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

Sterols. XLIX.* Isolation of Pregnanediols from Bull's Urine

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In a recent paper[1] from this Laboratory it was suggested that the parent male, female and cortical hormones arise from a common precursor, and that in the course of utilization of these hormones they are excreted as reduction products. For example, while the three isomeric pregnanediols, pregnanediol-3(α),20(α), *allo*-pregnanediol-3(α),-20(α) and *allo*-pregnanediol-3(β),20(α), which are the reduction products of progesterone, have been isolated from the urines of pregnant women, mares and cows,[2] they have been found to be absent or present only in traces in the urines of stallions[3] and non-pregnant women.[4] However, we have now found that bull's urine contains large amounts of the pregnanediols. The table below shows the approximate quantities of the pregnanediols present in various urines. Evidently bull's urine is the richest source of the pregnanediols yet investigated.

	Approximate quantities isolated, mg. per gallon of urine		
	Pregnanediol-3(α),-20(α)	*allo*-Pregnanediol-3(α),-20(α)	*allo*-Pregnanediol-3(β),-20(α)
Human pregnancy	50	25	6
Human non-pregnancy	8	4	.
Human male	.	.	.
Mare pregnancy	50	25	6
Cow pregnancy	25	15	3
Stallion	none	none	none
Bull	100	50	12

Thus there are now at least three instances of the apparently anomalous occurrence of steroids in urines, namely, the presence of estrone in stallion's urine,[5,6] of androsterone[7] in human pregnancy urine and of the pregnanediols in bull's urine. At present it seems that these results can best be explained by assuming, in accordance with the theory set forth recently,[1] that these compounds originate from cortical derivatives. In this connection it should be noted that cortical extracts have been found to have both estrogenic and progestational activity,[8,9] and recently Beall and Reichstein[10] have isolated progesterone and *allo*-pregnanol-3(β)-one-20 from this source. Also, Reichstein[11] recently has announced the isolation from cortical extracts of 21-hydroxyprogesterone. While it might be argued, since Reichstein's extracts are obtained from the suprarenals of both bulls and cows, that the progesterone he isolated was derived only from cows, the isolation of the pregnanediols from bull's urine nullifies this argument. Whether these pregnanediols come from the testes or the suprarenals is not yet known, but it is evident, since the bull has no ovaries, that progesterone is formed in other glands. If the pregnanediols from bull's urine are formed from cortical derivatives, it appears that either progesterone or Δ^4-pregnenol-20(α)-one-3 is an intermediate step in the reductive processes involved. Should the latter prove to be the case, it might suggest that Δ^4-pregnenol-20(α)-one-3 functions as a male hormone in the bull. There seems to be no doubt but that the occurrence of the pregnanediols is normal for the bull since the urine investigated was collected from sixteen bulls, and inordinately large amounts would have had to have been excreted if these steroids arose as the result of some disfunction in one of the bulls.

Besides the isomeric pregnanediols, a considerable amount of equistanol has also been found in bull's urine. Equistanol is present in the urines of mares, stallions, cows and bulls, but not in humans. On the other hand, human urine contains comparable amounts of cholesterol but only traces are present in the urines of mares, cows, bulls and stallions.

The mode of isolation of these steroids was essentially the same as that employed in other similar investigations reported from this Laboratory. The urine collected from sixteen mature bulls was hydrolyzed with hydrochloric acid, extracted with butanol and then hydrolyzed by alkali. It was filtered and extracted to yield an ether insoluble solid, and a sirupy concentrate. The former, after acetylation, yielded pregnanediol-3(α),20-

(*) Paper XLVIII, THIS JOURNAL, **60**, 2928 (1938).

(1) Marker, THIS JOURNAL, **60**, 1725 (1938).

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

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

(4) Marker, Rohrmann, Lawson and Wittle, *ibid.*, **60**, 1901 (1938).

(5) Deulofeu and Ferrari, *Z. physiol. Chem.*, **226**, 192 (1934).

(6) Haussler, *Helv. Chim. Acta*, **17**, 531 (1934).

(7) Marker and Lawson, Sterols. XLVII, THIS JOURNAL, **60**, 2927 (1938).

(8) Engehart, *Klin. Wochr.*, **9**, 2114 (1930).

(9) Callow and Parkes, *J. Physiol.*, **87**, 28P (1936).

(10) Beall and Reichstein, *Nature*, **142**, 479 (1938).

(11) Reichstein, *Helv. Chim. Acta*, **21**, 1197 (1938).

(α) diacetate, and a mother liquor which gave *allo*-pregnanediol-3(α),20(α) on hydrolysis. The sirupy concentrate after Girard's reagent and acid-succinate separations yielded a carbinol fraction which was treated with digitonin solution. The digitonide mixture gave, after decomposition, *allo*-pregnanediol-3(β),20(α), and β-equistanol. The results of our investigation of the other fractions obtained will be submitted soon for publication.

The authors wish to thank Dr. Oliver Kamm and Parke, Davis & Co. for their generous assistance in various phases of this work.

Experimental Part

The bull's urine used in this investigation was collected from 16 mature bulls. After hydrolyzing the urine by boiling with 10% hydrochloric acid for thirty minutes, a precipitate which formed was collected and the filtrate thoroughly extracted with butanol. The solvent was removed by vacuum distillation to a sirup and the latter dissolved in a large volume of ether and filtered from some solid material. The ether was evaporated to give 4500 cc. of sirup.[12]

The concentrate was dissolved in 10 liters of ether and shaken with sodium carbonate and sodium hydroxide solution to remove phenolic and acidic materials. The ether was evaporated and the residue steam distilled with an excess of aqueous potassium hydroxide (20 pounds (9.1 kg.) of potassium hydroxide per 1000 gallons (3800 liters) of urine extract) until no more volatile organic material came over. The mixture was cooled to room temperature, filtered and the residue washed well with water and ice cold ether. The filtrate was extracted well with ether and the solvent removed. The ether insoluble precipitate amounted to 175 mg. per gallon.

Pregnanediol-3(α),20(α) and ***allo*-Pregnanediol-3(α),20(α).**—The precipitate obtained as described above was refluxed for one hour with 7 volumes of acetic anhydride, the solution cooled to 0°, and filtered. The crystalline acetate was recrystallized from methanol to give a pure product melting at 180°. The yield was 100 mg. per gallon. Mixed with pregnanediol-3(α),20(α)-diacetate, this acetate gave no depression in melting point.

Anal. Calcd. for $C_{25}H_{40}O_4$: C, 74.2; H, 10.0. Found: C, 73.9; H, 10.0.

Hydrolysis of this pregnanediol-3(α),20(α) diacetate with alcoholic potassium hydroxide gave pregnanediol-3(α),20(α) melting at 240°. It gave no depression in melting point when mixed with an authentic sample.

Anal. Calcd. for $C_{21}H_{36}O_2$: C, 78.8; H, 11.3. Found: C, 78.6; H, 11.1.

The acetic anhydride mother liquors were evaporated to dryness and the residue hydrolyzed with alcoholic potassium hydroxide solution. Dilution of the solution with water gave a solid which was collected, washed well with water, and recrystallized from alcohol to a melting point of 243°. This was *allo*-pregnanediol-3(α),20(α) since it depressed to 215° with pregnanediol-3(α),20(α) but gave no depression with *allo*-pregnanediol-3(α),20(α). The yield was approximately 50 mg. per gallon of urine.

Anal. Calcd. for $C_{21}H_{36}O_2$: C, 78.8; H, 11.3. Found: C, 78.5; H, 11.2.

The diacetate prepared in the usual manner was crystallized from methanol. It melted at 141° and did not depress with an authentic sample of *allo*-pregnanediol-3(α),-20(α) diacetate.

Anal. Calcd. for $C_{25}H_{40}O_4$: C, 74.2; H, 10.0. Found: C, 74.0; H, 9.9.

The β-Steroids in Bull Urine.—The ether washings of the crude pregnanediols and the ether extract of the aqueous filtrate were combined and evaporated to a sirup. The sirup was dried by distilling 1 liter of benzene from it. The tarry residue was dissolved in 1 liter of alcohol and refluxed thirty minutes with 30 g. of Girard's reagent. The solution was diluted with ether and ice, the layers separated, and the aqueous layer extracted with ether. The aqueous layer was acidified with hydrochloric acid and heated on a steam-bath. The ketonic oil was extracted with ether and set aside for an examination now in progress.

The ethereal extract from the Girard's separation was evaporated to a sirup which was dried as before by distilling benzene from it. The residue was heated for one hour with 300 cc. of pyridine and 240 g. of succinic anhydride and then poured on ice. Ether was added and the pyridine removed by shaking with hydrochloric acid. The acid succinate mixture was then removed from the ethereal solution by shaking with sodium carbonate solution. The ethereal solution which contained hydrocarbons was reserved for a separate investigation now in progress. The carbonate solution was acidified, extracted with ether and after removal of the ether, hydrolyzed with an excess of alcoholic potassium hydroxide. The carbinol mixture was extracted with ether, and the solvent removed. The residue was dissolved in hot alcohol and an excess of a hot 2% alcoholic digitonin solution (200 mg. of digitonin per gallon of urine) was added. After standing overnight the digitonide was filtered and washed with cold alcohol. The filtrate was evaporated to 200 cc. and 5 liters of ether added. The suspension was filtered to remove the digitonin and soluble digitonides. The digitonin–digitonide mixture was combined giving 245 mg. per gallon of urine. The filtrate which contained sterols of the *epi*-OH configuration was reserved for a separate investigation now in progress.

The digitonides were dried and heated with 5 volumes of pyridine on a steam-bath for one hour. This was poured into ether, filtered, and the digitonin washed well with ether. The ethereal filtrate was freed of pyridine by shaking with hydrochloric acid solution, the solvent removed, and the residue dissolved in a small amount of benzene. On standing overnight a crystalline crop deposited. This was collected and recrystallized from methanol and acetone to give *allo*-pregnanediol-3(β),-20(α), m. p. 215°. When mixed with *allo*-pregnanediol-3(β),20(α), it gave no depression in melting point. Mixed with uranediol it depressed the melting point to 185°.

(12) We wish to thank the Parke, Davis & Co. for performing the above collection, hydrolysis and extraction at their laboratories and supplying us with the ethereal concentrate.

Anal. Calcd. for $C_{21}H_{36}O_2$: C, 78.8; H, 11.3. Found: C, 78.6; H, 11.2.

Upon refluxing with acetic anhydride it gave a diacetate melting at 168° which gave no depression in melting point when mixed with the diacetate of *allo*-pregnanediol-3(β),20(α). This compound was present in bull urine to the extent of 12 mg. per gallon.

The benzene was removed from the filtrate of the *allo*-pregnanediol-3(β),20(α) and the residue sublimed in high vacuum collecting a fraction distilling at 120–150°. This was crystallized from ethyl alcohol and methyl alcohol to a melting point of 137°. The product was saturated to bromine and when mixed with β-equistanol isolated from stallion, cow pregnancy, and mare pregnancy urine it gave no depression in melting point.

Upon refluxing with acetic anhydride it gave an acetate melting at 126° which did not depress the melting point of β-equistanol acetate. Equistanol was present in bull's urine to the extent of 8 mg. per gallon. As in the case of stallions, mares and cows pregnancy urine, the amount of cholesterol present was very small when compared to that in human urine. An investigation of the other steroid fractions will be reported in THIS JOURNAL in the near future.

Summary

Pregnanediol-3(α),20(α), *allo*-pregnanediol-3(α),20(α), *allo*-pregnanediol-3(β),20(α) were isolated from bull's urine in quantities about twice or more than that of human pregnancy urine. Equistanol also was isolated but very little cholesterol was found.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, WEST VIRGINIA UNIVERSITY]

β-Alkoxy Ethyl Esters of Chlorocarbonic and Carbamic Acids

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In order to study the relative narcotic powers of a series of carbamic acid esters containing an ethereal oxygen in the alcohol group, we have prepared a series of twelve esters of chlorocarbonic acid, ten of which are new, and from them their corresponding carbamic acid esters, eleven of which are new.

β-Alkoxyethanols.—The β-alkoxyethanols were prepared by treating ethylene oxide with the desired alcohol in the presence of a small amount of sulfuric acid as a catalyst,[1,2] except in the cases of the tertiary butoxy and tertiary amoxy ethanols for which aluminum fluosilicate was used as a catalyst.[3,4]

β-Alkoxyethyl Chlorocarbonates.—The chlorocarbonates were prepared by the action of phosgene upon the various β-alkoxyethanols.[5] A calculated excess of liquid phosgene was placed in a 500-cc. round-bottomed flask clamped in a freezing mixture. A reflux condenser, through which a freezing mixture was passed, was then attached. The alcohol was added drop by drop, the mixture being stirred mechanically, until all was added, and the reaction mixture allowed to come to room temperature gradually. The flask was opened and allowed to remain open for a day, at room temperature, in order to evaporate the excess phosgene. The mixture was poured into ice water, the ester separated and dried over anhydrous calcium chloride. It was then distilled *in vacuo* at 1–20 mm. pressure. The tertiary butoxy and tertiary amoxy chlorocarbonates could not be prepared by this reaction. *bis*-Ethylene chlorocarbonate was obtained in both cases.

The analyses were made in a few cases by digesting the sample with 10% sodium hydroxide, then titrating against standard silver nitrate, using potassium chromate as an indicator. In all cases the chlorocarbonates were converted into their respective carbamates and analyzed for nitrogen by the Kjeldahl method. Refractive indices were determined by means of an Abbe refractometer and surface tension values obtained by means of a du Nouy tensiometer at 25°. The Harkins correction for the ring method was applied. The yields are based upon the amount of the glycol ether used. Table I shows the boiling points, under reduced pressure, the refractive indices, surface tensions (corrected), yields, and the densities of the chlorocarbonates.

β-Alkoxyethyl Carbamates.—The carbamates were prepared from the corresponding chlorocarbonates by treating them with aqueous ammonia. The carbamates, with the exception of the β-methoxy, β-ethoxy- and β-isopropoxyethyl carbamates were purified by distillation *in vacuo*. These three were purified by crystallization from propylene chloride. The yield is based on the chlorocarbonate used. In Table II the yield, melting point or boiling point, and the analyses for the carbamates are given.

Narcotic Properties

Emerson and Abreu[6] have found that the β-ethoxyethyl carbamate is less active and less toxic than urethan. They also found that the propoxy and isopropoxy derivatives are roughly equivalent to urethan in narcotic activity and toxicity and

(1) Ashburn, Collett and Lazzell, THIS JOURNAL, **57**, 1862 (1935).
(2) German Patent 580,075, July 5, 1933; *C. A.*, **27**, 4814 (1933).
(3) Ashburn, Collett and Lazzell, THIS JOURNAL, **58**, 1549 (1936).
(4) French Patent 39,773, Feb. 17, 1931; *C. A.*, **26**, 4826 (1932).
(5) Dumas and Péligot, *Ann. chim.*, [2] **58**, 52 (1835); *Ann.*, **15**, 1–60 (1835); *Ann.*, **10**, 284 (1834); Hentschel, *Ber.*, **18**, 1177 (1885).

(6) From a report by Emerson and Abreu working in the Pharmacological Laboratory of the University of California Medical School, San Francisco, California.