[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY AND PHYSICS OF THE PENNSYLVANIA STATE COLLEGE AND THE PARKE, DAVIS AND CO. RESEARCH LABORATORIES]

Sterols. XXIII. Pregnandiols in Pregnancy Urine of Mares

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Although pregnandiol[1,2] and *allo*-pregnandiol[3] have been isolated from human pregnancy urine, their presence in the pregnancy urines of other animals has never been established.[4] Haslewood, Marrian, and Smith[5] were not able to isolate pregnandiol from mares' pregnancy urine by methods which had been applied successfully to human pregnancy urine. They were, however, able to isolate a pregnantriol of unknown structure. The results of the present work suggest that the failure to obtain pregnandiol by these precipitation methods may be attributed to the presence of large amounts of other carbinols in the mixture from pregnant mares' urine, whereas these are present only in small amounts in human pregnancy urine.

In the course of a systematic investigation of pregnancy urines in this Laboratory, we have obtained pregnandiol from mares' pregnancy urine. The carbinol fraction of mares' urine extract was distilled and the distillate was converted into an acetate mixture. This was precipitated several times from aqueous methyl alcohol, and the final residue upon hydrolysis and working up the product by the method used for human urine, gave pregnandiol. This is not a practical method of obtaining it, but we were able to obtain its oxidation product, pregnandione, as well as its isomer, *allo*-pregnandione, by first oxidizing the total carbinol fraction and separating the ketones as their semicarbazones. This is a practical method for obtaining these compounds. In this way we obtained yields of pregnandione and *allo*-pregnandione from mares' pregnancy urine of about the same magnitude as that of the pregnandiols obtained from human pregnancy urine. The disemicarbazones of the mixture of pregnandiones are very insoluble in alcohol, providing a good method for their separation from the other ketones formed. The disemicarbazone of pregnandione is more soluble than its isomer *allo*-pregnandione; so these compounds may be separated by repeated leaching with hot alcohol After hydrolysis of the mixture of semicarbazones, the *allo*-pregnandione is separated from the pregnandione by crystallization from acetone. It was found in the course of this work that it was not necessary to separate the carbinols from the hydrocarbons and other material before oxidation. Practically the same yields of the disemicarbazones were obtained by this simplified procedure.

Since the pregnandiols are reduction products of progesterone, their existence in the urine of pregnant mares suggests that progesterone is also the corpus luteum hormone in the mare.

Investigation of various other products of pregnant mares' urine is being continued. We wish to thank Dr. George H. Fleming of this Laboratory for the microanalyses reported in this paper.

Experimental

Preparation of Pregnandione and *allo*-Pregnandione from Urine of Pregnant Mares.—The non-phenolic extract from 1000 gallons of pregnant mares' urine was hydrolyzed by steam distilling with an excess of 20% aqueous sodium hydroxide solution. The product was extracted with ether and a small amount of ketones separated by means of alcoholic Girard's reagent. (This step was unnecessary but we desired the ketone fraction for a separate study.) The carbinols were then separated from the non-ketonic residue by means of their half-phthalic esters as described by us for human pregnancy urine in previous papers. The total carbinol fraction after removal of the solvent weighed 590 g. This was dissolved in 3.5 liters of glacial acetic acid and 300 g. of chromium trioxide in 1.5 liters of 80% acetic acid was added at 20° with stirring, over a period of two hours. Stirring was continued two hours longer. The excess chromium trioxide was destroyed by alcohol and the acetic acid solution was concentrated *in vacuo* to a volume of about 1.5 liters. The residue was stirred with ether and water until it was in solution. The ether layer was separated and washed free of acids with 10% sodium hydroxide solution. A considerable amount of insoluble salts of tarry acids separated. The ether layer was separated and distilled. The residue was dissolved in one liter of boiling alcohol and 50 g. of Girard's reagent was added. The product was heated twenty minutes and then poured on ice and extracted with ether. The aqueous layer was acidified with hydrochloric acid and heated on a steam-bath for thirty minutes. The ketones were extracted with ether. The ether was evaporated and the

(1) Marrian, *Biochem. J.*, **23**, 1090 (1929).
(2) Butenandt, *Ber.*, **63**, 659 (1930).
(3) Hartmann and Locher, *Helv. Chim. Acta*, **18**, 160 (1935).
(4) Fieser, "The Chemistry of Natural Products Related to Phenanthrene," Reinhold Pub. Corp., New York, N. Y., 1935, p. 204.
(5) Haslewood, Marrian and Smith, *Biochem. J.*, **28**, 1316 (1935).

residue was dissolved in one liter of alcohol. This solution was refluxed for one hour with 50 g. of semicarbazide hydrochloride and 60 g. of sodium acetate. After cooling, the precipitate was filtered and freed of sodium chloride by washing with boiling water. The residue was heated with one liter of boiling alcohol and filtered hot. This leaching process was repeated three times, giving 62 g. of a mixture of the disemicarbazones of the pregnandiones.

To a suspension of 62 g. of disemicarbazone in 2.5 liters of alcohol was added a solution of 250 cc. of sulfuric acid in 500 cc. of water. The mixture was heated two hours on a steam-bath and then poured into water and filtered. The solid was dissolved in ether and filtered to remove a small amount of insoluble material. The ether was distilled and the residue was dissolved in alcohol and treated with Norit. The alcohol was removed and the residue was crystallized from acetone. After three crystallizations a melting point of 199° was obtained. This product gave no depression in melting point when mixed with *allo*-pregnandione prepared from *allo*-pregnandiol of human pregnancy urine.

Anal. Calcd. for $C_{21}H_{32}O_2$: C, 79.7: H, 10.2. Found: C, 80.1; H, 10.2.

It gave a disemicarbazone which did not melt below 325°.

Anal. Calcd. for $C_{23}H_{38}O_2N_6$: C, 64.2; H, 8.9. Found: C, 64.8; H, 9.1.

The first filtrate from the *allo*-pregnandione was evaporated to dryness and the residue distilled using a mercury pump. The fraction distilling below 150° was crystallized from dilute acetone to give a product of melting point 119°. This substance gave no depression in melting point when mixed with pregnandione prepared from pregnandiol of human pregnancy urine.

Anal. Calcd. for $C_{21}H_{32}O_2$: C, 79.7; H, 10.2. Found: C, 80.1; H, 10.1.

It gave a disemicarbazone melting at 257° with decomposition.

Anal. Calcd. for $C_{23}H_{38}O_2N_6$: C, 64.2; H, 8.9. Found: C, 64.6; H, 9.2.

As the disemicarbazone of pregnandione is more soluble in alcohol than its isomer, *allo*-pregnandione, when the alcoholic filtrate from the mixture was concentrated and crystallized it yielded a product which consisted chiefly of the disemicarbazone of pregnandione.

A disemicarbazone mixture identical with that described above was also obtained from pregnant mares' urine by oxidizing the crude hydrolyzed neutral fraction containing both carbinols and hydrocarbons. The isolation of the ketonic oxidation products by the use of Girard's reagent was found unnecessary.

Pregnandiol from Mares' Pregnancy Urine.—A solution of 50 g. of the carbinol fraction of mares' urine extract was distilled using a mercury vapor pump and collecting the portion subliming between 150–210°. This portion was refluxed with 100 cc. of acetic anhydride for thirty minutes. The excess acetic anhydride was removed and the residue was dissolved in 200 cc. of methyl alcohol and then 25 cc. of water was added. The oil which came out was treated three times in this manner. It was then hydrolyzed with alcoholic potassium hydroxide solution and extracted with ether. The ether was evaporated to a small volume and set in a refrigerator. The crystals which formed were filtered, bone blacked and crystallized from acetone; m. p. 239°. When mixed with pregnandiol of human pregnancy urine it gave no depression in melting point.

Anal. Calcd. for $C_{21}H_{36}O_2$: C, 78.7; H, 11.3. Found: C, 78.9; H, 11.4.

It gave a diacetate melting at 179° which gave no depression in melting point with the diacetate of human pregnancy urine pregnandiol.

Anal. Calcd. for $C_{25}H_{40}O_4$: C, 74.2; H, 10.0. Found: C, 74.2; H, 9.8.

Summary

A practical method for the preparation of *allo*-pregnandione and pregnandione from pregnant mares' urine extracts is described.

Pregnandiol has been isolated from pregnant mares' urine.

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