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Summary

1. A successful synthesis has been accomplished for the triene, 3-[2'-methylene-cyclohexylidene-1']-propene-1, which possesses a conjugated system identical with that present in all antirachitic vitamins.

2. The molecular extinction coefficient of this triene is nearly identical with that reported for the antirachitic vitamins.

3. Work is being continued with the ultimate goal of synthesizing an antirachitic vitamin.

CAMBRIDGE, MASS.

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

Sterols. LXX.* The Steroid Content of Mares Pregnancy Urine

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In a previous paper¹ from this Laboratory it was pointed out that the ratios of pregnanediol-3(α)-20(α), *allo*-pregnanediol-3(α)-20(α) and *allo*-pregnanediol-3(β)-20(α) which occurred in human and cows pregnancy urine and in bulls urine appeared to be constant. The figures reported for mares pregnancy urine were arbitrarily based upon the amount of *allo*-pregnanedione² obtained from the oxidation of the total carbinol fraction and from this it was assumed that the observed ratio of the occurrence of the various pregnanediols held for all pregnancy urines. Both pregnanediol-3(α),20(α)² and *allo*-pregnanediol-3(β)-20(α)³ have been isolated while *allo*-pregnanediol-3(α),20(α) was assumed to be present from the amount of *allo*-pregnanedione obtained by oxidation of the total carbinol fraction.

Because of the large amount of tar present in mares pregnancy urine the isolation of the pregnanediols by the methods used to work up other urines is extremely difficult and this fact has greatly delayed a quantitative investigation of this urine. Recently we have obtained indications that mares pregnancy urine differs considerably from the other urines studied, especially in regard to the ratio of pregnanediols present. In order to obtain more quantitative information concerning these substances we have undertaken the present investigation of the carbinol fraction from 10,000 gallons of mares pregnancy urine.

(*) Paper LXIX, Marker and Rohrmann, *THIS JOURNAL*, **61**, 2072 (1939).

(1) Marker, Wittle and Lawson, *ibid.*, **60**, 2931 (1938).

(2) Marker, *et al.*, *ibid.*, **59**, 2297 (1937).

(3) Marker and Rohrmann, *ibid.*, **60**, 1565 (1938).

The results obtained show a remarkable difference between mares pregnancy urine and other urines as is shown in the following table.

APPROXIMATE AMOUNTS INDICATED IN MG. PER GALLON OF URINE

	Pregnanediol-3(α), 20(α)	<i>allo</i> - Pregnanediol-3(α), 20(α)	<i>allo</i> - Pregnanediol-3(β), 20(α)
Human pregnancy	50	25	6
Cow pregnancy	25	15	3
Bull	100	50	12
Mare pregnancy	3	2	25

The crude carbinols from 10,000 gallons of hydrolyzed mares pregnancy urine from which the ketones were previously removed with Girard's reagent were separated from the hydrocarbons by means of the half phthalates and the entire carbinol fraction precipitated with an excess of digitonin to separate the steroids epimeric at C-3. The material precipitated by digitonin consisted largely of uranediol-3(β),11⁴ and *allo*-pregnanediol-3(β),20(α) together with a new substance of the composition C₄₈H₈₂O₃. This latter substance appears to be a molecular compound of cholesterol and uranediol-3(β),11 since oxidation of the product yielded uranediolone while treatment with acetic anhydride yielded cholesteryl acetate.

The carbinol fraction which did not precipitate with digitonin was oxidized under mild conditions with chromic anhydride and the ketones separated with Girard's reagent. The ketonic material yielded some pregnanediolone and *allo*-pregnanediolone.

(4) Marker, Rohrmann and Wittle, *THIS JOURNAL*, **60**, 1561 (1938).

dione in addition to a new compound of the composition $C_{18}H_{16}O_2$, m. p. 214° . This substance which is evidently an unsaturated diketone formed only a monosemicarbazone, which suggests that the substance may be 3-desoxy-11-keto-equilenin. We are planning to investigate the nature of this compound and its possible origin more fully when more of the substance becomes available.

We have also obtained evidence of the presence of urenetriol and of a new *allo*-pregnanetriol in the digitonin precipitable fraction (*cf.* flow sheet, ref. 4, p. 1563). The bromination and mild oxidation with chromic anhydride of two fractions of non-sublimable carbinols⁴ gave in one case urenetriol identical with that prepared from uranetriol⁵ while the other fraction yielded a new triol, m. p. 129° . This triol upon Clemmensen reduction gave *allo*-pregnane.

Examination of the ligroin soluble fraction referred to in a previous paper (*cf.* flow sheet, ref. 4, p. 1563) yielded the characteristic urinary hydrocarbon of the composition $C_{28}H_{58}$ identical with that previously reported from other urines,^{6,7} together with cholesterol and equistanol.^{1,3,8}

It seems strange that cow pregnancy urine and bull urine should resemble human pregnancy urine so closely in regard to the ratio of the various pregnanediols present, while mares pregnancy urine, which one might expect to resemble cow pregnancy urine in composition, is so widely different from all the urines so far studied. Mares pregnancy urine differs from the other urines studied not only in regard to the ratio of the pregnanediols present but also by its extreme complexity, as is shown by the large number of substances which have been isolated from it and from the large amount of tarry products of unknown nature which are present. In view of these facts we feel that the investigation of the urines of a number of animal species may give highly interesting results. The fact that equistanol appears to be present in most animal urines but not in human urine may be of great importance and a further investigation of the occurrence and nature of this substance seems highly desirable.

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(5) Marker, *et al.*, *THIS JOURNAL*, **60**, 1061 (1938).

(6) Marker, *ibid.*, **60**, 2442 (1938).

(7) Marker, *ibid.*, **61**, 1287 (1939).

(8) Marker, Lawson, Rohrmann and Wittle, *ibid.*, **60**, 1555 (1938).

Sex, National Research Council. We wish to thank Parke, Davis and Company for the supply of urine concentrate used and their cooperation in various phases of this work.

Experimental Part

Digitonin Precipitation.—The neutral extract of 10,000 gallons of mares pregnancy urine after hydrolysis with hydrochloric acid, followed by steam distillation with an excess of sodium hydroxide and removal of the ketones by Girard's reagent, weighed 5.2 kg. The pregnanetriol and uranetriol were removed as previously described.⁹

The residue was dissolved in 2 liters of hot acetic acid and poured into 50 liters of ether. The ethereal solution was washed well with water and then stirred with an excess of aqueous sodium hydroxide solution. A large amount of tar, which upon burning left a large inorganic residue, separated out and was discarded. The ethereal solution was washed well with water and the ether removed. The residue was dried by distilling benzene from it. This was then heated on a steam-bath with 1500 cc. of pyridine and 1500 g. of phthalic anhydride for one hour. Ether and ice were added, and the pyridine removed by shaking with hydrochloric acid. The ethereal solution was shaken with an excess of potassium carbonate solution to remove the phthalic esters. These were hydrolyzed by refluxing with alcoholic potassium hydroxide. The carbinol fraction weighed 2.3 kg.

This was dissolved in alcohol and a 2% solution of alcoholic digitonin added until no more digitonide separated. This required a total of 2100 g. of digitonin for the complete precipitation. The digitonides were washed well, dried and decomposed by heating on a steam-bath with 10 liters of dry pyridine until dissolved. This was poured into an excess of ether and filtered from the precipitated digitonin. The pyridine was removed by washing with dilute hydrochloric acid and the ether was evaporated. The residue consisting of digitonin precipitable sterols was dissolved in 500 cc. of benzene.

The Carbinol Fraction Non-Precipitable by Digitonin.—The filtrate from the digitonide precipitation was evaporated, but due to the large amounts of aliphatic carbinols present resisted separation. The concentrate was dissolved in acetic acid and vacuum evaporated several times to remove any alcohol. The residue was then dissolved in 20 liters of acetic acid, cooled to 20° and 3 kg. of chromic anhydride dissolved in 3 liters of water and 5 liters of acetic acid slowly run in with stirring over a period of two hours, keeping the temperature at 20° . It was stirred for an additional hour. Ether and water were added and the ethereal solution washed with water and dilute alkali. The ether was removed and the ketones separated by means of Girard's reagent to give 215 g. of material. Upon steam distillation 75 g. of volatile ketones was removed. The ketones which did not steam distil were extracted with ether and upon concentration to 300 cc. and cooling 5.2 g. of material was obtained. This was sublimed in high vacuum at 140° and the sublimate was crystallized from acetone, methyl alcohol and ethyl alcohol to give a product of m. p. $212-214^\circ$. It

(9) Marker, *et al.*, *ibid.*, **60**, 210 (1938).

gave a depression in melting point when mixed with uranetrione and *allo*-pregnenedione.

Anal. Calcd. for $C_{18}H_{16}O_2$: C, 81.8; H, 6.1; mol. wt., 264. Found: C, 82.0, 81.6; H, 6.1, 6.0; mol. wt., (Rast), 248.

Upon heating with semicarbazide hydrochloride and sodium acetate in alcohol for two hours, it gave a semicarbazone which was crystallized from 80% ethanol to give a product, m. p. 255–260° dec. It analyzed for a monosemicarbazone.

Anal. Calcd. for $C_{19}H_{18}N_2O_2$: C, 71.0; H, 5.9. Found: C, 71.4; H, 5.9.

The ketones from the mother liquors of the above product were sublimed in high vacuum, collecting the fraction from 100–220°. These were dissolved in two liters of alcohol and refluxed for two hours with 100 g. of semicarbazide hydrochloride and 120 g. of sodium acetate. The extremely insoluble semicarbazone decomposed at about 320°. This product (21 g.) was hydrolyzed with sulfuric acid and the product crystallized from methanol, ethanol and acetone to give white crystals, m. p. 200–201°; yield 12 g. It gave no depression in melting point when mixed with *allo*-pregnenedione.

The filtrates from the above semicarbazone were combined and recrystallized. The first fraction upon acid hydrolysis gave a further quantity of 1.4 g. of *allo*-pregnenedione. The next fraction of semicarbazones gave only oily products which could not be separated. The following fraction, after hydrolysis of the semicarbazone and sublimation, was crystallized from dilute ethyl alcohol after seeding with a crystal of pregnenedione. It was recrystallized to give 21 g. of pregnenedione, m. p. 117–119.5°, which gave no depression in melting point when mixed with an authentic sample of pregnenedione.

The Digitonin Precipitable Sterols.—This fraction was dissolved in an equal quantity of benzene and allowed to stand at room temperature for one week. The precipitate which formed was filtered and crystallized from acetone. After treatment with Norite this product was crystallized from methanol to give 25 g. of compact white crystals, m. p. 210–212°. This gave no depression with a sample of uranediol obtained previously from mares urine.

The filtrate remaining after the separation of the crude uranediol was evaporated and the semicrystalline product crystallized repeatedly from ethyl acetate to give white flaky needles, m. p. 191–192.5°. The mixture with uranediol melted from 189 to 205°. The product sublimed unchanged in high vacuum at 120–160°.

Anal. Calcd. for $C_{21}H_{36}O_2 \cdot C_{27}H_{46}O$: C, 81.5; H, 11.7; mol. wt., 706. Found: C, 81.6, 81.5, 81.5, 81.4; H, 11.8, 11.5, 12.0, 11.9; mol. wt. (Rast), 363.

When refluxed for thirty minutes with acetic anhydride the product yielded an acetate which crystallized from aqueous acetone as white plates, m. p. 111–113°. This gave no depression with an authentic specimen of cholesteryl acetate.

Anal. Calcd. for $C_{25}H_{48}O_2$: C, 81.2; H, 11.3. Found: C, 81.2, 81.5, 81.2; H, 11.6, 11.3, 11.4.

When oxidized with chromic anhydride in acetic acid at room temperature for one hour the substance yielded a product which after treatment with Norite crystallized

from ether-pentane as white crystals, m. p. 177–178°. This gave no depression with a sample of uranediol.

Anal. Calcd. for $C_{21}H_{32}O_2$: C, 79.7; H, 10.2. Found: C, 79.4; H, 10.0.

The total sterol fraction remaining after removal of the uranediol and most of the uranediol-cholesterol compound was sublimed in high vacuum, the material distilling at 100–220° being collected. The sublimate was dissolved in 5 liters of acetic acid and the solution oxidized with 200 g. of chromic anhydride at 20° for three hours. The mixture was diluted with water and the precipitated solid taken up in ether and washed with sodium carbonate solution. Partial evaporation of the ether gave 205 g. of solid which after crystallization from methanol and acetone gave 165 g. of white crystals, m. p. 197–200°. This gave no depression in melting point with *allo*-pregnenedione.

The combined filtrates from the above yielded an additional 44 g. of *allo*-pregnenedione as well as a small amount of material which was crystallized from ethanol, acetic acid, methanol and acetone to give white crystals, m. p. 245–248°. Upon heating to 255° gas was evolved. The product, which analyzes for a hydroxy ketone, was recovered unchanged after refluxing with acetic anhydride.

Anal. Calcd. for $C_{21}H_{34}O_2$: C, 79.2; H, 10.8. Found: C, 79.2, 79.2; H, 11.1, 11.2.

Oxidation of Residue from Carbinol Mixture C. (*Cf.* flow sheet, ref. 4, p. 1563.)—The portion of carbinol mixture C which would not sublime at 200° was dissolved in acetic acid and a slight excess of bromine added to prevent oxidation of unsaturated compounds. To this solution was added an excess of chromic anhydride in 90% acetic acid and the mixture was allowed to stand at room temperature for ninety minutes, after which 10 cc. of methanol and 5 g. of zinc dust was added. After heating for thirty minutes on the steam-bath the mixture was filtered and the filtrate diluted with water. The precipitated solid was taken up in ether and the product sublimed in high vacuum, the product distilling at 140–160° being collected and crystallized from methanol to give short thick needles, m. p. 127–129°.

Anal. Calcd. for $C_{21}H_{30}O_3$: C, 76.3; H, 9.2. Found: C, 76.2; H, 9.1.

When treated with semicarbazide acetate under the usual conditions, the substance yielded a semicarbazone melting above 300°.

Anal. Calcd. for $C_{23}H_{36}O_3N_6 \cdot \frac{1}{2}H_2O$: C, 60.9; H, 8.3; N, 18.6. Found: C, 60.9, 60.8; H, 8.4, 8.3; N, 19.3.

A solution of 75 mg. of the ketone in 25 cc. of acetic acid was treated with 10 g. of amalgamated zinc and 25 cc. of concentrated hydrochloric acid and the mixture refluxed for three hours. The mixture was extracted with ether and the product crystallized from acetone-methanol to give white crystals, m. p. 80–82°. This gave no depression with an authentic sample of *allo*-pregnane.

Uranetrione from Residues of Carbinol Mixture A. (*Cf.* flow sheet, ref. 4, p. 1563.)—The residue remaining after the high vacuum sublimation of carbinol mixture A at 200° was dissolved in 50 cc. of glacial acetic acid. A slight excess of bromine was added and the solution was oxidized with 3 g. of chromic anhydride as described in the

previous experiments. The oxidation product was sublimed in high vacuum at 130° and the sublimate crystallized from methanol as pale yellow plates, m. p. 192°. This gave no depression with a sample of urenetrione (m. p. 195°).

Anal. Calcd. for $C_{21}H_{28}O_3$: C, 76.8; H, 8.6. Found: C, 76.7; H, 8.5.

Ligroin Soluble Fraction.—The ligroin soluble material corresponding to 1000 gallons (3700 liters) (*cf.* flow sheet, ref. 4, p. 1563) was used. The ligroin was evaporated *in vacuo* and the residual tar taken up in 350 cc. of hot 95% ethanol. The solution was cooled at 0° and the white crystals collected, washed with ethanol and crystallized from ethyl acetate after treatment with Norite to give greasy white plates, m. p. 64°. This gave no depression with samples previously reported from human and cows pregnancy urine.

Anal. Calcd. for $C_{28}H_{38}$: C, 85.3; H, 14.8. Found: C, 85.1; H, 14.8.

The filtrate from the above containing the ethanol soluble material was made up to a volume of 500 cc. with 95% ethanol and the solution was precipitated with digitonin (13 g.) to yield 12 g. of dried digitonide. This was de-

composed with pyridine in the usual manner and the product was crystallized from ethyl acetate to give white plates, m. p. 146°, which gave no depression with a sample of cholesterol.

The filtrate remaining after removal of part of the cholesterol was made up to a volume of 50 cc. with 95% ethanol and treated with a slight excess of bromine in ethanol solution. To the resulting solution at 25° was added a solution of 2 g. of digitonin in 40 cc. of ethanol and 8 cc. of water at 25°. The mixture was kept at 3° for twelve hours. The digitonide was collected, washed, dried and decomposed with pyridine. The product was crystallized from acetone to give white plates, m. p. 132°. This gave no depression with a sample of β -equistanol.

Summary

Mares pregnancy urine has been found to differ from other urines in the proportion of the three pregnanediols present, *allo*-pregnanediol-3(β),20-(α) being present in considerable amounts while only small amounts of pregnanediol-3(α),20(α) and *allo*-pregnanediol-3(α),20(α) are present.

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The Sorption of Methylene Blue by Curd Fibers of Sodium Palmitate

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Soap curd fibers account for the whiteness and much of the opacity of many commercial soaps. They are formed in characteristic manner when a solution of pure sodium palmitate is cooled and sets to a solid white mass, or curd, consisting of a fine mesh of these fibers of hydrated neutral soap enmeshing any excess of liquid or solution. They possess some remarkable properties. X-Ray examination clearly shows them to be crystalline, with rows of double molecules placed at right angles to the long axis of the fiber. However, although they may grow to be as much as several centimeters long, microscopic and ultramicroscopic examination shows them to be made up of bundles of very much finer fibers.

It is the purpose of the present study to obtain information as to the actual size of these curd fibers by measuring the superficial area available for the sorption of methylene blue from solution. It is also of interest to find whether such a dye is incorporated within the fibers or, as seems to be the case, lies upon their external surface. Furthermore, this method of study yields information as to changes, such as aging, that take place in curd fibers. Some progress also has been made toward

characterizing the differences between curd fibers separating from concentrated and from dilute solution.

Materials.—The sodium palmitate was the special preparation by Kahlbaum, found by analysis to be exactly neutral, that was used by McBain, Vold and Jameson¹ in their recent phase rule study. It was dried at 105° and all solutions were made up by weight. The methylene blue chloride was from the Eastman Kodak Company. Its concentration in the aqueous liquid was determined colorimetrically.

Sodium palmitate although highly soluble at temperatures above 60 to 70° is almost completely insoluble at room temperature, the mother liquor containing only the slightest trace of palmitate in addition to a few thousandths or a few hundredths normal sodium hydroxide as will be described later. Its hydration although large and variable does not appreciably affect the calculation of the amount of sorption. The mother liquor enmeshed in the curd is readily released by mechanical means when the concentration is 0.025 *N* or less. In higher concentrations it is generally possible to cause the apparently hard curd to soften and synerese by merely rotating the bottle to and fro for a short time, a striking (as yet unnamed)² phenomenon which is the opposite of "rheopexy."

(1) J. W. McBain, R. D. Vold and W. T. Jameson, *THIS JOURNAL*, **61**, 30 (1939).

(2) *Cf.* ultrasonic effects on sodium stearate curds; Freundlich and Gillings, *Trans. Faraday Soc.*, **34**, 657 (1938).