The Isolation of $5\alpha$-Pregnane-$3\beta,20\beta$-diol 20-Sulfate and Its Hydrolysis to Uranediol ($17\alpha$-Methyl-$\delta$-homo-$5\alpha$-androstane-$3\beta,17a\beta$-diol)*

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In 1938 Marker, Rohrmann, and Wittke (2) isolated from the urine of pregnant mares a new isomer of pregnanediol that they called uranediol. Ten years later, Klyne (3) obtained from the same source a sulfuric acid ester that yielded uranediol on acid hydrolysis. The structure of the free compound was determined by Klyne (4), who showed that it was a $17\alpha$-methyl-$\delta$-homo-$5\alpha$-androstan-3-$\beta$, 17-$\alpha$-diol (Fig. 2, VIa). The configuration at C-17a is not known with certainty but may well be $\beta$, as was proposed (5) on the basis of a comparison of optical rotations with those of $\alpha$-homosteroids that lack the adjacent asymmetrical center at C-17. Some interest attaches to the biological origin of uranediol because it is a major, if not the predominant, neutral steroid that has been obtained from the urine of pregnant mares (5). In spite of its unusual n-homostructure, uranediol was considered to be a natural product and not an artifact because relatively mild procedures were used in the isolation of the conjugate. This view (4) was accepted by others (6, 8).

The need to re-examine this problem arose when we observed the ready conversion of $3\beta$-acetoxoy-$5\alpha$-pregnan-$20\beta$-ol $p$-toluenesulfonate (Fig. 1, Ia) to uranediol $3\beta$-acetate $17a$-formate (Fig. 1, IIa) on heating with formic acid (9). This rearrangement on heating with formic acid (9). As this rearrangement gave almost exclusively a single stereoisomer, the substitution at C-20 most probably proceeds in a single stage (Fig. 1) rather than via the C-20 carbonium ion. The formation of a $17\alpha$-methyl-$\delta$-homosteroid (II) by a concerted process demands antiparallel orientations of the C-20 → oxygen and C-17 → C-16 bonds and a forward orientation of the hydrogen at C-20 in the starting compound (I). These conditions define the $\beta$ configuration in its most stable conformation (9). Therefore, if the reaction of the tosylate is a model for the formation of the uranediol recovered from urine, its precursor, like the tosylate, must possess the $\beta$ configuration at C-20. It was suspected, therefore, that the alleged uranediol sulfate was in fact the 20-sulfate of $5\alpha$-pregnan-$3\beta,20\beta$-diol (Fig. 2, IV). This view received some support from the observations that the 20-hydroxysteroids of mares' urine have predominantly the $\beta$ configuration (5) and that the rotary contribution of the sulfate group of Klyne's conjugate differed appreciably from that calculated for a sulfuric acid ester of a $3\beta$-hydroxy-$5\alpha$-steroid (10).

In order to test this hypothesis, we prepared the 20-sulfate of $5\alpha$-pregnan-$3\beta,20\beta$-diol. It was obtained by the reaction of the $3\beta$-acetate (Fig. 2, III) (11) with pyridine-sulfur trioxide in chloroform (12) and subsequent alkaline hydrolysis of the ester group at C-3 (Fig. 2). The product was characterized as the potassium and $p$-toluidinium salts. The melting point and optical rotation of the latter were in close accord with the constants reported for the natural product, and like the natural product, the synthetic toluidinium salt gave uranediol (Fig. 2, VIa) on heating with hydrochloric acid. As a direct comparison with the original preparation of the natural sulfate was no longer possible, we repeated its isolation from the urine of pregnant mares.

The "water-insoluble" potassium salts were isolated by the procedure of Klyne, Schachter, and Marrian (10) and extracted with 98% acetone. The extract gave a precipitate (3) which was subjected to countercurrent distribution between dilute ammonia and butanol-toluene (13). Material with the $R_f$ value of IV was recovered to the potassium salt and then recrystallized. Identity of the final product with the potassium salt of synthetic $5\alpha$-pregnan-$3\beta,20\beta$-diol 20-sulfate was established by comparison of optical rotations, infrared spectra (Fig. 3), chromatographic mobilities, and melting points, which were not depressed on admixture. Comparison of the $p$-toluidinium salts of both preparations confirmed their identity.

Although the mode of synthesis seemed to provide an unambiguous proof of the structure, the objection might be raised...
that the rearrangement that results in the formation of uranediol (Fig. 2, VIIa) occurred during the synthesis of the sulfate rather than during its acid hydrolysis. Actually we have observed a related phenomenon, the slow rearrangement of the 20-p-toluene-sulphonate of III (Fig. 2) to the 17a-toluenesulphonate 3-acetate of VIIa (Fig. 2) albeit under much more drastic conditions—prolonged exposure to boiling pyridine. To establish the structure of the synthetic sulfate, its potassium salt was heated in dioxane. This solvolysis procedure (12) has been shown to cleave only the oxygen-sulphur bond of steroidal 3-sulfates (12, 1) and can therefore be expected not to alter the carbon skeleton. The reaction product was 5α-pregnane-3β,20β-diol (Fig. 2, VIa), which was obtained free of any spectrographically detectable amounts of uranediol. Similar results were obtained on cleavage with ethyl acetate (16). It can be concluded, therefore, that no rearrangement occurred during the synthesis of the sulfate and that the synthetic and isolated products are both the 20-sulfate of 5α-pregnane-3β,20β-diol (Fig. 2, IV). To further verify their identity, the isolated sulfate was acetylated and then solvolyzed with dioxane. As anticipated, the product was identical with 5α-pregnane-3β,20β-diol 3-acetate (Fig. 2, III).

**EXPERIMENTAL PROCEDURE**

Preparation of 5α-Pregnane-3α,20β-diol 20-Sulfate (Fig. 2, IV)—A solution of 30.9 mg of 5α-pregnane-3α,20β-diol 3-acetate (Fig. 2, III) (melting at 172.5–174° (9)) in 3 ml of dry chloroform was shaken 1% with 220 mg of pyridine-sulfur trioxide (17) for 23 hours (12). After the addition of 1.5 ml of petroleum ether, the mixture was chilled and filtered through washed cellulose powder. The insoluble material was thoroughly washed with cold petroleum ether-chloroform. The filtrate was taken to dryness and the residue (55.7 mg) was taken up in 4 ml of hot chloroform. The precipitate (54.7 mg), which formed on the addition of 20 ml of petroleum ether, was separated, dissolved in 4.5 ml of methanol, and treated with 1.5 ml of 0.8 N potassium hydroxide in methanol. In addition to amorphous material, which precipitated immediately (potassium sulfate?), crystals formed at room temperature. To insure completeness of the reaction, these crystals were dissolved by three times bringing the mixture to a boil and maintaining it at this temperature for 1 minute. After 43 hours, the mixture was distributed between butanol and water. The butanol phase was washed free of alkali and taken to dryness in a vacuum. The residue (35.1 mg) on crystallization from methanol gave 5α-pregnane-3α,20β-diol potassium 20-sulfate, melting at 229–231° with decomposition. The yield was 32.9 mg, [α] 2° +22° (water, 4 mg per ml).

Calculated: C 55.23, H 8.17, K 8.56  
Found: C 55.33, H 8.20, K 8.61

Samples were dried for analysis and rotation for at least 6 hours in a vacuum at room temperature. Another sample, which was dried at 80°, gave C 55.50, H 8.59.

The potassium salt (26.7 mg) was dissolved with warming in 4 Unpublished data from this laboratory.

5 All melting points reported are corrected. A 2-dm tube was used for measuring rotations. Infrared spectra were recorded with a Perkin-Elmer grating spectrometer (model 421). Diacetates and monoacetates were examined as solutions in carbon disulfide, and diols and sulfates as pressings in potassium bromide. Paper chromatograms were examined after treatment with phosphomolybdic acid (9).
Samples were dried for rotation and analysis at 80° in a vacuum. The constants reported for Klyne’s p-toluidinium salt (3) were: melting point, 150-151° with decomposition, and [α] D 0 + 16.1° (ethanol).

Solvolyses of 5α-Pregnane-3β,20β-diol 20-Sulfate (Fig. 2, IV)---

1. In water: The synthetic p-toluidinium salt (9.5 mg) in 8 ml of 1N hydrochloric acid was heated on a steam bath for 1 hour as described by Klyne (3). The product (4.5 mg), which was isolated by ether extraction, was crystallized from dilute methanol. The melting point (216-217°) of the final product was not depressed by admixture with a reference sample 6 (m.p., 216.5-217°) of uranediol (Fig. 2, Vlb) prepared from 5α-pregnane-3β,20β-diol 3-acetate 20-tosylate (9). Of particular diagnostic significance are the strong bands at 963 cm⁻¹ (20P-hydroxy (20)) and at 978 cm⁻¹. No selective absorption could be detected at 978 cm⁻¹. The infrared spectrum agreed with that of a reference specimen of 5α-pregnane-3β,20β-diol diacetate (Fig. 2, Vb).

2. In dioxane: The synthetic potassium salt (6.6 mg) was heated with 4 ml of dioxane under reflux for 20 minutes. The product (4.5 mg), which was isolated by distribution of the reaction mixture between ether and water, was crystallized from dilute methanol and from acetone to yield 2.2 mg of 5α-pregnane-3β,20β-diol 3-acetate (9). Identity was established by mixture melting point with a reference specimen of 5α-pregnane-3β,20β-diol 3-acetate (9). The spectrum of the crude diacetate resembled quite closely that of the pure compound and gave no evidence of authentic 5α-pregnane-3β,20β-diol diacetate 20-tosylate (Fig. 2, Vb).

3. In ethyl acetate: Sodium chloride (2.5 g) and 1 ml of 1N sulfuric acid were added to a solution of 6.1 mg of the synthetic potassium salt in 9 ml of water. The mixture was promptly extracted with 40 ml of ethyl acetate. The organic phase was taken to dryness in a vacuum. The extracts (about 60 ml) were mixed with a sample of the synthetic potassium salt, did not depress its melting point. A comparison of the infrared spectra showed that the spectrum differed from the spectrum of the synthetic preparation in the positions of several of the major peaks, including those of the sulfate bands near 1200 cm⁻¹. The discrepancy disappeared upon crystallizing the natural product twice from water.

Isolation of 5α-Pregnane-3β,20β-diol 20-Sulfate (Fig. 2, IV)—Urine (14.6 liters) was collected from four mares 6 months to 1 week before foaling. Extraction and purification were done as described by Klyne et al. (10) and gave 1284 mg of the “water-insoluble fraction.” This material was extracted three times with boiling 98% acetone (3). The extract (about 60 ml) yielded 102 mg of precipitate when kept at 22° for 2 days. This precipitate was subjected to 24 transfers of the lower phases in countercurrent distribution in 10 separatory funnels. Equal volumes (30 ml) of the two phases, obtained from n-butanol, toluene, and 3% aqueous ammonia, 1:1:2 by volume, were used. The solids from funnels 3 to 9 were combined (29 mg), dissolved in 0.1 N potassium hydroxide and warmed to drive off ammonia. The solution was extracted with butanol, which was then washed with a potassium chloride solution and thoroughly with water. The residue of the butanol phase was repeatedly crystallized from methanol. A total of 18.8 mg of colorless needles was obtained; they melted at 229-231° with decomposition and, when mixed with a sample of the synthetic potassium salt, did not depress its melting point. A comparison of the infrared spectra showed that the spectrum differed from the spectrum of the synthetic preparation in the positions of several of the major peaks, including those of the sulfate bands near 1200 cm⁻¹. The discrepancy disappeared upon crystallizing the natural product twice from water.

Solvolysis of Acetate of Isolated Sulfate—The remainder of the p-toluidinium salt (about 10 mg of crystals and later mother liquors) was acetylated in 3 ml of pyridine with 1.5 ml of acetic anhydride for 16 hours at room temperature. The mixture was taken to dryness in a vacuum. This last step was repeated twice after the addition of toluene. The final residue was dissolved in water, washed with carbon tetrachloride, and after the addition of potassium chloride, extracted with butanol, which was then washed with potassium bicarbonate solution and repeatedly with water. Three crystallizations from methanol gave 0.9 mg of crystals that melted at 246-250° and gave a spectrum consistent with the proposed structure, 5α-pregnane-3β,20β-diol 3-acetate potassium 20-sulfate. The carboxyl peak was at 1720 cm⁻¹. Crystals and mother liquors were combined and heated with 4 ml of dioxane for 40 minutes. The solvolyzed product (4.8 mg) was isolated as described above and chromatographed on acid-washed alumina. The crystalline fractions were combined (2.3 mg) and crystallized thrice from acetone. The infrared spectrum agreed with that of a reference specimen of 5α-pregnane-3β,20β-diol 3-acetate (Fig. 2, III). Of particular diagnostic significance are the strong bands at 1025 (33-acetoxy in 5α-steroids (19)) and at 963 cm⁻¹ (20P-hydroxy (20)). The melting point (172-173.5°) was not depressed when a sample of the reaction product was mixed with one of the reference compound (Fig. 2, III).
DISCUSSION

The isolation of a sulfate with the properties of Klyne's sulfate, in comparable yield and by essentially the same procedure, leaves little room for doubt that our product is identical with Klyne's and that the only known naturally occurring sulfate that yields uranediol on acid hydrolysis is 5α-pregnane-3β,20β-diol 20-sulfate. The yield of this sulfate is of the same order as that of uranediol isolated after acid hydrolysis (2). Although this does not prove that all of the uranediol isolated from the urine of pregnant mares was formed by hydrolysis of the 20β-sulfate, it makes it probable that this was the case. The available data no longer justify the belief that a compound with the unusual α-homo structure of uranediol is formed in vitro by the pregnant mare.

The structure of the sulfate identified in this report does not conform to the usual pattern of conjugated steroids because the conjugation is not through the alcoholic hydroxyl function at C-3. Attachment of sulfuric or glucuronic acid to other hydroxyl groups has been noted before, but at least in the cases known to us, there is either no free hydroxyl group at C-3, as in testosterone 17-glucosiduronate (21) and corticosterone 21-sulfate (22), or the hydroxyl group at C-3 is aromatic, as in the glucosiduronates of estriol (23). The 20-sulfate cannot be the only form in which a 5α-pregnane-3β,20β-diol is excreted by the pregnant mare. Brooks et al. (5) have obtained rather large amounts of 5α-pregnane-3β,20β-diol along with uranediol from conjugates that were hydrolyzed with acid. One must conclude that the water-soluble moiety in this precursor (or precursors) of 5α-pregnane-3β,20β-diol must either be at C-3, or if it is at C-20, must be a group such as glucuronic acid, which is removed by acid without rupture of the steroid-oxygen bond.

The extensive rearrangement that was observed during the hydrolysis of the sulfate (Fig. 2, IV) in hot aqueous acid signifies carbon-oxygen cleavage. This may seem surprising since Lieberman, Hariton, and Fukushima (24) reported that 3α- and 3β-sulfates, in contrast to 3-toluenesulfonates, react under various conditions with rupture of the sulfur-oxygen linkage. It should be noted, therefore, that the conditions studied by these workers differ from those used in the present work.

A steroid conjugate has been isolated from the urine of pregnant mares. It agrees in its properties with a compound previously designated as uranediol sulfate and, like the latter, yielded uranediol (17α-methyl α-homo 5α androstan-3β,17αβ) on acid hydrolysis. The yield of this sulfate is of the same order as that of uranediol isolated after acid hydrolysis (2). Although this does not prove that all of the uranediol isolated from the urine of pregnant mares was formed by hydrolysis of the 20β-sulfate, it makes it probable that this was the case. The available data no longer justify the belief that a compound with the unusual α-homo structure of uranediol is formed in vitro by the pregnant mare.

SUMMARY

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