Anal. Calcd. for $C_{18}H_{24}O_2$: C, 79.3; H, 8.9. Found: C, 79.4; H, 8.9.

Summary

5,7,9-Oestratrienediol-3(α),17(α) and 5,7,9-oestratrienediol-3(α),17(β) were obtained in the non-phenolic fraction from the reduction of α- and β-dihydroequilenin with sodium in amyl alcohol.

STATE COLLEGE, PENNA. RECEIVED AUGUST 8, 1938

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

Sterols. XLVI. The Steroid Content of Cows' Pregnancy Urine*

BY RUSSELL E. MARKER

Although numerous investigations of the steroid content of human and mare urines have been described,[1] relatively little work has been done with other urines. In order to supply this deficiency we have undertaken to investigate the steroids present in the urines of other *mammalia*. A preliminary study[2] has revealed the presence in stallions' urine of β-equistanol, an *allo*-triol, $C_{21}H_{36}O_3$, an *allo*-tetrol, $C_{21}H_{36}O_4$, and a uranetriol-3,11,20. A similar preliminary study of cows' pregnancy urine is presented in this paper.

The butanol extract of 200 gallons (760 liters) of cows' pregnancy urine was extracted with alkali to remove phenols, and then hydrolyzed by steam distillation in the presence of an excess of strong alkali. From a concentrated ethereal solution of the resulting non-volatile tar, a crude pregnanediol mixture was obtained. Fractional crystallization of the acetate mixture from this crude crop yielded the diacetate of pregnanediol-3(α),20(α), m. p. 179°, and from this the diol was obtained. The soluble acetate mother liquor after hydrolysis yielded *allo*-pregnanediol-3(α),20(α). The mother liquor from the crude pregnanediol mixture was freed of traces of ketones by the use of Girard's reagent, and treated with digitonin. The insoluble digitonide was decomposed, and after treatment with bromine this 3(β)-OH sterol mixture was again treated with digitonin. The insoluble digitonide gave, after decomposition, *allo*-pregnanediol-3(β),20(α), and the mother liquor from this digitonide gave cholesterol. The mother liquor from the original digitonin treatment yielded the soluble digitonide of β-equistanol, from which the latter was obtained on decomposition, and an ethereal solution free of 3(β)-OH sterols. After the latter was separated into hydroxylated and non-hydroxylated fractions by means of the acid succinates, the non-hydroxylated fraction was distilled in a high vacuum, and yielded a hydrocarbon, $C_{28}H_{58}$, m. p. 63°. The carbinol fraction was epimerized with sodium in boiling xylene, and then treated with digitonin. The insoluble digitonide yielded *allo*-pregnanediol-3(β),20(α), which had been formed from some *allo*-pregnanediol-3(α),20(α) not removed in the original separation of the crude pregnanediol mixture. The soluble digitonide yielded β-equistanol, which must have been produced by the epimerization of α-equistanol originally present. The mother liquor from the digitonides, containing no 3(β)-OH sterols, was distilled in a high vacuum, and sirupy fractions collected at 115–150° and 150–200°. Since these fractions could not be crystallized readily, they were oxidized with chromic acid. The higher boiling fraction gave no definite products, while the lower boiling fraction gave an aliphatic acid, $C_{18}H_{36}O_2$, m. p. 55°, suggesting the presence of an aliphatic primary alcohol in the original urine extract.

The isolation from cows' pregnancy urine of the three pregnanediols, in about half the amounts present in human pregnancy urine, lends further support to the theory of the interrelationships of the sex hormones advanced in a paper of this series.[1] The occurrence of both the β- and α-equistanols in cows' pregnancy as well as in stallions, urine[2] and in mares' pregnancy urine[3] but not in human pregnancy urine seems to indicate, as suggested earlier,[2] that their presence may be traced to the herbivorous diet of these animals. The hydrocarbon $C_{28}H_{58}$, m. p. 63°, has been found to occur in human pregnancy urine,[4] in stallions' urine,[4] and in mares' pregnancy urine[4] as well as

(*) Paper XLV, THIS JOURNAL, **60**, 2440 (1938).

(1) These investigations are reviewed by Marker, THIS JOURNAL, **60**, 1725 (1938).

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

(3) Marker, Rohrmann and Wittle, *ibid.*, **60**, 1561 (1938).

(4) Unpublished results from this Laboratory.

in cows' pregnancy urine. Apparently other hydrocarbons are also present in some of these sources, for Hart and Northrup[5] found heptacosane, $C_{27}H_{56}$, m. p. 59°, and a hydrocarbon, $C_{25}H_{52}$, m. p. 52–54°, in large amounts in human pregnancy urine. Likewise, we find that the hydrocarbons in human and mares' pregnancy urine are rather complex mixtures. The acid, $C_{18}H_{36}O_2$, m. p. 55°, has been obtained by the oxidation of carbinol fractions from human[4] and mares' pregnancy urine[4] as well as from cows' urine. Besides the compounds discussed above, evidence has also been obtained for the presence in cows' pregnancy urine of polyhydroxysteroids, but in quantities too minute for investigation. A more exhaustive study on a much larger quantity of urine is in progress, and the results of this investigation will be reported later.

We wish to thank Dr. Oliver Kamm and Parke, Davis and Company for their generous support of this work.

Experimental Part

The butanol extract of 200 gallons (760 liters) of cows' pregnancy urine after removal of the phenolic fraction was hydrolyzed by steam distilling for three hours with an excess of aqueous 20% sodium hydroxide solution. The tarry product was filtered and washed with water. The residue was dissolved in a minimal amount of hot methanol and one liter of ether was added. The methanol was removed by several washings with water, and the ether was evaporated to 50 cc. The solution was cooled in ice–salt and the crystals collected and washed with a small amount of cold acetone. The resulting product, wt. 6.2 g., was treated in methanol with Norite and then crystallized from methanol and acetone to give a product melting unsharply at 215–228°.

Pregnanediol-3(α),20(α) and *allo*-Pregnanediol-3(α),20(α).—The crystalline product above was refluxed for thirty minutes with 35 cc. of acetic anhydride. The solution was cooled to 0°, and filtered. The product thus obtained was crystallized from methanol and melted at 179°. A mixture with pregnanediol diacetate gave no depression in melting point. Upon hydrolysis with alcoholic potassium hydroxide it gave pregnanediol-3(α),20(α), m. p. and mixed m. p. 242°.

Anal. Calcd. for $C_{21}H_{36}O_2$: C, 78.8; H. 11.3. Found: C, 78.8; H, 11.4.

The mother liquors from the crystallization of pregnanediol-3(α),20(α) diacetate were evaporated to dryness, and the residue hydrolyzed with alcoholic potassium hydroxide. The product was then crystallized from methanol, ethyl alcohol and acetone. It melted at 240° and gave no depression in melting point when mixed with *allo*-pregnanediol-3(α),20(α), but depressed the melting point of pregnanediol-3(α),20(α) to 215–220°.

Anal. Calcd. for $C_{21}H_{36}O_2$: C, 78.8; H, 11.3. Found: C, 78.6; H, 11.3.

The filtrate from the original pregnanediol(α),(α) mixture was treated in ethanol with Norite and filtered. The ketones were removed by the use of Girard's reagent. The non-ketonic fraction in alcohol was mixed with a solution of 20 g. of digitonin in 500 cc. of 95% alcohol, and the solution diluted to one liter. After standing overnight, the digitonide was filtered. When dry, it weighed 10 g. This digitonide was dissolved in 75 cc. of pyridine by heating on a steam-bath for fifteen minutes, and the solution poured into 500 cc. of ether and filtered. The pyridine was removed from the filtrate by shaking with dilute hydrochloric acid, the ether evaporated, and the residue, 2.2 g., sublimed in a high vacuum, taking the fraction distilling at 110–200°. This sublimate was dissolved in ethanol and enough bromine added at 0° to give a faint yellow color. Then an excess of 1% digitonin solution was added. The solution was filtered the next day. From the filtrate was obtained, after debromination in the usual manner, 320 mg. of cholesterol, m. p. 146°, which gave no depression in melting point when mixed with an authentic sample.

The digitonide was dried and then decomposed by heating for thirty minutes on a steam-bath with 30 cc. of dry pyridine. Ether was added and the digitonin filtered. The pyridine was removed from the ether solution, and the solvent was evaporated. The residue, after crystallization from dilute acetone, melted at 215°. Mixed with pregnanediol-3(α),20(α) and *allo*-pregnanediol-3(α),20(α) it gave a depression in melting point. When mixed with *allo*-pregnanediol-3(β),20(α) it gave no depression in melting point.

Anal. Calcd. for $C_{21}H_{36}O_2$: C, 78.8; H, 11.3. Found: C, 78.7; H, 11.3.

β-Equistanol.—The mother liquor from the original digitonin precipitation of the β-sterols was concentrated to 50 cc. and one liter of ether added. The digitonin–digitonide mixture was filtered and the dried product treated in the usual way with pyridine. The product thus obtained was sublimed in a high vacuum at 120–150°. After crystallization from a mixture of acetone and methanol, the sublimate gave a product melting at 132–134°. When mixed with equistanol obtained from mares' pregnancy urine or from stallions' urine it gave no depression in melting point; yield 1.2 g. of crude product.

The α-Sterol Fraction.—The filtrate from the digitonide from which the equistanol was obtained was evaporated to dryness and then dried by distilling benzene from it. The residual sirup was heated for thirty minutes with 10 g. of succinic anhydride and 15 cc. of pyridine. Ether was added and the pyridine was removed by shaking with hydrochloric acid. The acid-succinates were removed by shaking with sodium carbonate solution. The ether layer was reserved for investigation of the non-hydroxylated material. The alkaline solution was acidified and the succinate then hydrolyzed with alcoholic potassium hydroxide. The carbinol mixture was extracted with ether and the ether removed to give sirupy carbinols. Five and three-tenths grams of this sirup was refluxed with 10 g. of sodium and 150 cc. of xylene for eight hours, the excess product extracted with ether and washed well with water. The solvent was removed and the residue dissolved in 95%

(5) Hart and Northrup, THIS JOURNAL, 57, 2726 (1935).

alcohol and 5 g. of digitonin dissolved in 200 cc. of hot ethanol was added. After standing overnight, the digitonide was filtered, dried, and decomposed by warming with pyridine in the usual manner. The product thus obtained was distilled in a high vacuum, collecting the portion distilling below 200°. This fraction was crystallized from methanol to give *allo*-pregnanediol-3(β),20(α); m. p. and mixed m. p., 214°.

The digitonin mother liquors were evaporated to about 30 cc. and 500 cc. of ether added. The digitonide was filtered, dried and then decomposed with pyridine. The crude product was distilled and crystallized from methanol. It proved to be β-equistanol, m. p. 133°. This was formed by the epimerization of α-equistanol, as the β-form had been removed previously.

The filtrate from the equistanol digitonide was evaporated to dryness and sublimed *in vacuo*. Two fractions were taken, one at 115–150° and the other at 150–200°. The lower boiling fraction after separation of some crystalline pregnanediol-3(α),20(α) was oxidized in acetic acid with an equal weight of chromic acid for thirty minutes. The oxidation mixture yielded mostly acidic material, and only a very small neutral fraction. The acidic fraction was extracted with ether and removed from the ethereal solution with sodium carbonate solution. Acidification of the alkaline solution yielded a crystalline acid which was recrystallized from ethanol to a constant m. p., 55°. Calcd. for $C_{18}H_{36}O_2$: neut. equiv., 284; found. 277.

Anal. Calcd. for $C_{18}H_{36}O_2$: C, 75.9; H, 12.7. Found: C, 76.2; H, 12.9.

Although polyhydroxy sterols probably belonging to the cortical series were present, the quantities were too minute for separation from the small amount of urine processed.

Hydrocarbon Fraction.—The ether soluble material from the preparation of the succinates of the sterols was distilled *in vacuo*. The portion distilling at 80–110° was dissolved in acetone and methanol was added. It was allowed to stand for two days at room temperature in an unstoppered flask. The crystalline material was recrystallized from acetone to give a product of m. p. 63°. Molecular weight calcd. for $C_{28}H_{58}$, 394; found (Rast), 393. It gave no depression in melting point with a hydrocarbon isolated from human pregnancy urine.

Anal. Calcd. for $C_{28}H_{58}$: C, 85.3; H, 14.8. Found: C, 85.2; H, 14.6.

Summary

An investigation of the sterols present in cows' pregnancy urine gave pregnanediol-3(α),20(α), *allo*-pregnanediol-3(α),20(α), and *allo*-pregnanediol-3(β),20(α), in approximately one-half the quantities present in human pregnancy urine. β-Equistanol was also isolated, and the presence of α-equistanol was demonstrated. The carbinol fraction upon oxidation gave an acid of the aliphatic series with a molecular formula of $C_{18}H_{36}O_2$. There was also isolated a hydrocarbon of the aliphatic series melting at 63° and having the molecular formula $C_{28}H_{58}$.

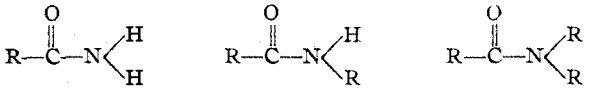
STATE COLLEGE, PENNA. RECEIVED AUGUST 8, 1938

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE UNIVERSITY OF ILLINOIS]

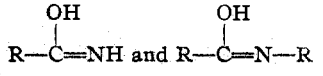
Infrared Absorption Studies. VI. Association in the Acid Amides and Oximes[1]

BY A. M. BUSWELL, W. H. RODEBUSH AND M. F. ROY

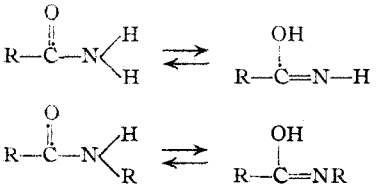
Structure of Acid Amides.—The classical structures assigned to amides and N-substituted amides are



However, certain reactions of the amides, *e. g.*, alkylation, indicate that compounds of the first two types above may have the alternative tautomeric structures



in that two isomeric reaction products are obtained in each case. Reactions of this type led to the belief that the amides probably existed in tautomeric equilibria of the two forms.



Attempts have been made to settle the question as to which of the two forms prevails by a study of the physical properties. Various authors[2] have found that the ultraviolet absorption spectrum indicates either tautomerism or the enolic form. Most of this work was in alcoholic solution but

(1) These studies are carried out with the aid of a grant from the Rockefeller Foundation. The main problem is the mechanism of hydration of biological substances ("bound water"). The present paper (VI) presents data on the absorption spectra of relatively simple organic nitrogen compounds whose functional groups resemble those found in proteins. Previous publications from this Laboratory dealing with association are II, *J. Chem. Phys.*, **5**, 501 (1937), and V, THIS JOURNAL, **60**, 2239 (1938).

(2) Ramart-Lucas and Gaunfeld, *Bull. soc. chim.*, [5] **4**, 478 (1937).