[16-^{3}H]Progesterone Metabolism in Advanced Pregnancy and in Oophorectomized–Hysterectomized Women

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The percentage recovery of administered progesterone as urinary pregnanediol has been reported to be much higher in pregnancy than in the non-pregnant state in the absence of luteal function (Venning & Browne, 1940; Sommerville & Marrian, 1950; Guterman, 1953). (The term 'pregnanediol' as used by some of the authors consisted largely, but not necessarily entirely, of 5β-pregnane-3α:20α-diol.) The extent of this metabolic conversion has similarly been related to the maintenance of pregnancy in patients with threatened abortion (Guterman, 1953). The validity of these observations might bear re-examination—indeed, the data on normal pregnancy could not be confirmed recently (Klopper & Michie, 1956)—in view of the crude, albeit useful, analytical methods employed in the early pioneer investigations; also, the observations of Sommerville & Marrian (1950) were subsequently revised (Marrian, Russell & Atherden, 1954). The determination of the additional amount of pregnanediol in pregnancy urine which arises from administered progesterone is subject to considerable error because (*a*) the control or base-line values for urinary pregnanediol fluctuate from day to day and progressively increase with advancing pregnancy (Venning, 1948), and (*b*) unless very large amounts of progesterone are administered, there is only a small increase in the urinary pregnanediol excretion. These difficulties may be obviated by administering isotopically labelled hormone (such as [16-^{3}H]progesterone) and a reinvestigation of the problem was accordingly undertaken; more information on progesterone metabolism was also expected with the isotope-tracer technique.

EXPERIMENTAL

Materials

[16-^{3}H]Progesterone (nominal specific activity 22·2 μc/mg.) was prepared in this Laboratory (Pearlman, 1957). A solution of the radioactive hormone in arachis oil was sterilized by heating at 120° for 1 hr.

Determination of tritium

By combustion of the sample to water, conversion into tritium–hydrogen gas, and gas counting. The procedures described by Avivi, Simpson, Tait & Whitehead (1954) were used. This time-consuming method was reserved chiefly for the analysis of the crude urinary ketonic and non-ketonic fractions.

By liquid scintillation counting. The apparatus was essentially that described by Avivi *et al.* (1954). The sample was dissolved in toluene (1·5 ml.) containing 0·5% (w/v) of the phosphor, *p*-terphenyl; the detection efficiency for counting tritium was about 7% and a reference standard of a steroid of known specific activity was employed on each occasion. This method was employed in the analysis of purified crystalline preparations or of colourless or only slightly coloured neutral urinary fractions. It was unsuitable for the accurate analysis of the crude urinary ketonic and non-ketonic fractions because of the presence of pigmented and fluorescent material. The isotope values for such fractions deviated by about $\pm$20% from those established by the combustion procedure. An attempt to introduce a correction by adding a known amount of colourless radioactive steroid to the crude urinary fractions was unsuccessful.

By counting in a windowless gas-flow counter. A few samples of high specific activity were analysed in a windowless counter (Tracerlab. Inc., Boston, Mass., U.S.A.) according to an unpublished procedure devised by Dr J. F. Tait and co-workers at the Middlesex Hospital Medical School, London. The sample (20 μg. or less of solids) was plated on a flat planchet containing many concentric grooves in order to promote uniform distribution of material over an area of 5 cm.^{2}. The count was corrected for self-absorption after the addition of a steroid standard of very high specific activity and negligible weight. The isotope values thus obtained were in good agreement with those established by the combustion method.

Administration of hormone and isolation of metabolic products from urine

After intramuscular injection of [16-^{3}H]progesterone as a single dose in each case, the urine was collected for 4 days (Expts. 1 and 2, Table 1) or 6 days (Expts. 3–5, Table 1), with toluene as preservative. The urinary excretion of isotope appeared to be practically complete by the end of the fourth day: thus, in Expt. 3, of the total amount of isotope eliminated as neutral material, 65, 22 and 13% appeared in the first, second and third 48 hr. collection periods; in Expt. 4 the corresponding figures were 60, 30 and 9% respectively.

The urine was usually worked up immediately, but in a few instances the specimens were stored in the refrigerator for 1 or 2 days. The urine specimen (for example, 1000 ml.) was brought to the boil and 10N-HCl (100 ml., A.R. quality) was cautiously added through a condenser;

refluxing was continued for exactly 10 min. and the solution was rapidly cooled. The hydrolysate was extracted with peroxide-free ether (1 × 1000 ml.; 2 × 500 ml.). The ether extract was washed with water (4 × 50 ml.) and evaporated. The residue was further purified by partitioning between benzene (100 ml.) and N-NaOH (4 × 50 ml.); with the pregnancy urines, the volumes of benzene and N-NaOH were larger in order to effect complete solution of the semi-crystalline residue (crude pregnanediol) which was obtained at this stage. The benzene phase was freed from alkali by repeated washing with water and then evaporated to dryness. The residue constituted the neutral fraction. It was partitioned into ketonic and non-ketonic moieties with the aid of Girard's reagent T (Girard & Sandulesco, 1936). A small amount of ketonic material was removed from the non-ketonic fraction on further treatment with Girard reagent; the ketonic products were combined and similarly retreated to give the final ketonic fraction. The tritium content (Table 1) of the final ketonic and non-ketonic fractions was determined on small aliquot portions.

The non-ketonic fraction was acetylated with pyridine–acetic anhydride (2:1, v/v, sufficient to effect solution on warming); after 48 hr. at room temperature the solution was diluted with water and extracted three times with ether. The ether extract, after washing successively with small portions of 2N-HCl (five times), 5% (w/v) Na_2CO_3 (twice), and water (four times), was evaporated. With the non-pregnancy non-ketonic fractions, to an aliquot of which inert 5β-pregnane-3α:20α-diol had been added before acetylation, the final acetylated product weighed less than 40 mg. It was dissolved in about 0·5 ml. of dry peroxide-free ether (stored over Na wire and redistilled over CaH_2 just before use) and 2 ml. of light petroleum (AnalaR, b.p. below 40°, treated with conc. H_2SO_4 in a separating funnel, carefully washed with water, dried over CaH_2 and distilled). The solution was filtered on a column (8 mm. × 80 mm.) containing 3·0 g. of Al_2O_3 (Type H, Peter Spence and Sons Ltd., Widnes, previously treated with acetic acid, exhaustively washed with water and dried at 150° overnight). Elution was made with four 10 ml. portions of light petroleum and finally with 20 ml. of dry ether. The crystalline solids from light-petroleum eluates were repeatedly recrystallized from ether–light petroleum or from aqueous methanol to give 5β-pregnane-3α:20α-diol 3:20-diacetate (3α:20α-diacetoxy-5β-pregnane). This product was present in large amounts in the late-pregnancy urine extracts and could be crystallized directly from the non-ketonic acetylated fraction (or after countercurrent distribution; see Expt. 3 below); it was further purified by chromatography as described above.

5β-Pregnane-3α:20α-diol demonstrated to be the major radioactive metabolite in the non-ketonic fraction

The procedure for estimating 5β-pregnane-3α:20α-diol was modified in Expt. 3 as follows.

The non-ketonic acetates (755·2 mg., 96% of original fraction) were dissolved in light petroleum–ether (2:1, v/v; 30 ml.), filtered through Al_2O_3 (6·0 g.) and eluted with the same solvent mixture, thereby removing a small amount of very dark pigment. The eluates furnished a red semi-crystalline mass (712·4 mg.) which was subjected to an 8-transfer countercurrent-distribution procedure with the aid of separating funnels; the solvent system was light petroleum–90% (v/v) methanol (50 ml.:156 ml.), which gave an effective partition coefficient (K) of 1·0 for 3α:20α-diacetoxy-5β-pregnane. [The solvents had been mutually saturated before use by shaking 1·0 vol. of light petroleum (b.p. below 40°) with 0·9 vol. of methanol and 0·1 vol. of water in a separating funnel.] The contents (both phases) of each funnel (0–8) were evaporated to dryness; the weight and specific activity of the residues are given in Table 3. The tritium determinations were made in a liquid scintillation counter. The contents of funnels 7 and 8 were highly pigmented and these determinations may have been in error by about ±20%; the contents of the remaining funnels were colourless or only slightly pigmented. According to Table 3 radio-inert, amorphous material appeared at both ends of the countercurrent distribution, and the central funnel, 4, contained the largest amount of white crystalline material with the highest specific activity; the partitioning behaviour of the radioactivity approximated to that of 3α:20α-diacetoxy-5β-pregnane. Indeed, as is described below, this progesterone metabolite accounted for about 84% of the isotope content of the non-ketonic acetates; a lower figure of 72% (Table 1) was based on the isotope content of the original non-ketonic fraction which was determined by a combustion procedure. The crystalline residue from funnel 4 (96·9 mg., 92% of original fraction; 3·67 μc) gave after repeated crystallization 33·5 mg. of the diacetate, specific activity 0·0482 μc/mg. The mother liquors were estimated by isotope-dilution procedure to contain 43·3 mg. of the same compound. Hence a total of 76·8 mg. of the diacetate was present, accounting for 79% of the weight of the residue from funnel 4 and 101% of its isotope content. The 3α:20α-diacetoxy-5β-pregnane content of the total non-ketonic fraction was obtained by dividing the weight of diacetate in funnel 4 by the factor 0·273, since K equals 1·0. The countercurrent-distribution procedure and calculations have been described by Williamson & Craig (1947).

RESULTS

According to Table 1 31% (average value; range 16–56%) of the isotope administered as [16-^{3}H]-progesterone was recovered in the neutral urinary extracts of all subjects; the isotope was distributed between the ketonic and non-ketonic fractions in a ratio of about 1:4. The percentage recovery of tritium as urinary 5β-pregnane-3α:20α-diol was 6, 15 and 14% in the pregnant women, and 27 and 14% in the oophorectomized–hysterectomized subjects. According to Table 2 the endogenous production of progesterone ranged from 212 to 284 mg./day in advanced pregnancy.

Determination of the percentage recovery of tritium as 5β-pregnane-3α:20α-diol

From urine of oophorectomized–hysterectomized subjects. To a measured fraction, a, of the non-ketonic fraction a weighed amount, y, of inert 5β-pregnane-3α:20α-diol was added. The mixture was acetylated, chromatographed and repeatedly crystallized to afford 3α:20α-diacetoxy-5β-pregnane, of constant melting point, 164·5–165° (uncorrected; Kofler-type hot-stage apparatus). This product was

Table 1. *Recovery of tritium after administration of* [16-^{3}H]*progesterone in urine*

The figures in columns 5–7 refer to the amount recovered after acid hydrolysis and ether extraction, as described in detail in the text. The specific activity of the [16-^{3}H]progesterone was 22·2 μc/mg.

		Amount of [16-^{3}H]-progesterone injected		Percentage of injected tritium recovered in urine				
Exptl. subjects	Expt. no.	(μc)	(mg.)	Neutral fraction (NK + K)	Non-ketonic fraction (NK)	Ketonic fraction (K)	Ratio $\left(\frac{K}{NK+K}\right)$	5β-Pregnane-3α:20α-diol
Oophorectomized–hysterectomized group	2	80·3	3·62	56	47	8·6	0·15	27
	5	102·0	4·60	24·5	20	4·5	0·18	14
Pregnant group								
25th week	1	185·0	8·31	16	10	5·5	0·34	5·7
36th week (twins)	3	106·0	4·77	36	22	14·0	0·39	15·0
34th week	4	103·0	4·65	22	17	5·0	0·23	14·0

Table 2. *Calculated daily endogenous production of progesterone and daily urinary 5β-pregnane-3α:20α-diol excretion in advanced pregnancy*

For method of calculation see text.

Stage of pregnancy (week)	Expt.	Urinary 5β-pregnane-3α:20α-diol excretion (mg./day)	Endogenous progesterone production (mg./day)
25	1	16	275
36 (twins)	3	42	284
34	4	30	212

Table 3. *Countercurrent distribution of acetylated non-ketonic fraction (713 mg.) from Expt. 3*

Solvent system: light petroleum (b.p. below 40°)–90% (v/v) methanol (50 ml.:156 ml.), giving an effective partition coefficient, K, of 1·0 for 3α:20α-diacetoxy-5β-pregnane; 8 transfers.

Funnel no.	Wt. of residue (mg.)	Specific activity (μc/mg.)	Total isotope content (μc)
0	105 (oil)	0·0049	0·52
1	41 (oil)	0·029	1·20
2	49 (semi-cryst.)	0·038	1·85
3	77 (cryst.)	0·036	2·80
4	105 (cryst.)	0·038	3·99
5	105 (cryst.)	0·036	3·73
6	85 (semi-cryst.)	0·026	2·19
7	72 (oil)	0·013	0·94
8	61 (oil)	0·0013	0·08

not in all instances examined for radiochemical purity; in Expt. 1, the specific activity of the isolated 3α:20α-diacetoxy-5β-pregnane remained unchanged on recrystallization. Possible contamination with ketonic radiometabolites appeared minimal in view of the rigorous fractionation of the neutral extract into ketonic and non-ketonic portions. Possible contamination with non-ketonic metabolites such as 3α:20α-diacetoxy-5α-pregnane was considered, but this also seemed negligible: analysis of the non-ketonic fraction (Expt. 2) by isotope dilution indicated that the respective 5β- and 5α-pregnanediols were present in a ratio of about 19:1. Kyle & Marrian (1951), employing conventional analytical methods, reported similarly a ratio of about 30:1 in a sample of human pregnancy urine. That 5β-pregnane-3α:20α-diol is the predominant non-ketonic radiometabolite was demonstrated in Expt. 3 by countercurrent-distribution procedures (see above).

The specific activity, C', of the isolated 3α:20α-diacetoxy-5β-pregnane was determined and the tritium content, z, of the free diol in the total non-ketonic fraction was obtained from the equation $z = (1{\cdot}26C')\ y/a$. The correction factor, 1·26, is the ratio of the molecular weights of the diacetate and the free diol (404·5 and 320·4 respectively). The percentage recovery of tritium as the urinary diol was readily obtained, the amount of isotope administered being known (Table 1). Undiluted urinary 5β-pregnane-3α:20α-diol was not isolated because the amount was too small, endogenous progesterone production being minimal in these subjects; since the amount by weight, x, of urinary diol in a was negligible by comparison with y, it was omitted from the calculations.

From pregnancy urine. The amount of 5β-pregnane-3α:20α-diol in the pregnancy-urine extracts being considerable, it was easily isolated as the diacetate from the acetylated non-ketonic fraction and its specific activity, C, determined. To a measured portion, a, of the acetylated non-ketonic fraction, a weighed amount, y, of inert 3α:20α-diacetoxy-5β-pregnane was added; the diacetate was isolated again and its specific activity, C', determined. The amount x of the diacetate in the entire non-ketonic fraction was obtained from the equation $x = yC'/a(C - C')$. The tritium content z of the total urinary 3α:20α-diacetoxy-5β-pregnane was obtained from

the equation $z = xC$. The daily urinary 5β-pregnane-3α:20α-diol excretion (Table 2) was obtained by dividing x by the number of days of the urine-collection period.

Estimation of the endogenous progesterone production in pregnancy

One need only determine the specific activity C of the undiluted urinary 3α:20α-diacetoxy-5β-pregnane in order to calculate the daily endogenous progesterone production P, since the amount by weight w of the [16-^{3}H]progesterone injected and its specific activity S were known. The equation employed was $P = 0{\cdot}98Sw/Cd$, where d is the number of days of the urine collection period; the correction factor, 0·98, is the ratio of the molecular weights of progesterone and 5β-pregnane-3α:20α-diol (314·4 and 320·4 respectively); the other symbols have been defined above. The same value for P would, of course, be obtained by dividing the daily urinary 5β-pregnane-3α:20α-diol excretion (Table 2) by the percentage recovery of tritium as the urinary diol (Table 1) and multiplying by the correction factor 0·98.

DISCUSSION

There is no support from the limited data (Table 1) in this study for the view (Venning & Browne, 1940; Sommerville & Marrian, 1950; Guterman, 1953) that the conversion of progesterone into pregnanediol is greatly increased (as much as threefold) in pregnancy as compared with that which obtains in the non-pregnant state in the absence of luteal function. Nor could such support be obtained in a recent study by Klopper & Michie (1956), who administered non-isotopic progesterone to three patients in early pregnancy: these workers stated that the analytical method they employed was new, precise, accurate and specific for pregnanediol, and that it was possible to estimate the additional amount in pregnancy urine in a way not available to the early workers. Indeed, from recent investigations (Marrian *et al.* 1954; Klopper & Michie, 1956) it appears that the course of progesterone metabolism with respect to pregnanediol formation is influenced neither by the uterus or the corpus luteum nor by the administration of oestrogen. Similarly the metabolism of oestrone in advanced pregnancy has been shown with the aid of deuterium-labelling (Pearlman, Pearlman & Rakoff, 1954) not to depart significantly from that in non-pregnancy. In view of the quantities of both progesterone and oestrogen produced in late pregnancy, it is noteworthy that these hormones do not appreciably affect each other in their metabolism, although they markedly modify their respective physiological activities (Courrier, 1950). The earlier observations that the metabolism of oestrogen and progesterone is influenced by the endocrine status of the organism, and hence is somehow linked with their physiological utilization, thus cannot be confirmed. In advanced pregnancy the body appears to be flooded with steroid sex hormones in a process which is wasteful in that they are rapidly metabolized into biologically inactive products in organs not associated with the reproductive process, notably the liver; these metabolic transformations overshadow any that might occur in the uterus or mammary glands.

The endogenous production of progesterone in advanced pregnancy, as calculated by the isotope-dilution method, is about 0·25 g./day (Table 2). It may be much higher in some women in view of the wide range among individuals in urinary pregnanediol excretion rates (Venning, 1948) and the varying degree of hormone metabolism. One wonders how much of this progesterone, which in the human species arises chiefly from the placenta with advancing pregnancy, undergoes transformation into corticoids in the maternal or foetal adrenal glands; the hormonal inter-relationships between these organs have recently been reviewed by Davis & Plotz (1956).

The question may be raised as to the validity of using [16-^{3}H]progesterone in the study of the intermediary metabolism of the hormone not involving loss of the C-17 side chain. It has been assumed in this study that no loss of tritium occurred in the *in vivo* conversion of progesterone into 5β-pregnane-3α:20α-diol. To establish this point a solution of [16-^{3}H]progesterone and [4-^{14}C]progesterone was injected into a cancer patient and 5β-pregnane-3α:20α-diol was subsequently isolated from the urine (unpublished experiments in collaboration with Dr J. F. Tait and others); the ^{3}H:^{14}C ratios of the urinary diol and of the hormone injected were identical within experimental error when determined by the combustion–gas-counting procedure. The recovery of radioactivity in the urine after [16-^{3}H]progesterone administration (Table 1) averaged 31% (range 16–56%), which is comparable with that obtained after the administration of [4-^{14}C]progesterone to human subjects (Bradlow & Gallagher, 1955; Wiest, Fujimoto & Sandberg, 1955); this agrees also with the finding that 5β-pregnane-3α:20α-diol is the major radioactive metabolite. Davis, Plotz, Le Roy, Gould & Werbin (1956) injected [4-^{14}C]progesterone into a pregnant patient and recovered 25% of the administered radioactivity from the urine and 28·5% from the faeces. (It is likely, in view of the latter observation, that the faeces in the present study contained tritium, but this was not ascertained.) Other examples of the effective application of hydrogen-isotope labelling in investigations of steroid-hormone metabolism may be mentioned, e.g. the conver-

sion of cholesterol into pregnanediol (Bloch, 1945), oestrone metabolism (Pearlman *et al.* 1954) and testosterone and cortisone metabolism (Gallagher *et al.* 1954). There are certain biochemical limitations in the valid use of deuterium or tritium, and broad aspects of the problem are discussed by Kamen (1951).

SUMMARY

1. [16-^{3}H]Progesterone was injected intramuscularly into three women in the last trimester of pregnancy and into two oophorectomized–hysterectomized patients who served as a control group. The urinary recovery of tritium in the neutral ketonic and non-ketonic fractions and as 5β-pregnane-3α:20α-diol was determined.

2. Of the radioactivity injected 31% (average value for all subjects) was recovered in the neutral urinary extracts; the isotope was distributed between the ketonic and non-ketonic fraction in a proportion of about 1:4. 5β-Pregnane-3α:20α-diol was the major radioactive metabolite.

3. The percentage recovery of progesterone as urinary 5β-pregnane-3α:20α-diol was calculated by isotope-dilution procedures to be 6, 15 and 14% in the pregnant subjects and 27 and 14% in the oophorectomized–hysterectomized subjects. These findings do not support earlier observations (Venning & Browne, 1940; Sommerville & Marrian, 1950; Guterman, 1953) by conventional analytical methods that the percentage recovery of the metabolite in pregnancy is increased about threefold.

4. The endogenous production of progesterone was similarly calculated to be of the order of 0·25 g./day in late pregnancy.

5. The validity of using [16-^{3}H]progesterone in the study of the intermediary metabolism of the hormone is discussed.

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The Metabolism of Thyroid Hormones

2. DETECTION OF THYROXINE AND TRI-IODOTHYRONINE IN HUMAN PLASMA*

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The introduction of paper chromatography and the availability of radio-iodide enabled Laidlaw (1949) to show that the circulating thyroid hormone was thyroxine and not thyroglobulin or a peptide, a conclusion supported by the work of Taurog, Chaikoff & Tong (1950) and Rosenberg (1951). After the discovery of tri-iodothyronine (Gross & Pitt-Rivers, 1952*a*, *b*, 1953*a*, *b*; Roche, Lissitzky & Michel, 1952*a*, *b*) it became clear that many of the earlier methods would not have been capable of differentiating between thyroxine and the new substance. The paper-chromatographic techniques

* Part 1: Sprott & Maclagan (1955).