REFERENCES

- Boas, I. (1933). *Dtsch. med. Wschr.* **59**, 126.
Gray, C. H. & Holt, L. B. (1947). *J. biol. Chem.* **169**, 235.
Rawlinson, W. A. & Hale, J. H. (1949). *Biochem. J.* **45**, 247.
Theorell, H. (1939). *Enzymologia*, **6**, 88.
Warburg, O. & Negelein, E. (1932). *Biochem. Z.* **244**, 9.

The Metabolic Reduction of Progesterone to *allo*Pregnane-3 α :20 α -diol in Human Subjects

By T. I. KYLE AND G. F. MARRIAN

Department of Biochemistry, University of Edinburgh

(Received 9 January 1951)

Since the original isolation of *allo*pregnane-3 α :20 α -diol from the urine of pregnant women by Hartmann & Locher (1935) it has been generally assumed that this steroid, like its C-5 stereoisomer pregnane-3 α :20 α -diol, is a metabolic reduction product of progesterone. Hitherto, however, no satisfactory experimental evidence to warrant this assumption has been published.*

The isolation of *allo*pregnane-3 α :20 α -diol from the urine of a post-menopausal woman receiving progesterone treatment has recently been reported from this laboratory (Kyle & Marrian, 1951), but since control urine collected from the same subject before or after the treatment was not examined, it could not be concluded that the isolated steroid had necessarily arisen by reduction of the administered hormone. The possibility that the *allo*pregnane-3 α :20 α -diol had originated from some endogenous source, and otherwise than by the reduction of progesterone, did not seem to be entirely remote in view of the reported findings of Dobriner, Lieberman, Rhoads & Taylor (1948). These workers, in a study of the ketosteroid excretion of a pregnant woman, showed that whereas the urinary pregnan-3 α -ol-20-one increased steadily from the third month until term, the excretion of *allo*pregnan-3 α -ol-20-one excretion remained relatively constant at about 1–2 mg./24 hr. throughout pregnancy. In view of these findings the possibility could not be entirely ignored that the urinary *allo*pregnane steroids, in contrast to those of the pregnane series, might arise from some precursor other than progesterone.

In the present work *allo*pregnane-3 α :20 α -diol has been isolated from the urine of one normal post-menopausal woman and one rheumatoid arthritic

man who were being treated daily with large doses of progesterone by intramuscular injection. In both cases urine specimens were collected over adequate control periods after the cessation of the hormone treatment and were examined by methods as nearly identical as possible with those used for the 'progesterone' urines. In neither instance could any *allo*pregnane-3 α :20 α -diol be isolated from these control urines, and it can therefore be concluded that the material isolated from the 'progesterone' urines had indeed been formed by the reduction of the administered progesterone.

It is of some considerable interest that the relative proportions of pregnane-3 α :20 α -diol and *allo*pregnane-3 α :20 α -diol isolated from the urine during periods of progesterone administration in these experiments (approx. 30:1) agree very closely with the relative proportion of these two steroids isolated from the urine of pregnant women by Kyle & Marrian (1951). This suggests that the *allo*pregnane-3 α :20 α -diol present in the urine during pregnancy arises largely by the metabolic reduction of endogenous progesterone.

EXPERIMENTAL

Methods

Melting points were determined as previously described (Kyle & Marrian, 1951). Al_2O_3 (Peter Spence and Sons) used for chromatography was activated to activity II, as determined by the method of Brockmann & Schodder (1941), by heating to 100° *in vacuo*. In most cases the amount of Al_2O_3 used was 30 times the weight of the fraction to be chromatographed.

All solvents were dried and redistilled before use.

Collection, extraction and fractionation of urine specimens

Urine was collected daily from each case using toluene as preservative, and hydrolysed by boiling under reflux for

* Since the completion of the present work Dorfman & Unger (1950) have reported that they have demonstrated the conversion of progesterone into *allo*pregnane-3 α :20 α -diol in human subjects. Experimental details of this work are not yet available, however.

10 min. after the addition of 10 vol. % of conc. HCl. The 'neutral ether-soluble fraction' was prepared daily from each hydrolysed specimen in the usual way by extraction with ether, washing the extract with aqueous NaOH and with water, and evaporation of the washed extract to dryness. The neutral ether-soluble fractions obtained from each case were bulked at the end of each experimental period ('progesterone' or control), before further working up.

Each bulked neutral ether-soluble fraction was dissolved in benzene and poured through a column of Al_2O_3 . After exhaustive washing with benzene and with ether, the column was eluted with acetone until evaporation of portions of the eluate yielded little or no solid or gummy material. Evaporation of the combined acetone eluates yielded the 'crude diol fraction'.

First experiment

The first experiment was carried out upon a normal post-menopausal woman who was treated with 50 mg. progesterone/day for 22 days by intramuscular injection.

'Progesterone' period. Complete 24 hr. specimens of urine were collected throughout the period of progesterone treatment. Two-fifths of each specimen were used for another purpose, so that the urine available for the present study was equivalent to 13.2 twenty-four hr. specimens only.

The crude diol fraction (180 mg.) which was a dark-purple semi-crystalline solid, was refluxed for 1 hr. with 25 ml. benzene, and after standing overnight at room temperature the solid material was filtered off. Evaporation of the benzene filtrate yielded 117 mg. of a dark-red gum which was dissolved in 2 ml. methanol. After standing for several days at room temperature the solution deposited crystalline material. The latter was filtered off and recrystallized from about 1.5 ml. methanol when 1.9 mg. of thick crystalline rods melting at 240–244° were obtained. The melting point was unchanged on admixture with authentic *allopregnane-3 α :20 α -diol* (Kyle & Marrian, 1951), but was depressed 20° on admixture with *pregnane-3 α :20 α -diol*. The identity of the isolated material was confirmed by the preparation of an acetate, the melting point of which (136–140°) was not depressed on admixture with authentic *allopregnane-3 α :20 α -diol diacetate* (m.p. 137–139°).

The white solid material filtered off after the treatment of the crude diol fraction with benzene (63 mg.), was treated with benzene a second time in the same manner as before. The residue obtained on evaporation of the second benzene filtrate yielded a small amount of *pregnane-3 α :20 α -diol* but no *allopregnane-3 α :20 α -diol*. The main bulk of the benzene-insoluble material was unfortunately not examined further, but since the corresponding fraction obtained in the 'progesterone' period of the second experiment (see below) proved to be nearly pure *pregnane-3 α :20 α -diol*, there is good reason to believe that this material also consisted largely of *pregnane-3 α :20 α -diol*. The ratio of the amounts of *pregnane-3 α :20 α -diol* and *allopregnane-3 α :20 α -diol* isolated in this experiment was therefore 63:1.9 = 33:1.

Control period. Commencing 1 week after the last dose of progesterone seventeen successive complete 24 hr. urine specimens were collected from the same patient.

The crude diol fraction (105 mg.) was a dark-purple oil. On refluxing with 25 ml. benzene a clear solution was obtained

from which no solid was deposited after standing overnight at room temperature.

Because of the oily character of the diol fraction it was decided to refractonate it chromatographically before examination for the presence of *allopregnane-3 α :20 α -diol*. Accordingly, the benzene solution was poured on to a column of Al_2O_3 which was then eluted successively with benzene (100 ml.), 25% (v/v) ether in benzene (30 ml.), 50% (v/v) ether in benzene (45 ml.), ether (75 ml.), 2% (v/v) acetone in ether (45 ml.), 5% (v/v) acetone in ether (45 ml.), 50% (v/v) acetone in ether (75 ml.), and acetone (60 ml.). Successive 15 ml. volumes of the eluates were evaporated to dryness and the residues examined for the presence of crystalline material by adding 1 or 2 drops of acetone and allowing to stand overnight.

Crystalline material was detected only in the first 15 ml. fraction eluted by 50% acetone in ether. After leaching this fraction with ice-cold acetone, a very small amount of white crystalline material remained which melted at 231–234°. The mixed melting point with authentic *pregnane-3 α :20 α -diol* was 231–235°, while that with authentic *allopregnane-3 α :20 α -diol* was 215–228°. High vacuum sublimation of the acetone leachings of this fraction yielded an additional small amount of crystalline material melting at 232–238°. The mixed melting point of this with authentic *pregnane-3 α :20 α -diol* was 230–235°, while that with authentic *allopregnane-3 α :20 α -diol* was 215–230°.

The isolation of a small amount of *pregnane-3 α :20 α -diol* from the control urine of this patient so long after the cessation of the progesterone treatment occasioned no surprise, since this steroid has been isolated in minute amounts from the urine of untreated men (Westphal, 1944) and from the urine of a post-menopausal women who had received no previous treatment with progesterone (Marrian, 1948).

No *allopregnane-3 α :20 α -diol* could be detected in any of the fractions obtained from the chromatogram.

Second experiment

The subject for this experiment was a rheumatoid arthritic man who was treated with 100 mg. progesterone/day for 14 days by intramuscular injection.

'Progesterone period'. Complete 24 hr. urine specimens were collected throughout the period of progesterone treatment. The crude diol fraction obtained (680 mg.) was refluxed for 1 hr. with 25 ml. benzene, and after standing overnight at room temperature the solid was filtered off. The benzene filtrate was poured onto a column of Al_2O_3 which was then developed with benzene (300 ml.) and ether (100 ml.). The washed column was then eluted with 5% (v/v) acetone in ether (50 ml.), 50% (v/v) acetone in ether (100 ml.), and acetone (150 ml.). Successive 25 ml. portions of the eluates were evaporated to dryness. Crystalline material was detected by 'spotting' with acetone in the last five fractions eluted by ether-acetone and in the first eluted by acetone. These were combined, dissolved in the minimum amount of hot methanol, and the solution set aside in the refrigerator. After several days the thick crystalline rods which had separated were filtered off. These weighed 4.5 mg. and melted at 243–244°. On admixture with authentic *allopregnane-3 α :20 α -diol* the melting point was unchanged, while on admixture with authentic *pregnane-3 α :20 α -diol* it

was depressed to 215–218°. No further *allopregnane-3 α :20 α* -diol could be obtained from the methanol mother liquors or from a second benzene leaching of the solid material in the diol fraction which had not dissolved in the first benzene treatment.

This benzene-insoluble material (120 mg.), although slightly coloured, appeared to consist largely of *pregnane-3 α :20 α* -diol, since it melted at 234–235° and since the mixed melting point with authentic *pregnane-3 α :20 α* -diol was 234–236°.

The ratio of *pregnane-3 α :20 α* -diol to *allopregnane-3 α :20 α* -diol isolated in this experiment was therefore 120:4.5 = 27:1.

First control period. Commencing 2 days after the cessation of the progesterone treatment fourteen complete 24 hr. urine samples were collected. The crude diol fraction (215 mg.), which was a red gum, was refluxed with 25 ml. benzene; it was completely soluble and no solid separated from the solution on standing overnight. This benzene solution was then chromatographed in an identical manner to that described above. Crystalline material was detected only in the second and third fractions eluted with 50% (v/v) ether-acetone. These were combined and crystallized from the minimum volume of methanol, when a few mg. of fine needles with m.p. 234–235° were obtained. A further small amount of crystalline material of m.p. 234–235° was obtained from the methanol mother liquor by high vacuum sublimation and crystallization of the sublimate from acetone. These melting points were not depressed on admixture with authentic *pregnane-3 α :20 α* -diol. No indication of the presence of even traces of *allopregnane-3 α :20 α* -diol could be detected in any of the chromatogram fractions.

Second control period. The failure to detect *allopregnane-3 α :20 α* -diol in the control period urines in both experiments was not considered to provide entirely conclusive evidence that the steroid was indeed not present in amounts comparable to those found in the 'progesterone' period urines: the possibility had to be considered that in the presence of large amounts of *pregnane-3 α :20 α* -diol the solubilities and elutability of *allopregnane-3 α :20 α* -diol might be so altered as to facilitate greatly its isolation. Accordingly, in this experiment it was decided to work up a second batch of control urine adding pure *pregnane-3 α :20 α* -diol to the neutral ether-soluble fraction before separating the diol fraction by chromatography.

Two months after the cessation of the progesterone treatment a further fourteen consecutive 24 hr. urine samples

were collected from the same patient. To the neutral ether-soluble fraction 300 mg. of carefully purified *pregnane-3 α :20 α* -diol was added.

The crude diol fraction was a light-brown crystalline solid weighing 790 mg. It was refluxed with 25 ml. benzene for 2 hr. and after standing overnight at room temperature the solid material was filtered off. The benzene filtrate was poured on to a column of Al_2O_3 which was then developed with benzene (200 ml.) and ether (200 ml.). The washed column was then eluted with 5% (v/v) acetone in ether (50 ml.), 8% (v/v) acetone in ether (50 ml.), 10% (v/v) acetone in ether (25 ml.), 50% (v/v) acetone in ether (100 ml.) and acetone (75 ml.). Successive 25 ml. portions of the eluates were evaporated to dryness and 'spotted' with acetone.

Crystalline material was detected only in the first and second portions of the 50% acetone in ether eluate. These were combined, dissolved in the minimum volume of methanol and the solution left in the refrigerator for 1 week to crystallize. The crystals which separated melted at 230–234° and on admixture with authentic *pregnane-3 α :20 α* -diol the melting point was 233–235°. It was clear, therefore, that in the control urine *allopregnane-3 α :20 α* -diol was not present in an amount comparable to that found in the urine of the same patient during treatment with progesterone.

SUMMARY

1. *alloPregnane-3 α :20 α* -diol has been isolated from the urines of one post-menopausal woman and one rheumatoid arthritic man collected during treatment with progesterone. The weights of *allopregnane-3 α :20 α* -diol isolated corresponded in each case to about 0.3% of the amount of progesterone administered.

2. No trace of *allopregnane-3 α :20 α* -diol could be detected in control urines collected from the same patients, and accordingly it is concluded that the *allopregnane-3 α :20 α* -diol was formed by the metabolic reduction of the administered progesterone.

3. The proportions of *pregnane-3 α :20 α* -diol and *allopregnane-3 α :20 α* -diol isolated after progesterone administration are similar to those found in the urine of pregnant women (Kyle & Marrian, 1951).

REFERENCES

- Brockmann, H. & Schodder, F. (1941). *Ber. dtsch. chem. Ges.* **74**, 73.
Dobriner, K., Lieberman, S., Rhoads, C. P. & Taylor, H. C. (1948). *Obstet. Gynec. Survey*, **3**, 677.
Dorfman, R. I. & Unger, F. (1950). *XVIIIth Int. Physiol. Congr. Abstr.* p. 178.
Hartman, M. & Locher, F. (1935). *Helv. chim. Acta*, **18**, 160.
Kyle, T. I. & Marrian, G. F. (1951). *Biochem. J.* (in the Press).
Marrian, G. F. (1948). Unpublished observation.
Westphal, U. (1944). *Hoppe-Seyl. Z.* **281**, 14.