steam-bath for two hours. The alcohol was distilled off and the residue dissolved or suspended in 75 cc. of water. The aqueous mixture was cooled and made alkaline with ammonium hydroxide and then slightly acid with acetic acid. The desired 4-aminobenzenesulfonanilide was obtained by filtration.

The derivatives of 4-aminobenzenesulfonanilide prepared as described above are insoluble in ether and benzene; slightly soluble in cold water but appreciably soluble in boiling water; soluble in alcohol, acetone, dioxane and acetic acid. They may be crystallized from diluted alcohol (50% for the nitro derivatives, 35–20% for the amino derivatives) or from water (hydroxy derivatives). Amino and hydroxy derivatives were crystallized in an atmosphere of carbon dioxide. All of these compounds are soluble in dilute sodium hydroxide solutions and in dilute mineral acids.

4,4'-Diaminobenzenesulfonanilide Dihydrochloride.—To a solution of 1.2 g. of the pure base in a mixture of 10 cc. of alcohol and 1 cc. of concd. hydrochloric acid an additional 5 cc. of concd. hydrochloric acid was added. The precipitated dihydrochloride was filtered and dried in a desiccator over calcium chloride and sodium hydroxide. The white crystals begin to decompose at 200°. A solution (1:1000) in water has *p*H 2.5. *Anal.*[10] Calcd. for $C_{12}H_{15}O_2N_3Cl_2S$: Cl: 21.10; Found: Cl, 21.27.

Diazotization of 4-Acetaminobenzenesulfon-2'-aminoanilide.—A solution of 3.05 g. (0.1 mole) of 4-acetaminobenzenesulfon-2'-aminoanilide in a mixture of 250 cc. of water and 5 cc. of concentrated sulfuric acid was diazotized with 0.7 g. (0.1 mole) of sodium nitrite, dissolved in 14 cc. of water, at room temperature. The *o*-diazoimide of 4-acetaminobenzenesulfonanilide is precipitated from the reaction mixture immediately.

The product was filtered from the reaction mixture and washed with hot water to remove inorganic compounds and recrystallized from 50% alcohol. The yield was 2.5 g. This compound was stable at the boiling temperature of the mixture but, when recrystallized and dried, it decomposed at 138–140°. The reaction is analogous to that reported by Morgan and Micklethwait[11] in the diazotization of benzenesulfon-2'-aminoanilide. *Anal.* Calcd. for $C_{14}H_{12}O_3N_4S$: N, 17.72; S, 10.14. Found: N (Dumas), 17.82; S, 10.07.

Diazotization of 4-Acetaminobenzenesulfon-3'-aminoanilide.—A solution of 1.5 g. of the 4-acetaminobenzenesulfon-3'-aminoanilide in a mixture of 5 cc. of concd. sulfuric acid and 25 cc. of water was diazotized with 0.35 g. of sodium nitrite in 7 cc. of water without cooling. The solution was heated on a steam-bath for one hour, cooled and filtered. After recrystallization from 20% alcohol, the compound melted at 217–218°. The mixed melting point with the product obtained from coupling 3-aminophenol with 4-acetaminobenzenesulfonchloride was unchanged.

Diazotization of 4-Acetaminobenzenesulfon-4'-aminoanilide.—Three grams of 4-acetaminobenzenesulfon-4'-aminoanilide dissolved in a mixture of 10 cc. of concd. sulfuric acid and 50 cc. of water was diazotized, without cooling, with 0.7 g. of sodium nitrite in 14 cc. of water. A precipitate formed which dissolved when the temperature of the solution was raised to 75°. The solution was heated at 90–95° for three hours and then briefly boiled. After cooling, the solution was made alkaline with ammonium hydroxide and then acid with acetic acid. Recrystallized from water the product melted at 195–196°. The mixed melting point with 4-aminobenzenesulfon-4'-hydroxyanilide was unchanged, indicating that hydrolysis of the acetyl group had taken place.

Summary

The preparation of a number of new nitro, amino and hydroxy derivatives of 4-aminobenzenesulfonanilide and 4-acetaminobenzenesulfonanilide has been described. Work is in progress on other derivatives of these compounds.

CHICAGO, ILLINOIS RECEIVED APRIL 23, 1938

(10) Method of Thompson and Oakdale, THIS JOURNAL, **52**, 1195 (1930).

(11) Morgan and Micklethwait, *J. Chem. Soc.*, **87**, 73 (1905).

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

Sterols. XXXV. Carbinols from Stallions' Urine

BY RUSSELL E. MARKER, ELMER J. LAWSON, EWALD ROHRMANN AND EUGENE L. WITTLE

Although the presence of oestrone in stallions' urine has been reported by several investigators,[1] apparently no work has been done on the neutral fractions from this source.

As part of an extensive investigation of the steroid substances in various urines, we have made a preliminary study of stallions' urine. After hydrolysis of the urine, and removal of the phenolic and acidic substances with alkali, the neutral extract was treated with Girard's reagent. Only a very small amount of ketonic material, amounting to 100 mg. per 200 gallons (760 liters) was obtained. The non-ketonic fraction was treated with phthalic anhydride and pyridine to separate the carbinols. The carbinol fraction was treated with digitonin to separate the β-sterols. From the digitonide a sterol of m. p. 134° was obtained. This sterol does not decolorize bromine in acetic

(1) For the literature on this subject, see Fieser, "Chemistry of Natural Products Related to Phenanthrene," 2nd ed., Reinhold Publishing Corp., New York, N. Y., 1936, pp. 198–199.

acid, yields an acetate melting at 124°, and remains largely unepimerized after treatment with sodium in boiling xylene. In view of its source, this sterol has been named β-equistanol. Oxidation of β-equistanol yields the corresponding ketone, equistanone, m. p. 115°. This ketone does not correspond in its solubility behavior or melting point to cholestanone, stigmastanone, ergostanone, or γ-sitostanone. The following table summarizes the properties of these compounds:

	M. p., °C.		M. p., °C.		M. p., °C.
β-Equistanol	134	β-Equistyl acetate	124	Equistanone	115
β-Cholestanol	142	β-Cholestyl acetate	115	Cholestanone	130
Stigmastanol	138.5	Stigmastyl acetate	135	Stigmastanone	160
γ-Sitostanol	144	γ-Sitostyl acetate	143	γ-Sitostanone	...
Ergostanol	151	Ergostyl acetate	144	Ergostanone	164

Evidently this sterol is different from any of the known sterols. Analytical data on β-equistanol, β-equistyl acetate and equistanone are in best agreement with the formula $C_{30}H_{54}O$ or $C_{31}H_{56}O$. We are inclined at present to regard β-equistanol as a phytosterol which is utilized incompletely, or not at all, in the metabolism of the stallion. In view of the herbivorous diet of the animal and of the widespread occurrence of β-sitosterol in plants, it is surprising that no β-sitosterol is present in stallions' urine.

Butenandt[2] has reported the presence of cholesterol in human male urine (4 mg./gal.), while Marker[3] has observed its presence in human pregnancy urine. We have found no evidence of cholesterol either in stallion urine or in mares' pregnancy urine. The occurrence of equistanol in both stallion urine and mares' pregnancy urine[4] indicates that it probably originates from the food and is not a product resulting from the condition of pregnancy.

We cannot overlook the fact that equistanol may be identical with dihydro-α-tritisterol prepared by Karrer, Salomon and Fritzsche.[5] Their compound has 30 carbon atoms with a melting point about the same as ours, but because of a lack of derivatives we are unable to make further comparisons.

The mother liquors from the digitonide were concentrated and the resulting carbinol mixture was epimerized with sodium and boiling xylene to convert any sterols of the *allo* series, originally present in the alpha form at C_3, to the corresponding beta compounds. Digitonin treatment of the epimerized mixture yielded an insoluble digitonide, a somewhat soluble digitonide and a carbinol mixture freed of compounds of the 3(β)-type. The soluble digitonide yielded β-equistanol, showing that the original carbinol mixture from stallions' urine contained, besides β-equistanol, about an equal amount of its epimer, α-equistanol. The latter compound could not, however, be isolated as such from any carbinol fractions.

The insoluble digitonide yielded a mixture of carbinols from which a triol, m. p. 295°, and a tetrol, m. p. 295°, were obtained by crystallization of the less volatile fractions obtained after vacuum sublimation of the mixture. The triol, m. p. 295°, yielded a triacetate, m. p. 140–145°. Analytical data agree best with the formula $C_{21}H_{36}O_3$ for this triol. It is evidently a compound of the *allo* series, and contains a 3(β)-hydroxyl group. As it existed in the urine the 3-OH group was in the epimeric form. We may suppose, by analogy to other carbinols present in urines, that one of the two remaining hydroxyls is at C_{20}, but the position of the third hydroxyl group is completely unknown. It should be noted that this carbinol is not present as such in stallions' urine, but rather is a transformation product of a triol of the *allo* series, having a 3(α)-hydroxyl group, which was originally present in the stallions' urine. These remarks on our knowledge of the triol, m. p. 295°, also apply to the tetrol, $C_{21}H_{36}O_4$, m. p. 295°. This tetrol may be a derivative of the products isolated from cortical extracts by Reichstein,[6] Wintersteiner and Pfiffner,[7] and Kendall.[8] Thus, it is possible that this compound may be a 3(β),11,20,21-*allo*-pregnanetetrol derived from corticosterone.

The epimerized carbinol mixture not precipitable with digitonin was oxidized, and the ketonic fraction distilled. A considerable amount of a mixture of ketones with a fruity odor was collected at 80°. After obtaining smaller fractions at 110–120°, and 120–160°, a fraction distilling at 160–200° was obtained. Crystallization of this

(2) Butenandt and Dannenbaum, *Z. physiol. Chem.*, **248**, 151 (1937).

(3) Unpublished results.

(4) Marker and Rohrmann, THIS JOURNAL, **60**, 1565 (1938).

(5) Karrer, Salomon and Fritzsche, *Helv. Chim. Acta*, **20**, 424, 1422 (1937).

(6) Reichstein, *ibid.*, **19**, 29 (1936).

(7) Wintersteiner and Pfiffner, *J. Biol. Chem.*, **111**, 599 (1935).

(8) Mason, Myers and Kendall, *ibid.*, **114**, 613 (1936).

fraction from methanol yielded the known uranetrione, m. p. 247°, as proved by direct comparison with an authentic sample. Since the carbinol fraction which yielded uranetrione contained sterols of the 3(α)-OH type belonging to the normal series in respect to C_5, it is likely that the uranetrione was formed from the same uranetriol which has been found in mares' pregnancy urine.[9] The probable existence of uranetriol in stallions' urine suggests that uranetriol may not be derived from compounds having the function of sex hormones. Rather, it is possible that uranetriol may be derived from the suprarenal cortex or from some hormone of unknown function. The latter view receives some support from the isolation of uranol-11-one-3[10] uranedione,[9] and uranediol-3(β)-11[11] from mares' pregnancy urine.

Experimental Part

Isolation of Carbinol Fraction from Stallions' Urine.—The concentrated extract from 200 gallons (760 liters) of stallions' urine weighed 40 lb. (18.2 kg.). It was steam distilled for twelve hours with 25 lb. (11.4 kg.) of sodium hydroxide in 30% aqueous solution, and then extracted with ether. The ether was evaporated, the residual tar steam distilled with 5 liters of concd. hydrochloric acid and 5 liters of water, and the non-volatile oil extracted with 10 liters of butanol. To the butanol extract was added 15 liters of heptane, causing the precipitation of much tar. The solution was decanted from the tar, treated with boneblack, and the solvents removed *in vacuo*. The sirupy residue was steam distilled with an excess of 30% aqueous sodium hydroxide for three hours, and extracted with ether. The ether was distilled and the residue treated with Girard's reagent to obtain about 100 mg. of ketonic material. The non-ketonic fraction was dried by distillation with benzene, and treated with 125 g. of phthalic anhydride and 150 cc. of pyridine to give 68 g. of carbinols. The carbinol mixture was dissolved in 1 liter of hot alcohol and a solution of digitonin added. The next day the mixture was filtered, and washed with alcohol and ether. There was obtained 8.3 g. of digitonide.

β-Equistanol.—The digitonide obtained as described above was decomposed by warming for fifteen minutes with 90 cc. of pyridine. The solution was poured into 500 cc. of ether and the precipitate collected and washed with ether. The ethereal filtrate was washed with dilute hydrochloric acid and water and evaporated to dryness. The residue was crystallized three times from methanol to give 250 mg. of β-equistanol as needles, m. p. 133°. This sterol is not very soluble in methanol, somewhat more soluble in acetone and readily soluble in ether. It does not decolorize bromine in acetic acid solution.

Anal. Calcd. for $C_{30}H_{54}O$: C, 83.7; H, 12.6. Found: C, 84.0; H, 12.3.

(9) Marker, Kamm, Oakwood, Wittle and Lawson, THIS JOURNAL, **60**, 1061 (1938).

(10) Marker, Lawson and Crooks, *ibid.*, **60**, 1559 (1938).

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

Twenty-five milligrams of β-equistanol was refluxed for twenty minutes with 2 cc. of acetic anhydride. On chilling the solution β-equistanyl acetate crystallized as shining plates, m. p. 124°.

Anal. Calcd. for $C_{32}H_{56}O_2$: C, 81.3; H, 12.0. Found: C, 81.4; H, 11.6. Mol. wt. calcd. for $C_{32}H_{56}O_2$, 472.5; calcd. for $C_{33}H_{58}O_2$, 486.5. Found: 485.

Nine milligrams of β-equistanol in 3 cc. of xylene was refluxed with 80 mg. of sodium for six hours. At the end of this time the sodium was decomposed with alcohol, the solution neutralized with dilute hydrochloric acid, and the organic matter extracted with ether. The ethereal solution was washed with water, and evaporated to dryness. The residue was dissolved in a little hot alcohol and to it was added a solution of 50 mg. of digitonin in 1.5 cc. of hot alcohol. The next day the precipitated digitonide was filtered and washed with alcohol and ether. The dried digitonide weighed 40 mg., corresponding to a recovery of 8 mg. of the sterol. This shows that β-equistanol is a sterol of the *allo* series.

β-Equistanone.—To a solution of 100 mg. of β-equistanol in 10 cc. of acetic acid was added a solution of 30 mg. of chromic acid in 5 cc. of 90% acetic acid. After standing for one hour, the solution was diluted with water, extracted with ether, and the ethereal extract washed with sodium carbonate solution and water. The ether was evaporated and the residue distilled in a high vacuum. The fraction distilling at a bath temperature of 120–150° was crystallized from diluted methanol to obtain β-equistanone, m. p. 115°. β-Equistanone is quite soluble in methanol, differing in this respect from cholestanone, β-sitostanone, and ergostanone.

Anal. Calcd. for $C_{30}H_{52}O$: C, 84.0; H, 12.2. Found: C, 84.2; H, 12.1.

β-Equistanol from α-Equistanol in Stallions' Urine.—The filtrate from the precipitation of β-equistanol digitonide was evaporated and extracted with ether to separate the α-sterols from some β-equistanol digitonide, which is somewhat soluble in alcohol. The ethereal solution was evaporated and the residue refluxed with 600 cc. of xylene and 25 g. of sodium for nine hours. The sodium was destroyed with alcohol, the solution neutralized with dilute hydrochloric acid, and the mixture extracted with ether. The ethereal extract was washed with water, evaporated, and the residue, in hot alcohol, treated with a solution of 25 g. of digitonin in 750 cc. of alcohol. After standing overnight in a refrigerator, the precipitated digitonide was filtered and washed thoroughly with alcohol and ether. The digitonide so obtained was set aside for further investigation. The filtrate was evaporated to dryness, leached with ether, and the soluble digitonide present decomposed by warming with 20 cc. of pyridine for thirty minutes. The resulting solution was poured into ether, the precipitated digitonin filtered, and the ethereal filtrate washed with dilute hydrochloric acid and water. After evaporating the ether, a crystalline residue remained. This residue was crystallized from acetone–methanol, and ethyl acetate, to give a product which proved to be β-equistanol, m. p. 134°.

Anal. Calcd. for $C_{30}H_{54}O$: C, 83.6; H, 12.6. Found: C, 83.8; H, 12.6.

The sterol did not decolorize bromine in acetic acid.

When warmed with concentrated sulfuric acid, it gave an orange-red color with a green fluorescence.

Ten mg. of the sterol was refluxed for thirty minutes with 5 cc. of acetic anhydride. On cooling, the acetate separated as shining plates. After recrystallization from methanol, the pure acetate was obtained as needles melting at 124°. This acetate did not depress the melting point of β-equistanyl acetate, but showed a marked depression with β-sitostanyl acetate.

Anal. Calcd. for $C_{32}H_{56}O_2$: C, 81.3; H, 12.0. Found: C, 81.4; H, 11.7. Mol. wt. calcd. for $C_{32}H_{56}O_2$, 472.5; for $C_{33}H_{58}O_2$, 486.5. Found: 485.

allo-Triol and allo-Tetrol from Stallions' Urine.—The insoluble digitonide from the epimerization of the α-sterols in stallions' urine was warmed with 50 cc. of pyridine for thirty minutes and the resulting solution poured into ether. After filtering and washing the precipitated digitonin, the filtrate was washed with dilute hydrochloric acid and water. The ether was evaporated giving 3.1 g. of residue. Crystallization of the residue from 15 cc. of methanol gave 120 mg. of a product melting at 295°. The analysis is that of an *allo*-pregnanetriol.

Anal. Calcd. for $C_{21}H_{36}O_3$: C, 74.9; H, 10.8. Found: C, 74.6; H, 10.9.

This compound gave a triacetate melting at 140–145°.

Anal. Calcd. for $C_{27}H_{42}O_6$: C, 70.1; H, 9.2; mol. wt., 462. Found: C, 70.3; H, 9.3; mol. wt., 457.

This compound originally existed in the urine as the *epi* form at C_3 but due to isomerization with sodium was converted and isolated as the β-form. It is saturated to bromine. The *allo*-configuration at C_5 is established by the isomerization with sodium to a product precipitated by digitonin.

The filtrate from the above triol was sublimed *in vacuo* and the portion that would not sublime below 200° was crystallized from methanol. This yielded 12 mg. of a carbinol melting at 290–295° which gave a depression in melting point when mixed with the first triol. The composition is that of a tetrol.

Anal. Calcd. for $C_{21}H_{36}O_4$: C, 71.6; H, 10.0; mol. wt., 352. Found: C, 71.1; H, 9.85, mol. wt., 362.

Uranetrione from Oxidation of Stallions' Urine Residues.—The ethereal leachings from the soluble digitonide described in the previous experiment contained chiefly α-sterols of the regular series at C_5. This ethereal solution was evaporated to dryness and the residue dissolved in 1 liter of acetic acid, and distilled to remove alcohol and other solvents. The residue was dissolved in 500 cc. of acetic acid and a solution of 20 g. of chromic acid in 90% acetic acid added with cooling over a period of fifteen minutes. The solution was diluted with water and extracted with ether. The ethereal solution was freed of acids by washing with sodium carbonate solution, and then with water. The ether was evaporated and the residue, dissolved in 500 cc. of alcohol, was heated for thirty minutes with 20 g. of Girard's reagent. Ice was added, and the solution diluted with water and extracted with ether. The aqueous layer was heated for thirty minutes on a steam-bath with an excess of hydrochloric acid. The cooled solution was thoroughly extracted with ether, and the ethereal solution evaporated. The residue was distilled in a high vacuum. A fraction, weighing 1.6 g. and distilling at 80°, had a fruity odor. However, it apparently was a mixture. A fraction weighing 500 mg. was collected at 110–120°, and another at 120–160°. A fourth fraction, distilling at 160–200°, was crystallized from methanol and washed with cold ether. The product melted at 247° and gave no depression with uranetrione.

Anal. Calcd. for $C_{21}H_{30}O_3$: C, 76.4; H, 9.1. Found: C, 76.4; H, 9.3.

We wish to thank Dr. Oliver Kamm and Parke, Davis and Company for their generous help and assistance in various phases of this work.

Summary

Stallions' urine yields a carbinol fraction from which a saturated β-sterol of the *allo* series may be obtained. This sterol, β-equistanol, yields β-equistanone on oxidation. The presence of the epimer, α-equistanol, and of two triols is also demonstrated. One of these triols yields uranetrione on oxidation, but the other triol is probably of the *allo* series. It also gave a compound of the composition of an *allo*-pregnanetetrol.

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