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The Steroids of Pregnant Mares' Urine

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

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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 I 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acids and have also been found to give crystalline salts with simple organic sulphates (Barton & Young, 1943; Klyne, unpublished observations), were as follows: *p*-toluidine, benzylamine, β -phenylethylamine and cyclohexylamine hydrochlorides, piperazine dihydrochloride and *S*-benzyl-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).

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98% acetone extraction. The acetone solutions from which potassium Y sulphate had been removed (see Klyne, 1948) were concentrated to a small volume and cooled, when amorphous solid, 'Fraction C', separated. This was filtered off and the mother liquors evaporated to dryness, giving a light-brown solid (2.5 g.). This material dissolved in the minimum volume of hot water was treated with piperazine dihydrochloride (1.2 g.), dissolved in the minimum volume of water. The light-brown precipitate which formed was recrystallized from water containing 10% (v/v) ethanol, giving 1.0 g. of a product, m.p. 232–234° (decomp.): further recrystallized three times from the same solvent, *piperazine X sulphate* formed small plates with m.p. constant at 239–241° (decomp.). A further quantity of the same compound was similarly obtained from the amorphous '98% acetone solids, Fraction C', mentioned above.

The salt was moderately soluble in water and rather more soluble in ethanol. (Found (after drying *in vacuo* at 80° for 1 hr.): C, 63.2; H, 8.9; N, 3.2; S, 7.7. $[C_{21}H_{33}O_3 \cdot SO_4]_2^- [C_4H_{12}N_4]^{++}$ requires: C, 62.6; H, 8.9; N, 3.2; S, 7.3%.)

Compound X and X acetate. Piperazine X sulphate (350 mg.) was dissolved in hot water (500 ml.) and ethanol (50 ml.), and conc. HCl (50 ml.) was added. The mixture was heated at 100° for 2 hr., cooled, and extracted with ether (1 × 300 ml., 3 × 150 ml.); the usual working up gave

aqueous methanol, followed by another sublimation it had m.p. 137–140°. X acetate gave no coloration with tetranitromethane, but gave a purple coloration in the Zimmermann ketosteroid test. With digitonin in 90% (v/v) ethanol a ppt. was slowly formed (cf. *allopregnan-3(β)-ol-20-one* acetate, Butenandt & Mamoli, 1935). (Found: C, 76.7, 77.0; H, 9.8, 10.2. Calc. for $C_{23}H_{35}O_3$: C, 76.6; H, 10.1%.)

More X acetate was prepared from another sample of piperazine X sulphate, which had been obtained from the '98% acetone solids, Fraction C', from the same batch of urine. The purification was similar to that described above, except that the crude free hydroxy compound was also submitted to a Girard separation.

Hydrolysis of X acetate. X acetate (18 mg.) in methanol (2 ml.) mixed with a solution of $KHCO_3$ (19 mg.) in water (1 ml.) was refluxed for 1.5 hr. After cooling, the mixture was extracted with ether (100 ml.) and worked up as usual. The product, crystallized from benzene-hexane, gave compound X (8 mg.), short rods, m.p. 190–194°, subliming at 140–150°/5 × 10⁻⁴ mm. (Found: C, 78.7; H, 10.6. Calc. for $C_{21}H_{34}O_3$: C, 79.2; H, 10.8%.) The properties of X and its acetate are compared with those of authentic *allopregnan-3(β)-ol-20-one* and its acetate (prepared by catalytic hydrogenation of *pregn-5-en-3(β)-ol-20-one* acetate (Plattner, Heusser & Angliker, 1946)) in Table 1.

Table 1. Comparison of compound X and *allopregnan-3(β)-ol-20-one*

Hydroxy compound:	X	<i>alloPregnanolone</i>
M.p.	192–195°	190–193°
Mixed m.p.		188–191°
$[\alpha]_D^{25}$ in chloroform (c, 1.0)	+89.7° ± 1.2°	+92.9° ± 0.7°
Acetate:		
M.p.	137–140°	138–140°
Mixed m.p.		137–139°
$[\alpha]_D^{25}$ in chloroform (c, 1.0)	+72.1° ± 0.8°	+70.9° ± 0.5°
Piperazine salt of sulphate:		
M.p. (decomp.)	239–241°	239–241°
Mixed m.p. (decomp.)		239–242°

Barton & Cox (1948) give the following values: *allopregnanolone*, m.p. 194–195° (uncorr.); $[\alpha]_D + 95°$ in chloroform (c, 1); acetate, m.p. 143.5–144.5° (uncorr.); $[\alpha]_D + 77°$ in chloroform (c, 1).

181 mg. product. Chromatography of this material on Al_2O_3 (activity I–II) failed to yield any fraction of sharp m.p. The bulk of the material (130 mg.), which was eluted with benzene-ether mixtures, was recombined, dissolved in 90% ethanol (5 ml.) and treated with digitonin (500 mg.) in 90% ethanol (5 ml.). The precipitate was centrifuged, washed twice with 90% ethanol and dried (530 mg.); it was then dissolved in dry pyridine (10 ml.) and the solution diluted with dry ether (100 ml.), yielding a ppt. which was centrifuged and washed twice with ether; the ethereal solution and washings after thorough washing with dilute acid and the usual working up gave the digitonin-precipitable fraction (105 mg.). This fraction on acetylation (acetic anhydride (5 ml.), pyridine (5 ml.); 18 hr. at 15–20°; usual working up) gave a colourless oil (120 mg.), which was chromatographed (Al_2O_3 , activity II). Most of the material was eluted with hexane-benzene (90:10 and 80:20) mixtures. These fractions, which had similar melting points, were combined and recrystallized from methanol-water (2:1, v/v) giving X acetate (24 mg.), m.p. 124–130°. After sublimation at 120°/4 × 10⁻⁵ mm., recrystallization from

Preparation of piperazine *allopregnan-3(β)-ol-20-one* sulphate. *alloPregnan-3(β)-ol-20-one* (320 mg.) in dry benzene (14 ml.) was mixed with pyridine-sulphur trioxide reagent (320 mg.) (Baumgarten, 1926; Baumgarten & Marggraff, 1931) and heated at 100° for 1.5 hr. After cooling, light petroleum (b.p. 60–80°, 140 ml.) was added and the mixture kept at 0° for 1 hr. The white solid which separated was centrifuged and washed twice with small quantities of light petroleum. The solid was then extracted six times with small quantities of warm $CHCl_3$, which, when filtered and evaporated, yielded a whitish solid. This was taken up in $CHCl_3$, and hexane (approx. 1.5 vol.) added. On cooling at 0° *pyridine allopregnanolone sulphate* was obtained as small rods, 195 mg., m.p. 182–187° (decomp.). This pyridine salt (17 mg.) was dissolved in the minimum volume of water and treated with piperazine dihydrochloride (9 mg.) in the minimum volume of water. The precipitate which formed was recrystallized twice from water containing 10% (v/v) ethanol to give *piperazine allopregnan-3(β)-ol-20-one sulphate* (9 mg.), m.p. 239–241° (decomp.). (Found (after drying *in vacuo* at 80° for 6 hr.): C, 60.3, 60.1; H, 8.5, 8.5;

N, 3.0; S, 6.8. $[C_{21}H_{33}O.SO_4^-]_2 [C_4H_{12}N_2^{++}]$, $2H_2O$ requires: C, 60.1; H, 9.0; N, 3.1; S, 7.1. $[C_{21}H_{33}O.SO_4^-]_2 [C_4H_{12}N_2]$ requires: C, 62.6; H, 8.9; N, 3.2; S, 7.3%. For comparison with piperazine X sulphate, see Table 1.

SUMMARY

*allo*Pregnan-3(β)-ol-20-one sulphate has been isolated from pregnant mares' urine and identified as its piperazine salt.

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Carbon Dioxide Fixation in Animal Tissues

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It is now firmly established that carbon dioxide can be incorporated into organic compounds by animal tissues. Two primary reactions are known, viz.

- (1) Pyruvate + $CO_2 \rightarrow$ oxaloacetate (Wood & Werkman, 1940).
- (2) α -Ketoglutarate + $CO_2 \rightarrow$ oxalosuccinate (Ochoa, 1945).

Previous work on CO_2 fixation was concerned mainly with establishing its existence and studying the enzymic mechanisms involved; information on the rates of reactions (1) and (2) in animal tissues has remained scanty. The work reported in this paper was undertaken in order to collect data on the rates of these two reactions in various animal tissues and to assess their quantitative significance in metabolism.

METHODS

Owing to the instability and reactivity of the initial products the measurement of the rates of reactions (1) and (2) offers great experimental difficulties. Direct determination of oxaloacetate or of oxalosuccinate would not indicate the extent of reactions (1) or (2) because these compounds

undergo rapid secondary changes. Oxaloacetate yields fumarate, malate, succinate, citrate and α -ketoglutarate; oxalosuccinate yields isocitrate, cisaconitate and citrate. These substances can arise under aerobic as well as anaerobic conditions.

Reaction (1) was examined in two ways. First, the sum of α -ketoglutarate and succinate formed on anaerobic incubation with pyruvate and CO_2 was determined. Data published by Krebs, Eggleston, Kleinzeller & Smyth (1940), on the anaerobic products formed from oxaloacetate in various tissues, showed that the sum of succinate plus α -ketoglutarate formed was fairly constant, amounting to 15–25% of the oxaloacetate utilized. The other products showed much greater variation. The sum of succinate plus α -ketoglutarate formed, therefore, may be taken to represent approximately one fifth of the CO_2 fixed by reaction (1).

A second indirect method of studying the rate of reaction (1), applicable in O_2 only, is based on the fact that the oxidation of succinate is largely prevented by the addition of malonate. The interruption of the tricarboxylic acid cycle at the stage of succinate causes the bulk of the di- and tricarboxylic acids to accumulate as succinate provided the rate of respiration is somewhat greater than that of the carboxylation of pyruvate. The amount of succinate found