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The metabolism of 16α-ethylprogesterone by rat liver *in vitro*

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The metabolism of 16α-ethylprogesterone by rat liver homogenate at different steroid to tissue ratios has been examined. The products, identified by thin-layer chromatography, gas-liquid chromatography and combined gas-liquid chromatography-mass spectrometry were 16α-ethyl-5α-pregnane-3,20-dione and 16α-ethyl-3α-hydroxy-5α-pregnan-20-one. The yield of these metabolites was 14 and 48% respectively. The results are discussed in relation to the liver *in vitro* metabolism of progesterone and 16α-hydroxyprogesterone.

PARTIAL regression of carcinoma of the endometrium has been obtained in some patients treated with progesterone (Kelley & Baker, 1961) or with the synthetic progesterone analogues 17α-hydroxyprogesterone caproate (Kistner, Griffiths & Craig, 1965; Mussey & Malkesian, 1966), 6α-methyl-17α-hydroxyprogesterone acetate (medroxyprogesterone acetate) or 6α,17α-dimethyl-6-dehydroprogesterone (Smith, Rutledge & Soffar, 1966). The latter authors noted that progesterone analogues showed a higher degree of endometrial activity than progesterone itself. Substituents present in such analogues also confer on the progesterone molecule an increased resistance to enzymic attack (Cooke & Vallance, 1965). Thus the increased biological stability of synthetic progesterone derivatives may be correlated with their increased endometrial activity and wider use in the management of endometrial carcinoma. Where regression is observed on administration of these synthetic steroids only biologically slow growing tumours are affected; tumours of rapid growth remain largely unaffected (Kennedy, 1963; Frick II, 1965). The mechanism of such compounds is unknown.

The metabolism of progesterone by liver preparations has been examined by several authors. In a study with human liver Atherden (1959) identified 5α- and 5β-pregnane-3,20-dione, 3α-hydroxy- and 3β-hydroxy-5α-pregnan-20-one, 3α-hydroxy-5β-pregnan-20-one and 5β-pregnane-3α,20α-diol. With the exception of 5β-pregnane-3,20-dione, Taylor (1955) isolated a similar range of products from the metabolism of progesterone by rabbit liver preparations. In contrast, the same author (Taylor, 1954) isolated only 5α-pregnane-3,20-dione and 3α-hydroxy-5α-pregnan-20-one from the incubation of progesterone with rat liver. From the metabolism of 16α-hydroxyprogesterone by rat liver preprations, however, Wettstein, Neher & Urech (1959) identified 3α,16α-dihydroxy-5β-pregnan-20-one and 3α,16α-dihydroxy- and 3β,16α-dihydroxy-5α-pregnan-20-one. Thus progesterone and 16α-hydroxyprogesterone appear to be metabolized by different routes in rat liver. The results of a study of the metabolism of 16α-ethylprogesterone by female rat liver homogenate are presented here.

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Experimental

MATERIALS AND APPARATUS

Solvents were of A.R. grade or were redistilled.

Absorbance measurements were made on a Unicam SP500 spectrophotometer.

Thin-layer chromatography plates were prepared, 0·25 mm thick, from Merck silica gel G to which was added zinc silicate (2%) as a phosphor. The plates were activated by heating for 0·5 hr at 110°, and stored over silica gel. As the method of analysis was "internally compensating", the use of either non-treated silica gel or that which had been refluxed with methanol was without effect on the results. The plates were examined under light of 254 and 350 mμ wavelength.

Gas-liquid chromatography was performed on two instruments. A Pye Panchromatograph equipped with an argon ionization detector contained a 5 ft × 4 mm i.d. glass column packed with 1% cyclohexane dimethanol succinate plus 1% polyvinyl pyrrolidone on chromosorb G (60–80 mesh); column temperature 225° and argon flow rate 50 ml/min. A Pye 104 series instrument, equipped with flame ionization detectors, contained two 5 ft × 4 mm i.d. glass columns packed with (*a*) 3% SE30 on Celite (85–100 mesh); column temperature 224° and nitrogen flow rate 50 ml/min: and (*b*) 3% QF1 on Celite (85–100 mesh); column temperature 224° for free steroids and 209° for steroid trimethylsilyl ethers, nitrogen flow rate in both cases was 60 ml/min.

Mass spectral data was recorded on an LKB 9000 gas chromatograph-mass spectrometer. Two 5 ft × ¼ o.d. glass columns were used in this instrument, (*a*) 1% SE30 on gas chrom Q (100–120 mesh); column temperature 230°, helium flow rate 30 ml/min: and (*b*) 1% QF1 on gas chrom Q (100–120 mesh); column temperature 200°, helium flow rate 30 ml/min. Mass spectrometer ionization voltage 70 eV.

METHODS

Incubation media. Female Wistar rats (120 to 150 g weight) were stunned, exsanguinated, and the livers were removed and placed in ice-cold phosphate buffer (0·1M, pH 7·0). Connective tissue was removed and liver (approximately 5 ml by displacement) was homogenized with two volumes of phosphate buffer (0·1M, pH 7·0) for 2 min at full speed by an MSE homogenizer. The homogenate was filtered through four layers of muslin. All operations were at 0° to 2°, and the homogenate was stored briefly at this temperature till used. Incubation media consisted of glucose-6-phosphate (10 μmole), NADP (0·1 μmole), nicotinamide (15 μmole), magnesium chloride (20 μmole), phosphate buffer (0·2M, pH 7·4, 0·5 ml) and homogenate (1 ml) in a total volume of 3 ml. Substrate was added in solution (7·24 μg/μl) in propylene glycol. Incubation was effected aerobically with shaking for 2 hr at 37°.

Determination of the extent of metabolism with different substrate to tissue ratios. For each substrate concentration three incubation media were prepared: (1) *active metabolism*—incubation of substrate plus media;

(2) *maximum recovery determination*—incubation of media alone with addition of substrate after incubation and immediately before protein precipitation; (3) *background value*—incubation of media plus the relevant aliquot of propylene glycol.

The last determination (3) was used as a spectrophotometric blank in determining the absorbance of (1) and (2). Loss of substrate as given by the difference between values for (2) and (1) was taken as the amount of 16α-ethylprogesterone metabolized.

Immediately following incubation, protein was precipitated with methylene chloride–methanol (2:1; 12 ml) (McKerns & Nordstrand, 1965). The mixture was shaken (45 min) then centrifuged at 2000 *g* (5 min); the liquid phases were removed by aspiration, and the residual pellicle washed with methylene chloride–methanol (2:1; 12 ml). The combined liquid phases were evaporated to dryness under vacuum at a temperature not greater than 55°, and the residue was dried over phosphorus pentoxide. Acetone (1 ml) was added, and an aliquot (100 μl) of the acetone solution was placed on a thin-layer plate, which was developed with chloroform-acetone (95:5) for a distance of 15 cm. The area corresponding to 16α-ethylprogesterone, defined by a dark spot when it was irradiated at 254 mμ, was scraped from the plate. An equal area was taken from the chromatogram of the *background value*—defined as (3) above. The silica gel was extracted with ether (1 × 6 ml and 1 × 2 ml) the ether extract evaporated to dryness under a stream of nitrogen, and the residue was dissolved in ethanol (5 ml; increased to 10 ml in the more concentrated substrate determinations). The absorbance was measured at 240 mμ.

Identification of metabolites by thin-layer chromatography. Aliquots (20 μl) of the acetone solutions of the crude extracts from the incubation media were run on thin-layer plates in the solvent systems chloroform-acetone (95:5) and benzene–ethyl acetate (60:40) (Neher & Wettstein, 1960). 16α-Ethylprogesterone was located on the plate by irradiating it at 254 mμ: other steroids were visualized by heating the plate at 110° (3 min) then spraying either with sulphuric acid (sp.gr. 1·84)–water (1:1) or sulphuric acid (sp.gr. 1·84)–ethanol (1:1) (Neher, 1964). The plates were further heated at 110° (10 min) and the Rf values of the spots noted together with their colours when viewed in daylight or ultraviolet light (350 mμ). Reference steroids were run on the same plates as the extracts.

Identification of metabolites by gas-liquid chromatography. By direct injection: aliquots (1–2 μl) of the acetone solutions of the crude extracts were used.

By trimethylsilyl ether formation: an aliquot (0·9 ml) of the acetone solution of the crude extracts was evaporated to dryness under a stream of nitrogen. Pyridine (1·0 ml), hexamethyldisilazane (0·2 ml) and trimethylchlorosilane (100 μl), were added to the residue, and the mixture left at room temperature (1 hr) before evaporation to dryness under a stream of nitrogen. The residue was dissolved in n-hexane (100 μl), and aliquots (1–2 μl) were injected. Trimethylsilyl ethers of reference steroids (1 mg) were prepared and injected in a similar manner.

By combined thin-layer and gas-liquid chromatography: an aliquot (100 μl) of the acetone solution of the crude extracts and appropriate reference steroids were subjected to thin-layer chromatography as described above. After development, the reference steroids only were visualized and those areas from the extract which corresponded to the reference steroids were removed. The silica gel was extracted with ether (1 × 6 ml and 1 × 2 ml), and the ether extract was evaporated to dryness under nitrogen. The residue *per se* or in the form of the trimethylsilyl ethers (see above) was analysed by gas-liquid chromatography alone and in combination with mass spectrometry.

Quantitative determination of metabolites. Acetone solutions of the extracts from the active metabolism determination and from the maximum recovery determination of the same experiment were analysed by gas-liquid chromatography. Peak areas corresponding to 16α-ethylprogesterone (peak height × width at half height), were compared for equivalent amounts of the solutions injected. The difference in peak area between the maximum recovery determination and the active metabolism determination indicated the loss of 16α-ethylprogesterone resulting from metabolism. The areas of the remaining two peaks corresponding to metabolites in the active metabolism extract (the cholesterol peak was disregarded) were compared with that of the 16α-ethylprogesterone peak, and the percentage of each metabolite relative to recovered 16α-ethyl progesterone calculated. The calculation requires a similar molar response of the flame ionisation detector to the three steroids concerned. Application of the method of Sternberg, Gallaway & Jones (1962) indicates a maximum difference in molar response of 3%. The observed differences in molar response over a range of steroid concentrations were within the limits of experimental error.

Results

Metabolism of 16α-ethylprogesterone by female rat liver homogenate fortified with NADP, nicotinamide, glucose-6-phosphate and magnesium chloride was observed to an extent of about 60% over the range of steroid to tissue ratios studied (Table 1). These results are based on measurement of the absorption of the Δ^4-3-keto-chromophore at 240 mμ, and represent only metabolism resulting in loss of this chromophore. Identification of the metabolites (below) proved this to be a valid assessment of

TABLE 1. PERCENTAGE METABOLISM OF 16α-ETHYLPROGESTERONE BY RAT LIVER HOMOGENATE AT DIFFERENT STEROID TO TISSUE RATIOS

Experiment	Amount of steroid in incubation medium (μg)	Steroid : tissue ratio	Metabolism %		
			Series I*	Series II*	Mean value
1	180	1 : 1854	64	61	62·5
2	288·3	1 : 1156	67	60	63·5
3	370·4	1 : 900	59	47	53
4	520·0	1 : 641	72	64	68
5	1333·3	1 : 250	62	63	62·5

* Each value mean of three determinations.

total metabolism. Recovery of 16α-ethylprogesterone from an incubation medium in the absence of metabolism is 68%. No interference with the spectrophotometric determination (240 mμ) was observed, and Beer's law was obeyed over the concentration range 0 to 0·18 μmole of steroid per 5 ml ethanol.

Extracts from the experiments with higher steroid to tissue ratios (3, 4 and 5, Table 1) were separately analysed for metabolites. Relative retention values on thin-layer chromatograms and colour development with sulphuric acid–ethanol of reference 16α-ethyl-steroids are shown in Table 2. The limit of detectability of 16α-ethylprogesterone on fluorescent silica gel when irradiated with light of 254 mμ is 0·25 μg; that of 16α-ethyl-3α-hydroxy-5α-pregnan-20-one is 0·05 μg when a sulphuric acid–ethanol spray is used for visualisation. Cholesterol was chosen as an internal standard as it was an endogenous constituent of the extracts. Reference steroids were chromatographed on the same plates as the extracts, or were developed separately with the addition of cholesterol as reference standard. Table 2 shows that in addition to starting material

TABLE 2. THIN-LAYER CHROMATOGRAPHY RELATIVE RETENTION VALUES (CHOLESTEROL = 1·0) OF REFERENCE STEROIDS AND OF COMPONENTS PRESENT IN EXTRACTS OF INCUBATION MEDIA

Reference steroids		Relative retention in solvent system 1	Relative retention in solvent system 2	Colour in daylight (a)	Colour at 350 mμ (a)
16α-Ethylprogesterone		1·33	1·20	Light brown	Absorbs at 254 mμ (b)
16α-Ethyl-5α-pregnane-3,20-dione		1·39	1·23	Light brown	Pale blue
16α-Ethyl-5β-pregnane-3,20-dione		1·35	1·20	Light brown	Grey
16α-Ethyl-3α-hydroxy-5α-pregnan-20-one		0·98	0·96	Red-brown	Brown
16α-Ethyl-3β-hydroxy-5α-pregnan-20-one		0·83	0·93	Pink-brown	Brown
16α-Ethyl-3α-hydroxy-5β-pregnan-20-one		0·88	0·95	Red-brown	Brown
16α-Ethyl-5α-pregnane-3α,20β-diol		0·61	0·64	Pink-brown	Blue-grey
16α-Ethyl-20β-hydroxy-pregn-4-ene-3-one		0·76	0·78	Yellow	Yellow
16α-Ethyl-20β-hydroxy-5β-pregnan-3-one		0·93	0·96	Yellow-green	Blue
Cholesterol (reference standard)		1·00	1·00	Purple	Red
Components detected on chromatograms of extracts of incubation media	1	0·96	0·95	Red-brown	Brown
	2	1·00	1·00	Red-purple	Red
	3	1·30	1·18	Light brown	Absorbs at 254 mμ (b)
	4	1·38	1·23	Brown	Blue

Solvent system 1. Chloroform–acetone (95 : 5); cholesterol Rf = 0·54.
Solvent system 2. Benzene–ethyl acetate (60 : 40); cholesterol Rf = 0·56.
(a) Colours observed after spraying with sulphuric acid-ethanol.
(b) Absorption at 254 mμ determined before spraying.

(16α-ethylprogesterone), 16α-ethyl-5ε-pregnane-3,20-dione, 16α-ethyl-3ε-hydroxy-5ε-pregnan-20-one, and endogenous cholesterol were the only components present in extracts of incubation media. The relative retention data from both solvent systems suggested that the configuration at C-5 of the pregnanedione present (component 4) was alpha rather than beta: this was supported by the distinctive colour reactions of the two isomers. The epimeric 3-hydroxy-5α-pregnanones were clearly separated by the chloroform-acetone solvent system, as were the 3α-hydroxy-5α- and 5β-pregnanones. Thus component (1) of the incubation extract was probably 16α-ethyl-3α-hydroxy-5α-pregnan-20-one.

This assignment was confirmed by gas-liquid chromatographic analysis

(Table 3) which also excludes the 3β-hydroxy-5α- and 3α-hydroxy-5β-isomers. Table 3 also indicates that the relative retention values of components (2) and (3) of the extracts correspond with those of 16α-ethyl-5α-pregnane-3,20-dione and 16α-ethylprogesterone, respectively, again confirming thin-layer chromatography evidence. Retention data of the steroid trimethylsilyl-ethers gave further proof of the presence of the 3α-hydroxy-5α-pregnan-20-one, in the incubation extract.

Final confirmation of the structures of the isolated steroids was by subjecting the extracts to combined gas chromatography-mass spectrometry after a preliminary "clean-up" by thin-layer chromatography. The m/e values of the main fragment ions of components 3, 2 and 1 (Table 3) of the extract coincided with those of 16α-ethylprogesterone, 16α-ethyl-5α-pregnane-3,20-dione and 16α-ethyl-3α-hydroxy-5α-pregnan-20-one, respectively (Table 4). Minor differences in the percentage abundance figures can probably be attributed to extract-derived background in the effluent from gas-liquid chromatography. Where possible the mass spectrum of the background adjacent to the relevant peak was determined and subtracted from that of the peak. No major peak was present in the spectrum of any extract component for which there was not a corresponding peak in the spectrum of the relevant reference steroid.

Quantitative comparison of the 16α-ethylprogesterone peak areas obtained from gas-liquid chromatography of both the maximum recovery

TABLE 3. GAS-LIQUID CHROMATOGRAPHY RELATIVE RETENTION VALUES (CHOLESTEROL = 1·00) OF REFERENCE STEROIDS AND OF COMPONENTS PRESENT IN EXTRACTS OF INCUBATION MEDIA, AND OF THEIR TMS DERIVATIVES

Reference steroids		Relative retention in system 1	Relative retention in system 2A	Relative retention in system 3
16α-Ethylprogesterone		0·57	3·40	2·89
16α-Ethyl-5α-pregnane-3,20-dione		0·49	2·12	1·89
16α-Ethyl-5β-pregnane-3,20-dione		0·43	1·94	—
16α-Ethyl-3α-hydroxy-5α-pregnan-20-one		0·43	1·01	0·93
16α-Ethyl-3β-hydroxy-5α-pregnan-20-one		0·45	1·29	1·03
16α-Ethyl-3α-hydroxy-5β-pregnan-20-one		0·39	1·06	—
16α-Ethyl-5α-pregnane-3α,20β-diol		0·65	0·89	—
Cholesterol (reference standard)		1·00	1·00	1·00
Peaks observed on chromatograms of extracts of incubation media	1	0·43	1·01	0·92
	2	0·49	2·10	1·92
	3	0·56	3·50	2·86
	4	1·00	1·00	1·00
TMS Ethers of reference hydroxy steroids		Relative retention in system 1	Relative retention in system 2B	
16α-Ethyl-3α-hydroxy-5α-pregnan-20-one		0·33	0·81	
16α-Ethyl-3β-hydroxy-5α-pregnan-20-one		0·43	1·16	
16α-Ethyl-3α-hydroxy-5β-pregnan-20-one		0·34	0·83	
16α-Ethyl-5α-pregnane-3α,20β-diol		0·47	0·44	
Cholesterol		1·00	1·00	
Peaks observed on chromatograms of extracts of incubation media subject to TMS derivitization	1	0·34	0·81	
	2	1·00	1·00	

System 1. 3% SE30, column temp. 222°; Cholesterol Rt = 36·40 min; Cholesterol TMS ether Rt = 43·70 min.
System 2. 3% QFI, A. column temp. 224°; Cholesterol Rt = 12·30 min.
B. column temp. 209°; Cholesterol TMS ether Rt = 15·20 min.
System 3. 1% CDMS + 1% PVP, column temp. 225°; Cholesterol Rt = 9·20 min.

TABLE 4. MASS SPECTRAL DATA OF REFERENCE STEROIDS AND OF COMPONENTS PRESENT IN EXTRACTS OF INCUBATION MEDIA

m/e value	% of base peak: 16α-Ethyl progesterone	% of base peak: GLC peak 3 Table 3	m/e value	% of base peak: 16α-Ethyl-5α-pregnane-3,20-dione	% of base peak: GLC peak 2 Table 3	m/e value	% of base peak: 16α-Ethyl-3α-OH-5α-pregnan-20-one	% of base peak: GLC peak 1 Table 3
342[1]	56·2	53·3	344[1]	36·5	35·1	346[1]	36·8	35·0
327	17·6	17·6	329	33·2	34·8	331	38·7	32·4
300	29·1	22·8	315	63·3	64·2	317	62·3	55·2
257	17·9	20·2	301	16·8	21·6	288	32·1	29·5
244	30·3	29·9	286	37·9	36·1	285	21·7	21·8
219	20·2	17·5	246	59·0	56·5	248	52·8	54·8
175	16·7	18·8	231	53·1	53·7	233	54·7	51·9
161	16·8	20·5	147	27·0	26·9	147	24·9	24·7
135	19·7	21·1	135	23·8	26·6	135	29·6	25·3
124[2]	100·0	100·0	124	19·7	21·9	133	30·2	23·3
123	26·3	32·9	123	53·0	59·5	123	41·5	44·6
121	30·8	38·9	121	41·1	48·0	122	54·3	57·9
199	19·3	27·2	119	26·7	39·8	121	45·3	41·4
112	17·8	20·1	112[2]	100·0	100·0	112[2]	100·0	100·0

[1] Molecular ion. [2] Base peak of spectrum.

determination and active metabolism determination in experiment 5 (Table 1) confirmed the extent of metabolism as 62%. Comparison of the peak areas of the metabolites with that of recovered 16α-ethylprogesterone on the same chromatogram showed the amount of each metabolite formed to be 14% for 16α-ethyl-5α-pregnane-3,20-dione and 48% for 16α-ethyl-3α-hydroxy-5α-pregnan-20-one.

Discussion

A species difference is apparent in the nature of the products identified from the catabolism of progesterone by liver preparations (Table 5). Human liver and rabbit liver possess enzyme systems which catalyse the reduction of the C-4 double bond, yielding pregnanes of the 5α- and 5β-series, as well as reduction of the C-3 carbonyl function, giving both epimeric C-3 hydroxyl groups. On the other hand, reduction of the C-20

TABLE 5. *In vitro* LIVER METABOLISM OF PROGESTERONE, 16α-HYDROXYPROGESTERONE AND 16α-ETHYLPROGESTERONE

Metabolites identified				
Pregnane derivatives			16α-HO-pregnane derivatives	16α-Ethylpregnane derivatives
Human liver Progesterone (1)	Rabbit liver Progesterone (2)	Rat liver Progesterone (3)	Rat liver 16α-hydroxy-Progesterone (4)	Rat liver 16α-ethyl-Progesterone
3-one, 5α, 20-one	3-one, 5α, 20-one	3-one, 5α, 20-one	—	3-one, 5α, 20-one (a)
3-one, 5β, 20-one	—	—	—	—
3α-OH, 5α, 20-one	3α-OH, 5α, 20-one	3α-OH, 5α, 20-one	3α-OH, 5α, 20-one (a)	3α-OH, 5α, 20-one (b)
3β-OH, 5α, 20-one	3β-OH, 5α, 20-one	—	3β-OH, 5α, 20-one (b)	—
3α-OH, 5β, 20-one	3α-OH, 5β, 20-one	—	3α-OH, 5β, 20-one (c)	—
3α-OH, 5β, 20α-OH	3α-OH, 5β, 20α-OH	—	—	—
Major metabolites of 5β-Series	Major metabolites of 5β-Series	Above metabolites plus recovered progesterone in 28% yield	% yield of metabolites (a) 39·3% (b) 11·4% (c) 15·9%	% yield of metabolites (a) 14% (b) 48%

(1) Atherden (1959). (2) Taylor (1955).
(3) Taylor (1954). (4) Wettstein & others (1959).

carbonyl function yielded only the C-20α-hydroxyl group. Pregnanes of the 5α- and 5β- series have also been reported as metabolic products of 16α-hydroxyprogesterone by rat liver *in vitro* (Wettstein & others, 1959). Reduction of the C-3 carbonyl function gave both epimeric hydroxyl groups, although attack on the C-20 carbonyl function was not observed.

In the present investigation only 3α-hydroxy and 5α-steroids resulted, no evidence being obtained for either a 3β-hydroxy group or 5β-pregnanes. These observations are similar to those of Taylor (1954), who found that 5α-pregnane-3,20-dione and 3α-hydroxy-5α-pregnan-20-one were the only products of the catabolism of progesterone by rat liver preparations. In this tissue therefore 16α-ethylprogesterone and progesterone are metabolized by similar enzyme systems, whereas the 16α-hydroxy-analogue is subject further to reductase systems which produce β-isomerism at C-3 and C-5. Thus the metabolism of the progesterone molecule by rat liver homogenate would appear to be profoundly influenced by a 16α-hydroxyl substituent although the substituent itself is not metabolized. In addition in presence of a 16α-hydroxy-group but not a 16α-ethyl-group, the progesterone molecule is subject to enzymic change more similar to that encountered in rabbit liver than in rat liver.

Note added in proof. Recent evidence (Shirley & Cooke, 1968) indicates that 3β-hydroxy-5α- pregnan-20-one (5·8%) is a product of the metabolism of progesterone by female rat liver homogenate. A difference is therefore apparent in the metabolism of progesterone and 16α-ethylprogesterone by this tissue, as in the metabolism of the latter no product having the 3β-hydroxy-configuration was identified. The 16α-ethyl group may thus be significant in suppressing metabolism by this route. 5β-Pregnane-derivatives were not observed as metabolites (Shirley, I. M. & Cooke, B. A., 1968, *J. Endocr.*, **40** (4), 477–483).

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