

[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY AND PHYSICS OF THE PENNSYLVANIA STATE COLLEGE]

**Sterols. XCIX. Sterols from Various Sources\***

BY RUSSELL E. MARKER AND ANTHONY C. SHABICA

It has been observed by Stern<sup>1</sup> that a subcutaneous injection of cantharidin contained in the genital gland of Spanish flies produces an estrogenic activity. He attributed this activity to the presence of traces of sex hormones.

This observation warranted the investigation of "Cantharides Russian" (Spanish Flies) to determine the possible presence of androstane and pregnane derivatives already found in the urine of mammals.<sup>2</sup>

This work was followed by the investigation of mares non-pregnancy urine, Mexican flies, ant eggs, chicken feces and sheep feces.

Since no androstane or pregnane derivatives were isolated these investigations were combined for publication.

The mode of isolation was essentially the same as that described in earlier papers on urine. The pulverized Spanish flies were extracted with ethanol and hydrolyzed with alkali to yield a neutral fraction which was separated by means of digitonin and succinic anhydride into  $\beta$ -sterols, carbinols and inert substances. The neutral fraction from hydrolysis gave a methanol insoluble fraction which was identified as a hydrocarbon of the aliphatic series melting at 63°. This hydrocarbon is identical with one found in pregnancy urines.<sup>2</sup>

The insoluble digitonide fraction yielded a sterol identified as sitosterol. Since this sterol had a sharp melting point on hydrolysis of the digitonide no attempt was made to show the presence or absence of cholesterol. On treatment of the residue from the digitonin precipitation with succinic anhydride, two unidentified carbinols were isolated, melting at 69° and 201° and having the molecular weights of 256 and 381, respectively.

The mares' non-pregnancy urine and the ant eggs on treatment analogous to the cantharides yielded cholesterol as the only product from the digitonin precipitation. No crystalline carbinols or hydrocarbons were obtained from the succinic anhydride treatment. An attempt to oxidize

the carbinols from the mares' non-pregnancy urine resulted in a neutral oily substance which did not give a crystalline semicarbazone, showing the absence of *allo*-pregnenedione.

The investigation of Mexican flies yielded the same aliphatic hydrocarbon found in the cantharides. In addition an unidentified sterol was isolated which has been shown to be identical with one obtained from chicken feces. Since this unidentified sterol gave no depression in melting point with cholesterol it has been concluded that the compound either has a structure very similar to that of cholesterol or it is a molecular compound of cholesterol. Lack of material prevented further investigation at the present time. Sitosterol also was isolated from the chicken feces.

With the failure to detect any pregnane and androstane derivatives commonly found in urine, or coprosterol found in feces, it was deemed worth while to extend this work to the investigation of the steroid content of other vertebrates. For this purpose, sheep feces was used.

The insoluble digitonide on decomposition yielded a sterol identified as sitostanol. The remaining residue on treatment with succinic anhydride gave an aliphatic hydrocarbon identical to the one found in "Cantharides Russian." A very small amount of a carbinol melting at 75-79° was isolated but not identified.

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**Experimental Part**

**Cantharides Russian.**—The ethanol extract of two pounds (909 g.) of cantharides, Russian (S. B. Penick and Co., New York City), was hydrolyzed with an excess of alcoholic potassium hydroxide solution. The neutral fraction was thoroughly extracted with ether which on evaporation yielded a brown solid. This residue was dissolved in a minimum amount of hot methanol and allowed to stand overnight. The insoluble material was filtered and recrystallized from acetone, yielding 100 mg. of product melting at 64° which gave no depression in melting point when mixed with a hydrocarbon obtained from cows' pregnancy urine,<sup>2</sup> m. p. 63°.

*Anal.* Found: C, 85.1; H, 14.7.

The sterol obtained from the digitonin precipitation,

\* Paper XCVIII, Marker, Rohrmann and Jones, *THIS JOURNAL*, 62, 1162 (1940).

(1) Stern, *Arch. expl. Path.*, 166, 395 (1932).

(2) Marker, *THIS JOURNAL*, 60, 2442 (1938).

1.98 g., on recrystallization from acetone melted at 144°. When mixed with *beta*-sitosterol it gave no depression in melting point.

*Anal.* Calcd. for  $C_{29}H_{50}O$ : C, 83.97; H, 12.1. Found: C, 83.6; H, 12.1.

When refluxed with acetic anhydride this sample gave an acetate melting at 125° which gave no depression with sitosterol acetate, m. p. 125°.

The carbinols obtained from succinic anhydride treatment when sublimed under high vacuum at 100–135° gave two crystalline products. After crystallization from acetone of the fraction subliming at 100°, an unidentified product was obtained, m. p. 69°.

*Anal.* Found: C, 73.3; H, 12.6; mol. wt. (Rast), 256.

The product subliming at 135° was crystallized from acetone and melted at 201°.

*Anal.* Found: C, 77.12; H, 10.71; mol. wt. (Rast), 381.4.

The non-carbinol fraction (0.73 g.) from the succinic anhydride treatment gave no crystalline product on sublimation in a high vacuum.

**Mexican Flies.**—The ethanol extract of 10 lb. of Mexican Flies (Penick) was treated in the same manner as the *Cantharides* Russian. The methanol insoluble material, 1.4 g., on crystallization from acetone gave a waxy compound melting at 62° which gave no depression when mixed with a hydrocarbon of the aliphatic series obtained from cows' pregnancy urine, m. p. 63°.

*Anal.* Found: C, 85.7; H, 15.1.

The sterol obtained from the digitonin precipitation, 1.7 g., on recrystallization from acetone melted at 149–151°. This has not been identified.

*Anal.* Found: C, 83.74; H, 12.13.

When refluxed with acetic anhydride this sample gave an acetate melting at 130°.

*Anal.* Found: C, 81.58; H, 11.47.

**Ant Eggs.**—The ethanol extract of 10 lb. of pulverized ant eggs (Penick) was treated according to the method used in the isolation of the sterols from *Cantharides* Russian. The methanol insoluble material (2.0 g.) was sublimed under high vacuum at 115°. This fraction after repeated recrystallization from acetone gave the same hydrocarbon obtained from the previous products.

The sterol obtained from the digitonin precipitation, 1.77 g., on recrystallization from acetone melted at 147–148°. When mixed with cholesterol it gave no depression in melting point.

*Anal.* Calcd. for  $C_{27}H_{46}O$ : C, 83.85; H, 12.00. Found: C, 84.2; H, 11.9.

When refluxed with acetic anhydride an acetate was obtained melting at 116° which gave no depression in melting point when mixed with cholesterol acetate.

*Anal.* Calcd. for  $C_{29}H_{48}O_2$ : C, 81.2; H, 11.3. Found: C, 81.1; H, 11.2.

**Chicken Feces.**—The ethanol extract of 50 lb. of partially dried chicken feces was hydrolyzed with an excess of alcoholic potassium hydroxide solution. The neutral fraction was extracted with ether, which on evaporation yielded orange colored plates. The crude crystals were dissolved

in a minimum amount of hot ethyl alcohol and allowed to stand overnight. The insoluble material was filtered and recrystallized from acetone, yielding 16.0 g. of product melting at 136° which gave no depression in melting point when mixed with a known sample of sitosterol, m. p. 136°.

*Anal.* Calcd. for  $C_{29}H_{50}O$ : C, 83.97; H, 12.1. Found: C, 84.04; H, 12.13.

When refluxed with acetic anhydride this compound gave an acetate melting at 133° which gave no depression in melting point when mixed with a known sample.

*Anal.* Calcd. for  $C_{31}H_{52}O_2$ : C, 81.5; H, 11.4. Found: C, 82.3; H, 11.5.

The filtrate from the above product was treated with digitonin in alcohol. The digitonide was decomposed in the usual manner and the residue recrystallized from acetone. The product melting at 148° gave no depression in melting point when mixed with cholesterol, m. p. 148°, and the unidentified sterol obtained from Mexican Flies, m. p. 149–151°. A depression of 10° was observed with *beta*-sitosterol.

*Anal.* Found: C, 83.72; H, 12.27.

When refluxed with acetic anhydride this sample gave an acetate melting at 131° which gave a 30° depression when mixed with cholesterol acetate.

*Anal.* Found: C, 81.1; H, 11.8.

On reduction of the unidentified sterol with platinum oxide catalyst, a product was obtained melting at 136° which when mixed with sitostanol gave no depression in melting point. A mixed melting point with the starting material gave a depression of 10°.

*Anal.* Found: C, 83.4; H, 12.7.

When refluxed with acetic anhydride this sample gave an acetate melting at 136° which gave a depression of 35° when mixed with the acetate of the starting material. When mixed with sitostanol acetate a 60° depression in melting point was observed.

*Anal.* Found: C, 80.9; H, 11.9.

**Sheep Feces.**—The ethanol extract of 50 lb. of dried pulverized sheep feces was treated in the same manner as the chicken feces. The residue from the hydrolysis (32.2 g.) was dissolved in 750 cc. of ethanol, treated with Norit and filtered. The sterol obtained from the digitonin precipitation (1.4 g.) on crystallization from acetone melted at 139°, which when mixed with sitostanol, m. p. 140–141°, gave no depression in melting point.

*Anal.* Calcd. for  $C_{29}H_{52}O$ : C, 83.57; H, 12.58. Found: C, 84.2; H, 12.2.

When refluxed with acetic anhydride this product gave an acetate melting at 130–132° which gave no depression in melting point when mixed with sitostanol acetate.

*Anal.* Calcd. for  $C_{31}H_{54}O_2$ : C, 81.1; H, 11.86. Found: C, 81.4; H, 12.0.

A hydrocarbon was obtained by crystallizing the non-carbinol fraction from acetone, m. p. 64°, which gave no depression in melting point when mixed with an aliphatic hydrocarbon obtained from cows' pregnancy urine.

The carbinol obtained from the succinic anhydride treatment was sublimed under vacuum at 135°. A very small amount of crystalline material melting at 75–79° was obtained. Identification was not completed.

**Mares' Non-Pregnancy Urine.**—The butanol extract of 300 gallons of mares' non-pregnancy urine (previously hydrolyzed with hydrochloric acid) was hydrolyzed with an excess of aqueous potassium hydroxide solution. The neutral fraction was extracted with ether, which on evaporation yielded 209 g. of tarry residue. This residue was dissolved in a minimum amount of ethyl alcohol and treated with a 2% digitonin ethyl alcohol solution. On decomposition of the digitonide and sublimation of the residue under high vacuum at 130–140°, 0.34 g. of cholesterol was obtained, m. p. 144–146°. There was no depression in melting point when mixed with an authentic sample of cholesterol.

*Anal.* Calcd. for  $C_{27}H_{46}O$ : C, 83.85; H, 12.00. Found: C, 83.62; H, 11.69.

When refluxed with acetic anhydride this sample gave an acetate melting at 119° which gave no depression when mixed with an authentic sample.

*Anal.* Calcd. for  $C_{29}H_{48}O_2$ : C, 81.2; H, 11.3. Found: C, 81.27; H, 11.1.

The neutral fraction (42.5 g.) from the succinic anhydride

treatment gave no crystalline products when sublimed under high vacuum at 100–170°.

The carbinol fraction (30 g.) from the succinic anhydride treatment gave 25.19 g. of a very viscous oil on sublimation under high vacuum. This oil was oxidized by dissolving in 100 cc. of acetic acid and adding 20 g. of chromic anhydride in 20 cc. of water and 200 cc. of acetic acid. The mixture was kept at room temperature for thirty minutes. The ether extract was washed well with water and then with potassium carbonate to remove any acids present. The neutral fraction (8.8 g.) in 100 cc. of ethanol was added to 7.2 g. of semicarbazide hydrochloride and 10.8 g. of sodium acetate in 140 cc. of ethanol and refluxed for three hours. No crystalline semicarbazones could be isolated, indicating the absence of *allo*-pregnenedione.

### Summary

The androstane and pregnane content of *Cantharides*, Mexican flies, ant eggs, sheep feces, chicken feces and mares' non-pregnancy urine was investigated.

STATE COLLEGE, PENNA.

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## Sterols. C. Diosgenin<sup>1</sup>

BY RUSSELL E. MARKER, TAKEO TSUKAMOTO AND D. L. TURNER

Diosgenin (I) has been isolated from an extract of *Dioscorea tokoro* (Makino), and its nuclear structure proven by Tsukamoto, *et al.*<sup>2,3,4</sup> It has been shown to have a 5,6 double bond and one nuclear hydroxyl group in the 3-position having the beta-configuration as in cholesterol. By catalytic reduction, Tsukamoto obtained tigenin, showing that it has the same side chain characteristic of the saturated steroidal sapogenins.

The ready availability of diosgenin and the fact that it contains a double bond in its nucleus suggested that it would be an ideal compound to apply the reactions<sup>5,6</sup> which have been applied to sarsasapogenin and other steroidal sapogenins for the removal of the side chain leading to derivatives of the pregnane and androstane series.

(1) The isolation of the diosgenin was carried out in the Japanese laboratories and the experimental work reported in this paper was carried out at The Pennsylvania State College.

(2) Tsukamoto, Ueno and Ohta, *J. Pharm. Soc., Japan*, **56**, 135 (1936).

(3) Tsukamoto, Ueno and Ohta, *ibid.*, **57**, 9 (1937).

(4) Tsukamoto, Ueno, Ohta and Tschesche, *ibid.*, **57**, 283 (1937).

(5) Marker and Rohrmann, *THIS JOURNAL*, **61**, 3592 (1939), **62**, 518, 521, 896, 898 (1940).

(6) Marker, Rohrmann and Jones, *ibid.*, **62**, 648 (1940).

It is also a compound which can be correlated readily with the other sapogenins.

When diosgenin (I) is heated at 200° with acetic anhydride it is converted into pseudodiosgenin (II). As is characteristic of the other pseudosapogenins, pseudodiosgenin is easily reconverted to diosgenin by alcoholic hydrochloric acid. In preliminary experiments on pseudosarsasapogenin<sup>7</sup> it was found that bromine in acetic acid converted much of the pseudosapogenin back to the sapogenin. This probably was due to the catalytic effect of hydrobromic acid formed in the reaction. To overcome this difficulty, we acetylated pseudodiosgenin and brominated it at a low temperature in acetic acid containing sodium acetate. We then added chromic acid to this solution. After debromination and hydrolysis we obtained  $\Delta^{5,16}$ -pregnadienol-3-one-20 (V). However, the yield in this case was much lower than that obtained in the oxidation of the saturated pseudosapogenins.<sup>5,6</sup> This is probably due to the difficulty of bromination of the pseudo compound without converting it back to the original sapogenin. When

(7) Unpublished results from this Laboratory.