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

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The following experiments are an extension of those described by Pearlman (1957) in which [16-^{3}H]progesterone (nominal specific activity 22·2 μc/mg.) was injected intramuscularly into oophorectomized–hysterectomized patients and into women in an advanced stage of pregnancy. The urine was subsequently collected for 4–6 days, hydrolysed, and extracted. The neutral urinary extracts contained 31% (average) of the radioactivity injected; the isotope was distributed between the ketonic and non-ketonic fractions in a proportion of about 1:4. From the latter fraction 5β-pregnane-3α:20α-diol was isolated as the diacetyl derivative; determination of the specific activity of this major progesterone metabolite permitted the estimation of the daily endogenous production of progesterone in pregnancy. The studies made on the ketonic fraction of the neutral urinary extracts employing partition column chromatography are now described.

EXPERIMENTAL

Determination of tritium

Most of the radioassays were performed by counting in a windowless gas-flow counter by a procedure essentially that described by Ayres, Pearlman, Tait & Tait (1958): 20 μg. or less of the tritium-containing sample and 160 μg. of beeswax were deposited from 0·2 ml. of a chloroform solution on a planchet, 2·5 cm. in diameter, which had a large number of circulation grooves, about 0·05 mm. deep, scored on its surface, to promote uniform distribution of the sample. As a standard in counting, a known amount of [16-^{3}H]progesterone, negligible in weight, and 160 μg. of beeswax were similarly deposited on a planchet.

A few samples were counted by combustion of the sample to water, conversion into tritium–hydrogen gas, and gas counting as described by Avivi, Simpson, Tait & Whitehead (1954).

Reverse-phase partition column chromatography

The performance of the partition columns used in this study could be predicted from the equations developed by Carpenter & Hess (1956). For reverse-phase chromatography the derived equation $V_E = V_s K + V_H$ applies, where V_E, the elution volume, is equal to the total volume of eluate collected from the time the solute is introduced on the column, up to and including the eluted fraction of maximum concentration, V_s is the total volume of the stationary phase used in preparing the column, K is the partition coefficient of the solute, and V_H, the hold-up volume, is the total volume of moving phase held in the column. V_H can be calculated from the equation:

$$V_H = \pi r^2 h - (V_s + V_{sup}),$$

where r is the radius of the column, h is the height of the packed column and V_{sup} is the volume of the inert support, i.e. the silicone-treated Celite used in preparing the column. V_{sup} can be calculated from the weight and density measurements of the silicone-treated Celite. K can be calculated by partitioning the solute between equal volumes of the stationary phase (upper layer) and the moving phase (lower layer) and determining the ratio of the amount of the solute in the upper phase to that in the lower phase.

The number, N, of 'theoretical plates' on the column was calculated from the equation $N = 5{\cdot}5[V_E^2 - V_E V_H]/V_B^2$, where V_B is the width of the elution band in volume units between the two points on the elution curve of concentration equal to one-half the maximum concentration; V_E and V_B were experimentally determined.

Model experiment. Hyflo Super-Cel (Johns-Manville Products, New York) was stirred with 2N-HCl and then washed free of acid. The dried Celite was spread thinly on the bottom of a desiccator and exposed for 48 hr. to the vapours of about 5–10 ml. of organo-silicone chlorides (M441, obtained from Imperial Chemical Industries Ltd., Glasgow) contained in a glass evaporating dish. The Celite was then washed free of acid with methanol and dried. The silicone-treated Celite could not be wetted by water; its density was determined by measuring the displacement in toluene in a density bottle.

In the solvent system used, the stationary phase was light petroleum (b.p. 90°)–toluene (3:2, v/v) and the moving phase methanol–water (4:1, v/v); equal volumes of both phases were shaken in a separating funnel and separated before use. This solvent system was most suitable for the separation of progesterone and its metabolites, particularly as the acetyl derivatives. Such steroids are highly soluble in the stationary phase, which factor permits the loading of the reverse-phase partition column with large quantities of compounds.

The column was prepared by adding to 10 g. of silicone-treated Celite (density 1·40) in a 100 ml. round-bottom flask 6·0 ml. (V_s) of stationary phase, followed by about 50 ml. of moving phase. The flask was stoppered; the contents were shaken for 1 hr. and then stirred for 0·5 hr. with

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a magnetic stirrer. This treatment ensured uniform distribution of the stationary phase in the Celite. The Celite mixture was transferred to a column (πr^2 = 1·24 cm.^{2}) and packed: a slow, uniform downward movement of the Martin packer (Howard & Martin, 1950) facilitated the rate of packing, which is usually difficult to accomplish with silicone-treated Celite. The height, h, of the packed column was 17·5 cm., V_{sup} was calculated to be 7·1 ml., and hence V_H was 8·6 ml.

A solution (0·3 ml. of stationary phase) containing 14·1 mg. of pregnanedione (5β-pregnane-3:20-dione), 14·0 mg. of dehydro*epi*androsterone acetate, 33·6 mg. of pregnenolone acetate (3β-acetoxypregn-5-en-20-one) and 28·1 mg. of pregnanediol diacetate (3α:20α-diacetoxy-5β-pregnane) was placed on the column. The moving phase flowed at 10 ml./hr. The elution curve is given in Fig. 1. The elution volumes (V_E, theoretical and experimental) and the K values of the respective steroids are listed in Table 1. The number, N, of theoretical plates in this column was calculated on the basis of the experimental findings to be about 100.

Oophorectomized–hysterectomized group

Experiment no. 5, isotope dilution analysis of ketonic fraction. To a 56·2 mg. portion of the ketonic fraction (140·5 mg.) was added 21·3 mg. of non-isotopic pregnanedione (5β-pregnane-3:20-dione) and 21·6 mg. of non-isotopic pregnanolone (3α-hydroxy-5β-pregnan-20-one). The mixture was acetylated with pyridine–acetic anhydride (2:1, v/v, sufficient to effect solution on warming); after

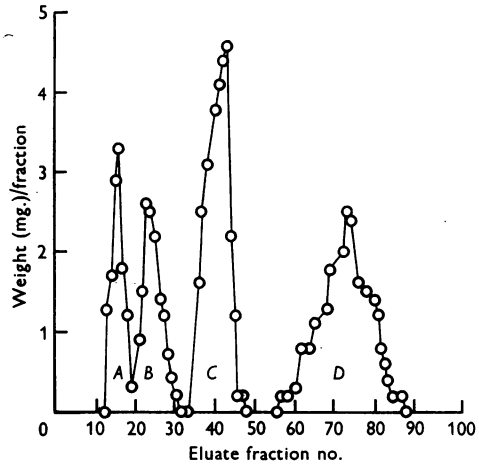

Fig. 1. Elution curve for reverse-phase partition column chromatography in a model experiment (see text and Table 1). Fraction nos. 1–32 are 2 ml. each; fraction nos. 33–90, 5 ml. each. *A*, *B*, *C*, and *D* correspond to pregnanedione, dehydro*epi*androsterone acetate, pregnenolone acetate and pregnanediol diacetate respectively.

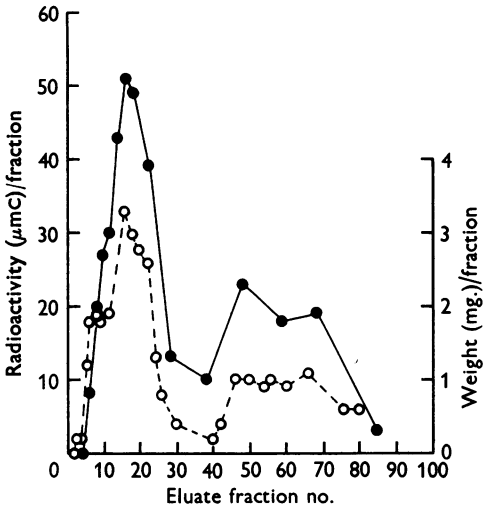

Fig. 2. Elution curves for reverse-phase partition column chromatography in experiment no. 5 (isotope dilution analysis). Eluate fraction nos. 12–28 and nos. 29–84 are 2 ml. each and constitute the 'pregnanedione' and 'pregnanolone acetate' fractions respectively. ●, Radioactivity; ○, weight.

Table 1. *Elution volume (V_E) and partition coefficient (K) of steroids in reverse-phase partition column chromatography in model experiment*

See Fig. 1 for elution curve: stationary phase, light petroleum (b.p. 90°)–toluene (3:2, v/v); moving phase, methanol–water (4:1, v/v). Theoretical values of V_E were determined from the equation $V_E = V_s K + V_H$, see text. Values of K were determined by distributing the steroid between equal volumes of the two immiscible phases; the ratio of the weight of the residue in the upper phase to that in the lower phase gives the value of K.

Compound	V_E Theoretical	V_E Experimental	K
Dehydro*epi*androsterone	10	—	0·28
Progesterone	16	—	1·26
5β-Pregnane-3:20-dione	31	31	3·65
Dehydro*epi*androsterone acetate	49	46	6·72
3α-Acetoxy-5β-pregnan-20-one	97	—*	14·7
3α-Acetoxy-5α-pregnan-20-one	99	—	15·0
3β-Acetoxy-pregn-5-en-20-one	106	121	16·3
3α:20α-Diacetoxy-5β-pregnane	328	269	53

* V_E (experimental) for this compound was determined in experiment no. 5 and found to be in good agreement with the theoretical value.

48 hr. at room temperature, the solution was diluted with water and extracted three times with ether. The ether extract, after washing with small portions of 2N-HCl (five times), 5% (w/v) Na_2CO_3 (twice) and water (four times), was evaporated. The residue (106 mg.) was subjected to reverse-phase partition column chromatography under the conditions of the model experiment described above. The elution curves (radioactivity and weight against eluate fraction no.) are given in Fig. 2. Fraction nos. 12–28, constituting the 'pregnanedione' fraction (V_E, theoretical 32 ml., experimental 31 ml.), were pooled to give 32·2 mg. of material of which 30·6 mg. was chromatographed on 3·0 g. of Al_2O_3 (Savory and Moore) in a column (1·0 cm., internal diameter). Eluate fractions nos. 1–4, each 50 ml., were mixtures of light petroleum (b.p. below 40°)–benzene [8:2, 7:3, 6:4 and 4:6 (v/v) respectively]. Fractions nos. 3 and 4 on evaporation gave 16·0 mg. of crystals, m.p. 118°; on recrystallization from ethanol, 8·0 mg. of 5β-pregnane-3:20-dione, m.p. 122°, was obtained. Its specific activity, C', was 9·18 μmc/mg., which was unchanged on recrystallization. The tritium content, z, of pregnanedione in the total ketonic fraction was calculated as described below to be 0·484 μc.

Fraction nos. 29–84 (Fig. 2), constituting the 'pregnanolone acetate' fraction (V_E, theoretical 99 ml., experimental 95 ml.), were pooled to give 31·4 mg. of material of which 30·0 mg. was chromatographed on 3·0 g. of Al_2O_3, as was the 'pregnanedione' fraction. Eluate fraction no. 1 gave 4·8 mg. of crystals, m.p. 89–91°; fraction no. 2, 17·2 mg., m.p. 93–95°, raised to 99–100° on two recrystallizations from light petroleum. The specific activity, C', of the 3α-acetoxy-5β-pregnan-20-one thus isolated was 26·0 μmc/mg.; it remained unchanged on recrystallization. The tritium content, z, of 3α-hydroxy-5β-pregnan-20-one in the total ketonic fraction was calculated to be 1·59 μc.

Experiment 2. Isotope dilution analysis of the ketonic fraction was carried out as in Expt. 5.

Pregnant group

Experiment 4. A portion (62·0 mg.) of the total ketonic fraction (106·9 mg.), acetylated as described above, was subjected to reverse-phase partition column chromatography: the conditions were the same as those in the model experiment except that 14·0 g. of silicone-treated Celite was used; V_s was 8·4 ml., πr^2 1·04 cm.², h 34·3 cm. and V_H 17 ml. The elution curves are given in Fig. 3 and the data on the major pooled eluate fractions summarized in Table 2.

The 'pregnanolone acetate' fraction (Fig. 3, Table 2) was purified as in Expt. 5 by chromatographing 15·6 mg. of this material on 3 g. of Al_2O_3. The consecutive eluate fractions, each 50 ml., were mixtures of light petroleum (b.p. below 40°)–benzene [8:2, 6:4 and 2:8 (v/v)]. The second fraction gave on evaporation 6·3 mg. of crystals, m.p. 92–94°, and on recrystallization from light petroleum gave 1·8 mg. of 3α-acetoxy-5β-pregnan-20-one, m.p. 97–99°. The specific activity, C, was 89·5 μmc/mg.

Isotope dilution analysis for pregnanolone was carried out on 18 mg. of the total ketonic fraction, to which was

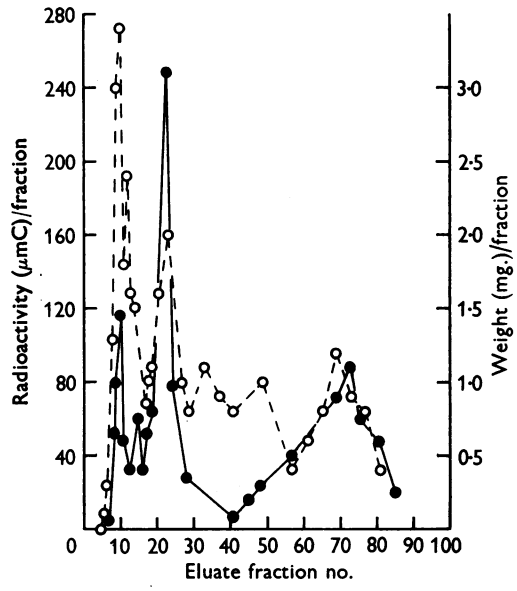

Fig. 3. Elution curves for reverse-phase partition column chromatography in experiment no. 4. Eluate fraction nos. 17–40 and nos. 41–85 are 2 ml. each and constitute the 'pregnanedione' and 'pregnanolone acetate' fractions respectively; eluate fractions nos. 1–16 are unidentified ('X') fractions (see text and Table 2). ●, Radioactivity; ○, weight.

Table 2. *Major radioactive fractions, obtained on reverse-phase partition column chromatography of 62·0 mg. (2·90 μc) of the acetylated ketonic fraction, Expt. 4*

See Fig. 3 for elution curve.

Major fraction	Fraction nos.	V_E Theoretical (ml.)	V_E Experimental (ml.)	Weight (mg.)	Radioactivity (μc)
X	1–16	—	21; 31	15·8	0·307
'Pregnanedione'	17–40	47	45	19·2	0·841
'Pregnanolone acetate'	41–85	145	141	16·9	1·08*
Stripping†	86–	—	—	9·4	0·093
Total				61·3	2·32

* The radioactivity of the 3α-acetoxy-5β-pregnan-20-one present in this fraction was determined by isotope dilution analysis.

† The column was stripped of its radioactive contents by elution with ether.

added 21·8 mg. of non-isotopic 3α-hydroxy-5β-pregnan-20-one. The mixture was acetylated and fractionated by reverse-phase partition column chromatography as described above with 10 g. of silicone-treated Celite. The 'pregnanolone acetate' fraction (23·4 mg.) obtained was chromatographed on 3·0 g. of Al_2O_3; 7·1 mg. of 3α-acetoxy-5β-pregnan-20-one, m.p. 97–98°, was obtained. Its specific activity, C', was 12·2 μmc/mg. The tritium content, z, of 3α-hydroxy-5β-pregnan-20-one in the total ketonic fraction was calculated to be 1·81 μc.

Experiment 3. The ketonic fraction was analysed as in Expt. 4. Also, an attempt was made to isolate pregnanedione from the 'pregnanedione' fraction which was obtained in reverse-phase partition column chromatography, but the amounts were inadequate for isolation. The presence of radioactive pregnanedione in this fraction was, however, demonstrated by isotope dilution analysis. A highly polar 'X' fraction, similar to that obtained in Expt. 4 (see Fig. 3), was also obtained in this experiment. This material, on reverse-phase partition column chromatography in a different solvent system, appeared to consist of at least three radioactive components, none of which could be identified.

RESULTS AND DISCUSSION

Table 3 gives the percentage of tritium injected as [16-^{3}H]progesterone which was recovered in the neutral ketonic fraction in the urine of oophorectomized–hysterectomized subjects and of pregnant women. Of the radioactive components of this fraction, 3α-hydroxy-5β-pregnan-20-one is present in major proportion and 5β-pregnane-3:20-dione (and possibly other ketones of similar partition coefficient) in minor proportion. A significant portion, 10–14 %, of the radioactivity in the ketonic fraction in pregnancy urine is in unidentified, highly polar material called the 'X' fraction (see Fig. 3 and Table 2). This material was not detected in the neutral ketonic fraction in the urine of oophorectomized–hysterectomized subjects (compare Fig. 2 with Fig. 3).

Further investigation is required to ascertain whether there is a significant difference in the course of progesterone metabolism in the pregnant as compared with the non-pregnant luteal-deficient state. Davis & Plotz (1958), who administered [^{14}C]progesterone to 10 pregnant and 5 non-pregnant patients, found no apparent relationship between the urinary recovery of radioactivity and the pregnant or the non-pregnant state, or the viability of the foetus; the urinary recoveries varied widely, however, among individuals of either group. The metabolic fate of [^{14}C]progesterone in women has been recently investigated by several groups of workers: progesterone radiometabolites are excreted in considerable amounts in the bile and stool as well as in urine, according to Bradlow & Gallagher (1955), Davis, Plotz, LeRoy, Gould & Werbin (1956), Davis & Plotz (1958), Sandberg & Slaunwhite (1958) and Wiest, Fujimoto & Sandberg (1958); the identification of the various metabolites in the excreta has not been completed, except for major products such as 5β-pregnane-3α:20α-diol and 3α-hydroxy-5β-pregnan-20-one.

Table 4 gives the endogenous production rate of progesterone in advanced pregnancy based on the measurement of urinary 3α-acetoxy-5β-pregnan-20-one, also the urinary excretion rate of this metabolite based on isotope dilution analysis. However, it is more convenient to estimate endogenous progesterone production by measuring the specific activity of urinary 5β-pregnane-3α:20α-diol (Pearlman, 1957) rather than that of 3α-hydroxy-5β-pregnan-20-one, because the former metabolite is present in larger amounts, permitting its ready isolation. Considerably less than 100 μc of [16-^{3}H]-progesterone need be injected to determine the

Table 3. *Recovery of tritium after administration of* [16-^{3}H]*progesterone in the urinary neutral ketonic fraction*

The numbering of the experiments corresponds to those described by Pearlman (1957), of which these are an extension. The fractionation of the ketonic material is described in detail in the text. The specific activity of the [16-^{3}H]progesterone was 22·2 μc/mg. The 'X' fraction contained unidentified radiometabolites more polar than pregnanedione. It was obtained on reverse-phase partition column chromatography of the acetylated ketonic fraction, see text.

			Percentage of injected tritium recovered as urinary ketonic metabolites			
Experimental subjects	Expt.	Administered dose (μc)	Total ketonic fraction	3α-Hydroxy-5β-pregnan-20-one	5β-Pregnane-3:20-dione	'X' fraction
Oophorectomized–hysterectomized group	2	80·3	8·6	3·8	0·91	—
	5	102·0	4·5	1·6	0·48	—
Pregnant group						
36th week (twins)	3	106·0	14·0	5·1	1·9*	1·9
34th week	4	103·0	5·0	1·8	1·4*	0·54

* This refers to a 'pregnanedione' fraction containing 5β-pregnane-3:20-dione and possibly other radiometabolites of the same partition coefficient.

Table 4. *Calculated daily endogenous production of progesterone and daily urinary 3α-hydroxy-5β-pregnan-20-one excretion in advanced pregnancy*

For method of calculation see text.

Stage of pregnancy (week)	Expt. no.	Urinary 3α-hydroxy-5β-pregnan-20-one excretion (mg./day)	Endogenous progesterone production (mg./day)
36 (twins)	3	12·6*	240†
34	4	3·2	167

* Based on radioassay by combustion of sample and gas counting; a slightly lower figure, 10·9, was derived from radioassay by windowless gas-flow counting.

† Based on radioassay by combustion of sample and gas counting; a much lower figure, 156, was derived from a radioassay by windowless gas-flow counting which may have been incorrect in this instance. The figure 240 is in fair agreement with the figure 284 obtained by Pearlman (1957) on the basis of the specific activity of urinary 3α:20α-diacetoxy-5β-pregnane.

rate of endogenous secretion of hormone. Moreover, the recent advances in instrumentation particularly in liquid scintillation counting (Arnold, 1957) make possible a rapid estimation of tritium in several milligrams of pregnanediol diacetate—it readily dissolves in the toluene-phosphor—whereas the windowless gas-flow counting technique, as described here, cannot be performed on more than 20 μg. of the steroid with a precision of more than about ±10%. Certain properties of tritium make it safer for studies on human subjects, especially in pregnancy, than ^{14}C.

Determination of the percentage recovery of tritium as ketonic progesterone metabolites

From urine of oophorectomized–hysterectomized subjects. The quantities of 5β-pregnane-3:20-dione and 3α-hydroxy-5β-pregnan-20-one present in these urinary extracts are negligible by comparison with the quantities of the non-isotopic steroids added, and are omitted in the following calculations.

To a measured portion, a, of the ketonic fraction a weighed amount, y, of non-isotopic progesterone metabolite (5β-pregnane-3:20-dione, also 3α-hydroxy-5β-pregnan-20-one) was added. The acetylated mixture was purified as described above (Expt. 5, isotope dilution analysis) and the specific activity, C', of the reisolated steroids determined.

The tritium content, z, of 5β-pregnane-3:20-dione in the total ketonic fraction was obtained from the equation $z = yC'/a$. The tritium content, z, of 3α-hydroxy-5β-pregnan-20-one in the total ketonic fraction was obtained by multiplying yC'/a by a correction factor, 1·13, which is the ratio of the molecular weights of acetylated and free 3α-hydroxy-5β-pregnan-20-one (360 and 318 respectively).

From pregnancy urine. To a measured portion, a, of the ketonic fraction was added a weighed amount, y, of non-isotopic 3α-hydroxy-5β-pregnan-20-one. The acetylated mixture was purified as described above (Expt. 4, isotope dilution analysis), and the specific activity, C', of the reisolated 3α-acetoxy-5β-pregnan-20-one determined. The amount, x, of 3α-hydroxy-5β-pregnan-20-one in the total ketonic fraction was obtained from the equation $x = yC'/a(C - C')$. The specific activity, C, is that of 3α-acetoxy-5β-pregnan-20-one which had been isolated from the acetylated ketonic fraction before dilution. The tritium content, z, of 3α-hydroxy-5β-pregnan-20-one in the total ketonic fraction was obtained from the equation $z = x(1{\cdot}13C)$.

The tritium content of 5β-pregnane-3:20-dione in the total ketonic fraction could not be estimated because the quantity of this metabolite in the urinary extracts was inadequate for direct isolation and determination of its specific activity. The tritium content of the 'X' and 'pregnanedione' fractions as described above was, however, determined. This does not of course indicate the isotope content of any of the individual metabolites present in these fractions but only their total isotope contribution.

Estimation of the endogenous progesterone production in pregnancy

For this one need determine only the specific activity of a urinary progesterone metabolite. The dilution undergone by the administered [16-^{3}H]-progesterone (specific activity S) with endogenous progesterone is equal to $S/1{\cdot}15C$, where C is the specific activity of the urinary 3α-acetoxy-5β-pregnan-20-one, and 1·15 a correction factor equal to the ratio of the molecular weights of 3α-acetoxy-5β-pregnan-20-one and progesterone (360 and 314 respectively). The daily endogenous progesterone production, P, is obtained by multiplying the dilution by w/d, where w is the weight of [16-^{3}H]progesterone injected and d the number of days of the urine collection period. It is assumed that the rate of progesterone metabolism remains uniform throughout the urine collection period. It is essential that the collection be continued until the urinary excretion of radiometabolites becomes negligible, and that all the urine is obtained and pooled. A collection period of 4–6 days is usually required, but it may be considerably shorter if the radioactive hormone is injected intravenously rather than intramuscularly, as the excretion studies of Davis & Plotz (1958) suggest; these authors administered [^{14}C]progesterone.

The same value for P is derived by dividing the figure for the daily urinary excretion of 3α-hydroxy-5β-pregnan-20-one (Table 4) by the percentage

recovery of tritium as this metabolite (Table 3) and multiplying by 100 and by 0·99. The latter figure is a correction factor equal to the ratio of the molecular weights of progesterone and 3α-hydroxy-5β-pregnan-20-one (314 and 318 respectively).

In the equation employed by Pearlman (1957) for calculating P on the basis of the specific activity of urinary 3α:20α-diacetoxy-5β-pregnane, the correction factor was incorrectly given as 0·98, although the values for P were correctly stated. The correction factor in that particular form of the equation should be 1·28, which is the ratio of the molecular weights of 3α:20α-diacetoxy-5β-pregnane and progesterone (404 and 314 respectively).

SUMMARY

1. [16-^{3}H]Progesterone was injected intramuscularly into two 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 various components of the neutral ketonic fraction is reported.

2. The major ketonic radiometabolite in both experimental groups is 3α-hydroxy-5β-pregnan-20-one; a minor constituent is 5β-pregnane-3:20-dione.

3. A significant portion, 10–14%, of the radioactivity in the ketonic fraction in pregnancy urine is in an unidentified highly polar material. This radioactive material was not detected in the urine of oophorectomized–hysterectomized subjects.

4. The daily endogenous production of progesterone in advanced pregnancy was estimated from measurements of the specific activity of urinary 3α-hydroxy-5β-pregnan-20-one; these estimates were in fair agreement with those previously obtained on the basis of the specific activity of urinary 5β-pregnane-3α:20α-diol.

5. Procedures for the separation of certain urinary progesterone metabolites by reverse-phase partition column chromatography are described.

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The Effect of *Plasmodium berghei* Malaria on Mouse-Liver Mitochondria

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Recent investigations into the pharmacological or toxic actions of various drugs, hormones and steroids have been concerned with a possible correlation of such actions with impairment of the normal functions of the mitochondria of tissue cells. To this end, the properties of isolated liver mitochondria, after treatment *in vivo* or *in vitro* with the substance under investigation, have been employed as test systems by comparison with the same properties of untreated mitochondria. By means of this approach, for example, Christie & Judah (1954) have shown that fatty degeneration and necrosis caused by oral administration of carbon tetrachloride are accompanied by