The Steroids of Pregnant Mares' Urine

2. ISOLATION OF URANEDIOL SULPHATE*

By W. KLYNE,† *Department of Biochemistry, University of Edinburgh*

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The first paper of this series (Klyne, Schachter & Marrian, 1948) describes a general method for the extraction of steroid conjugates from mares' urine and the isolation of an *allopregnenolone* sulphate. The present paper deals with the isolation from the 'water-insoluble' fraction of the conjugates of a compound, *Y* sulphate, which appears to be the sulphate of the steroid 'uranediol', $C_{21}H_{36}O_2$, isolated from pregnant mares' urine by Marker, Rohrmann & Wittle (1938). Derivatives of 'urane', which may be a hydrocarbon isomeric with pregnane and *allo*-pregnane, have never been reported hitherto except by Marker and his co-workers (Marker, Kamm, Crooks, Oakwood, Wittle & Lawson, 1938; Marker, Kamm, Oakwood, Wittle & Lawson, 1938; Marker, Lawson, Wittle & Crooks, 1938; Marker, Rohrmann & Wittle, 1938; Marker, Wittle & Oakwood, 1938; Marker & Rohrmann, 1939; Marker, 1941 *a, b*, 1944; see also Parke, Davis and Co. 1940).

Subsequent to the isolation of *Y* sulphate Klyne & Paterson (1948) obtained from pregnant mares' urine a triol (*compound T*), apparently identical with 'uranetriol' (Marker, Kamm, Oakwood, Wittle & Lawson, 1938).

RESULTS

The '98% acetone solids, Fractions *A* and *B*', obtained by the general method of extraction (Klyne *et al.* 1948) from two large batches of late pregnancy urine, consisted of the potassium salt of a new organic sulphate (*potassium Y* sulphate) in fairly pure form. The yields from the two batches were c. 4 and 1 mg./l. urine respectively. The potassium salt was transformed into *p*-toluidine *Y* sulphate, the analyses and equivalent of which indicated that it was probably the salt of the monosulphate of an isomer of pregnanediol,

The *p*-toluidine salt was hydrolyzed by hot acid to the free hydroxy compound, *Y*, analytical results for which agreed fairly well with those required for a saturated dihydroxy steroid, $C_{21}H_{36}O_2$. Compound

Y absorbed no hydrogen on shaking with Adams PtO_2 catalyst in glacial acetic acid for 2 hr., and gave negative tetranitromethane, Zimmermann and Millon reactions. It formed a precipitate with digitonin in 90% ethanol, indicating the probable presence of a 3(β)-hydroxyl group. In concentrated H_2SO_4 , it gave an immediate orange-red colour with a slight green fluorescence, and in the Liebermann-Burchardt test an immediate deep crimson colour.

Compound *Y* gave with acetic anhydride and pyridine at 15–20° *Y* diacetate, $C_{25}H_{40}O_4$, and with CrO_3 in glacial acetic acid at 15–20° an oxidation product *Y* ketone, $C_{21}H_{32}O_2$. The latter, on treatment with excess semicarbazide hydrochloride and sodium acetate at 15–20°, yielded a *monosemicarbazone*. *Y* ketone was recovered substantially unchanged after boiling with aqueous ethanolic HCl for 15 min.

Y ketone on Clemmensen reduction gave in c. 50% yield a compound provisionally called *Y* hydrocarbon, which was saturated to tetranitromethane. A single elementary analysis on this compound gave results which differed considerably from those calculated for the expected formula. (Found: C, 84.4; H, 14.8. $C_{21}H_{36}$ requires: C, 87.4; H, 12.6%.) Lack of material prevented repetition of the Clemmensen reduction, and the author prefers to regard the nature of the product as doubtful, although Marker, Kamm, Oakwood, Wittle & Lawson (1938) gave for a product, obtained in the same way from 'uranetriol', analytical figures in good agreement with those required by $C_{21}H_{36}$.

EXPERIMENTAL

Methods

Melting points. These were determined on the improvised Kofler hot-stage apparatus of Klyne & Rankeillor (1947) and are corrected. Speed of heating at m.p., 3–4°/min. Limits of error, $\pm 2^\circ$.

Specific rotations. See Klyne *et al.* (1948).

Micro-analyses. These are by Dr J. W. Minnis unless otherwise stated.

'*Usual working-up.*' This expression indicates the following operations: A solution of the reaction product in an organic solvent (usually ether) was washed with aqueous $N-Na_2CO_3$, aqueous $N-H_2SO_4$ and three times with water; it was then dried over anhydrous Na_2SO_4 , the latter filtered off, and the solution evaporated to dryness.

* Preliminary accounts of this work have been given to the Biochemical Society: see Klyne (1946); Klyne & Paterson (1948).

† Present address: Postgraduate Medical School, London, W. 12.

Isolation of *p*-toluidine *Y* sulphate

The 'water-insoluble' fraction (19.4 g.) from 270 l. late pregnancy urine was submitted to the '98% acetone' treatment (Klyne *et al.* 1948) using 1×100 , 7×200 , 3×100 ml. fractions. The acetone solutions yielded no solid on cooling, but on heating *without concentration* gave crystalline precipitates, Fraction *B* (1090 mg.), which did not redissolve on cooling and were filtered off.

The 'water-insoluble' fraction (9.9 g.) from another batch of late pregnancy urine (530 l.) was submitted to the '98% acetone' treatment (1×50 , 8×100 , 3×50 ml. fractions). In this case the acetone solutions yielded on cooling to room temperature some dirty-white amorphous material (360 mg.) from which no pure compound could be obtained. The filtrates from this material on standing at 0° gave a crisp white amorphous solid, Fraction *A* (360 mg.). After this fraction had been removed by filtration the mother liquors were heated without concentration, but no Fraction *B* separated.

The above Fractions *A* and *B* (crude potassium *Y* sulphate), on treatment with *p*-toluidine hydrochloride in aqueous solution as described for *Z* sulphate (Klyne *et al.* 1948), yielded fairly pure *p*-toluidine *Y* sulphate, which when recrystallized from water formed very fine white needles, m.p. $181-183^\circ$ (decomp.); $[\alpha]_D^{20} + 16.1 \pm 0.3^\circ$ in ethanol (*c.* 3.0). (Found: C, 66.6, 66.1; H, 9.1, 9.0; N, 2.8; S, 6.4; equivalent, 521, 522. $[\text{C}_{21}\text{H}_{25}\text{OSO}_4]^- [\text{CH}_3 \cdot \text{C}_6\text{H}_4 \cdot \text{NH}_2]^+$ requires: C, 66.2; H, 8.9; N, 2.8; S, 6.3%; equivalent 507.)

Compound *Y*

p-Toluidine *Y* sulphate was hydrolyzed by hot N-HCl as described for *Z* sulphate (Klyne *et al.* 1948), except that 1 hr. heating at 100° gave a better yield than 2 hr. heating (88 as against 67%). Compound *Y* after repeated crystallization from ethanol (2 vol.): water (1 vol.) formed needles, m.p. $211-213^\circ$, subliming at $180^\circ/0.06-0.1$ mm.; $[\alpha]_D^{15} + 3.7 \pm 0.8^\circ$ in ethanol (*c.* 1.8). (Found: C, 78.3, 77.9; H, 11.4, 11.2. $\text{C}_{21}\text{H}_{25}\text{O}_2$ requires: C, 78.7; H, 11.3%.)

Compound *Y* (12 mg.) was treated with acetic anhydride (2 ml.) and pyridine (2 ml.) for 12 hr. at $15-20^\circ$. The product, *Y* diacetate, after the usual working up and recrystallization from aqueous methanol formed glistening plates, m.p. $159.5-160.5^\circ$, subliming at $190^\circ/0.1$ mm.; $[\alpha]_D^{20} - 30.4 \pm 0.7^\circ$ in chloroform (*c.* 1.4); $[\alpha]_D^{22} - 29.6 \pm 1.1^\circ$ in ethanol (*c.* 1.0). (Found: C, 74.0, 74.4; H, 10.2, 10.1. $\text{C}_{25}\text{H}_{30}\text{O}_4$ requires: C, 74.2; H, 10.0%.)

Table 1. Comparison of compound *Y* and its derivatives with urane compounds

	<i>Y</i> series (m.p.)	Urane series, Marker (m.p. (uncorr.))
Diol, $\text{C}_{21}\text{H}_{34}(\text{OH})_2$	211-213°	210°
Dioldiacetate, $\text{C}_{21}\text{H}_{34}(\text{O} \cdot \text{CO} \cdot \text{CH}_3)_2$	159.5-160.5°	160°
Ketone, $\text{C}_{21}\text{H}_{32}\text{O}_2$	171-173°	177.5°
Ketone semicarbazone, $\text{C}_{21}\text{H}_{32}\text{O}(\text{N} \cdot \text{NH} \cdot \text{CO} \cdot \text{NH}_2)$	217-221°	245°
Hydrocarbon, $\text{C}_{21}\text{H}_{36}$ (?)	122-124°	128°

Y ketone

Compound *Y* (24 mg.), dissolved in glacial acetic acid (7.0 ml.), was treated with a solution of CrO_3 (16 mg. $\equiv 3$ atoms oxygen) in 98% acetic acid (1.2 ml.) and allowed to stand for 18 hr. at $15-20^\circ$. Excess CrO_3 was reduced by

adding a few drops of ethanol and the mixture diluted with ether. The usual working up gave *Y* ketone, which after crystallization from ethanol-water (2:1) formed plates, m.p. $171-173^\circ$, subliming at $130^\circ/2 \times 10^{-4}$ mm.; $[\alpha]_D^{20} - 21.3 \pm 0.9^\circ$ in chloroform (*c.* 1.2). (Found: C, 79.4, 79.4; H, 10.2, 10.0. $\text{C}_{21}\text{H}_{32}\text{O}_2$ requires: C, 79.7; H, 10.2%.)

Y ketone (4.5 mg.) in ethanol (5 ml.) was treated with semicarbazide hydrochloride (3.2 mg.) and hydrated sodium acetate (4.5 mg.) in water (0.5 ml.), and allowed to stand at $15-20^\circ$. After 18 hr. more semicarbazide hydrochloride (6.4 mg.) and hydrated sodium acetate (10.0 mg.) were added. After 3 days most of the ethanol was evaporated and the semicarbazone was precipitated with water, filtered, washed and dried. After recrystallization from ethanol it formed a white powder, m.p. $217-221^\circ$ with browning, after softening $206-216^\circ$. (Found: N, 12.1. $\text{C}_{22}\text{H}_{35}\text{O}_2\text{N}_3$ (monosemicarbazone) requires N, 11.1. $\text{C}_{22}\text{H}_{35}\text{O}_2\text{N}_6$ (disemicarbazone) requires N, 19.5%.)

Clemmensen reduction of *Y* ketone

Y ketone (6.0 mg.) was dissolved in glacial acetic acid (2.5 ml.) and conc. HCl (2.5 ml.). Amalgamated zinc wool (1 g.) was added, and the mixture was refluxed for 7 hr., 1 ml. of a mixture of glacial acetic acid and conc. HCl (1:1) being added after each hour. The reaction mixture was diluted with hexane, and the usual working up gave a white solid (4.0 mg., m.p. not less than 90°). This material was chromatographed on Al_2O_3 (activity II, Brockmann & Schodder, 1941); pentane eluted 3 mg. of a product which melted at $110-115^\circ$ after softening from 103° , and recrystallized in long prisms on cooling to 109° . Recrystallization from acetone (*c.* 1 ml.) gave '*Y* hydrocarbon', glistening leaflets, m.p. $122-124^\circ$ after softening at 116° (recrystallized partly on cooling to 123°). (Found: C, 84.4; H, 14.8. $\text{C}_{21}\text{H}_{36}$ requires: C, 87.4; H, 12.6%.) (This analysis was carried out on 1.8 mg. of substance in the Chemistry Department, University of Glasgow.)

DISCUSSION

Comparison of the properties of compound *Y* and its derivatives with those reported by Marker, Rohrmann & Wittle (1938) for uranediol and its derivatives indicates that the two series of compounds are almost certainly identical (see Table 1), although the present author has been unable to obtain samples of Marker's materials for direct comparison.

The only connexion between the urane series and compounds of known structure is a supposed transformation of uranetrione into pregnane-3:20-dione and uranedione reported by Marker, Kamm, Oakwood, Wittle & Lawson (1938) (see also Parke,

Davies and Co. 1940; Marker, 1941*a*). The transformation involved six stages; no intermediates were purified, and the final yield of pregnanediol was c. 5%. The author feels that little weight can be given to such evidence and that the uranetriol, from which the uranetrione was originally made, may have been contaminated with a little pregnanediol.

Marker, Kamm, Oakwood, Wittle & Lawson (1938) suggested that urane was epimeric with pregnane at C₉, and that uranetrione was 9-*isopreg*nan-3:11:20-trione (I). They originally suggested that uranediol was the 3:20-diketone, but later Marker,

Rohrmann & Wittle (1938) formulated it as the 3:11-diketone (II). The partial transformation of uranetrione into pregnane-3:20-dione was thought to involve epimerization at C₉, at some stage (see also Marker, Wittle & Oakwood, 1938).

No evidence for or against a 9-*iso*-structure for urane is available either from Marker's or from the present work, and the only evidence in favour of an 11-keto group in uranediol (Y ketone) and uranetrione is the inertness of one keto group in these compounds. Marker's suggested 11-keto-9-*iso*-structure for these compounds is probably incorrect. Such a structure would be of *cis*- α -decalone type, and would be isomerized by heating with acid to the corresponding *trans*- α -decalone type (cf. coprostane-3:6-dione, which gives with acid cholestane-3:6-dione; Butenandt, Schramm & Kudzusz, 1937); however, uranetrione (Marker, Kamm, Oakwood, Wittle & Lawson 1938) and Y ketone are stable to acid. The stability of these ketones to acid also excludes the possibility of a 14-*n*-17-*iso*-20-keto structure, since such compounds are isomerized by acid to the corresponding 14-*n*-17-*n* compounds (Butenandt, Schmidt-Thomé & Paul, 1939; Shoppee, 1940; Shoppee & Reichstein, 1940).

If 'urane' is a saturated C₂₁ steroid hydrocarbon it is certainly different from any known compound. Shoppee (1944) gave a list of C₂₁ hydrocarbons known at that date. His dignane is now known to be 5-*allo*-14-*iso*-17-*isopreg*nan-3 (Press & Reichstein, 1947); 5-*n*-14-*iso*-17-*isopreg*nan-3 has also been made (Meyer, 1947). The possibility that 'urane' is a C₂₀ or C₂₂ hydrocarbon should be considered, since analytical figures for compounds of these series do not differ greatly from those for the C₂₁ series.

The constitution of 'urane' will be investigated further when more material is available.

SUMMARY

1. A new organic sulphate has been isolated from pregnant mares' urine as its *p*-toluidine salt, which on acid hydrolysis yields compound Y, C₂₁H₃₆O₃, apparently identical with uranediol.

2. The reactions of compound Y have been studied.

3. Previous views on the structure of urane compounds are discussed.

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The Steroids of Pregnant Mares' Urine

3. ISOLATION OF ALLOPREGNAN-3(β)-OL-20-ONE SULPHATE*

By J. Y. F. PATERSON AND W. KLYNE,† *Department of Biochemistry, University of Edinburgh*

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alloPregnan-3(β)-ol-20-one has been isolated from pregnant mares' urine after hydrolysis by Marker, Lawson, Wittle & Crooks (1938), by Heard & McKay (1939), and by Oppenauer (1941). The present paper describes the isolation of this compound as its sulphate.

RESULTS

Pregnant mares' urine was extracted by the method described in Part 1 of this series (Klyne, Schachter & Marrian, 1948), and the crude conjugates obtained as potassium salts in certain '98 % acetone mother liquors' and '98 % acetone solids' were treated in aqueous solution with the hydrochlorides of various organic bases in an attempt to obtain crystalline derivatives. The hydrochlorides used, which have been employed for the characterization of carboxylic and sulphonic acids and have also been found to give crystalline salts with simple organic sulphates (Barton & Young, 1943; Klyne, unpublished observations), were as follows: *p*-toluidine, benzylamine, β-phenylethylamine and cyclohexylamine hydrochlorides, piperazine dihydrochloride and *S*-benzyl-ψ-thiuronium chloride. Piperazine dihydrochloride gave with the potassium salts of the crude conjugates a crystalline salt $[C_{21}H_{33}O \cdot SO_4]_2^- \cdot [C_4H_{12}N_2]^{++}$, piperazine *X* sulphate.

This sulphate on acid hydrolysis gave a product (compound *X*), which, after purification as the

acetate, was shown to be identical with allopregnan-3(β)-ol-20-one. Authentic allopregnan-3(β)-ol-20-one was converted into its sulphate (pyridine salt) by treatment with pyridine-sulphur trioxide (cf. Sobel & Spoerri, 1941), and the pyridine salt transformed into the piperazine salt. This synthetic piperazine allopregnanolone sulphate and the piperazine *X* sulphate from mares' urine had identical melting points and mixed melting point. However, analysis showed that the synthetic sulphate contained 2 molecules of water of crystallization even after drying *in vacuo* at 80° for 6 hr., while the mares' urine sulphate was anhydrous after drying for 1 hr. in the same conditions. Each sample had been crystallized from ethanol-water, and no explanation of the difference in composition can be given.

EXPERIMENTAL

Melting points. These are corrected (see Klyne, 1948).

Micro-analyses. These are by Dr J. W. Minnis.

Specific rotations. See Klyne *et al.* (1948).

'Usual working up.' See Klyne (1948).

Chromatograms. These were carried out with Al_2O_3 , supplied by Peter Spence and Co. Ltd., Widnes (Grade H, 100–200 mesh), the activity being determined according to Brockmann & Schodder (1941). The proportions used were as follows: material to be fractionated, 1 g.; Al_2O_3 , 30 g.; eluent (each fraction), 100 ml. In all cases the height of the Al_2O_3 column was 1–1.5 times its diameter. The proportions of mixed solvents are given as % (v/v).

Isolation of X sulphate (allopregnan-3(β)-ol-20-one sulphate) as piperazine salt. Late pregnancy urine (530 l.) was extracted by the procedure previously described (Klyne *et al.* 1948) and the 'water-insoluble' fraction submitted to

* For a preliminary account of this work see Paterson & Klyne (1948).

† Present address: Postgraduate Medical School, London, W. 12.