***allo*Pregnane-3 α :20 α -diol in the Urine of Pregnant Women**

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*allo*Pregnane-3 α :20 α -diol was first isolated from human pregnancy urine by Hartmann & Locher (1935). The method employed consisted essentially of separating pregnane-3 α :20 α -diol diacetate from an acetylated crude 'pregnanediol' fraction as completely as possible by crystallization from ethanol, saponifying the mother liquor, and crystallizing from ethanol. A similar method was employed later by Beall (1937). A method for the estimation of the amount of *allo*pregnane-3 α :20 α -diol in urine, involving oxidation of the crude 'pregnanediol' fraction and separation of the mixed diketones by fractional crystallization of their disemicarbazones, was suggested by Marker, Kamm, Crooks, Oakwood, Lawson & Wittle (1937), and later Marker, Wittle & Lawson (1938) published figures indicating that the ratio of pregnane-3 α :20 α -diol to *allo*pregnane-3 α :20 α -diol in the urine of pregnant women is about 2:1.

In the course of other work in this laboratory the chance isolation of *allo*pregnane-3 α :20 α -diol in a state of high purity by a relatively simple procedure suggested the possibility of developing a method which would be more satisfactory for semi-quantitative work than the isolation process of Hartmann & Locher or the somewhat drastic procedure of Marker *et al.* In the present paper such a method is described and a re-examination of the problem of the ratio of pregnane-3 α :20 α -diol to *allo*pregnane-3 α :20 α -diol in the urine of pregnant women is reported. Some preliminary observations upon the conjugated form of *allo*pregnane-3 α :20 α -diol in human pregnancy urine are also reported.

EXPERIMENTAL

Methods. All melting points were determined on an improvised hot-stage apparatus of the Kofler type (Klyne & Rankellor, 1947). The apparatus was calibrated with pure substances of known melting point. The melting points recorded are therefore corrected.

Al₂O₃ used for chromatography was obtained from Peter Spence and Sons and was activated before use to activity II, as determined by the method of Brockmann & Schodder (1941), by heating *in vacuo* at 100°.

All solvents used were carefully dried and redistilled. Samples for analysis were dried over P₂O₅ *in vacuo* for 6 hr. at 80° unless stated otherwise.

Initial isolation of allopregnane-3 α :20 α -diol

The starting material was the 95% acetone-soluble fraction from the preparation of 'sodium pregnanediol glucuronidate' by the method of Venning & Browne (1936) from about 450 l. of the urine of pregnant women. (The term 'sodium pregnanediol glucuronidate' is here used to denote the mixture of sodium glucuronides which can be obtained from human pregnancy urine by the method of Venning & Browne.) After hydrolysis with HCl the ether-soluble neutral fraction (28.6 g.) was prepared in the usual way and separated into ketonic and non-ketonic fractions by means of Girard's reagent T (Girard & Sandulesco, 1936). The non-ketonic fraction (15.4 g.) was dissolved in benzene and poured on a column containing 450 g. Al₂O₃. The column was then eluted successively with 2600 ml. benzene, 3000 ml. ether and 4000 ml. acetone. The acetone eluate on evaporation to dryness yielded a brown crystalline mass weighing 7.5 g. This material on recrystallization from methanol yielded 220 mg. of colourless stout crystalline rods melting at 245–245.5°. On admixture with authentic pregnane-3 α :20 α -diol the melting point was 214–216°. (Found: C, 78.7, 78.7; H, 11.1, 10.9. Calc. for C₂₇H₄₆O₂: C, 78.8; H, 11.2%.)

An acetate of the substance was prepared in the usual manner with acetic anhydride and pyridine. After recrystallization from methanol a product melting at 141.5° was obtained. (Found: C, 74.6; H, 9.9. Calc. for C₂₈H₄₈O₄: C, 74.3; H, 9.9%.) $[\alpha]_D^{19} + 15.5 \pm 0.1^\circ$ in ethanol (c, 0.5608).

A portion of the original substance was oxidized at room temperature overnight with CrO₃ in 90% aqueous acetic acid. The product after crystallizing once from aqueous ethanol and once from acetone melted at 200–202° with preliminary softening at 195°.

On treatment with semicarbazide hydrochloride in aqueous pyridine the oxidation product yielded a semicarbazone which after washing with hot ethanol did not melt below 300°. The sample for analysis was dried for 8 hr. at 130°. (Found: N, 19.6. Calc. for C₂₃H₃₈O₂N₂: N, 19.6%.)

A comparison of the properties of the substance isolated and its derivatives with those of *allo*pregnane-3 α :20 α -diol and its corresponding derivatives which are reported in the literature provided convincing proof that the substance was *allo*pregnane-3 α :20 α -diol (see Table 1).

Solubilities of pregnane-3 α :20 α -diol and allopregnane-3 α :20 α -diol

Mixtures consisting largely of pregnane-3 α :20 α -diol and *allo*pregnane-3 α :20 α -diol can be obtained from the neutral ether-soluble fraction of acid-hydrolysed human pregnancy urine by the ethanol-

Table 1. *Comparison of substance isolated with allopregnane-3 α :20 α -diol*

Substance isolated			<i>allo</i> Pregnane-3 α :20 α -diol		
Original substance	m.p.	245–245.5°	Diol	m.p.	248–248.5° (a)
	Analysis (found)	C, 78.7% H, 11.0%		Analysis (calc.)	C, 78.8% H, 11.2%
Acetylated product	m.p. [α] _D ¹⁹ ° (ethanol)	141.5–142.5° + 15.5°	Diacetate	m.p. [α] _D ¹⁹ ° (benzene) [α] _D ²⁴ ° (ethanol)	141.5–142.5° (a) + 18.8° (a) + 16.0° (c)
	Analysis (found)	C, 74.6% H, 9.9%		Analysis (calc.)	C, 74.3% H, 9.9%
Oxidation product	m.p.	200–202°	Diketone	m.p.	204–204.5° (a)
Semicarbazone of oxidation product	m.p. Analysis (found)	>300° N, 19.6%	Disemicarbazone of diketone	m.p. Analysis (calc.)	>325 (b) N, 19.6%

(a) Hartmann & Locher (1935).

(b) Marker *et al.* (1937).

(c) Hirschmann & Hirschmann (1950).

Table 2. *Solubilities of pregnane-3 α :20 α -diol and allopregnane-3 α :20 α -diol*

(The values shown are in g./100 ml.; each is the mean of duplicate determinations.)

	Pregnane-3 α :20 α -diol			<i>allo</i> Pregnane-3 α :20 α -diol		
	At b.p. of solvent	At 20°	At 0°	At b.p. of solvent	At 20°	At 0°
Methanol	1.8	—	0.33	0.45	—	0.07
Ethanol	2.6	—	0.50	1.0	—	0.18
Benzene	0.55	0.06	—	0.28	0.06	—
Acetone	—	—	0.03	—	—	0.02
Chloroform	—	0.23	—	—	0.13	—

water precipitation process of Astwood & Jones (1941), or by chromatography on Al₂O₃. As a first step towards developing a simple procedure for the isolation in high yield of *allopregnane-3 α :20 α -diol* from such mixtures, the approximate solubilities of both diols were determined at various temperatures, in various solvents with the aid of the apparatus described by Klyne & Bell (1946). The results of the determinations are shown in Table 2.

Separation of a mixture of pregnane-3 α :20 α -diol and allopregnane-3 α :20 α -diol

The fact that the solubilities of the two diols in benzene at 20° are about the same, whereas *pregnane-3 α :20 α -diol* is about five times more soluble than *allopregnane-3 α :20 α -diol* in methanol at 0° indicated that it should be possible to isolate the latter in high yield from a pure binary mixture by leaching with a suitable volume of benzene and crystallizing the benzene-soluble fraction at 0° from methanol. To test this possibility the following experiment was performed.

Pregnane-3 α :20 α -diol. (700 mg., m.p. 236–237°) was refluxed for 1 hr. with 100 ml. benzene. After standing overnight at room temperature the insoluble material was filtered off and dried, and the benzene filtrate evaporated to dryness. The benzene-soluble material was dissolved in 7 ml. methanol and the mixture set aside in the refrigerator to crystallize. After 3 days fine white needles melting at 237° were deposited. The melting point was unchanged on admixture

with authentic *pregnane-3 α :20 α -diol*. Since the benzene-soluble fraction yielded only *pregnane-3 α :20 α -diol* on crystallization from methanol, it was assumed with some confidence that the benzene-insoluble material was *pregnane-3 α :20 α -diol* uncontaminated with any traces of *allopregnane-3 α :20 α -diol*.

This material (670 mg.) and *allopregnane-3 α :20 α -diol* (13.5 mg.) were dissolved in ethanol and the solution evaporated to dryness. The residue was refluxed for 2 hr. with 100 ml. benzene and after standing overnight the mixture was filtered. The benzene-soluble material obtained by evaporation of the filtrate was dissolved in 5 ml. methanol and the solution allowed to stand for 7 days. The thick crystalline rods which separated (7.0 mg.) melted at 242–243° and did not depress the melting point of authentic *allopregnane-3 α :20 α -diol*.

A further 1.5 mg. of *allopregnane-3 α :20 α -diol* (m.p. 238–241°) was obtained by treating the benzene-insoluble material again with a further 100 ml. benzene.

From the mixture of 670 mg. of *pregnane-3 α :20 α -diol* and 13.5 mg. of *allopregnane-3 α :20 α -diol*, a total of 8.5 mg. (63%) of the latter was recovered.

Recovery of allopregnane-3 α :20 α -diol added to the neutral ether-soluble fraction of human urine

Although the separation of *allopregnane-3 α :20 α -diol* in high yield from a pure binary mixture of both diols had been accomplished, it could not be assumed that the procedure would necessarily give such satisfactory results when applied to fractions obtained from human urine. To test this point a recovery experiment was carried out using the

neutral ether-soluble fraction from the acid-hydrolysed urine obtained from a post-menopausal woman receiving 100 mg. progesterone/day by intramuscular injection for 15 days.

All the urine excreted during the 15-day period of treatment was collected and hydrolysed by boiling for 10 min. after the addition of one-tenth of its volume of concentrated HCl. The neutral ether-soluble fraction (1.24 g.) was obtained in the usual way and after solution in ethanol was divided into two equal portions. To one of these portions 10 mg. of *allopregnane-3 α :20 α -diol* was added. Both portions were then separately and simultaneously treated as follows: After evaporation of the ethanol the residue was dissolved in 100 ml. benzene and the solution poured on to a column containing 20 g. Al_2O_3 . The column was eluted in succession with 1000 ml. benzene, 1000 ml. ether and 350 ml. 15% (v/v) methanol in acetone.

The residue obtained by the evaporation of the methanol-acetone eluate was refluxed with 25 ml. benzene, and after standing overnight at room temperature the mixture was filtered. The benzene filtrate was then poured on to a column containing 5 g. Al_2O_3 . The column was eluted successively with 200 ml. benzene, 100 ml. 50% (v/v) ether-benzene, 100 ml. ether, and 75 ml. 5% (v/v) methanol in ether. The methanol-ether eluate was evaporated to dryness and the semi-crystalline residue crystallized twice from methanol.

The portion of the starting material to which no *allopregnane-3 α :20 α -diol* had been added yielded 1.0 mg. of thick crystalline rods melting at 238–244° which did not significantly depress the melting point of authentic *allopregnane-3 α :20 α -diol*. The portion to which 10 mg. of *allopregnane-3 α :20 α -diol* had been added yielded 8.0 mg. of similarly shaped crystals melting at 239–242° which also did not depress the melting point of the authentic compound. Thus 70% of the *allopregnane-3 α :20 α -diol* added to the neutral ether-soluble urinary fraction was recovered.

The isolation of *allopregnane-3 α :20 α -diol* from the control urine in this experiment does not constitute conclusive evidence that this substance is a metabolic reduction product of progesterone. Urine collected from the same woman before or after the progesterone treatment was not examined, and accordingly the possibility that the small amount of *allopregnane-3 α :20 α -diol* isolated had some endogenous source could not be excluded.

Isolation of pregnane-3 α :20 α -diol and allopregnane-3 α :20 α -diol from the urine of pregnant women

The result of the above-mentioned experiment indicated that the application of similar methods to the urine of pregnant women might yield reliable information concerning the relative amounts of *pregnane-3 α :20 α -diol* and *allopregnane-3 α :20 α -diol* present.

The neutral ether-soluble fraction from 98 l. of pooled acid-hydrolysed late pregnancy urine was prepared in the usual manner. This fraction (10.5 g.) was dissolved in 500 ml. benzene and poured through a column containing 300 g. Al_2O_3 . After exhaustive elution with benzene and with ether the desired fraction was eluted with acetone. The residue obtained on evaporation of the acetone was deeply pigmented and somewhat oily; accordingly it was decided to purify it further by the precipitation procedure of Astwood & Jones

(1941) as modified by Sommerville, Marrian & Kellar (1948) before proceeding to the benzene extraction.

The diol fraction was dissolved in 1000 ml. ethanol and 4000 ml. water was added slowly while maintaining the temperature of the mixture at 75°. The mixture was then allowed to stand in the incubator at 37° overnight. After the addition of 6 g. Supercel filter aid the mixture was shaken vigorously, and 2 hr. later filtered with suction through a pad of Supercel on a sintered-glass funnel. The diol fraction obtained by the elution of the Supercel pad with boiling ethanol and evaporation of the latter was still highly pigmented; accordingly it was treated with Norit charcoal in boiling ethanolic solution. The ethanolic filtrate on evaporation to dryness yielded 2.55 g. of a brown crystalline solid.

This material was refluxed for 2 hr. with 200 ml. benzene, and after standing at room temperature overnight the solid was filtered off. The latter was returned to the original flask while the benzene filtrate was poured through a column containing 1 g. Al_2O_3 . The desired fraction was obtained by elution with 5% (v/v) methanol in ether after exhaustive elution with benzene and with ether. The material obtained by evaporation of the methanol-ether eluate was dissolved in a small volume of methanol and set aside to crystallize at room temperature over a period of 10 days. The solid crystalline material thus obtained was recrystallized from methanol and yielded 21 mg. of thick crystalline rods melting at 235–242°. This material did not significantly depress the melting point of authentic *allopregnane-3 α :20 α -diol* and was evidently a somewhat impure specimen of the latter.

By further treatments of the benzene-insoluble fraction in the same manner with benzene and by reworking the methanol mother liquors, additional crops of thick crystalline rods melting at 237–242° (not depressing the m.p. of authentic *allopregnane-3 α :20 α -diol*) and amounting to 31.5 mg., were obtained.

The final benzene-insoluble fraction was purified by the modified Astwood-Jones precipitation method as previously described, using 250 ml. ethanol and 1000 ml. water. The precipitated material after a further leaching with benzene weighed 1.70 g., melted at 235–237° and on admixture with authentic *pregnane-3 α :20 α -diol* did not depress the melting point of the latter.

Altogether the 98 l. of urine yielded about 1700 mg. of *pregnane-3 α :20 α -diol* and 52.5 mg. of *allopregnane-3 α :20 α -diol*, and accordingly the ratio of the amounts of these two steroids isolated was about 30:1. Since the isolation of neither steroid was strictly quantitative, this ratio may not represent the exact relative proportion of the two substances actually present in the urine. Nevertheless, it is improbable that it is grossly in error.

It will be seen that this approximate ratio differs strikingly from that of 2:1 given by Marker *et al.* (1938). Quite apart from this discrepancy, however, consideration of the solubility figures for the two diols presented in the present paper seems to make it quite clear that the latter ratio must be incorrect. If the ratio of the two diols in a crude urinary diol fraction were indeed 2:1, then the solubility figures indicate that the first crop of crystals obtained by crystallization from methanol or ethanol should

consist largely of *allopregnane-3 α :20 α -diol*: common experience, however, is that the first crop of crystals is invariably *pregnane-3 α :20 α -diol*.

Some observations on the conjugated form of allopregnane-3 α :20 α -diol in human pregnancy urine

The ready isolation of *allopregnane-3 α :20 α -diol*, uncontaminated by *pregnane-3 α :20 α -diol*, from the hydrolysis products of the 95 % aqueous acetone filtrate obtained in the preparation of 'sodium pregnanediol glucuronide', clearly showed that the ratio of *pregnane-3 α :20 α -diol* to *allopregnane-3 α :20 α -diol* must be considerably lower than 30:1 in this fraction. The relative enrichment of this fraction in *allopregnane-3 α :20 α -diol* suggested either that the latter is present in urine in the free state or that its conjugated form is less completely precipitated from aqueous solution by acetone than is 'sodium pregnanediol glucuronide'.

To test the first alternative the neutral ether-soluble fraction from 30 l. of fresh unhydrolysed human pregnancy urine was examined for the presence of *allopregnane-3 α :20 α -diol*. None was detected, and it is accordingly concluded that the substance is not present in urine in the free unconjugated state.

To test the second alternative two batches of 'sodium pregnanediol glucuronide' were hydrolysed with acid and the neutral acid-soluble hydrolysis products examined for *allopregnane-3 α :20 α -diol*. The first batch, which had been purified by two acetone purifications, yielded significant amounts of *allopregnane-3 α :20 α -diol*. A second batch, made up from various samples of 'sodium pregnanediol glucuronide', each of which had been purified by at least two acetone precipitations, was subjected to three further acetone precipitations. The hydrolysis

products of the material finally precipitated yielded no *allopregnane-3 α :20 α -diol*.

This experiment is admittedly inconclusive since the two batches of 'sodium pregnanediol glucuronide' which were examined had different origins. However, the results obtained, considered together with the initial isolation of *allopregnane-3 α :20 α -diol* from the 95 % acetone-soluble fraction, suggest that the conjugated form of *allopregnane-3 α :20 α -diol* is more soluble in 95 % acetone than 'sodium pregnanediol glucuronide' and can be separated from the latter by repeated precipitation from water with acetone.

SUMMARY

1. A simple method for the isolation of *allopregnane-3 α :20 α -diol* from human urine has been devised. Evidence is presented to suggest that about 70 % of the *allopregnanediol* actually present can be isolated by this method.

2. The ratio of *pregnane-3 α :20 α -diol* to *allopregnane-3 α :20 α -diol* in a mixed sample of the urine of pregnant women has been found to be about 30:1.

3. *allopregnane-3 α :20 α -diol* is not present in the free state in human pregnancy urine. Preliminary evidence suggests that the *allopregnane-3 α :20 α -diol* conjugate in crude 'sodium pregnanediol glucuronide' may be completely removed by repeated precipitation from aqueous solution with 20 vol. of acetone.

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