3. 2:4:6-Triiodophenol, the aldehyde and thyroxine are all formed during the hot peroxide oxidation of diiodotyrosine.

4. 3:5-Diiodo-4-hydroxybenzaldehyde is formed during the iodination of proteins to give products with thyroid-like activity; the aldehyde itself has no biological activity as measured by the tadpole metamorphosis test.

5. *N*-Acetyl-L-thyroxine is formed in substantial yield during aerobic incubation of *N*-acetyl-L-diiodotyrosine. Acid hydrolysis of the thyroxine derivative yields L-thyroxine.

6. *N*-Acetyl-DL-diiodotyrosylglutamic acid has been prepared. Aerobic incubation of this peptide leads to the formation of good yields of *N*-acetyl-

DL-thyroxylglutamic acid which, on acid hydrolysis, yields thyroxine and glutamic acid.

7. Alanine has been chromatographically identified among the reaction products obtained during thyroxine synthesis from diiodotyrosine. This finding is briefly discussed.

I wish to express my indebtedness to Dr C. R. Harington for his interest in this work. I have to thank Dr R. L. M. Syngé for doing the chromatographic identifications of alanine; Dr W. J. Elford for his help in doing the potentiometric titration of *N*-acetyl-L-diiodotyrosine, and Dr A. S. Parkes for testing the biological activity of 3:5-diiodo-4-hydroxybenzaldehyde. I have also to thank Imperial Chemical Industries (Explosives) Ltd. for gifts of iodinated proteins.

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The Steroids of Pregnant Mares' Urine

1. A METHOD FOR THE EXTRACTION OF STEROID SULPHATES AND THE ISOLATION OF *allo*PREGN-16-EN-3(β)-OL-20-ONE SULPHATE*

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(Received 23 October 1947)

In a previous paper (Schachter & Marrian, 1938) dealing with the isolation of oestrone sulphate from the urine of pregnant mares, the isolation from the same source of what appeared to be the sulphate of a non-phenolic steroid was briefly noted. Fuller details were not reported at that time since concordant analyses upon the new sulphate and the parent steroid could not be obtained, and since there

was some evidence to suggest that the material isolated might be a mixture of closely related sulphates varying in composition from batch to batch. More recently, it has been possible to commence a reinvestigation of the conjugated steroids in pregnant mares' urine, and in the present paper the methods used for the extraction and concentration of a steroid-sulphate fraction and the isolation and identification of one pure component of this fraction are described.

* For preliminary accounts of this work see *Biochem. J.* 1945, **39**, xlv; 1946, **40**, lv; 1948, **42**, i.

RESULTS

The method used for the extraction and concentration of the steroid-sulphate fraction was based on that of Schachter & Marrian (1938). It consisted of the following stages: (i) Extraction of the urine with *n*-butanol. (ii) Removal of much acidic matter from the material extracted with butanol, giving the 'crude conjugate fraction'. (iii) Treatment of an aqueous solution of the crude conjugate fraction with an equal volume of saturated potassium acetate, giving 'potassium acetate-soluble' and 'potassium acetate-insoluble' fractions. This stage served the double purpose of ensuring that K^+ was the only cation present in the insoluble fraction and of partially removing in the soluble fraction certain simpler organic sulphates such as that of *p*-cresol. (iv) Separation of the 'potassium acetate-insoluble' fraction into fractions readily and sparingly soluble in water, so-called 'water-soluble' and 'water-insoluble' fractions. (v) Repeated extraction of the 'water-insoluble' fraction with boiling acetone containing water (2% (v/v); '98% acetone'). On concentration and cooling to 0° these extracts yielded white solid material, '98% acetone solids, fraction C', which in some cases was crystalline. (In one batch of urine solid material (fraction A) separated from the 98% acetone extracts before concentration on cooling to 0°. In another batch, although no solid was obtained on cooling the unconcentrated extracts, crystalline material (fraction B) separated immediately when these extracts were heated in order to concentrate them. Fractions A and B will be dealt with in subsequent papers.)

The weights of certain fractions at the different stages of concentration for a number of batches of urine are summarized in Table 1.

Table 1. *Weights of conjugate fractions in standard extraction method*

(All batches of urine from mares in 9th-11th months of pregnancy. Results expressed as mg./l. urine.)

	No. of results	Mean	S.E. of mean	Range
Crude conjugate fraction	11	440	50	210-810
Potassium acetate-soluble fraction	8	40	8	20-80
Potassium acetate-insoluble fraction	10	280	30	140-450
Water-soluble fraction	2	175	—	160-190
Water-insoluble fraction	10	120	20	50-210

Fraction C from certain batches of urine was found to consist largely of a single substance (*potassium Z sulphate*), which was obtained pure either directly by crystallization from water and from ethanol or via its *p*-toluidine salt (cf. Barton & Young, 1943). Analyses indicated the formulae

for the potassium and *p*-toluidine salts, respectively.

On acid hydrolysis of the potassium or *p*-toluidine salt an ether-soluble substance (Z), the analysis of which agreed with the formula $C_{21}H_{32}O_2$, was obtained. Z yielded a monoacetate on treatment with acetic anhydride and pyridine, and gave a precipitate with digitonin in 90% (v/v) ethanol. It thus appeared to be a 3(β)-hydroxysteroid. No colour was given with tetranitromethane.

In the Zimmermann test Z gave a purple colour, although the development of the colour was slower than with neutral 17-ketosteroids, indicating the presence of a ketone group at some position other than C₁₇. Z did not, however, form a semicarbazone. The failure of Z to form a semicarbazone and the lack of colour with tetranitromethane were not inconsistent with the presence of an αβ-unsaturated ketone group (cf. Ruzicka, Huyser, Pfeiffer & Seidel (1929) on tetranitromethane reactions, and Allen & Blatt (1943) on semicarbazide reactions), and subsequently Z acetate was found to show the 240 and 320 mμ. absorption bands characteristic of αβ-unsaturated ketones.

The above evidence suggested that Z might be a pregnen- or *allopregnen*-3(β)-ol-one having the double bond conjugated with the ketone group; its melting point and that of its acetate indicated that it might be *allopregn*-16-en-3(β)-ol-20-one, which was obtained by Marker & Turner (1940) from dihydropseudotigogenin diacetate.

Samples of this *allopregnenolone* acetate and of the 16:17-epoxy compound obtained from it by treatment with perbenzoic acid (Plattner, Ruzicka, Heusser & Angliker, 1947) were kindly supplied by Prof. Plattner of Zürich, and a comparison of these samples with Z acetate and its epoxy compound, respectively, showed that Z was indeed *allopregn*-16-en-3(β)-ol-20-one. This appears to be the first reported isolation of a Δ¹⁶-20-ketosteroid from urine.

The batches of urine from which Z sulphate was readily isolated were collected 3 weeks or less before foaling, the yield of crude potassium Z sulphate being c. 25 mg./l. Other batches collected 4-16 weeks before foaling gave smaller quantities of cruder material, from which it was more difficult to obtain Z sulphate. This suggests that the amount of Z sulphate excreted, and the ratio of Z sulphate to related sulphates, both increase in the last few weeks before foaling.

EXPERIMENTAL

Melting points. These are corrected. Speed of heating at melting point, 3–4°/min.

Micro-analyses. We are indebted to Mrs G. F. Marrian for all micro-analyses, except those of compound Z which are by Drs Weiler and Strauss (Oxford).

Specific rotations. These are for the sodium D line; a 0.5 dm. micro-tube was used. $[\alpha]_D$ is given as

$$X^\circ \pm Y^\circ = \frac{100}{lc} (x^\circ \pm y^\circ),$$

where x° is the difference between the mean of twenty readings with the solution and the mean of twenty readings with a solvent blank; y° is the standard error of this difference. Allowance is thus made for the variation of the polarimeter readings, but not for errors in the weighing of solute or solvent, which are much smaller.

Extraction of urine and preparation of fraction C. Many modifications of the extraction procedure were tried (see Klyne, 1946). The following are details for a batch of urine from which Z sulphate was obtained.

Urine (22.2 l., collected in the last 3 weeks before foaling) was extracted with *n*-butanol (4 × 6 l.). Emulsions were broken by allowing to stand with gentle shaking, centrifuging or adding ethanol (1–5% of vol. of urine). The butanol extracts were washed with *N*-aqueous KOH (4 × 6 l.) and with water (4 × 1.5 l.). Emulsions in the water-washing stage were broken rapidly by the addition of saturated aqueous potassium acetate (10% of vol. of aqueous layer).

The washed butanol extracts were evaporated to dryness *in vacuo* at >100° and the residue dissolved in water (500 ml.). The aqueous solution so obtained was extracted with ether (4 × 200 ml.) and cooled to 0°; its pH was adjusted to c. 2 with HCl and it was again extracted with ether (2 × 300 ml., 4 × 200 ml.). It was then made alkaline to litmus with KOH, warmed to remove ether, cooled, and extracted with butanol (4 × 200 ml.). The butanol extracts were washed with water (3 × 100 ml.) and evaporated to dryness giving the 'crude conjugate fraction' as a brown friable powder (12.3 g.).

This material was dissolved in boiling water (90 ml.), saturated aqueous potassium acetate (90 ml.) was added, the mixture was cooled to 15–20° and the pasty precipitate centrifuged. The precipitate was washed twice by centrifuging with its own volume of half-saturated aqueous potassium acetate and dissolved in moist butanol (250 ml.). This solution was washed with water (3 × 50 ml.) and evaporated to dryness giving the 'potassium acetate-insoluble fraction' as a yellow powder (8.8 g.).

The supernatant and washings from the potassium acetate precipitation were extracted with butanol (4 × 100 ml.); the extracts were washed with water (3 × 50 ml.) and evaporated to dryness giving the 'potassium acetate-soluble fraction' as a nearly white powder (2.0 g.).

The 'potassium acetate-insoluble fraction' was dissolved in the minimum volume of boiling water (140 ml.) and cooled to 0°. The sticky precipitate which formed was centrifuged, washed once by centrifuging with its own volume of ice-cold water and dissolved in moist butanol (200 ml.). The butanol solution on evaporation to dryness gave the 'water-insoluble fraction' as a yellow powder (4.7 g.).

The supernatant and washings from the above separation were extracted with butanol (3 × 100 ml.); the extracts were washed with water (3 × 20 ml.) and evaporated to dryness giving the 'water-soluble fraction' as a pale brown powder (3.9 g.).

The 'water-insoluble fraction' was then extracted repeatedly with boiling 98% acetone (2 × 200, 6 × 100 ml.). Each extract was filtered hot and the undissolved solid used for the next extraction. The extracts, which on cooling gave no precipitate, were concentrated on the water bath under a gentle air blast until solid began to separate; on cooling again, a nearly white, partly crystalline precipitate separated (98% acetone solids, fraction C; 620 mg.). The melting points of the fractions ranged from 218–219° (decomp.) (first fraction) to 235–240° (decomp.) (last three fractions).

Isolation of Z sulphate as the p-toluidine salt. The 98% acetone solids, fraction C, from the above separation (301 mg.) were dissolved in boiling water (50 ml.) and a solution of *p*-toluidine hydrochloride (185 mg., 2 equiv.) in water (5 ml.) was added. The copious precipitate which formed was just dissolved by adding more water (35 ml.) at 100°, and the solution was cooled to 0° giving *p*-toluidine Z sulphate (293 mg., m.p. 190–192°). After two recrystallizations from boiling water this salt formed very fine white needles, m.p. 195–197°, after softening at 186°, $[\alpha]_D^{19} + 31.7^\circ \pm 0.6^\circ$ in chloroform (c, 3). Approximate solubilities (in mg./ml.) were as follows: in water, at b.p., 3.5; at 15–20°, 0.5; in chloroform, at 15–20°, >30. The salt showed no loss in weight on drying at 80° *in vacuo*. (Found: C, 67.2, 67.2; H, 8.1, 8.1; N, 2.8, 2.9; S, 6.4, 6.4. $[C_{21}H_{31}OSO_4]^- [CH_3 \cdot C_6H_4 \cdot NH_3]^+$ requires C, 66.8; H, 8.2; N, 2.8; S, 6.4%.)

Potassium Z sulphate. This was obtained from the 98% acetone solids, fraction C, from two batches of urine by repeated crystallization from water and then from ethanol. It was best obtained pure from the *p*-toluidine salt as follows.

p-Toluidine Z sulphate (100 mg.) in warm water (100 ml.) was treated with KOH (1.0 g.) in water (5 ml.), and *p*-toluidine was extracted with ether (4 × 25 ml.). The aqueous solution was heated to drive off dissolved ether, cooled, and extracted with butanol (2 × 50, 2 × 25 ml.). The extracts on evaporation gave crude *potassium Z sulphate*, 86 mg. (90% of theory), m.p. 222–224° (decomp.), which was then crystallized from water and from ethanol giving rosettes of needles, m.p. 244–245° (decomp.) after browning at 234° and sintering at 241°.

Analyses of the potassium salt, when compared with those of the *p*-toluidine salt and of the free steroid, showed that it was a *monohydrate*, which did not lose its water of crystallization on drying to constant weight at 80° *in vacuo* over P_2O_5 . (Found: C, 55.8, 55.8; H, 7.5, 7.5; K, 8.8, 8.5. $C_{21}H_{31}OSO_4K \cdot H_2O$ requires C, 55.7; H, 7.4; K, 8.6. $C_{21}H_{31}OSO_4K$ requires C, 58.0; H, 7.2; K, 9.0%.)

Approximate solubilities (in mg./ml.) were as follows: in water at b.p., 10–11; at 0°, 3; in ethanol at b.p., 9; at 0°, 2.5; in butanol at b.p., 10; at 0°, 7; in chloroform at b.p. or at 0°, <0.1.

The potassium salt was dimorphous, clusters of prisms, m.p. 216–217° (decomp.) sometimes appearing when the compound was crystallized from water. (The quantity obtained was insufficient for analysis.) This prismatic form on treatment with *p*-toluidine hydrochloride gave the *p*-toluidine salt described above.

Table 2. Comparison of *Z* acetate and allopregn-16-en-3(β)-ol-20-one acetate

	<i>Z</i> acetate	alloPregnenolone acetate
M.p.	165–166.5°	165–167°*
Mixed m.p.		163–165.5°
$[\alpha]_D^{22}$ in chloroform (c, 1.4)	+33.9° ± 0.6°	+36.3° ± 0.7°*
Ultraviolet absorption†		
$\lambda_{\max}$	240, 320 m μ .	240, 320 m μ .
log $\epsilon_{\max}$	3.95, 1.78	3.93, 1.92*
16:17-Epoxy compound		
M.p.	185–187°	183–185°*
Mixed m.p.		183–185°

* These values were determined on Prof. Plattner's samples in Edinburgh and Liverpool. Plattner *et al.* (1947) give the following values: allopregnenolone acetate, m.p. 166–167°; $[\alpha]_D^{20}$ +42.2° (c, 1.42; chloroform); log $\epsilon_{\max}$ = 4.2, 2.1 (solvent not stated). Epoxy compound, m.p. 186–187°.

† In ethanol containing a little ether. We are indebted to Prof. R. A. Morton, Dr A. L. Stubbs and Mr T. W. Goodwin (University of Liverpool) for these measurements.

Compound Z. *p*-Toluidine *Z* sulphate (154 mg.) was dissolved in hot water (100 ml.), 2*N*-HCl (100 ml.) was added, and the mixture was heated in the boiling water bath. The initially clear solution became cloudy after 10 min. heating, and a precipitate formed, which gradually increased in bulk. After 2 hr. heating the mixture was cooled and extracted with ether (1 × 100 ml. and 3 × 50 ml.). The ether extracts were washed with *N*-HCl (2 × 30 ml.), 0.5*M*-NaHCO₃ (30 ml.) and water (3 × 30 ml.). The extracts on evaporation to dryness yielded crude compound *Z*, 88 mg. (91% of theory), m.p. 197–202° after sintering at 184°. After two recrystallizations from aqueous ethanol, *Z* formed glistening leaflets, m.p. 205–207°, $[\alpha]_D^{17}$ +50.2° ± 4.2° in ethanol (c, 1.4). (Found: C, 79.5, 79.8; H, 10.3, 10.1. Mol. wt. (Rast) 307, 325. Calc. for C₂₁H₃₂O₂: C, 79.7; H, 10.2%. Mol. wt. 316.)

***Z* acetate.** Compound *Z* (7.8 mg.) was heated at 100° for 2 hr. with acetic anhydride (1 ml.) and pyridine (1 ml.). Much water was added, and the white flocculent precipitate obtained was filtered, washed and dried. This material was recrystallized twice from aqueous ethanol (approx. 1:1 by vol.) giving pure *Z* acetate in elongated hexagonal leaflets, m.p. 165–166.5°. (Found: C, 76.7, 76.4; H, 9.5, 9.8. Calc. for C₂₃H₃₄O₃: C, 77.1; H, 9.6%.)

The epoxy compound of *Z* acetate was prepared by treatment with perbenzoic acid as described by Plattner *et al.*

(1947). The properties of *Z* acetate and allopregn-16-en-3(β)-ol-20-one acetate are compared in Table 2.

SUMMARY

1. A general method for the extraction of steroid and other conjugates from mares' urine is described.
2. A new organic sulphate has been isolated from pregnant mares' urine as its *p*-toluidine salt, which on acid hydrolysis yielded allopregn-16-en-3(β)-ol-20-one.

We are indebted to the Agricultural Research Council for a grant which defrayed the greater part of the expenses of this work; also to the Moray Fund and the Lewis Cameron Fund of the University of Edinburgh for other grants. We are grateful to N. V. Organon, Oss, Holland, for the gift of a large volume of mares' urine and facilities for working this up; also to British Drug Houses Ltd., the Ovaltine Research Laboratories and the Animal Diseases Research Institute, Moredun, Edinburgh, for gifts of urine. Without their willing co-operation this research could not have been carried out. We are also indebted to Goodlass Wall and Lead Industries Ltd. for the loan of large-scale apparatus, to Mrs G. F. Marrian for micro-analyses and to Mr A. Purdie for much valuable help in stable and laboratory.

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