], which did not depress the melting point when mixed with an authentic sample of the 2,4-DNP of pregnenolone. $\lambda_{max.}^{CHCl_3}$ 369 mμ, ϵ = 25,300 [lit. $\lambda_{max.}^{CHCl_3}$ 368 mμ (16)].

Anal.—Calcd. for $C_{27}H_{36}N_4O_5$: C, 65.29; H, 7.31; N, 11.28. Found: C, 65.37; H, 7.40; N, 11.30.

The 2,4-DNP of dehydroepiandrosterone was recrystallized from a mixture of chloroform and absolute ethanol to give bright yellow plates, m.p. 235–237° dec., which did not depress the melting point when mixed with an authentic sample of the 2,4-DNP of dehydroepiandrosterone. $\lambda_{max.}^{CHCl_3}$ 366 mμ, ϵ = 25,300 [lit. $\lambda_{max.}^{CHCl_3}$ 366 mμ (16)].

Anal.—Calcd. for $C_{25}H_{32}N_4O_5$: C, 64.08; H, 6.89; N, 11.96. Found: C, 63.73; H, 6.90; N, 12.18.

Several general methods for the regeneration of the corresponding carbonyl compounds have been reported (18–20). The Mattox-Kendal method (18, 19) which utilizes pyruvic acid and hydrobromic acid and the Demaeker-Martin method (20) which utilizes acetone, hydrochloric acid, and stannous chloride were employed to cleave the above isolated 2,4-DNPs.

The bis-2,4-DNP of progesterone was cleaved to give a white solid product. Recrystallization from dilute ethanol gave white needles, m.p. 127.5–128° [lit. m.p. 128.5° (21)], which did not depress the melting point when mixed with an authentic sample of progesterone. $[\alpha]_D^{18}$ + 191.1° in ethanol [lit. $[\alpha]_D^{18}$ + 192° in ethanol (21)]. $\lambda_{max.}^{EtOH}$ 240 mμ, ϵ = 19,600 [lit. $\lambda_{max.}^{EtOH}$ 240 mμ) (21)].

Anal.—Calcd. for $C_{21}H_{30}O_2$: C, 80.20; H, 9.54. Found: C, 80.68; H, 9.44.

The 2,4-DNP of pregnenolone was cleaved to give a white solid product. Recrystallization from dilute ethanol gave white needles, m.p. 188.5–190° [lit. m.p. 189–190° (21)], which did not depress the melting point when mixed with an authentic sample of pregnenolone. $[\alpha]_D^{18}$ + 30.8° in ethanol [lit. $[\alpha]_D^{18}$ + 30° in ethanol (21)].

Anal.—Calcd. for $C_{21}H_{32}O_2$: C, 79.70; H, 10.19. Found: C, 79.43; H, 10.04.

The 2,4-DNP of dehydroepiandrosterone was cleaved to give a white solid product. Recrystallization from a mixture of chloroform and skellysolve B gave white needles, m.p. 140–142° [lit. m.p. 140–141° (21)], which did not depress the melting point when mixed with an authentic sample of dehydroepiandrosterone. $[\alpha]_D^{18}$ + 11.3° in ethanol [lit. $[\alpha]_D^{18}$ + 10.9° in ethanol (21)].

Anal.—Calcd. for $C_{19}H_{28}O_2$: C, 79.12; H, 9.79. Found: C, 79.38; H, 10.32.

DISCUSSION

The isolation and characterization of dehydroepiandrosterone, pregnenolone, and progesterone as metabolic products of normal sterol metabolism in *T. confusum* larvae indicates that certain insects possess enzyme systems capable of altering the side chain of dietary sterols. As part of a study concerned with the metabolic degradation of cholesterol, mammalian tissue was used to cleave the side chain

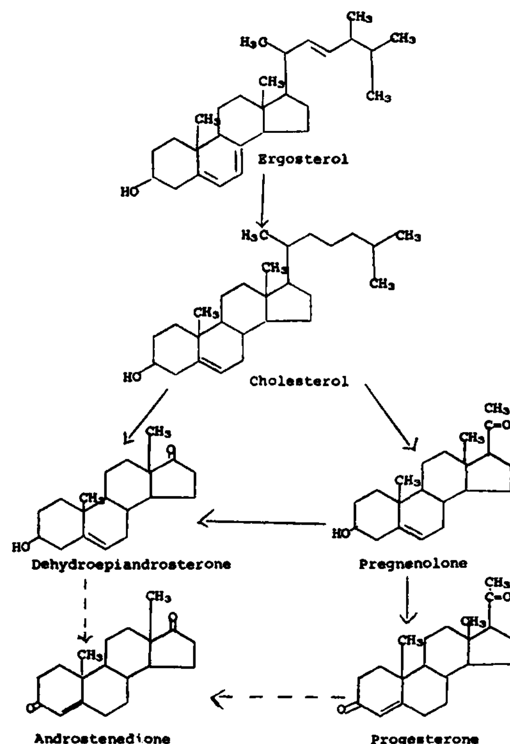

Fig. 1.—Proposed biogenetic breakdown of dietary sterols in the larvae of the confused flour beetle, *T. confusum*.

of cholesterol to yield isocaproic acid and pregnenolone (22) which is further oxidized to progesterone (23). Thus, mammalian enzymes capable of cleaving the cholesterol side chain are known. The action of this insect's enzyme system parallels that of enzyme systems in higher animals (24), and the proposed biogenetic breakdown of dietary sterols by *T. confusum* larvae is outlined in Fig. 1. Since it has been shown that many dietary sterols, including ergosterol, are converted to cholesterol and 7-dehydrocholesterol in *T. confusum* larvae, the conclusion may be made that alteration of the side chain of dietary sterols occurred after conversion to cholesterol.

During the isolation of these metabolic products, a number of other products was obtained whose structures could not be elucidated due to the paucity of material. Androstenedione may be included among these unidentified products, since its presence is indicated by physical methods of characterization, and it would be a normal conversion product from progesterone and dehydroepiandrosterone.

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Synthetic Polymers as Potential Sustained-Release Coatings

By JOHN W. KLEBER, J. FRANK NASH, and CHENG-CHUN LEE*

The characteristics of the absorption of prednisolone were studied both in intact dogs and in ligated segments of the intestinal tract. Absorption of uncoated prednisolone was essentially complete in 2 hours. Following the administration of copolymer-coated pellets, absorption was extended over a period of 10–12 hours.

FOLLOWING THE oral administration of a given drug, effective concentrations in the blood and target tissue are dependent on several factors—solubility, site of absorption, stability in the gastrointestinal tract, rate of metabolism, and excretion. In many instances maintenance of therapeutic effect requires repeated administration at 4–6-hour intervals. Longer duration of a single dose is often desirable to permit uninterrupted rest during the night or to diminish the possibility of missed dosage.

Various coatings have been investigated to delay the release of drug into the intestinal tract. The use of a pH-dependent coating to obtain sustained release of prednisolone has been reported (1); however, the chemical nature of the coating substance was not disclosed. Several copolymers have been discovered (2) that differ in their rate of solution in water at different pH values. An earlier report (3) on these synthetic copolymers described their use as enteric coatings on acetylsalicylic acid tablets. The current study also provides information about the potential of these copolymers to delay the absorption of a drug. Prednisolone served as the model drug.

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EXPERIMENTAL

Materials

Nonpareil sugar pellets, 16–18 mesh, prednisolone U.S.P., prednisolone acetate U.S.P., cetyl alcohol N.F., magnesium stearate-talc dusting powder (15:85), $^1/_2$ *n*-butyl ethylene maleic acid copolymer (butyl EMA), $^1/_2$ isopropyl polymethylvinylether/maleic acid copolymer (isopropyl PVM/MA), and $^1/_2$ *n*-butyl polymethylvinylether/maleic acid copolymer (butyl PVM/MA) were utilized.