Metabolism of Megestrol Acetate by Rat Adrenal Glands *in vitro*

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Megestrol acetate is metabolized by homogenates of rat adrenal glands to three more polar metabolites Y₂, Y₃ and Y₄. Their structures were investigated by microchemical reactions, paper chromatography, and ultraviolet and infrared spectroscopy. Metabolite Y₃ was identified as 17 α -acetoxy-11 β -hydroxy-6-methylpregna-4,6-diene-3,20-dione, its oxidation product being identical with authentic 17 α -acetoxy-6-methylpregna-4,6-diene-3,11,20-trione. It is suggested that metabolite Y₂ is 17,18-dihydroxy-6-methylpregna-4,6-diene-3,20-dione and that it also exists in the form of an α -ketol formed between the 18-hydroxyl and 20-oxo groups. Metabolite Y₄ is probably a tautomeric form of metabolite Y₂ as they were partially interconverted on chromatography and also have similar properties.

Previous studies have shown that megestrol acetate (17 α -acetoxy-6-methylpregna-4,6-diene-3,20-dione), an orally active ovulation inhibitor (David, Edwards, Fellowes & Plummer, 1963), is resistant to metabolism by liver preparations *in vitro* (Cooke & Vallance, 1965). It was concluded that this steroid is metabolized with difficulty, if at all, by the known normal pathways of progesterone metabolism because of interference by the Δ^6 , 6-methyl and 17 α -acetoxy groups, the effect of these groups being additive.

Cooke, McDonald & Vallance (1965) have reported that the adrenal gland contained a high concentration of radioactivity after oral administration of [6-Me-¹⁴C]megestrol acetate plus ethinyl-oestradiol [17 α -ethinyloestra-1,3,5(10)-triene-3,17 β -diol] to the female rat. It has now been found that megestrol acetate is readily metabolized *in vitro* by this tissue; the nature of the metabolites was therefore investigated.

METHODS

Solvents were purified by the methods described by Bush & Willoughby (1957).

Preparation of rat adrenal homogenate. Mature female Sprague-Dawley rats [B.D.H. (Research) Ltd. specific-pathogen-free colony] were killed by a blow on the head. The adrenal glands were quickly removed, chilled in 0.154M-KCl at 0–4° and dried between filter papers. The glands were homogenized in an incubation medium (6.0 ml. per adrenal) consisting of KH₂PO₄ (50 mM) adjusted to pH 7.4 with 2N-NaOH, KCl (50 mM), MgSO₄ (7 mM), nicotinamide (25 mM) and EDTA (1 mM) adjusted to pH 7.4 with 2N-NaOH. An all-glass motor-driven Potter-

type homogenizer (Aimer; medium fit) (600 rev./min.; eight complete strokes) was used.

Incubation procedure. Portions (3 ml.) of the homogenate were incubated at 37° with 100 μ g. of megestrol acetate dissolved in 0.1 ml. of *NN*-dimethylacetamide in 25 ml. stoppered flasks in a shaking incubator. An NADPH-generating system was also added, this consisting of NADP⁺ (0.6 μ mole), 0.1 M-glucose 6-phosphate (0.1 ml.) and glucose 6-phosphate dehydrogenase (0.5 unit).

Extraction procedure. At the end of the incubation period 0.7 M-Na₂HPO₄ (5 ml.) was added and three extractions were carried out with equal volumes of methylene dichloride-ethyl acetate (1:1, v/v). The combined extracts were washed with water and then filtered through Whatman no. 1 filter papers after addition of 1 drop of acetic acid. They were finally evaporated to dryness under reduced pressure on a rotary evaporator, or by a stream of N₂ at 45°.

Chromatography. The general chromatographic procedure, paper washing and solvent systems used were as described by Bush (1952, 1961). The residues obtained as described above were chromatographed at 25 $\pm$ 1° on washed Whatman no. 2 paper (22 in. $\times$ 2 in.) in the system light petroleum (b.p. 60–80°)-methanol-water (4:3:1, by vol.) (L/75). The u.v.-positive area corresponding to megestrol acetate was eluted and the amount of unchanged compound then determined as described by Cooke & Vallance (1965). The u.v.-positive areas near the origin were also eluted and the eluates re-run in light petroleum (b.p. 60–80°)-benzene-methanol-water (40:20:51:9, by vol.) (LB 21/85). The resulting u.v.-positive areas were individually eluted and determined as megestrol acetate.

Microchemical methods. Acetylations with acetic anhydride and pyridine, hydrolyses and oxidations with chromic acid, periodate and sodium bismuthate were carried out on the metabolites by the methods described by Bush (1961, pp. 358–365). For the hydrolyses 0.1 N-KOH in 70% (v/v) methanol was used, and chromic acid oxidations were carried out for 20 min. at room temperature. The blue tetrazolium, Zimmermann and phosphomolybdic acid reactions were carried out on paper as described by Bush (1961, pp. 374–379).

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Table 1. *Rate of formation of megestrol acetate metabolites during incubation with rat adrenal homogenates*

Experimental conditions are given in the Methods section. Amounts of metabolites formed are expressed as percentages of the megestrol acetate metabolized.

Incubation time (min.)	Megestrol acetate metabolized (%)	Metabolites formed (%)		
		Y ₂	Y ₃	Y ₄
15	2.8	3.7	3.1	0.4
45	23.2	8.3	9.4	1.7
120	49.6	22.3	16.0	2.6
240	57.7	26.1	16.8	5.3

The u.v.-absorption spectra of the metabolites and their derivatives were recorded on a Beckman DK-2 ratio recording spectrophotometer and i.r.-absorption spectra were obtained in CS₂ on a Hilger and Watts H 800 double-beam spectrophotometer with NaCl optics by using a micro-cell attachment.

R_F values of the metabolites and their derivatives were measured and the corresponding R_M values were calculated [$R_M = \log(1/R_F - 1)$] (Bate-Smith & Westall, 1950; Bush, 1961, pp. 74-132).

Preparation of 17 α -acetoxy-6-methylpregna-4,6-diene-3,11,20-trione. The experimental procedure adopted was an extension of the Vilsmeier and subsequent reactions described by Burn *et al.* (1964) and Burn, Kirk & Petrow (1965). Starting from 17 α -hydroxypregn-4-ene-3,11,20-trione (Bernstein, Brown, Feldman & Rigler, 1959), 17 α -acetoxy-6-methylpregna-4,6-diene-3,11,20-trione was

obtained as solvated plates (dichloromethane-methanol) m.p. 216.5-217°, [α_D^{25} +177° (c 1.0 in chloroform), $\lambda_{\text{max}}^{\text{EtOH}}$ 283 m μ (ϵ 22700) (Found: C, 70.2; H, 7.6; C₂₄H₃₀O₅, CH₃OH requires C, 69.75; H, 7.95%).

RESULTS

It was found that on incubating megestrol acetate with rat adrenal homogenates for up to 4 hr. three more polar u.v.-positive metabolites were obtained. These compounds were absent from control incubations in which megestrol acetate was added just before the ethyl acetate-methylene dichloride after incubating the adrenal homogenate for 3.5 hr. The compounds are referred to as metabolites Y₂, Y₃ and Y₄. A fourth u.v.-positive band (R_F 0.08 in LB 21/85) (metabolite Y₁), detected on chromatograms of extracts of incubations carried out with or without megestrol acetate, was disregarded.

Table 1 shows the rate of megestrol acetate metabolism by rat adrenal homogenates and the rate of formation of the metabolites. Metabolites Y₂ and Y₃ are the predominant metabolites, and approx. 83% of the megestrol acetate metabolized in 4 hr. was accounted for.

To obtain sufficient material for identification of the metabolites, replicate incubations were carried out for 3.5 hr. in the same way as single incubations. After separation of the metabolites as described in the Methods section, the metabolites with the same R_F were combined and rechromatographed two or three times in LB 21/85. The structures of these

Table 2. *Properties and microchemical reactions of megestrol acetate adrenal metabolites*

Solvent E4, methyleyclohexane-2-methylpropan-2-ol-methanol-water (20:9:9:2, by vol.) (Eberlein & Bongiovanni, 1955). N. D., Not determined.

Metabolite	Y ₂	Y ₄	Y ₃
Paper chromatography			
LB 21/85	R_F 0.24	R_F 0.82	R_F 0.38
E4	R_F 0.24	R_F 0.61	R_F 0.37
Absorption maximum	287.5 m μ	287.5 m μ	287.5 m μ
Hydrolysis (KOH-methanol)	Not hydrolysed	Not hydrolysed	Hydrolysed, R_F 0.10 in LB 21/85
Oxidation (chromic acid)	Oxidized, R_F 0.63 in LB 21/85	Oxidized, R_F 0.66 in LB 21/85	Oxidized, R_F 0.66 in LB 21/85
Oxidation (periodate)	Oxidized, R_F 0.63 in LB 21/85	Oxidized, R_F 0.66 in LB 21/85	Not oxidized
Oxidation (bismuthate)	Oxidized to two compounds, R_F 0.47 and 0.64 in LB 21/85	N.D.	N.D.
Acetylation (acetic anhydride-pyridine)	Not acetylated	Not acetylated	Not acetylated
Zimmermann reaction	Blue colour	Blue colour	Grey-blue colour
Blue tetrazolium reaction	Faint blue colour	Negative	Negative
Phosphomolybdc acid reaction	Negative	Negative	Negative
Sulphuric acid	Strong absorption at 361 m μ	N.D.	Lime-coloured fluorescent solution, absorption at 292 and 417 m μ

Table 3. *Infrared-spectra data of megestrol acetate adrenal metabolites and derivatives*

Metabolite ...	Frequency (cm. ⁻¹)		
	Y ₂	Y ₄	Y ₃
Unchanged metabolite	3625	3620	3635
	3585	3570 (weak)	1743
	1713	1712	1720 (shoulder)
	1667	1667	1713
	1628	1628	1668
Chromic acid oxidation product			1630
	3605 (weak)	3605 (weak)	3605 (weak)
	1748 (double-intensity peak)	1749	1746
	1716 (weak)	1711 (shoulder)	1719 (double-intensity peak)
	1668	1666	1666
Hydrolysis product			1629
	Not hydrolysed	Not hydrolysed	3583
			1736 (weak)
			1710
			1690 (weak)
			1664
			1626

metabolites and various derivatives were then investigated by paper chromatography and i.r. and u.v. spectroscopy. The data obtained by these methods are summarized in Tables 2, 3 and 4.

Metabolites Y₂ and Y₄. The u.v.-absorption spectra ($\lambda_{\max}$, 287.5 m μ) and i.r. spectra ($\nu_{\max}^{\text{CS}}$, 1667 and 1628 cm.⁻¹) of metabolites Y₂ and Y₄ indicated that the Δ^{4-6} -3-oxo group was unchanged. Neither metabolite was hydrolysed by methanolic potassium hydroxide under conditions that completely hydrolysed similar amounts of megestrol acetate and the i.r. spectra also showed the absence of the acetate peak at 1743 cm.⁻¹. Thus it may be concluded that the 17 α -acetoxy group present in megestrol acetate is absent from the two metabolites.

Metabolite Y₂ was readily oxidized by chromic oxide to one product (R_F 0.63 in LB 21/85) and by bismuthate to two products (R_F 0.47 and 0.64 in LB 21/85). Metabolite Y₂ was also oxidized by periodate (R_F 0.63 in LB 21/85) and had two peaks in the i.r. spectrum at 3625 and 3585 cm.⁻¹, suggesting the presence of two adjacent hydroxyl groups, though metabolite Y₂ was not acetylated by acetic anhydride in pyridine. There was also a peak at 1713 cm.⁻¹, indicating the presence of a six-membered-ring ketone or a 20-oxo group. However, the i.r. spectra of the chromic acid-oxidized product showed an intense peak at 1748 cm.⁻¹ and a very small peak at 1716 cm.⁻¹, which would seem to contraindicate the presence of a six-membered-ring ketone. It is, on the other hand, difficult to account for a 20-oxo group in the presence of two hydroxyl groups, presuming these to be on C-17 and C-20, unless metabolite Y₂ exists in two isomeric forms. The i.r. spectrum of oxidized metabolite Y₂ in the

'fingerprint' region revealed a peak at 1233 cm.⁻¹ and a peak of double intensity at 1748 cm.⁻¹, strongly indicating the presence of an acetate group. This could only have arisen by the formation of a ring structure with a group adjacent to the side chain, i.e. at C-18, and its subsequent fission to form the acetate and a 17-oxo group ($\nu_{\max}^{\text{CS}}$, 1748 cm.⁻¹). As 18-hydroxylation is a well-documented reaction in rat adrenal glands (Péron, 1962; Sandor & Lanthier, 1963) it is suggested that the structures shown in Scheme 1 fit the available data.

The formation of an α -ketol from the 18-hydroxyl and 20-oxo group has been reported also with

Table 4. *Predicted and found R_M values for megestrol acetate adrenal metabolite Y_3*

Solvent LT 11/70, light petroleum-toluene-methanol-water (5:5:7:3, by vol.).

	Solvent system	Found R_{Mg}	Predicted R_{Mg}	Group introduced
Megestrol acetate	L/75	1.64	1.27-1.31*	11-OH
↓ Y_3				
Megestrol acetate	LB 21/85	0.99	0.86-0.99*	
↓ Y_3				
	Solvent system	Found R_{Mr}	Predicted R_{Mr}	Reaction
↓ Y_3	LB 21/85	0.48	0.18-0.47† (LT 11/70)	11-OH
Oxidized Y_3				↓
↓ Y_3	LB 21/85	0.75	0.75	11-Oxo group 17-O·CO·CH ₃
Hydrolysed Y_3				↓
				17-OH

* Bush (1961, p. 87).

† Bush (1961, p. 106).

18-hydroxycorticosterone (Péron, 1962; Sandor & Lanthier, 1963). The presence of two adjacent hydroxyl groups as well as the 20-oxo group indicated in the i.r. spectra would be explained if metabolite Y_2 were a mixture of both forms. On oxidation, the bond between C-17 and C-20 would be split with the formation of an 18-acetoxy group and a 17-oxo group.

It is concluded that metabolite Y_2 is most probably the 17,18-dihydroxy-6-methylpregna-4,6-diene-3,20-dione and that it also exists in the form of an α -ketol resulting from ring closure between the 18-hydroxyl and 20-oxo groups.

It was found that metabolites Y_2 and Y_4 were partially interconverted when subjected to paper chromatography, approx. 25% of metabolite Y_2 being converted into metabolite Y_4 in the system LB 21/85. Metabolite Y_4 was also found to have very similar properties to those of metabolite Y_2 , the i.r. spectra of the unchanged metabolites and the chromic acid oxidation products being identical. It is probable that metabolite Y_4 is a tautomeric form of metabolite Y_2 , though it is difficult to postulate a structure for it in view of their large difference in polarity on paper chromatograms (R_F 0.82 and R_F 0.24 respectively in LB 21/85). Metabolite Y_4 is most probably not the α -ketol form of metabolite Y_2 because this could be expected to have a similar polarity to the 18-hydroxy-20-oxo form. It is noteworthy that Péron (1962) and Sandor & Lanthier (1963) found that 18-hydroxycorticosterone also formed a less polar compound on chromatography, the difference in polarity between the two compounds being approximately the same as that found between metabolites Y_2 and Y_4 .

Metabolite Y_3 . The u.v.-absorption spectra ($\lambda_{\max}$,

287.5m μ) and i.r. spectra ($\nu_{\max}^{CS_2}$, 1713, 1668 and 1630cm.⁻¹) of metabolite Y_3 indicate the presence of the Δ^{4-6} , 3-oxo and 20-oxo groups. A peak was also present at 1743cm.⁻¹, indicating a 17-acetate. This was confirmed by hydrolysis with methanolic potassium hydroxide, the peak being absent from the spectrum of the hydrolysed product. The change in ΔR_{Mg} also agrees with the found value for the hydrolysis of the 17 α -acetoxy group of megestrol acetate (see Table 4).

A peak was present at 3635cm.⁻¹, indicating the presence of a hydroxyl group. As metabolite Y_3 was not acetylated by acetic anhydride in pyridine but was oxidized by chromic acid though not by periodic acid, it would appear that the hydroxyl group is in a hindered position. This was further substantiated by the presence of a peak at 1719cm.⁻¹ in the i.r. spectrum of oxidized metabolite Y_3 having double intensity, thus indicating the presence of a six-membered-ring ketone in addition to the 20-oxo group. These data, together with the observed changes in ΔR_{Mr} and ΔR_{Mg} (see Table 4), could be explained by the occurrence of 11 β -hydroxylation, a reaction known to occur in adrenal glands. Consequently the reference compound 17 α -acetoxy-6-methylpregna-4,6-dione-3,11,20-trione was prepared chemically (see the Methods section) and compared with oxidized metabolite Y_3 . It was found that these two compounds have identical i.r. (ranges scanned: 1400-650, 2000-1650 and 4000-2700cm.⁻¹) and u.v. ($\lambda_{\max}$, 283.0m μ) spectra and the same R_F in LB 21/85. It is concluded that oxidized metabolite Y_3 is 17 α -acetoxy-6-methylpregna-4,6-diene-3,11,20-trione and that metabolite Y_3 is therefore most probably 17 α -acetoxy-11 β -hydroxy-6-methylpregna-4,6-diene-3,20-dione.

DISCUSSION

Hydroxylation of naturally occurring steroids *in vitro* at the 11 and 18 positions by rat adrenal glands has been established by various workers (e.g. Johnson, Snell, Francois & Heftmann, 1961; Birmingham & Ward, 1961). It would appear from the present experiments that 11- and 18-hydroxylation also occurs when megestrol acetate is incubated with rat adrenal tissue, i.e. neither of these normal biosynthetic pathways in the adrenal gland is impeded by the presence of Δ^6 , 6-methyl and 17 α -acetoxy groups in this compound. This is of interest in that the presence of these groups has a marked inhibitory effect on the metabolism of megestrol acetate by rat and rabbit liver preparations (Cooke & Vallance, 1965).

Other synthetic steroids have also been shown to be hydroxylated by adrenal tissue, e.g. 11 β -hydroxylation of 17 β -acetoxy-17 α -ethynyltestosterone and 17 β -acetoxy-17 α -vinyltestosterone occurs on perfusion through surviving bovine adrenal tissue (Marshall, Ralls, Saunders & Riegel, 1957). The 17 β -acetoxy groups of these two compounds were not hydrolysed by the perfusion and it was concluded by the authors that this was probably due to their hindered position, primary and secondary alcohol acetates being easily hydrolysed by adrenal tissue. It was found in the present study that the tertiary 17 α -acetoxy group of megestrol acetate does undergo hydrolysis with adrenal tissue; it was absent from metabolites Y₂ and Y₄ though not from metabolite Y₃. Metabolites isolated from rabbit urine (Cooper, Jones & Kellie, 1965) and rat bile (B. A. Cooke & D. K. Vallance, unpublished work) after oral administration of megestrol acetate had the 17 α -acetoxy group intact.

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