4. An amount of malonaldehyde greater than that in oxidized erythrocyte fatty acids had no haemolytic action.

This work was made possible by a grant from the National Health and Medical Research Council. The author wishes to thank Dr E. Eden and other members of the Biochemistry Department, University of Sydney, for much helpful criticism and advice, and the Red Cross Blood Transfusion Service for generous gifts of material.

REFERENCES


Biochem. J. (1962) 83, 450

Ketonic Metabolites of Progesterone and 19-Norprogesterone in Rabbit Urine

BY G. H. THOMAS

Department of Anatomy, The Medical School, Birmingham 15

(Received 26 October 1961)

20α-Hydroxy pregn-4-en-3-one and its 20β-epimer have been found in the blood and tissues of several animal species as well as in man. This fact, together with their endocrine activities and biochemical relationship to progesterone, led Zander, Forbes, Münstermann & Neher (1958) to propose that these compounds should be classified as naturally occurring gestogens. The present paper describes the detection of the 20α-hydroxy compound in the urine of progesterone-treated rabbits. Despite the widespread occurrence of the two pregnenolones, neither has previously been reported as a metabolite excreted in urine. Consequently this finding would appear to indicate that progesterone is metabolized by a novel pathway in the rabbit. It was decided, therefore, to study also the ketonic metabolites of 19-norprogesterone (Djerassi, Miramontes & Rosenkranz, 1953) to determine whether this steroid was metabolized in a manner similar to that for progesterone, despite the absence of the methyl group flanking the Δ4-3-ketone system.

EXPERIMENTAL

Administration of steroids. Dutch-strain rabbits were injected subcutaneously with a solution of the steroid in arachis oil (40 mg./ml.). Urine was then collected for 3 days in vessels containing 5 ml. of toluene as preservative.

Fractionation of urine. Steroids were extracted from the urine with 3 vol. of butan-1-ol. The butanol extract was evaporated to dryness in vacuo and the residue partitioned between water (100 ml.) and ether (4 × 40 ml.). The ether layer (unconjugated metabolites) was devoid of steroids. Part of the aqueous layer (90 ml.) (the conjugated-metabolite fraction) was hydrolysed by heating under reflux with conc. hydrochloric acid (13 ml.) for 30 min. The remainder of the conjugated fraction (10 ml.) was buffered in acetate to pH 4·6 and then incubated with 5000 Fishman units of β-glucuronidase (Ketodase; Warner-Chilcott Laboratories) at 37° for 3 days. The two hydrolysates were then fractionated separately as follows: The hydrolysate was saturated with sodium chloride and extracted with ether. The extracted material was fractionated by the method of Brown (1955) up to the point where a solution of the metabolites in 50 ml. of light petroleum–benzene...
Scheme 1. Synthesis of 20α- and 20β-hydroxy-19-norpregn-4-en-3-one. 20α-ol: R' = OH, R'' = H; 20β-ol: R' = H, R'' = OH.

(1:1, v/v) was shaken first with water (4 × 25 ml.) and then with 2N-sodium hydroxide (4 × 25 ml). This procedure was preferred to extraction with toluene (e.g., Verley, Sommerville & Marrian, 1950) in that relatively polar metabolites, such as trihydroxypregnanes, partitioned into the aqueous phase (equivalent to Brown's oestriol fraction). The neutral residue, obtained on evaporation of the light petroleum-benzene solution to dryness, was treated with Girard T reagent. The ketonic and non-ketonic fractions so obtained were each separated into digitonin-non-precipitable (α) and digitonin-precipitable (β) fractions.

**Paper chromatography.** Unless otherwise stated, ketonic metabolites were chromatographed on Whatman no. 1 paper with solvent system A (Bush, 1952) at 37°. The equilibration period was limited to about 2 hr. and under these conditions there were often marked variations in Rf values. This difficulty was obviated by including standards on every paper. A Hanovia Chromatolite was used for locating the ultraviolet-absorbing steroids; keto steroids were detected with 2,4-dinitrophenylhydrazine. The sodium hydroxide-fluorescence reaction (Bush, 1952) was used for the identification of Δ4-3-oxo steroids. The chromatogram (Fig. 1c) was obtained with 3% of the ketonic α-fraction from the urine of a female rabbit injected with 50 mg. of progesterone. Similar patterns were obtained for a male rabbit given doses of 50 mg. and 500 mg. of progesterone and also for a pregnant rabbit after 50 mg. of progesterone. No steroid metabolites were detected at comparable concentrations in the ketonic α-fraction of the urine of untreated rabbits. Fig. 1e was obtained with 0.6% of the ketonic α-fraction from the urine of a male rabbit injected with 300 mg. of 19-norprogesterone. Eluted metabolites were oxidized in acetone solution by the dropwise addition of a 26% (w/v) solution of chromium trioxide in

<table>
<thead>
<tr>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
<th>e</th>
<th>f</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>α</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>α</td>
<td></td>
<td>β</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>β</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Solvent front

Fig. 1. Chromatography of ketonic metabolites in solvent system A at 37°. Time of run, 4 hr. a, 3α-Hydroxy-5β-pregnan-20-one; b, the ketonic fraction from human-pregnancy urine; c, α-ketonic metabolites of progesterone in the rabbit; d, 20α- and 20β-hydroxyprogren-4-en-3-one; e, α-ketonic metabolites of 19-norprogesterone in the rabbit; f, 20α- and 20β-hydroxy-19-norpregn-4-en-3-one.

29-2
8-2x-sulphuric acid until the first permanent brown colour was observed (Bowden, Heilbron, Jones & Weedon, 1946).

Purification and physical properties of compounds. Neutral Woelm alumina (L. Light and Co. Ltd.) was used for absorption chromatography; its activity was adjusted to Brockmann grade II by the addition of water (3 ml./100 g.). Melting points were uncorrected. Light-absorption maxima were determined in ethanol and infrared spectra in chloroform.

Isolation of 20x-hydroxy-19-norpregn-4-en-3-one from the urine of a rabbit treated with 19-norpregesterone

An adult male rabbit was injected subcutaneously with 19-norpregesterone (390 mg.) in arachis oil (10 ml.). The x-ketonic fraction of the urine was chromatographed on Whatman no. 3 MM paper in solvent system A at 37°. The Δ-3-oxo steroid band was located under ultraviolet light, cut out and eluted by continuous extraction with hot methanol. The residue obtained on evaporation of the methanol was leached with hot benzene (25 ml.). The benzene extract was concentrated to a small volume and adsorbed on alumina (2 g.). Elution with chloroform-benzene (1:1, v/v), followed by one crystallization from acetone-hexane, yielded 2 mg. of 20x-hydroxy-19-norpregn-4-en-3-one, m.p. 119-123°; [x]D +50.2° (c 0.79 in chloroform), light-absorption max. 240 μ (log ε 4.25) (Found: C, 79-4; H, 10-0%). The infrared-absorption spectrum showed peaks at 1657 and 1615 cm.-1 (αβ-un saturated ketone system).

The less mobile component. A benzene extract was chromatographed on alumina (Grade II, 3 g.). Elution with benzene–chloroform (4:1, v/v), followed by crystallization from acetone–hexane, yielded 20α-hydroxy-19-norpregn-4-en-3-one (10 mg.), m.p. 128-129°; [x]D +50.2° (c 0.79 in chloroform), light-absorption max. 240 μ (log ε 4.25) (Found: C, 79-4; H, 10-0%). The infrared-absorption spectrum showed peaks at 1653 and 1616 cm.-1 (αβ-un saturated ketone system).

Djerassi et al. (1953) reported the isolation of a mixture of the epimers (IV) as a stage in the synthesis of 19-norpregesterone but did not separate them. From the p.m. (174-177°) and [x]D +42° of the mixture it would appear that the 20β-ol was the predominant component.

Preparation of 20α-hydroxyprogren-4-en-3-one and its 20β-epimer from progesterone

Treatment of a methanolic solution of progesterone (250 mg. in 2 ml.) with pyrrolidine (0.2 ml.) gave 273 mg. of the 3-N-pyrrolidine enamine. After reduction with lithium aluminium hydride (200 mg.) and hydrolysis of the pyrrolidino group, part of the crude reaction product (170 mg.) was applied to Whatman 3 MM paper. The epimers were separated by chromatography in solvent system A and then purified by alumina chromatography. In this way the more mobile fraction from the paper chromatograms yielded 65 mg. of 20β-hydroxyprogren-4-en-3-one, m.p. 172-174°, light-absorption max. 240 μ (log ε 4.21), and the less mobile fraction 19 mg. of 20α-hydroxyprogren-4-en-3-one, m.p. 155-160°, light-absorption max. 240 μ (log ε 4.18). For both compounds, proof of identity was established by mixed-melting-point determination with an authentic sample.

RESULTS

The ketonic x- and β-fractions of both the conjugated and unconjugated metabolites from urine of progesterone-treated rabbits were examined for steroids. The ketonic x-fraction, derived from the conjugated metabolites, was the only one of these four fractions in which metabolites of exogenously administered progesterone could be detected. The pattern of metabolites revealed on paper chromatography (Fig. 1c) was qualitatively unaffected either by the sex of the animal, the dose level (50-500 mg.) of progesterone administered or the method of hydrolysis (i.e. hydrochloric acid or β-glucuronidase). The most noticeable difference from the ketonic fraction of human-pregnancy urine (Fig. 1b) was the presence in the rabbit urine of an ultraviolet-absorbing steroid having mobility 0-51 relative to 3α-hydroxy-5β-pregnan-20-one. A positive response to the sodium hydroxide-fluorescence reaction and the formation of an orange-coloured derivative with 2,4-dinitrophenylhydrazine were indicative of a Δ4-3-oxo steroid. The metabolite was eluted with methanol and the eluate had the expected absorption maximum at 240 μ. Since oxidation of the eluted material...
gave a substance having the same $R_p$ as progesterone, the possible structures for the metabolite were limited to that of 20$\alpha$-hydroxyprogren-4-en-3-one and its 20$\beta$-epimer. These two pregnenolones are readily differentiated by paper chromatography (Fig. 1d) and the metabolite had the same $R_p$ as that of 20$\alpha$-hydroxyprogren-4-en-3-one. Examination of the products of acid hydrolysis of the conjugated metabolites of 19-norprogesterone showed that an ultraviolet-absorbing steroid was a major component of the ketonic $\alpha$-fraction (Fig. 1e). It gave the colour reactions characteristic of a $\Delta^4$-3-oxo steroid and on oxidation it gave material having the same $R_p$ as 19-norprogesterone. The metabolite was isolated and shown to be the expected 20$\alpha$-hydroxy-19-norpregn-4-en-3-one by comparison of $R_p$ values and by mixed-melting-point determination with the synthesized compound (IV).

Each of the chromatograms (Fig. 1c and 1e) showed, in addition to an ultraviolet-absorbing steroid, a second, more mobile component which was located as a diffuse, faint-yellow spot on staining the paper with 2,4-dinitrophenylhydrazine. The progesterone metabolite was only slightly less mobile than 3$\alpha$-hydroxy-5$\beta$-pregnan-20-one but it has been confirmed by isopredilution methods (unpublished) that 3$\alpha$-hydroxy-5$\beta$-pregnan-20-one is not present to any significant extent.

**DISCUSSION**

*Structural assignment.* The preparation of the two reference compounds (IV) was based on the method used by Heyl & Herr (1953) for the conversion of androst-4-ene-3,17-dione into testosterone. The infrared and ultraviolet spectra and elementary analyses of the two purified compounds were in accord with their formulation as 20$\alpha$-hydroxy-19-norpregn-4-en-3-ones. Although lithium aluminium hydride reduction of these structural assignments to the nor-compounds. 20$\beta$-Hydroxyprogren-4-en-3-one and 20$\beta$-hydroxy-19-norpregn-4-en-3-one are both less polar than their respective 20$\alpha$-epimers, the mobility ratios being 0:61 for the two nor-compounds and 0:69 for their C-10 methyl analogues. This difference in mobility of 20$\alpha$- and 20$\beta$-ols can be correlated with the degree of steric hindrance to which the hydroxyl groups are subject. Dreding models show that a 20$\beta$-ol differs from a 20$\alpha$-ol in that it is shielded not only by the common C-21 methyl groups, but also by the C-18 (axial) methyl groups and 12$\beta$- (equatorial) hydrogen atom. In effect, the 20$\beta$-ol is encased by neighbouring groups to a greater extent than is its epimer and, consequently, would be expected to be the less polar of the two. The fact that 20$\beta$-hydroxy-19-norpregn-4-en-3-one is less polar than 20$\alpha$-hydroxyprogren-4-en-3-one would also tend to support the validity of the structural assignment given to the norsteroid; were it, instead, the 20$\alpha$-ol, this would be contrary to general experience that 19-norsteroids are more polar than their C-10 methyl analogues (Zaffaroni et al. 1958).

(3) In Table 1 the molecular rotations of the 19-norpregnenolones are compared with those of the

<table>
<thead>
<tr>
<th>Compound</th>
<th>$[M]_D$</th>
<th>$[M]_D$ (nor)</th>
<th>$[M]_D - [M]_D$ (nor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20$\alpha$-Hydroxyprogren-4-en-3-one</td>
<td>+330°*</td>
<td>+162°</td>
<td>+168°</td>
</tr>
<tr>
<td>20$\beta$-Hydroxyprogren-4-en-3-one</td>
<td>+272°*</td>
<td>+105°</td>
<td>+167°</td>
</tr>
<tr>
<td>Progesterone</td>
<td>+640°</td>
<td>+441°</td>
<td>+199°†</td>
</tr>
</tbody>
</table>

corresponding 19-methyl compounds. In both series the 20α-ols are the more dextrorotatory of the two epimers. The molecular-rotation contribution for the C-19 methyl group, [M]Dα-[M]Dβ(nor), calculated from both sets of 20-ols, falls within the range of values +150° to +220° determined for other Δ4-3-oxo 19-norsteroids (Sandoval, Thomas, Djerassi, Rosenkranz & Sondheimer, 1955; Zaffaroni et al. 1958). The molecular-rotation contribution for a C-18 methyl group (+199°, calculated from 18-norprogesterone) is of the same sign and order of magnitude as that for a C-19 methyl group (Anliker, Muller, Perelman, Wohlfahrt & Heusser, 1959).

Metabolism of progesterone. Previous studies on the metabolism of progesterone in man and in the rabbit suggest that both species have the catabolic pathway: Progesterone → 5β-pregnane-3α,20α-diol → 3α-hydroxy-5β-pregn-20-one = 5β-pregnane 3α,20α-diol, for the following reasons:

1. 3α-Hydroxy-5β-pregn-20-one and 5β-pregnane 3α,20α-diol are found together in urine as metabolites of exogenously administered progesterone in the human (Dorfman, Ross & Shipley, 1948) as well as in human-pregnancy urine (Marrian & Gough, 1946). The rabbit also excretes 5β-pregnane 3α,20α-diol as a metabolic end-product of both endogenous (Verley et al. 1950) and exogenous (Hoffman & Browne, 1942; Hoffman, 1942; Westphal, 1942) progesterone.

2. There is a marked similarity in the results obtained on incubation of progesterone with rabbit liver (Taylor, 1955) and human liver (Atherden, 1959). In both experiments 3α-hydroxy-5β-pregn-20-one and 5β-pregnane 3α,20α-diol were isolated in relatively high yields. One difference was that 5β-pregnane 3α,20α-diol was found in the experiment with human liver, but not in that with rabbit liver. However, the types of metabolites found in both experiments were in accord with the concept that reduction of ring A preceded reduction of the 20-ketone.

3. That reduction of ring A involves irreversible saturation of the double bond followed by reduction of the 3-oxo group (Tomkins & Isselbacher, 1954) has been demonstrated for the rabbit in so far as 3α-hydroxy-5β-pregn-20-one and 5β-pregnane 3α,20α-dione, but not progesterone, have been found on incubation of 5β-pregnane 3α,20α-diol with rabbit-liver homogenate (Taylor, 1956).

The surprising occurrence of 20α-hydroxyprogren-4-en-3-one as a metabolite of progesterone in rabbits, together with the failure to find 3α-hydroxy-5β-pregn-20-one, suggests that there may be different metabolic pathways for progesterone in the two species. Although one cannot preclude the possibility that 3α-hydroxy-5β-pregn-20-one is an intermediary metabolite in the formation of 5β-pregnane 3α,20α-diol in vivo in the rabbit, the alternative pathway in which reduction of the 20-ketone precedes ring-A reduction is worthy of consideration.

The absence in 19-norprogesterone of the protective methyl group at C-10 does not appear to make the αβ-unsaturated ketone system any more susceptible to reduction in the rabbit, as judged by the presence of 20α-hydroxy-19-norpregn-4-en-3-one in the urine. Girardi, Jadrijević, Iglesias & Lipschutz (1958), in comparing the antifibrinogenic activities of 19-norprogesterone and 17α-ethynyl-19-nortestosterone in the guinea pig with the activities of their respective C-10 methyl analogues, concluded that ‘the 19-nor configuration is of no consequence with regard to resistance in the liver (19-nor-ethinyltestosterone) and neither is it of any consequence as to non-resistance (19-norprogesterone)’. Also, the stereochemical course of reduction of ring A of 19-nortestosterone to saturated neutral metabolites does not appear to be significantly influenced by the absence of the angular methyl group either in vivo on administration to a female patient (Engel, Alexander & Wheeler, 1958) or on incubation with female rat-liver homogenate (Kupfer, Forchielli & Dorfman, 1960).

SUMMARY

1. The ketonic fractions of the conjugated metabolites in urine of rabbits treated with progesterone and 19-norprogesterone have been examined.

2. The occurrence of 20α-hydroxyprogren-4-en-3-one as a metabolite of exogenously administered progesterone, together with the absence of 3α-hydroxy-5β-pregn-20-one, suggests that the catabolic pathway leading to 5β-pregnane 3α,20α-diol is different in the rabbit from that in the human.

3. 20α-Hydroxy-19-norpregn-4-en-3-one has been identified as a metabolite of 19-norpregesterone. The former compounds and its 20β-epimer have been synthesized.

The author is happy to acknowledge his thanks to Professor C. Djerassi for a generous gift of 19-norprogesterone and to Dr R. E. Bowman and Dr D. D. Evans (Parke Davis and Co., Hounslow, Middlesex) for the infrared spectra and elementary analyses of the two 19-norpregnenolones. Samples of 20α- and 20β-hydroxyprogren-4-en-3-one were obtained from the Medical Research Council Steroid Reference Collection through the courtesy of Professor W. Klyne. The technical assistance of Miss A. M. Cooke is most gratefully acknowledged.

REFERENCES


Fractionation of the Products of the Direct Sulphation of Monosaccharides on Anion-Exchange Resin

BY A. G. LLOYD
Department of Biochemistry, University of Wales, Newport Road, Cardiff

(Received 20 November 1961)

Previous methods for the preparation of monosaccharide sulphate esters have involved either the use of definitive synthetic routes (Percival & Soutar, 1940; Lloyd, 1959; Peat, Turvey, Clancy & Williams, 1960; Guiseley & Ruoff, 1961) or the fractionation of the products of the direct sulphation of monosaccharides by chromatography on cellulose columns (Lloyd, 1959; Turvey & Clancy, 1959; Peat et al. 1960), by electrophoresis on cellulose columns (Lloyd, 1960) or by repeated recrystallization of the crude salt (Guiseley & Ruoff, 1961). The present report describes the use of anion-exchange chromatography as an alternative general method for the isolation of the monosaccharide sulphate products of direct esterification procedures.

MATERIALS AND METHODS

Monosaccharide 6-O-sulphate esters. Authentic samples of the potassium salts of the 6-O-sulphate esters of D-glucose, D-galactose and N-acetyl-D-glucosamine were prepared by the definitive routes described previously (Lloyd, 1959; 1960). Preparations of the potassium salts of D-glucose 6-O-sulphate, D-galactose 6-O-sulphate, N-acetyl-D-glucosamine 6-O-sulphate and N-acetyl-D-galactosamine 6-O-sulphate also were obtained from the products of direct esterification of the appropriate monosaccharides by cellulose-column chromatography (Lloyd, 1959a) or cellulose-column electrophoresis (Lloyd, 1960). Similar methods were employed for the isolation of the corresponding disulphate esters.

Sulphation procedures. Preparations of crude monosaccharide sulphate esters for the present fractionation studies were obtained in two ways. In procedure I the monosaccharide (0.01 mole) was dissolved in the minimum amount of dry pyridine in the presence of anhydrous CaSO₄ (200 mg) before cooling to 5°C and the dropwise addition of 0.01 mole of chlorosulphonic acid in 0.9 ml of dry CHCl₃. The temperature of the reaction mixture was maintained at 5°C during the course of the addition. The mixture was stirred at 5°C for 30 min., at 25°C for 2 hr. and then allowed to stand at 25°C for 2 hr., when the crude sulphate ester separated as an oily lower layer. The upper pyridine layer was removed by decantation and the oil dissolved in the minimum amount of water with vigorous stirring. The remainder of the procedure for the isolation of the potassium salts, including recrystallization via the brucine salt, was according to Lloyd (1960).

For comparative purposes preparations were also obtained from D-glucose and D-galactose by procedure II, namely that of Peat et al. (1960), in which the monosaccharide (0.01 mole) dissolved in the minimum amount of dry pyridine was treated with an excess (0.03 mole) of the