Metabolism of Megestrol Acetate and Related Progesterone Analogues by Liver Preparations *in vitro*

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1. The rates of metabolism of megestrol acetate, 17 α -acetoxy-6 α -methylprogesterone, 6 α -methylprogesterone, 17 α -acetoxyprogesterone and progesterone by liver microsome-supernatant fractions from female rats and rabbits have been compared. 2. Introduction of the 6 α -methyl group into the progesterone molecule markedly decreased the metabolism by liver microsome-supernatant preparations in the rat but not in the rabbit. When 17 α -acetoxy was the substituting group, metabolism was diminished in the rabbit but not in the rat preparations. Introduction of both the 6 α -methyl and 17 α -acetoxy groups into the progesterone molecule markedly decreased the rate of metabolism by liver microsome-supernatant preparations of both species. The Δ^6 -bond in megestrol acetate confers additional resistance to metabolism by rabbit-liver preparations. 3. It is proposed that the observed inhibition of metabolism by these substituents in the progesterone molecule partly or wholly explains the enhanced activity of megestrol acetate.

It has been suggested that the remarkably enhanced hormonal activity of progesterone when substituted at C-6 and C-17 in the steroid nucleus is due to increased resistance to metabolizing enzymes (Bush, 1962; Glenn, Richardson & Bowman, 1959). Preliminary experiments with one of these substituted progesterones, megestrol acetate (17 α -acetoxy-6-methylpregna-4,6-diene-3,20-dione), a potent orally active ovulation inhibitor (David, Edwards, Fellowes & Plummer, 1963), indicated that it was very resistant to metabolism *in vitro* by rat liver as compared with progesterone. It was therefore decided to identify the structural features in megestrol acetate that prevent its rapid metabolism. This has been accomplished by measuring the rates of metabolism of progesterone (pregn-4-ene-3,20-dione), 6 α -methylprogesterone, 17 α -acetoxyprogesterone, 17 α -acetoxy-6 α -methylprogesterone and megestrol acetate by microsome-supernatant fractions of liver obtained from female rats and rabbits. A preliminary communication of this work has been published (Cooke & Vallance, 1964).

METHODS

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

Preparation of liver microsome-supernatant fraction. Mature female Sprague-Dawley rats (BDH-SPF colony) or female New Zealand White rabbits were killed by a blow on the head. The liver was quickly removed, chilled in 0.154M-

KCl at 0–4°, dried between filter papers, minced and weighed. A 20% homogenate was prepared in an incubation medium (4.5 ml./g.) consisting of KH₂PO₄ (50 mM) adjusted to pH 7.4 with 2N-NaOH, KCl (50 mM), MgSO₄ (7 mM), nicotinamide (25 mM), EDTA (1 mM) adjusted to pH 7.4 with 2N-NaOH, and sucrose (0.25 M), by using an all-glass motor-driven Potter-type homogenizer (Aimer; medium fit) (600 rev./min.; 8 complete strokes). The homogenate was centrifuged at 900g for 20 min. at 0°, diluted with an equal volume of the incubation medium and then centrifuged at 10000g in an MSE centrifuge fitted with a Super-Speed unit for 10 min. at 0° to remove mitochondria.

Incubation procedure. Varying quantities (3 ml., 1 ml. or 0.25 ml.) of the microsome-supernatant preparation remaining after removal of mitochondria were added immediately to the incubation vessels containing an appropriate amount of the incubation medium together with a NADPH-generating system to give a final volume of 3.3 ml. The NADPH-generating system consisted of NADP⁺ (0.6 μ -mole), 0.1 M-glucose 6-phosphate (0.1 ml.) and glucose 6-phosphate dehydrogenase (0.5 unit). Finally, steroid dissolved in *N,N*-dimethylacetamide (1 mg./ml.) was added (all the steroids used in these experiments were soluble in the incubation medium at this concentration) to give a final tissue/steroid ratio 15000:1 (with 20 μ g. of steroid), 1000:1 (with 100 μ g. of steroid) or 250:1 (with 100 μ g. of steroid). The tissue/steroid ratios were calculated from the original weight of liver mince. Incubation was carried out at 37° in 25 ml. stoppered flasks in a shaking incubator and was terminated by the addition of acetone at –15° at suitable time-intervals. Each incubation was carried out in duplicate, and those incubations carried out at the same time and with the same microsome-supernatant preparation are given the same experiment number in the Tables.

Extraction procedure. On termination of the incubation, extraction was performed as described by Cooke & Taylor (1963a) except that methylene dichloride-ethyl acetate (1:1, v/v) was used instead of hexane-chloroform. One drop of acetic acid was added to the final combined extracts, which were then filtered through Whatman no. 1 filter paper and 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, 1963). The extracts obtained as described above were defatted on washed Whatman no. 2 paper (22 in. × 2 in.) with 75% (v/v) methanol, run up to the origin with ethyl acetate-methanol (3:1, v/v) and then chromatographed in the following systems at 25 ± 1°: light petroleum (b.p. 60–80°)-acetic acid-water (10:9:1, by vol.) for extracts containing 6 α -methylprogesterone, and light petroleum (b.p. 60–80°)-methanol-water (4:3:1, by vol.) for extracts containing progesterone, megestrol acetate, 17 α -acetoxyprogesterone and 17 α -acetoxy-6 α -methylprogesterone.

Steroid estimations. Appropriate areas of chromatograms corresponding to unmetabolized steroids were eluted with ethyl acetate-methanol (3:1, v/v). Steroids that had λ_{max} at 240 m μ were estimated by their extinctions at 292, 302 and 312 m μ after the thiosemicarbazone derivatives had been formed as described by Sommerville, Pickett, Collins & Denyer (1963). Results were corrected for background absorption by applying the Allen (1950) correction. Megestrol acetate was estimated by its extinctions at 267.5, 287.5 and 307.5 m μ , the values obtained again being corrected for background absorption by the method of Allen (1950). Paper blanks and incubation extracts containing no steroid gave values equivalent to less than 0.6 μ g. of steroid thiosemicarbazone or megestrol acetate.

The percentage of steroid unmetabolized was calculated, a correction for extraction losses being applied. The latter was based on the percentage recovery from controls in which

steroid was added to previously incubated microsome-supernatant preparations before the precipitation of protein by acetone. The mean recovery from these controls, which were carried out with each steroid in all the experiments, was 72 ± 10% (21) (s.d.). The difference between duplicate determinations was less than 5%.

RESULTS

Preliminary experiments in which megestrol acetate, 6 α -methylprogesterone and 17 α -acetoxy-6 α -methylprogesterone were incubated with rat-liver homogenate at a tissue/steroid ratio 2000:1 showed that less than 5% of each steroid was metabolized during periods of up to 3.5 hr. at 37°. Progesterone, on the other hand, was completely metabolized within 15 min. under the same conditions.

The results in Table 1 show that progesterone was also metabolized very rapidly by microsome-supernatant fractions of rat and rabbit liver. A tissue/steroid ratio 250:1 was used for the rat-liver preparation, since progesterone was completely metabolized within a few minutes when higher ratios were used. A comparable rate of metabolism by rabbit liver was obtained at a tissue/steroid ratio 1000:1, i.e. rabbit liver was less active than rat liver (w/w). Similar results were obtained by Taylor (1955) with homogenates. Relative to progesterone, 6 α -methylprogesterone was slowly metabolized by rat liver at a tissue/steroid ratio 250:1 but at a similar rate by rabbit liver at a tissue/steroid ratio 1000:1 (Table 1). 17 α -Acetoxyprogesterone, on the other hand, was metabolized

Table 1. *Metabolism of progesterone, 6 α -methylprogesterone and 17 α -acetoxyprogesterone by rat- and rabbit-liver microsome-supernatant fractions*

Experimental details are given in the Methods section. N.D., Not determined.

Steroid	Rat liver (tissue/steroid ratio 250:1)			Rabbit liver (tissue/steroid ratio 1000:1)		
	Incubation time (min.)	% metabolized		Incubation time (min.)	% metabolized	
		Expt. 1	Expt. 2		Expt. 3	Expt. 4
Progesterone	5	24	27	10	22	32
	10	69	70	20	N.D.	48
	20	98	94	43	84	73
	30	100	98	120	N.D.	93
17 α -Acetoxyprogesterone	5	17	23	10	13	None
	10	54	51	20	N.D.	None
	20	87	93	43	10	2
	30	99	100	120	N.D.	39
6 α -Methylprogesterone	5	None	N.D.	10	N.D.	18
	15	None	6	20	N.D.	37
	30	2	N.D.	45	N.D.	59
	120	9	N.D.	120	N.D.	80

as rapidly as progesterone by rat liver but more slowly by rabbit liver at tissue/steroid ratios 250:1 and 1000:1 respectively (Table 1).

Megestrol acetate and 17 α -acetoxy-6 α -methylprogesterone were very slowly metabolized at tissue/steroid ratios 250:1 and 1000:1 by rat and rabbit liver respectively. At the higher ratio 15000:1 more metabolism took place (Table 2) but neither steroid was completely metabolized by rat liver even after 4 hr. incubation. 17 α -Acetoxy-6 α -methylprogesterone was metabolized more rapidly than megestrol acetate by rabbit liver and possibly also by rat liver. 17 α -Acetoxyprogesterone and 6 α -methylprogesterone were rapidly metabolized at this higher tissue/steroid ratio 15000:1 by rabbit and rat liver respectively (Table 2).

To confirm that the loss of steroids that occurred during these experiments was due to metabolism, experiments were carried out in which NADPH and nicotinamide were omitted from the incubation medium. NADPH is one of the essential cofactors for steroid metabolism, and nicotinamide protects the nicotinamide nucleotides from destruction by nucleotidases (Bassham, Birt, Hems & Loening, 1959). Table 3 shows that in the absence of these cofactors the rates of metabolism of megestrol acetate and 17 α -acetoxy-6 α -methylprogesterone by rat-liver microsome-supernatant fraction were decreased, although 6 α -methylprogesterone was

still rapidly metabolized at a tissue/steroid ratio 15000:1. As this may have been due to the presence of endogenous NADPH, a further experiment was carried out with a microsome-supernatant preparation that had been heated at 37° for 20 min. in the absence of nicotinamide with the object of destroying the endogenous nicotinamide nucleotide (Bassham *et al.* 1959). Little or no metabolism of megestrol acetate, 17 α -acetoxy-6 α -methylprogesterone or 6 α -methylprogesterone was then found to occur if no NADPH and nicotinamide were added to the incubation medium (Table 3). Although pre-incubation of the liver preparation will also destroy endogenous conjugating systems (Cooke & Taylor, 1963a), no suitable groups are available in the unmetabolized steroids for conjugation. It may be concluded therefore that the observed loss of steroid during the main experiments was the result of metabolism.

It has been reported by Glenn *et al.* (1959) that 6 α -methylprogesterone inhibits the generation of NADPH from NADP⁺ by glucose 6-phosphate and glucose 6-phosphate dehydrogenase. As this regenerating system has been used in the present series of experiments, additional amounts of NADP⁺ plus NADPH-regenerating system have been added during incubation of megestrol acetate with rabbit-liver microsome-supernatant fractions to determine whether the amount of metabolism could thereby be

Table 2. *Metabolism of megestrol acetate, 17 α -acetoxy-6 α -methylprogesterone, 6 α -methylprogesterone and 17 α -acetoxyprogesterone by rat- and rabbit-liver microsome-supernatant fractions*

Experimental details are given in the Methods section. N. D., Not determined.

Steroid	Rat liver (tissue/steroid ratio 15000:1)				Rabbit liver (tissue/steroid ratio 15000:1)		
	Incubation time (min.)	% metabolized			Incubation time (min.)	% metabolized	
		Expt. 5	Expt. 6	Expt. 7		Expt. 3	Expt. 8
Megestrol acetate	15	15	N.D.	N.D.	5	N.D.	None
	30	N.D.	25	9	20	N.D.	15
	60	40	36	N.D.	30	23	N.D.
	120	33	36	13	45	N.D.	16
	240	33	46	20	120	49	44
17 α -Acetoxy-6 α -methyl- progesterone	15	42	N.D.	N.D.	5		26
	30	N.D.	40	32	20		66
	60	44	46	N.D.	45		89
	120	54	44	65	120		89
	240	49	55	73			
6 α -Methylprogesterone	5	N.D.	34				
	20	N.D.	69				
	45	N.D.	100				
	60	46	100				
	120	73	N.D.				
	240	100	N.D.				
17 α -Acetoxyprogesterone					15	87	
					45	100	
					120	100	

Table 3. *Effect of preincubation and/or omission of NADPH and nicotinamide from the incubation medium on the rate of metabolism of megestrol acetate, 17 α -acetoxy-6 α -methylprogesterone and 6 α -methylprogesterone by a microsome-supernatant preparation of rat liver*

Experimental details are given in the Methods section. The tissue/steroid ratio was 15000:1.

Incubation conditions	Steroid	Incubation time (min.)	% metabolized	
			Expt. 9	Expt. 10
NADPH and nicotinamide present	Megestrol acetate	240	79	
	17 α -Acetoxy-6 α -methylprogesterone	240	77	
	6 α -Methylprogesterone	60	100	
NADPH and nicotinamide omitted	Megestrol acetate	240	33	
	17 α -Acetoxy-6 α -methylprogesterone	240	31	
	6 α -Methylprogesterone	60	100	
NADPH and nicotinamide present	Megestrol acetate	15		21
		180		71
	17 α -Acetoxy-6 α -methylprogesterone	15		1
		180		51
NADPH and nicotinamide omitted; microsome-supernatant preparation pre-incubated at 37° for 20 min.	Megestrol acetate	15		5
		180		6
	17 α -Acetoxy-6 α -methylprogesterone	15		None
		180		None
	6 α -Methylprogesterone	15		None

Table 4. *Effect of adding NADPH during the incubation of megestrol acetate with a rabbit-liver microsome-supernatant preparation*

The tissue/steroid ratio was 15000:1.

Steroid	Incubation conditions	Incubation time (min.)	% metabolized
			Expt. 11
Megestrol acetate	See the Methods section	45	36
		120	42
	Further NADP ⁺	45	31
	+NADPH-generating system added 20 min. after incubation had commenced	120	39

increased. The NADP⁺ and NADPH-regenerating system were incubated at 37° for 5 min. before addition to incubation mixtures to ensure that all the NADP⁺ had been reduced to NADPH. The results in Table 4 show that the amount of megestrol acetate metabolized was not increased by the further addition of NADPH, and thus the relatively slow rate of metabolism of megestrol acetate cannot be explained on the basis of inhibition of the NADPH-generating system by the steroid or its metabolic products.

As described in the Methods section, steroids were added to the incubation vessels as solutions in *NN*-dimethylacetamide, megestrol acetate being

Table 5. *Effect of varying amounts of dimethylacetamide on the rate of progesterone metabolism by microsome-supernatant fractions of rat liver*

Experimental details are given in the Methods section. The tissue/steroid ratio was 250:1

Steroid	Dimethylacetamide added (ml.)	Incubation time (min.)	% metabolized	
			Expt. 12	Expt. 1
Progesterone	0.02	15	82	
	0.065	15	90	
	0.12	15	92	
Progesterone	0.02	10		78
	0.07	10		74
	0.18	10		79

more soluble in *NN*-dimethylacetamide than in the solvents more commonly used, e.g. ethanol and propylene glycol. The latter are also subject to the disadvantage that they may inhibit conjugation, as demonstrated for [4-¹⁴C]progesterone metabolism by rat-liver homogenates *in vitro* by Cooke & Taylor (1963b). As the effect of *NN*-dimethylacetamide on metabolism has not previously been investigated, and as different amounts were used in the experiments described above depending on the tissue/steroid ratio employed, the effect of varying quantities of this solvent on progesterone metabolism was investigated. No effect on the rate of metabolism was found (Table 5).

DISCUSSION

The results show that the introduction of both a 6 α -methyl and a 17 α -acetoxy group into the progesterone molecule produces a marked resistance to metabolism by liver microsome-supernatant fractions from female rats and rabbits. Glenn *et al.* (1959) obtained similar results although their rat-liver microsome-supernatant preparation (sex not given) was more active in metabolizing 17 α -acetoxy-6 α -methylprogesterone and less active in metabolizing progesterone. When only one of the substituent groups is introduced, there is a marked species difference in the effect on the rate of metabolism. It is probable that the 6 α -methyl and 17 α -acetoxy groups block metabolism by inhibiting reduction of the Δ^4 -3-oxo and C-20 groups respectively, and thus the difference in the metabolism of 6 α -methylprogesterone and 17 α -acetoxyprogesterone may be due to the different amounts of metabolizing enzymes present in the two species.

In female-rat liver, the Δ^4 -3-oxo steroid reductases have been shown to be very active, whereas reduction of progesterone at C-20 has not been demonstrated. Taylor (1954) found no C-20-reduced steroid products after incubating progesterone with female-rat-liver homogenate. Rechnagel (1957) showed that the steroid C-20 reductase in the microsome fraction of male-rat liver had a marked preference for steroids with a dihydroxyacetone side chain and was relatively inactive towards steroids lacking a 17-hydroxyl group. Cooke (1961) and Cooke & Taylor (1962) found that 87% of progesterone undergoing metabolism by female-rat-liver homogenate could be accounted for as 5 α -pregnane-3,20-dione and 3 α - and 3 β -hydroxy-5 α -pregnan-20-one. From the evidence available, it would seem that ring A reduction of progesterone is the initial step in female-rat liver whereas C-20 reduction is a minor step, if it occurs at all. Reduction of both the C-20-oxo and Δ^4 -3-oxo groups of progesterone by rabbit-liver homogenate has been shown to occur (Taylor, 1955), but the relative activities of the enzymes have not been investigated. Taylor showed that progesterone is converted into 5 β -pregnane-3,20-dione, 3 α - and 3 β -hydroxy-5 α -pregnan-20-one, 3 α -hydroxy-5 β -pregnan-20-one and 5 β -pregnane-3 α ,20 α -diol by rabbit-liver homogenates. No sex differences were found. The last-named compound is excreted naturally in rabbit urine (Verley, Sommerville & Marrian, 1950) and is also excreted after the injection of progesterone (Hoffman, 1942; Hoffman & Browne, 1942; Westphal, 1942). Thomas (1962) and Cooke, Rogers & Thomas (1963) found 20 α -hydroxy-pregn-4-en-3-one but not 3 α -hydroxy-5 β -pregnan-20-one in rabbit urine after the administration of progesterone. It appears therefore that reduction

of progesterone by rabbit liver can occur initially at C-20 or in ring A.

Introduction of a Δ^6 -bond, in addition to 6-methyl and 17 α -acetoxy groups, into the progesterone molecule, as in megestrol acetate, gives further protection to metabolizing enzymes, possibly by inhibiting hydroxylation at the C-6 position. Such a reaction has been shown to occur with 17 α -acetoxy-6 α -methylprogesterone in man, the metabolites isolated from human urine after the administration of this steroid being 6 β ,17 α ,21-trihydroxy-6 α -methylpregn-4-ene-3,20-dione (21-acetate) (Castegnaro & Sala, 1962) and 6 β ,17 α ,21-trihydroxy-6 α -methylpregn-4-ene-3,20-dione (17-acetate) (Helmreich & Huseby, 1962). Cooper, Jones & Kellie (1965) have isolated 17 α -acetoxy-6-hydroxymethylpregna-4,6-diene-3,20-dione and 17 α -acetoxy-2 α -hydroxy-6-hydroxymethylpregna-4,6-diene-3,20-dione from rabbit urine after the oral administration of [1,2- 3 H₂]megestrol acetate, again indicating that reduction and subsequent hydroxylation of the C-20 and Δ^4 -3-oxo groups is blocked in this compound.

In conclusion, therefore, it seems probable that the various modifications of the steroid molecule illustrated by the compounds included in these investigations block the normal pathways of progesterone metabolism by liver microsome-supernatant fractions. As the liver is probably the major site of steroid metabolism and compounds given orally pass through the liver before entering the general circulation, the results obtained may well be a true reflection of the fate of these progesterone analogues in the intact animal. Further, this resistance to metabolism could explain the high oral progestational activity of megestrol acetate as compared with the parent compound.

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