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

Sterols. XLII. The Isolation of Oestranediols from Human Non-Pregnancy Urine

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In an earlier paper in this series[1] it has been proposed that the numerous steroids present in urines and glandular extracts arise by reductive processes in the course of utilization of the sex and cortical hormones. On the basis of new experimental information to be presented in this paper, it now appears that the oestrogenic hormones suffer these same reductive processes when they are utilized as hormones in the non-pregnant woman, for from the urines of such subjects we have isolated two isomeric oestranediols.[2]

The mode of isolation of these diols was essentially the same as that described in earlier papers. Two hundred gallons (755 liters) of non-pregnancy human urine was extracted with butanol, and hydrolyzed with acid and alkali to yield a neutral fraction which was separated by means of succinic anhydride and Girard's reagent into carbinols, ketones, and inert substances. The carbinol fraction, corresponding to 40 mg. per gallon of urine, was treated with digitonin to separate the 3-βOH and 3-αOH sterols. The 3-βOH sterol fraction proved to be mainly cholesterol. From the carbinol fraction not precipitable by digitonin two alcohols, $C_{18}H_{30}O_2$, m. p. 242 and 204°, were isolated by fractional crystallization after removal of a small amount of pregnanediol. The structures of these diols as stereoisomeric oestranediols are proved by the following facts. Both diols form diacetates melting at 160° which show a decided depression in admixture. Both diols, when heated with platinum black, are dehydrogenated to give equilenin, although in poor yields because of the polymerization of the latter to equilenin red. Furthermore, the catalytic hydrogenation of oestrone in alcoholic solution containing a little hydrochloric acid[3] gives an oestranediol, m. p. 205°, identical with oestranediol-B, m. p. 204°. Since this oestranediol yields a different diketone, 170°,[3a] from the diketone, m. p. 124°, formed by oestranediol-A the two carbinols must differ at least in regard to their configuration at C_5 or C_{10}. Ample opportunity for isomerism is of course possible since the reduction of oestrone generates new asymmetric centers at C_3, C_5, and C_{10}. We have, indeed, indications of the presence of other possibly isomeric carbinols in the mother liquors from which the diols were isolated. Neither oestranediol-A, nor the less abundant and more soluble oestranediol-B, precipitates with digitonin, but this cannot be taken as indicating that these diols contain 3-αOH groups, for neither neoergosterol nor *epi*-neoergosterol form insoluble digitonides,[4] apparently, the presence of an angular methyl group at C_{10} is essential for the formation of insoluble digitonides.

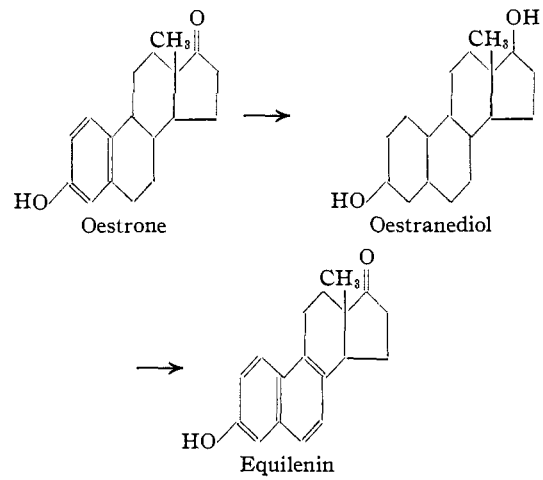

Fig. 1.

The isolation of these stereoisomeric oestranediols from the urine of non-pregnant women throws considerable light on the manner of utilization of oestrone when it functions as a hormone in the organism. Large quantities of oestrone are excreted by the pregnant woman, probably because the hormone is not being utilized while the oestrus cycle is suspended. During this same period, however, no appreciable amount of progesterone is excreted for the latter is being used to control the development of pregnancy; instead large quantities of carbinols and ketones, which are reduction products of progesterone, are elimi-

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

(2) The name oestrane for the saturated hydrocarbon related to oestrone has been suggested by Adam, Danielli, Dodd, King, Marrian, Parkes and Rosenheim, *Nature*, **132**, 205 (1933).

(3) Dirscherl, *Z. physiol. Chem.*, **239**, 53 (1936).

(3a) The value of 148° reported in Sterols XXXIV, THIS JOURNAL, **60**, 1512 (1938), should be 170°.

(4) Unpublished observations in this Laboratory.

nated. In the mature non-pregnant female, on the other hand, oestrone is being utilized to control the oestrus cycle, so almost none of this hormone is excreted in the urine;[5] instead, its reduction products, the oestranediols formed in the course of utilization of the hormone are excreted. In complete harmony with these facts is the small amount of pregnanediols in non-pregnancy urine. Furthermore, the oestranediols are not present in pregnancy urine, for their relative ease of isolation makes it highly improbable that they could have been overlooked in our extensive investigation of its steroid fraction.[6]

The presence of the oestranediols in non-pregnancy urine but not in pregnancy urine shows that they are formed, not by merely enzymatic processes, but in the actual utilization of oestrone. This view is supported by the results of some experiments on the enzymatic reduction of oestrone by the methods of Mamoli, Vercellone and Ercoli.[7] Enzyme extracts of hog ovaries, beef suprarenal glands and bull testes were mixed with oestrone and allowed to stand for thirty days. In none of these cases was any reduction product formed, the oestrone being recovered unchanged.

The investigation of steroids from non-pregnancy urine is being continued on a much larger scale. We wish to thank Dr. Oliver Kamm and Parke, Davis & Company for their generous help and assistance in various phases of this work. The micro-analyses were done by Dr. George H. Fleming of this Laboratory.

Experimental Part

Isolation of Total Sterol Fraction.—Two hundred gallons (756 liters) of human non-pregnancy urine was extracted thoroughly with butanol before hydrolysis. The aqueous portion was then hydrolyzed by heating with hydrochloric acid, and after hydrolysis this was again thoroughly extracted with butanol. The butanol was evaporated *in vacuo* from both extracts and the products worked up separately.

The product from the extraction of the non-hydrolyzed urine was heated on a steam-bath with 5 kg. of sodium hydroxide dissolved in 20 liters of water for one hour, then steam distilled until no more volatile material came over. This required six hours. The residue remaining in the flask was thoroughly extracted with ether, and the solvent evaporated. The residue was rehydrolyzed with alcoholic potassium hydroxide, and the neutral product so obtained weighed 17 g. after removal of the solvent.

The residue was dried by distillation with benzene. To this was added 25 g. of succinic anhydride and 50 cc. of pyridine and the mixture was heated for one hour on a steam-bath. Ice and ether were added and the pyridine was removed by shaking with dilute hydrochloric acid. The succinic esters were then removed from the ether solution by shaking with sodium carbonate solution. The aqueous layer was acidified and the succinic esters of the carbinols were extracted with ether. The ether was evaporated and the residue was hydrolyzed by heating with an excess of alcoholic potassium hydroxide solution. The hydrolyzed carbinol fraction weighed 8 g. after removal of the solvent. This residue was dissolved in 100 cc. of ethyl alcohol and 5 g. of Girard's reagent was added. The solution was heated for twenty minutes on a steam-bath, water was added and the product was well extracted with ether. The aqueous layer upon heating with hydrochloric acid gave only a small amount of ketones which were reserved for future investigation. The solvent was removed from the ethereal extract from the Girard's reagent treatment and the residue was dissolved in a small amount of ethyl alcohol. To this was added 250 cc. of a 2% digitonin solution in 90% ethyl alcohol, and the mixture allowed to stand overnight. The digitonide was filtered and dried. It gave 3.7 g. of digitonide which upon hydrolysis gave mainly cholesterol. The alcohol was distilled from the filtrate from the digitonide and the residue was thoroughly extracted with ether and filtered. The ether was evaporated giving a residue weighing 5 g.

The sterol fraction from the acid hydrolyzed fraction of the non-pregnancy urine was worked up to this point in a similar manner. This fraction was similar to the other in all respects, except that the cholesterol content appeared to be less. Four grams of carbinols remained after removal of the cholesterol, ketones and non-carbinol material.

Oestranediol-A.—The above sterol fractions were sublimed separately using a mercury vapor pump and the fraction subliming at 110–135° was collected over a period of twenty hours. The distillates upon cooling in acetone gave in both cases a crude crystalline product melting at 200–210° which did not depress in melting point when mixed, so the total distillates, weighing 3.9 g., were combined. This was dissolved in 15 cc. of acetone, cooled overnight in a refrigerator and then filtered. This fraction was principally pregnanediol. The filtrate was cooled in a mixture of ice and hydrochloric acid for two hours. The crystalline product was filtered and the mother liquors reserved for future investigation.

The crystalline material (1.2 g.) was recrystallized from acetone to give a substance of a constant melting point 242°; yield 295 mg. This, when pure, is quite insoluble in acetone and can also be crystallized from a mixture of benzene and ligroin. It gave a depression in melting point of 30° when mixed with pregnanediol and 25° when mixed with *allo*-pregnanediol.

Anal. Calcd. for $C_{18}H_{30}O_2$: C, 77.6; H, 10.9. Found: C, 77.4; H, 10.8.

(5) Loews and Lange, *Klin. Wochr.*, **5**, 1038 (1926), found very little oestrogenic activity in concentrates from non-pregnancy urine.

(6) At present we have worked over the steroid fraction of over 100,000 gallons (378,000 liters) of human pregnancy urine, whereas the oestranediols are easily isolated from small amounts of non-pregnancy urine.

(7) Mamoli and Vercellone, *Z. physiol. Chem.*, **245**, 93 (1937); *Ber.*, **70B**, 470 (1937); Vercellone and Mamoli, *Z. physiol. Chem.*, **248**, 277 (1937); Ercoli and Mamoli, *Ber.*, **71**, 156 (1938); Ercoli, *Ber.*, **71**, 650 (1938).

Diacetate of Oestranediol-A.—A solution of 25 mg. of oestranediol-A in 5 cc. of acetic anhydride was refluxed for thirty minutes. The acetic anhydride was evaporated and the residue was recrystallized from methanol to a constant melting point of 160°.

Anal. Calcd. for $C_{22}H_{34}O_4$: C, 72.9; H, 9.5. Found: C, 73.2; H, 9.7.

Oestranedione-A.—To a solution of 50 mg. of oestranediol-A in 20 cc. of acetic acid was added 40 mg. of chromic anhydride in 5 cc. of 90% acetic acid. After standing at room temperature for one hour, water was added and the product was extracted with ether. The ethereal solution was freed of acids by shaking with water and 2% sodium hydroxide solution, and evaporated. The residue was sublimed in high vacuum and the fraction distilling at 120° was recrystallized from 50% acetone to give 36 mg. of oestranedione-A, m. p. 124°.

Anal. Calcd. for $C_{18}H_{26}O_2$: C, 78.8; H, 9.5. Found: C, 79.2; H, 9.8.

Equilenin from Oestranediol-A.—A mixture of 150 mg. of oestranediol-A and 100 mg. of platinum black was melted in a stream of nitrogen. The temperature was then lowered to 215–225° and kept there for one hour, during which time bubbles of hydrogen were given off. The product was dissolved in ether and filtered. The ether was evaporated and the residue dissolved in 10 cc. of benzene. To this was added 10 cc. of a benzene solution saturated with picric acid and the solution concentrated to 10 cc. After standing in a refrigerator overnight the red crystalline picrate was collected and dissolved in ether. The ether solution was shaken with ammonia diluted with an equal volume of water until the color of picric acid was no longer present. The ether solution was then shaken with 5% sodium hydroxide solution. The alkaline solution was acidified and the product was filtered and washed with water. It was recrystallized from 50% alcohol to give 14 mg. of a product melting at 247–249°, giving a bright red color characteristic of equilenin when melted in the presence of oxygen. It gave no depression in melting point when mixed with an authentic sample of equilenin, m. p. 256°. It gave a depression in melting point to 233° when mixed with oestrone.

Oestranediol-B.—The mother liquors from the crystallization of oestranediol-A were concentrated to about 20 cc. and cooled in a refrigerator overnight. The crystalline material was filtered and the filtrate was evaporated to about 10 cc. and cooled in ice–hydrochloric acid mixture. The crystalline product was filtered and crystallized from benzene–ligroin mixture. It was then crystallized from acetone to a constant melting point of 204°, yield 43 mg.

A sample of octahydro-oestrone was prepared by the method of Dirscherl[3] by the catalytic reduction of oestrone in alcoholic–hydrochloric acid solution using platinum oxide as a catalyst. This melted at 204–205°, and gave no depression in melting point when mixed with oestranediol-B isolated from non-pregnancy urine. Oestranediols-A and -B gave a depression in melting point when mixed with each other.

Anal. Calcd. for $C_{18}H_{30}O_2$: C, 77.6; H, 10.9. Found: C, 77.6; H, 10.8.

Diacetate of Oestranediol-B.—A solution of 25 mg. of oestranediol-B in 5 cc. of acetic anhydride was refluxed for thirty minutes. The acetic anhydride was evaporated and the residue was crystallized from methanol to a constant melting point of 160°.

Anal. Calcd. for $C_{22}H_{34}O_4$: C, 72.9; H, 9.5. Found: C, 72.8; H, 9.5.

Oestranedione-B.—A solution of 30 mg. of oestranediol-B (prepared by the reduction of oestrone) was oxidized by 30 mg. of chromic anhydride as described for oestranediol-A. The product was sublimed *in vacuo* and the sublimate was recrystallized from 50% acetone to a melting point of 170°.

Anal. Calcd. for $C_{18}H_{26}O_2$: C, 78.8; H, 9.5. Found: C, 79.0; H, 9.6.

Equilenin from Oestranediol-B.—One hundred and fifty mg. of oestranediol-B (from oestrone) was dehydrogenated as described for oestranediol-A. The product was isolated and purified as described before. It melted at 247° with the formation of a red color. Mixed with equilenin of known purity its melting point was raised to 250–254°. Mixed with oestrone it depressed to 230°.

Attempted Enzymatic Reduction of Oestrone.—Fresh hog ovaries were autolyzed according to the procedure used by Mamoli with stallion testes.[7] Three hundred cc. of the resulting extract (equivalent to 150 g. of ovaries) was mixed with 200 mg. of oestrone and the mixture was allowed to stand at 37° with frequent shaking for a period of thirty days. Then the mixture was extracted with ether, the solvent was evaporated to a volume of 50 cc. and washed with a 5% sodium hydroxide solution. The separated aqueous washings were then acidified with hydrochloric acid and the crystalline precipitate filtered, thoroughly washed with water and dried. The weight of the dried precipitate was 140 mg. It melted at 240–250° and showed no depression in melting point with an authentic sample of oestrone.

Similar results were obtained with extracts from suprarenal glands of beef, and bull testes.

Summary

Two stereoisomeric oestranediols have been isolated from non-pregnancy urine. The occurrence of these oestranediols in non-pregnancy urine but not in pregnancy urine indicates that they are formed by non-enzymatic reductive processes in the course of utilization of oestrone as a hormone.

STATE COLLEGE, PA. RECEIVED JUNE 15, 1938