solute ethanol acidified with 2 cc. of concentrated hydrochloric acid with 300 mg. of Adams catalyst at 45 pounds pressure (3 atm.) at room temperature for six hours gave only the lower melting lactone (100 mg.), m. p. 186–188°.

Anal. Calcd. for $C_{22}H_{34}O_3$: C, 76.25; H, 9.9. Found: C, 76.1; H, 9.9.

The sodium carbonate washings in both of these hydrogenations yielded only traces of acidic material which was not investigated further.

(b) **By Sodium and Ethanol.**—To a boiling solution of 200 mg. of the keto acid in 75 cc. of absolute ethanol was added 5 g. of sodium in small pieces over a period of ninety minutes. The solution was cooled, diluted with water and acidified with hydrochloric acid. The separated solid was extracted with ether and the ethereal extract washed with 3% sodium hydroxide solution. Evaporation of the ether gave a crystalline residue which crystallized from ether–pentane as white needles, m. p. 197–199°. This gave no depression with an authentic sample of the hydroxy lactone, m. p. 198–200°.

The filtrate upon further crystallization from ether–pentane yielded the lower melting form of the lactone, m. p. 185–187°. This gave no depression with either the higher melting form or the lower melting form obtained above by catalytic hydrogenation.

The sodium hydroxide washings yielded only a trace of acids upon acidification with hydrochloric acid. This was not investigated further.

(c) **Attempted Clemmensen Reduction.**—A boiling mixture of 500 mg. of the acid, 75 cc. of 95% ethanol and 20 g. of amalgamated 20-mesh zinc was treated with 16 cc. of concentrated hydrochloric acid, added over a period of nine hours. The resulting solution was diluted with water and extracted with ether. The ethereal extract after washing with water was shaken with 3% sodium hydroxide solution. The remaining ethereal extract was washed with water and the ether evaporated. The crystalline residue was crystallized from ether–pentane to give white needles, m. p. 163–164°. This gave a 20° depression with the lactone, m. p. 200°.

Anal. Calcd. for $C_{24}H_{38}O_4$: C, 73.8; H, 9.8. Found: C, 73.6; H, 9.8.

A sample of the material when refluxed for fifteen minutes with an excess of alcoholic potassium hydroxide solution yielded, upon crystallization from ether, compact white crystals, m. p. 285° dec. This gave no depression with the original keto acid, indicating that the substance is the ethyl ester of the keto acid.

The sodium hydroxide washings containing the acidic fraction from the Clemmensen reduction was acidified with hydrochloric acid and extracted with ether. The ether was evaporated and the residue crystallized from methanol to give compact white crystals, m. p. 284° dec. This gave no depression with the original keto acid. No other acidic products appeared to be present.

Semicarbazone of Keto Acid.—A solution of 100 mg. of the keto acid, 100 mg. of semicarbazide hydrochloride and 150 mg. of sodium acetate in 10 cc. of 95% ethanol and 2 cc. of water was refluxed on the steam-bath for one hour. The solution was then diluted with water and the white solid collected and crystallized from ether to give a product of m. p. 204–207° dec.

Anal. Calcd. for $C_{23}H_{37}O_4N_3$: C, 65.8; H, 8.9. Found: C, 65.40; H, 8.9.

Summary

A monobasic keto acid of the composition $C_{22}H_{34}O_4$ has been obtained by the chromic anhydride oxidation of sarsasapogenin acetate. Catalytic reduction of the acid yields the C_{22} hydroxy lactone previously obtained by the chromic anhydride oxidation of sarsasapogenin acetate. Reduction of the acid with sodium and alcohol gives similar results.

STATE COLLEGE, PENNA. RECEIVED MARCH 6, 1939

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

Sterols. LXI. The Steroidal Content of Steers' Urine

BY RUSSELL E. MARKER

The occurrence of large amounts of the pregnanediols in bulls' urine[1] has prompted us to investigate the steroid content of animals with various sex organs removed in order to show the effect of their removal upon the type of sterols present. For this purpose our first investigation was carried out on the urine of steers that had their testes removed in infancy.

The steers' urine was hydrolyzed and separated into phenolic and neutral fractions and these examined in the usual manner. The phenolic fraction, after treatment with Girard's reagent, yielded a ketonic oil from which a small amount of estrone, characterized by its benzoate, was obtained. The neutral fraction was separated into hydroxylated and non-hydroxylated portions, and the former further separated into ketonic and non-ketonic parts. From the non-hydroxylated fraction the characteristic urinary hydrocarbon, $C_{28}H_{58}$, m. p. 62–63°, was obtained. The hydroxyketone fraction yielded androsterone and dehydroisoandrosterone in amounts comparable to those present in bulls' urine.[2] An examination of the carbinol fraction revealed the absence of the

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

(2) Marker, *ibid.*, **61**, 944 (1939).

pregnanediols. The carbinol fraction was treated with digitonin. Cholesterol, but no equistanol, was obtained from the digitonide. The non-precipitable carbinol mixture was distilled and the various fractions subjected to the usual crystallization technique. Although the pregnanediols are rather insoluble in acetone, no crystalline material could be obtained; nor did the combined distilled carbinol mixture yield any appreciable amount of digitonide after epimerization with sodium in xylene. The mixture on oxidation yielded only a small ketonic fraction, the chief product being acids. These results show that no appreciable quantities of the pregnanediols are present in steers' urine. The non-distillable carbinol fraction gave, on oxidation, some androstanedione, showing the probable presence of steroids related to those isolated from suprarenal glands.

The occurrence in steers' urine of large amounts of cholesterol (60 mg. per gallon compared to 4 mg. per gallon in human urines) and the corresponding absence of equistanol is rather surprising for in the urines of stallions,[3] pregnant mares,[4] cows,[5] and bulls there are large amounts of equistanol, but little cholesterol. The hypothesis, suggested in earlier papers, that equistanol may be derived from the herbivorous diet of these animals needs investigation as our present work indicates that this sterol may be a product of metabolism in the animal. That it is secreted in the urine rather than in the feces of the animal also suggests the latter course. Its origin and function remains obscure, as does that of the urinary hydrocarbon, $C_{28}H_{58}$.

The presence of androsterone, dehydroisoandrosterone, and estrone in steers' urine in amounts comparable to those present in bulls' urine strongly suggests that these substances are derived from cortical steroids by a mechanism such as we have discussed in an earlier paper,[6] either in the animal or during the acid or alkaline hydrolysis of the urines. It seems that androsterone does not function as a true male hormone, since approximately equal amounts occur in human male urine,[7] female urine,[8] and pregnancy urines.[9] Its male hormone properties may be purely fortuitous. This is in harmony with the lack of parallelism in the action of androsterone, testosterone, and other C_{19} male hormones on primary and secondary sex characteristics.

The absence of the pregnanediols from steers' urine is in marked contrast to the large amounts present in bulls' urine and suggests that these steroids are formed in the testes, or by the influence of the testes on some other gland; for it is well known that the absence of a gland seriously affects the functioning of other glands. While it is possible that the testes may contain an enzyme capable of reducing the cortical steroids almost quantitatively to the pregnanediols, it is also possible that the bull may utilize a male hormone of 21 carbon atoms, which like progesterone is reduced to the pregnanediols in the course of its functioning as a hormone. This warrants further investigation.

The finding of estrone in steers' urine is in accord with its anomalous distribution in other male urines such as stallion urine,[10] and human male urine,[11] as well as its presence in cortical extracts.[12]

In analyzing the results of the investigations of the steroids of urines the possibility of the occurrence of abnormal products due to some disfunction of one of the animals must always be borne in mind. This possibility is greatly lessened when the urine is collected from a large number of animals as in our investigations on human and mares' urines.

We wish to thank Dr. Oliver Kamm and Parke, Davis and Company for their generous support and assistance in various phases of this work, and Dr. Elmer J. Lawson for assisting in preparing this manuscript.

Experimental Part

The urine used in these experiments was collected from mature steers that had their testes removed before sexual adolescence.

One hundred gallons (380 liters) of urine was refluxed with 10% hydrochloric acid for thirty minutes. It was well extracted with butanol, and then the latter was evaporated to give a tarry residue of 8.5 kg. To this was added 4.5 kg. of sodium hydroxide in 10 liters of water and the mixture was steam distilled for six hours. It was then well extracted with ether and the alkaline layer reserved for an investigation of the phenols present.

The ethereal extract was evaporated to dryness, giving a

(3) Marker, Lawson, Rohrmann and Wittle, THIS JOURNAL, **60**, 1555 (1938).
(4) Marker and Rohrmann, *ibid.*, **60**, 1565 (1938).
(5) Marker, THIS JOURNAL, **60**, 2442 (1938).
(6) Marker, *ibid.*, **60**, 1725 (1938).
(7) Callow, Callow and Emmens, *Biochem. J.*, **32**, 1312 (1938).
(8) Callow and Callow, *ibid.*, **32**, 1759 (1938).
(9) Marker and Lawson, THIS JOURNAL, **60**, 2928 (1938).
(10) Deulofeu and Ferrari, *Z. physiol. Chem.*, **226**, 192 (1934); Haussler, *Helv. Chim. Acta*, **17**, 531 (1934).
(11) Dingemanse, Lacquer and Muhlbock, *Nature*, **141**, 927 (1938).
(12) Englehart, *Klin. Wochr.*, **9**, 2114 (1930); Callow and Parkes, *J. Physiol.*, **87**, 288 (1936).

residue weighing 63 g. This sirup was treated with 50 cc. of dry pyridine and 40 g. of succinic anhydride for forty minutes on a steam-bath. Ice was added and the succinates extracted with ether. The pyridine was removed by shaking with dilute hydrochloric acid and the succinates removed by shaking with sodium carbonate solution. The ethereal solution was reserved for an investigation of the non-hydroxylated compounds.

The aqueous layer was acidified with dilute hydrochloric acid and extracted with ether. After removal of the ether the residue was hydrolyzed by refluxing for forty-five minutes with alcoholic potassium hydroxide. The carbinol fraction was dissolved in 200 cc. of alcohol and refluxed for twenty minutes with 5 g. of Girard's reagent. Water was added and the solution extracted well with ether. The ethereal solution was reserved for an investigation of the non-ketonic carbinols.

Isolation of **Androsterone and Dehydroisoandrosterone.**—The aqueous layer obtained after Girard's treatment was acidified with dilute hydrochloric acid, heated one hour on a steam-bath, cooled and extracted with ether. Upon evaporation of the solvent a residue of 2.1 g. of hydroxy ketones was obtained. This was sublimed in high vacuum and a fraction (1.6 g.) collected at 100–180°. The distillate was dissolved in 25 cc. of ethyl alcohol and 1 g. of digitonin in 50 cc. of boiling ethyl alcohol was added. After standing overnight, the digitonide was collected and dried; weight 490 mg.

The digitonide was dissolved in 10 cc. of dry pyridine and heated on a steam-bath for one hour. Ether was added and the mixture filtered. The ethereal solution was freed of pyridine and the ether evaporated to leave 110 mg. of residue which crystallized on standing. This was dissolved in 2 cc. of pyridine, and 5 drops of benzoyl chloride was added. The next day the solution was heated on a steam-bath for ten minutes, diluted with water, and the product extracted with ether. After washing the ethereal extract with dilute hydrochloric acid and sodium carbonate solution the solvent was removed. The residue was dissolved in 1 cc. of methyl alcohol and let stand overnight in a refrigerator. The crystals were collected and recrystallized from methanol to give 14 mg., m. p. 220–241°. After recrystallization from methanol the benzoate melted at 247–249° and gave no depression in melting point when mixed with an authentic sample of the benzoate of dehydroisoandrosterone.

The benzoate was mixed with some less pure product and hydrolyzed with alcoholic potassium hydroxide. The product was sublimed in a high vacuum and crystallized from ligroin to give dehydroisoandrosterone, m. p. 145–147°, which did not depress with an authentic sample.

Anal. Calcd. for $C_{19}H_{28}O_2$: C, 79.1; H, 9.8. Found: C, 79.4; H, 9.9.

The filtrate from the digitonide of the ketonic fraction was evaporated to 5 cc., diluted with ether, filtered and washed with water. After removal of the solvent from the filtrate 1.2 g. of residue remained. This was dissolved in 15 cc. of alcohol and refluxed for four hours with 500 mg. of hydroxylamine hydrochloride and 500 mg. of sodium acetate. The addition of water caused the separation of a gum which adhered to the walls of the flask; so the liquor was decanted and the gum dissolved in 10 cc. of alcohol. To this was added 3 cc. of water. After standing in a refrigerator overnight crystalline material separated. This was recrystallized 3 times from 80% acetone to a melting point of 214–215°; yield 18 mg. The oxime gave no depression in melting point when mixed with an authentic sample of androsterone oxime.

Anal. Calcd. for $C_{19}H_{31}O_2N$: C, 74.7; H, 10.2. Found: C, 74.4; H, 10.2.

The oxime was hydrolyzed with alcoholic sulfuric acid to give androsterone, m. p. 177–180°, which when mixed with an authentic sample melted at 178–182°.

Anal. Calcd. for $C_{19}H_{30}O_2$: C, 78.6; H, 10.4. Found: C, 78.3; H, 10.4.

Estrone.—The alkaline solution from the hydrolysis of the butanol extract of steer urine was treated with carbon dioxide until no more tar precipitated. The tar was extracted with ether, dissolved in sodium hydroxide solution, and shaken well with ether. Carbon dioxide was passed through the aqueous layer until no more precipitate was formed. It was extracted again with ether and after removal of the solvent the residue was refluxed for thirty minutes with 20 g. of Girard's reagent in 1 liter of alcohol. Ice was added and the solution extracted well with ether. The aqueous layer was acidified and heated on a steam-bath for thirty minutes. It was extracted with ether and after removal of the solvent was treated again with Girard's reagent. This procedure yielded 340 mg. of an oil which upon standing for two days deposited crystals. These were shaken quickly with 3 cc. of cold ether and rapidly filtered. After sublimation at 160–170° the product was crystallized from 50% ethanol to give 6 mg. of a product melting at 257–259° which gave no depression in melting point when mixed with estrone.

Anal. Calcd. for $C_{18}H_{22}O_2$: C, 79.9; H, 8.3. Found: C, 80.2; H, 8.4.

The mother liquors of the distillate upon treatment with benzoyl chloride and sodium hydroxide gave a product melting at 200–205° which gave no depression in melting point when mixed with estrone benzoate.

Hydrocarbon Fraction.—The non-hydroxylated fraction of the neutral portion was sublimed *in vacuo*. The product distilling from 90–100° was dissolved in alcohol. Upon cooling an oil was deposited. This was dissolved in acetone from which it crystallized, melting at 62–63°. This substance gave no depression in melting point with the crystalline hydrocarbon obtained from stallions', bulls', cow pregnancy, or human pregnancy urines.

Non-Ketonic Carbinol Fraction.—The non-ketonic carbinol fraction (44 g.) was dissolved in methanol and cooled. The crystalline portion was recrystallized from methanol to yield 5.2 g. of product, m. p. 148–149°, which gave no depression in melting point when mixed with cholesterol. Upon acetylation it gave cholesteryl acetate.

The filtrate from the cholesterol was dissolved in a small amount of alcohol and a solution of 10 g. of digitonin was added. The digitonide which formed gave 1.1 g. additional cholesterol, but no other products could be isolated from this fraction.

The filtrate from the digitonide was evaporated to 50 cc. and 1 liter of ether was added. The product was filtered, washed with water, and distilled in a high vacuum.

Four almost equal fractions were obtained: 80–100°, 100–120°, 120–200°, and a non-distillable residue. The sublimate distilling at 120–200° was in the range in which one would expect to find the pregnanediols if present. As these are quite insoluble in acetone, each of the four fractions was dissolved in an equal volume of this solvent, but no product crystallized. The fraction also resisted crystallization from other solvents. Therefore, the total sublimate was combined and refluxed for ten hours with an equal weight of sodium in 100 cc. of dry xylene. If *allo*-pregnanediol had been present it would have been converted to the configuration at the 3-hydroxyl which would precipitate with digitonin. But when the isomerized product was treated with alcoholic digitonin, less than 100 mg. of insoluble digitonide was formed, showing the absence of *allo*-pregnanediol. This fraction was then oxidized with chromic acid in acetic acid at room temperature for thirty minutes. Only 4 g. of ketonic materials was obtained from 24 g. of carbinol, the remainder being acids. Upon sublimation of the ketones no crystalline product could be obtained.

The non-distillable carbinol fraction (9 g.) was oxidized by dissolving in 150 cc. of acetic acid and adding 4 g. of chromic anhydride in 25 cc. of 80% acetic acid. After standing for thirty minutes, water was added. The product, isolated in the usual manner, was treated with Girard's reagent and the ketonic fraction (1.3 g.) sublimed in a high vacuum. The fraction distilling at 100–120° crystallized from dilute methanol, giving androstanedione, m. p. 125–128°, which gave no depression in melting point when mixed with an authentic sample.

Anal. Calcd. for $C_{19}H_{28}O_2$: C, 79.1; H, 9.8. Found: C, 79.0; H, 9.9.

Summary

The steroidal content of steers' urine was investigated. Unlike bulls' urine, steers' urine contains no pregnanediols. A relatively large amount of cholesterol but no equistanol was found. The ketonic fraction gave androsterone, dehydroisoandrosterone and estrone in approximately the same proportions as found in bulls' urine. The non-distillable carbinols gave a small amount of androstanedione on oxidation. The characteristic urinary hydrocarbon, $C_{28}H_{58}$, was found.

STATE COLLEGE, PENNA. RECEIVED FEBRUARY 27, 1939

NOTES

Esterification of Highly Hindered Acids

BY REYNOLD C. FUSON, JOSEPH CORSE AND E. C. HORNING

Esterification of highly hindered acids is generally very difficult to accomplish by direct methods. Satisfactory yields of methyl esters have been obtained, however, by thermal decomposition of the corresponding tetramethylammonium salts according to the procedure of Prelog and Piantanida.[1] The method is based on observations of Lawson and Collie[2] and involves a reaction which is closely related to that which occurs when betaines are decomposed thermally.[3]

The method has now been applied to 2,4,6-trimethyl- and 2,4,6-triethylbenzoic acids to determine the influence of excessive hindrance. Tetramethylammonium hydroxide made by the method of Walker and Johnson[4] was employed to make the tetramethylammonium salts. These were decomposed by heating to 200–250°. The yields of pure methyl esters varied from 63 to 90% of the theoretical amounts.

The methyl 2,4,6-triethylbenzoate is a new compound. It boils at 114–115° (5 mm.); $n^{20}D$ 1.5012; d^{20}_4 0.982.

Anal. Calcd. for $C_{14}H_{20}O_2$: C, 76.86; H, 9.47. Found: C, 76.59; H, 9.48.

CHEMICAL LABORATORY
UNIVERSITY OF ILLINOIS
URBANA, ILLINOIS RECEIVED FEBRUARY 6, 1939

(1) Prelog and Piantanida, *Z. physiol. Chem.*, **244**, 56 (1936).
(2) Lawson and Collie, *J. Chem. Soc.*, **53**, 631 (1888).
(3) Willstätter, *Ber.*, **35**, 587 (1902); Willstätter and Kahn, *ibid.*, **37**, 401, 1853 (1904); Prelog, *Coll. trav. chim. Tchech.*, **2**, 712 (1930); Kuhn and Giral, *Ber.*, **68**, 387 (1935).
(4) Walker and Johnson, *J. Chem. Soc.*, **87**, 955 (1905).

The Isolation of a Crystalline Substance from Starches Oxidized by Periodate

BY D. H. GRANGAARD, J. H. MICHELL AND C. B. PURVES

By degrading various periodate oxy-starches with acid methyl alcohol,[1] we have isolated a white, crystalline, levorotatory compound with the formula $C_{13}H_{16}O_8(OCH_3)_4$ and m. p. 150–150.5° (corr.). Although stable to further oxidation with periodate or Fehling's solution, the substance

(1) Jackson and Hudson, THIS JOURNAL, **60**, 989 (1938).