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The Quantitative Determination and Identification of Pregnane-3α:20α-diol* in the Urine of the Pregnant Rabbit

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Although it has been shown by Heard, Bauld & Hoffman (1941), Hoffman & Browne (1942), Hoffman (1942) and Westphal (1942) that pregnanediol is present in the urine of rabbits following the administration of progesterone, the presence of pregnanediol of endogenous origin in the urine of pregnant rabbits has hitherto not been reported. In fact, Buxton & Westphal (1939) and Westphal (1942) examined the urine of pregnant rabbits for this end product of progesterone metabolism, but were unable to detect its presence. In the belief that previous failures to detect pregnanediol in the urine of rabbits during pregnancy might be due as much to shortcomings in the methods employed as to a low level of excretion, a reinvestigation of the problem was undertaken.

Recently, a sensitive method for the determination of pregnanediol in human urine has been described by Sommerville, Gough & Marrian (1948), and it seemed possible that this method might be applicable to rabbit urine. A series of preliminary experiments on the urine of male rabbits injected with progesterone and on acid-hydrolysed urine from male rabbits after the addition of pure pregnane-3α:20α-diol in varying amounts, showed that the method was, indeed, suitable for the purpose and that as little as 0·25 mg. of pregnanediol could be determined with a satisfactory degree of accuracy in one-half of a 24 hr. specimen of rabbit urine.

The application of this method to the urine of mated female rabbits yielded surprising results. In every instance in which the urine collected immediately after mating was examined, an excretion of 'pregnanediol' varying from about 0·5 to 2·8 mg./24 hr. and lasting for 1–3 days was observed. Following this transient post-copulatory excretion of 'pregnanediol', the values fell and remained at the 'blank' level of about 0·10–0·15 mg./24 hr. until the eight to tenth day, when the 'pregnanediol' rose again and was maintained at a level varying between 0·25 and 0·70 mg./24 hr. until shortly before parturition.

* *Editorial note.* The indices α and β for substituents of known orientation in steroids will in future be shown in the *Biochemical Journal* without parentheses. This practice, suggested by Fieser & Fieser (1949), has recently been adopted by the *Journal of the Chemical Society* and by *British Abstracts*.

Since the sulphuric acid colour reaction, by which pregnanediol is finally determined in this procedure, is not specific for this steroid, it could not be assumed at this stage of the work that the 'pregnanediol' excreted immediately after copulation or that excreted later in pregnancy was, in fact, pregnane-3α:20α-diol. Accordingly, attempts were made to isolate the sulphuric acid chromogen from the pooled extracts of urine specimens from a large number of rabbits. Urine was collected during the 24 hr. following mating and also during the last 20 days of pregnancy. From both batches of pooled extract pregnane-3α:20α-diol was isolated and identified as such unequivocally. Furthermore, the amounts isolated were of the same order of magnitude as those which were anticipated to be present on the basis of the previous colorimetric determinations. There can be little doubt therefore that most, if not all, of the 'pregnanediol' excreted at both periods is indeed pregnane-3α:20α-diol.

Experiments which throw some light on the origin of the pregnanediol excreted immediately after mating have been carried out by one of the present authors (W. G. V.) and will be published elsewhere.

EXPERIMENTAL

Determination of pregnanediol in rabbit urine

Collection of urine and application of the method of Sommerville et al. (1948). Rabbits fed on a pellet diet (Diet 43, Associated London Flour Millers Ltd.) and receiving 200–300 ml. of water daily were housed in metabolism cages, and the urine collected over 24 hr. periods in vessels containing 0·5 ml. toluene as a preservative. Catheterization was not employed and accordingly the specimens obtained did not represent 24 hr. collections exactly.

Since rabbit urine is usually alkaline, the specimens were made faintly acid to litmus with HCl in order that the pH should be similar to that of human urine. Each specimen was then filtered through glass wool, made up to 500 ml. with water, and divided into two 250 ml. samples for the duplicate determinations. Since this volume is half that employed in the original method, all volumes of reagents and solvents used in the determination were halved.

The only other modifications of the original method which were introduced were the following: (i) In the precipitation procedure for the purification of the pregnanediol the times of incubation at 37° were increased to 24 hr. in every case. (ii) The charcoal treatment for the removal of interfering

pigment was found to be unnecessary with rabbit urine. Accordingly, it was replaced by a simple filtration in ethanolic solution through a Whatman no. 1 paper. (Miss E. Sutherland has informed the authors that material giving a yellow colour with H_2SO_4 may sometimes be eluted from filter paper by ethanol. Accordingly, it is now considered to be essential that all filter papers used in the determination of pregnanediol by this method should be thoroughly washed with hot ethanol before use.)

Hydrolysis of the conjugated pregnanediol in rabbit urine. Since Hoffman (1942) has found that after the administration of progesterone to rabbits the pregnanediol in the urine is 'for the most part if not entirely' present as the glucuronide, it seemed probable that conditions of acid hydrolysis giving the maximum yield of free pregnanediol from human urine would also be optimal for rabbit urine containing conjugated pregnanediol. However, it could not be assumed that this was necessarily the case, and, accordingly, experimental verification was sought.

In a preliminary experiment several male rabbits were injected with progesterone and the urine collected during the following 48 hr. was pooled and diluted. Samples of the pooled urine were boiled for 5, 10, 15 and 20 min. after the addition of 0·1 vol. of conc. HCl and the pregnanediol determined in the usual manner. The results indicated that 10 min. boiling gives optimal hydrolysis of the conjugated pregnanediol as it does with human urine.

Later in the course of this work, after it had been demonstrated that pregnanediol of endogenous origin is present in the urine of pregnant rabbits, a similar experiment was carried out on pooled 24 hr. urine specimens obtained from four rabbits 13–15 days pregnant. The results, shown in Table 1, clearly support the conclusion arrived at from the preliminary experiment with the urine of progesterone-injected male rabbits.

Table 1. *Hydrolysis of conjugated pregnanediol in the urine of pregnant rabbits*

Time of boiling with 0·1 vol. of conc. HCl (min.)	Free pregnanediol found (mg./250 ml. diluted urine)
5	0·243
5	0·238
10	0·256
10	0·263
15	0·243
15	0·258
20	0·232
20	0·258

Recovery experiments. The sensitivity of the method and the accuracy of the procedures for extraction of the urine and purification of the pregnanediol were investigated in a series of recovery experiments in which pregnanediol was added to acid-hydrolysed urine from male rabbits. Three 24 hr. urine specimens were collected from three male rabbits, pooled, made up to 1500 ml. with water, and divided into six samples of 250 ml. Each sample was boiled under reflux for 10 min. after the addition of 25 ml. conc. HCl and then cooled. Two of the acid-treated samples were retained as 'blanks', while to the remaining four, pure pregnane-3α:20α-diol in ethanolic solution was added in two different known amounts in duplicate. This procedure was repeated on five occasions and the amounts of added pregnanediol were varied between 0·13 and 2·51 mg. The results of these experiments are shown in Table 2. It will be seen that whereas the recovery was unsatisfactory when 0·13 mg. was added to half a 24 hr. specimen, good recoveries resulted when 0·23 mg. or more was added.

Pregnanediol excretion of rabbits after mating and during pregnancy

The pregnanediol excretion was followed daily in three rabbits from 1 to 2 days before mating until 2 days after the birth of the litter (Figs. 1–3). In these three cases the transient excretion of pregnanediol immediately after mating and the prolonged excretion of the steroid during the latter two-thirds of pregnancy were well marked. In two rabbits (Figs. 4 and 5) urine collections were not commenced

Table 2. *Recovery of pregnanediol added to acid-hydrolysed male rabbit urine*

Pregnanediol added to equiv. of half of 24 hr. urine (mg.)	Apparent pregnanediol recovery (mg.)	Pregnanediol recovery corr. for 'blank' (mg.)	(%)
0·00	0·045	—	—
0·00	0·030	—	—
2·51	2·50	2·46	98·4
2·51	2·47	2·43	97·1
1·26	1·28	1·24	99·0
1·26	1·24	1·20	96·2
0·00	0·018	—	—
0·00	0·023	—	—
1·00	0·99	0·98	97·5
1·00	1·05	1·03	102·5
0·50	0·491	0·471	94·2
0·50	0·484	0·464	92·8
0·00	0·033	—	—
0·00	0·037	—	—
0·50	0·540	0·505	101·0
0·50	0·523	0·488	97·6
0·25	0·295	0·250	100·0
0·25	0·272	0·237	94·8
0·00	0·030	—	—
0·00	—	—	—
0·45	0·448	0·418	92·1
0·45	0·447	0·417	92·1
0·23	0·236	0·206	90·0
0·23	0·235	0·205	90·0
0·00	0·060	—	—
0·00	0·037	—	—
0·25	0·266	0·218	87·2
0·25	0·275	0·222	88·8
0·13	0·120	0·072	57·6
0·13	0·118	0·070	56·0

until 24 hr. after mating, and in these cases the transient post-copulatory excretion of pregnanediol was missed. In order to confirm the transient excretion of pregnanediol after mating, the pregnanediol excretion levels of two rabbits were followed from the day before mating until a few days after (Figs. 6 and 7).

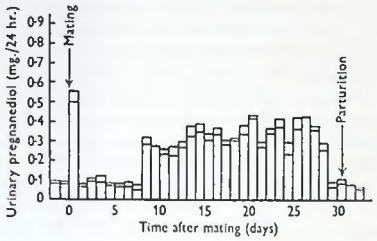

Fig. 1. Pregnanediol excretion of pregnant rabbit.

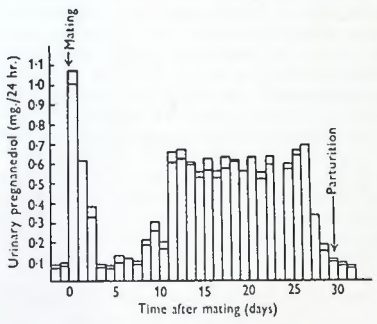

Fig. 2. Pregnanediol excretion of pregnant rabbit.

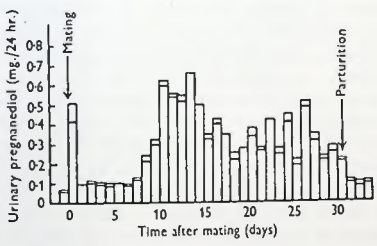

Fig. 3. Pregnanediol excretion of pregnant rabbit.

In these Figures the values for the duplicate determinations are shown by the double lines at the tops of the columns. It should be noted that values of less than 0·50 mg./24 hr. have no strict quantitative significance.

Isolation of pregnane-3α: 20α-diol from the urine of pregnant rabbits (twelfth day until term)

Complete 24 hr. urine samples were collected with toluene as preservative from five pregnant Chinchilla

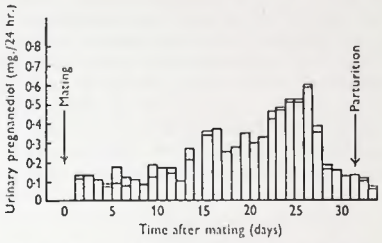

Fig. 4. Pregnanediol excretion of pregnant rabbit.

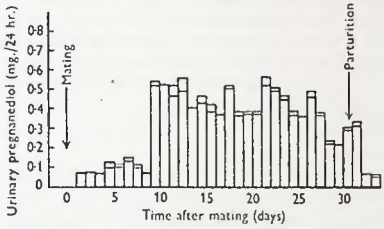

Fig. 5. Pregnanediol excretion of pregnant rabbit.

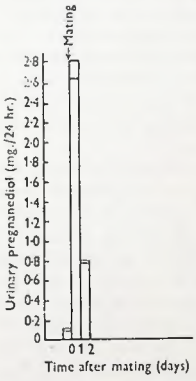

Fig. 6.

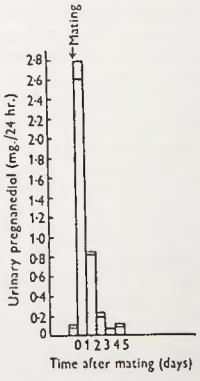

Fig 7.

Fig. 6. Pregnanediol excretion of rabbit (female) after mating.

Fig. 7. Pregnanediol excretion of rabbit (female) after mating.

rabbits from the twelfth day after mating until term. The samples were stored in the refrigerator and worked up at intervals of 3 or 4 days in the following

manner: three or four successive samples from each rabbit were pooled and diluted to 1000 ml. Each diluted, pooled sample was made just acid to litmus with HCl and heated to boiling under reflux with 200 ml. of toluene. Concentrated HCl (100 ml.) was then added and the boiling continued for 10 min. After cooling, the mixture was transferred to a separating funnel, shaken, and the aqueous layer run off from the upper toluene-emulsion layer. The aqueous layer was re-extracted twice more with 200 ml. vol. of toluene and the combined toluene emulsions filtered through a Büchner funnel with gentle suction. The filtrate was then transferred to a separating funnel and the aqueous phase run off. The toluene extract, after washing with 2 × 200 ml. of N-NaOH and 2 × 200 ml. of water, was evaporated to a small volume on a hot plate and then to dryness under reduced pressure in a boiling water bath. The 'toluene-soluble neutral fractions' so obtained were stored in a vacuum desiccator until the completion of the working up of all the urine samples.

The 'toluene-soluble neutral fractions' were combined, and after treatment in the usual manner with trimethylammoniumacetohydrazide chloride (Girard & Sandulesco, 1936) yielded 0·739 g. of non-ketonic material.

An attempt to purify the pregnanediol assumed to be in this material by the Astwood & Jones (1941) precipitation procedure was unsuccessful, since after two successive precipitations from ethanolic solution by 0·1 N-NaOH and water respectively, only 4·9 mg. of solid material were obtained. Accordingly, the combined filtrates from these precipitations, after removal of most of the ethanol by distillation under reduced pressure, were extracted with ether, and the extract evaporated to dryness after washing with water. The non-ketonic material recovered in this manner weighed only 0·309 g.

This material, dissolved in 20 ml. of benzene, was poured on to a column of 9 g. of Al_2O_3 (Peter Spence, Type H; dried at 100° *in vacuo*) and the column eluted successively with eight 20 ml. portions of benzene, twelve 20 ml. portions of anhydrous ether, and seven 20 ml. portions of anhydrous acetone. The residues obtained by evaporation of the second and third acetone eluates contained considerable amounts of white crystalline material, and this was separated from the accompanying gum by chilling overnight in a small volume of acetone. A further small quantity of crystalline material was obtained in a similar manner from the first acetone eluate.

An attempt was made to purify this material by sublimation at 130°/10^{-5} mm. The product (23·6 mg.) was, however, grossly impure. Purification was effected by warming for a few minutes with 2 ml. of benzene, allowing to stand at room temperature for 2 hr. and then filtering off the solid. The benzene-insoluble solid so obtained weighed 17·7 mg. and melted at 232–237° (corr.). Crystallization of this from 1·5 ml. of methanol yielded 7·5 mg. of fine white needles, m.p. 234–237° (corr.). Mixed with authentic pregnane-3α:20α-diol (m.p. 237–238·5°, corr.), the melting point was 233–236° (corr.). Analysis of material dried at 80° for 2 hr. *in vacuo* over P_2O_5: C, 78·3; H, 11·1. Calculated for $C_{21}H_{36}O_2$: C, 78·8; H, 11·2%.

A further quantity of material (m.p. 233–236°, corr.) was obtained from the methanolic mother liquor after evaporation to dryness by two successive treatments with benzene in the manner described above. This was acetylated in the usual manner with acetic anhydride and pyridine, and the product crystallized from hexane. Well-formed stout needles melting at 162–163° and 176–179° (corr.) were obtained. Mixed with authentic pregnane-3α:20α-diol diacetate (m.p. 165–166° and 180–181°) the melting points were 162–163° and 176–179° (corr.).

It has been suggested that the double melting point of pregnane-3α:20α-diol diacetate, which was first observed by Marrian (1929), is characteristic of impure samples (Heard, Hoffman & Mack, 1944). In the experience of the present authors, this is not the case, since the diacetate, even after repeated recrystallizations from methanol and from hexane, still exhibits this phenomenon. Recrystallized samples may melt completely at the lower temperature, or after slight softening at the lower temperature may not melt completely until the higher temperature is reached. Samples melting at the lower temperature, if held at this temperature for a few minutes before melting is quite complete, may resolidify and then do not melt below the higher temperature. Melted samples which do not resolidify before they have supercooled below the lower melting point invariably melt completely at the lower temperature on reheating.

Isolation of pregnane-3α:20 α-diol from the urine of female rabbits after mating

Urine was collected from twenty-one female rabbits during the 24 hr. period immediately following mating. According to the number of specimens obtained on any 1 day, batches of two to five were pooled for hydrolysis and extraction with toluene. In order to avoid the difficulties encountered in the previously described isolation of pregnanediol from rabbit urine (see preceding section), the toluene-soluble fractions from each batch were purified immediately by the precipitation technique as described by Somerville *et al.* (1948). It was evident that the previous failure of the precipitation technique must have been due to the prolonged storage of the neutral fraction, or to the subsequently used Girard separation, or to both, since in the present experiment each batch of neutral fraction yielded light coloured solid material in about the expected amount.

On combination of the solid products obtained from the different batches, 21·8 mg. of slightly pigmented solid contaminated with traces of gum

were obtained. Some of the pigment and most of the gum was removed by leaching the solid first with about 1 ml. of ice-cold acetone and then with about 1 ml. of benzene. The material, after treatment with charcoal in boiling ethanol to remove the remaining pigment and crystallization from 1·25 ml. of methanol, yielded 7·8 mg. of fine white needles, m.p. 232–237° (corr.). Mixed with authentic pregnane-3α:20α-diol (m.p. 237–238·5°, corr.) the melting point was 233–237° (corr.).

Acetylation with acetic anhydride and pyridine yielded a product which, after treatment with charcoal in boiling *n*-hexane followed by crystallization from *n*-hexane, was in the form of stout needles, m.p. 163–165° and 179–181° (corr.). Mixed with authentic pregnane-3α:20α-diol diacetate (m.p. 165–166° and 180–181°, corr.) the melting point was 162–163° and 178–181° (corr.).

SUMMARY

1. It has been shown that, using the method of Sommerville *et al.* (1948), as little as about 0·25 mg. of pregnanediol can be determined with a satisfactory degree of accuracy in one-half of a 24 hr. specimen of rabbit urine.

2. By this procedure the excretion of 'pregnanediol' in the urine of pregnant rabbits has been detected and studied quantitatively. Immediately after mating a transient excretion of 'pregnanediol' lasting for 1–3 days occurs. This is followed at about the eighth to tenth day of pregnancy by a prolonged excretion of 'pregnanediol' which is maintained until just before parturition.

3. The 'pregnanediol' excreted immediately after mating and that excreted later in pregnancy in the rabbit have been isolated and identified as pregnane-3α:20α-diol.

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REFERENCES

Astwood, E. B. & Jones, G. E. S. (1941) *J. biol. Chem.* **137**, 397.

Buxton, C. L. & Westphal, U. (1939). *Proc. Soc. exp. Biol., N.Y.*, **42**, 749.

Fieser, L. F. & Fieser, M. (1949). *Natural Products related to Phenanthrene.* 3rd ed. New York: Reinhold.

Girard, A. & Sandulesco, G (1936). *Helv. chim. Acta*, **19**, 1095.

Heard, R. D. H., Bauld, W. S. & Hoffman, M. M. (1941). *J. biol. Chem.* **141**, 709.

Heard, R. D. H., Hoffman, M. M. & Mack, G. E. (1944). *J. biol. Chem.* **155**, 607.

Hoffman, M. M. (1942). *Canad. med. Ass. J.* **47**, 424.

Hoffman, M. M. & Browne, J. S. L. (1942). *Fed. Proc.* **1**, 41.

Marrian, G. F. (1929). *Biochem. J.* **23**, 1090.

Sommerville, I. F., Gough, N. & Marrian, G. F. (1948). *J. Endocrinol.* **5**, 247.

Westphal, U. (1942). *Hoppe-Seyl. Z.* **271**, 1.