Steroid Metabolism in the Rabbit

BILIARY AND URINARY EXCRETION OF METABOLITES OF [4-¹⁴C]PROGESTERONE

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1. [4-¹⁴C]Progesterone was administered intravenously to anaesthetized male and female New Zealand White rabbits as a single injection or as a 45–60 min. infusion. 2. After a single dose about 60% of the radioactivity was recovered in 6 hr., and twice as much radioactivity was present in bile as in urine. After infusion total recovery of radioactivity was only about 40% in 6 hr., but the relative proportions of metabolites in bile and urine were about the same as after a single dose. 3. Bile and urine samples were hydrolysed successively by β -glucuronidase, cold acid and hot acid. 4. In bile the major proportion of metabolites appeared in the glucuronide fraction; in urine β -glucuronidase hydrolysis yielded the greatest amounts of ether-extractable radioactivity, but the greatest proportion of radioactivity could not be extracted by ether from an alkaline solution of the hydrolysed urine. 5. There was no apparent difference in the quantity or distribution of metabolites excreted by male and female animals.

The anaesthetized cat has been used for the study of biliary and urinary excretion of ¹⁴C-labelled steroids. Relatively small amounts (less than 100 μ g.) of steroid can be administered intravenously and excretion of metabolites followed for up to 6 hr. (Taylor & Scratcherd, 1961, 1963; Archer, Scratcherd & Taylor, 1965). The results obtained correlate well with those obtained with intact animals (Taylor & Scratcherd, 1962). The excretion of metabolites of [4-¹⁴C]progesterone in the bile and urine of anaesthetized rabbits has therefore been investigated. A preliminary account of part of this work has been published (Taylor, 1964, 1965).

MATERIALS AND METHODS

[4-¹⁴C]Progesterone (pregn-4-ene-3,20-dione) (20.8 mc/mole) was obtained from The Radiochemical Centre, Amersham, Bucks., and used without further purification. The stock solution in ethanol was stored at -15° : as judged by paper chromatography at least 95% of the radioactivity had the same R_F as authentic progesterone even after storage for 10 weeks. The steroid was administered either as a single dose (0.4 ml. of stock solution in 0.6 ml. of 0.15 M-NaCl) or by continuous infusion over 45–60 min. (0.8 ml. of stock solution in 20–25 ml. of 0.15 M-NaCl). New Zealand White rabbits were anaesthetized with urethane (1 g./kg.) after preliminary induction with ether. The operative procedure was slightly different from that used on cats. All injections were made through a cannula in the external jugular vein. The splanchnic nerves were not cut, and the pylorus was not tied off since it has been shown that choleresis is not induced

in the rabbit by acid entering the duodenum (Scratcherd, 1965). Bile was collected by inserting a stainless-steel cannula into the common bile duct, and the cystic duct was ligated. The urinary bladder was intubated as described for cats (Taylor & Scratcherd, 1961). Because of the small volume of urine flow in some animals 2 ml. of 0.15 M-NaCl was passed through a fine polythene cannula inserted in the lumen of the larger cannula. The urine and saline washings were expelled from the bladder and collecting tube by gently forcing air through the fine cannula by means of a syringe. In those animals in which spontaneous urine flow occurred the final urine sample was obtained by passing air through the fine cannula until no fluid remained in the collecting tube. Descriptions of materials, methods of hydrolysis and of radioactive assay were as described by Taylor & Scratcherd (1961, 1963), except that the 'free steroid' fraction was not measured since preliminary experiments showed that less than 0.5% of the radioactivity in bile and urine samples was extractable by ether from alkaline solutions before hydrolysis.

RESULTS

Table 1 shows the excretion of radioactivity in the bile of male and female rabbits after a single intravenous injection (animals 1 and 2) and after 45–60 min. infusion (animals 3–6) of a dose (less than 100 μ g.) of [4-¹⁴C]progesterone. Also included in Table 1 are the volumes of bile collected and the concentrations of total solids to show the degree of biliary function maintained over the experimental period. The mean concentration of total solids in

Table 1. *Excretion of radioactivity in bile of male and female rabbits after intravenous injection of [4-¹⁴C]progesterone*
 A single dose was given to rabbits 1 and 2, and rabbits 3-6 were dosed for 45-60 min. Experimental details are given in the text.

Animal no.	Sex	Wt. (kg.)	Dose of steroid		Collec- tion period (hr.)	Vol. of bile (ml.)	Bile		Distribution of radioactivity (% of total in sample)					
			(μg.)	(10 ⁻⁶ × counts/min.)			Total solids (mg.)	Radioactivity (10 ⁻⁶ × counts/min.)	(% of dose)	Neutral ether-soluble radioactivity				
										Glucuronide	Cold-acid hydrolysed	Hot-acid hydrolysed	Radio-activity unaccounted for	
1	Female	1.95	43.2	6.00	1	6.5	123.0	1.9	1.372	22.9	76.4	16.0	3.8	3.8
					1	6.5	113.2	1.7	0.348	5.8	57.4	15.5	8.6	18.5
					1	6.1	106.6	1.7	0.200	3.3	56.0	11.0	12.0	21.0
					1	5.1	89.6	1.8	0.019	2.0	50.4	10.5	10.5	28.6
					1	5.1	80.5	1.6	0.075	1.3	45.4	13.4	12.0	29.2
									Total	35.3				
2	Male	2.0	40.0	5.31	1	6.2	118.8	1.9	1.415	26.8	82.4	13.6	3.0	1.0
					1	5.7	105.0	1.8	0.316	6.0	74.6	11.6	6.0	7.8
					1	6.1	105.6	1.7	0.190	3.6	70.1	8.3	5.3	16.3
					1	6.9	121.3	1.8	0.116	2.2	69.0	10.3	3.5	17.2
					1	7.0	123.3	1.8	0.086	1.6	69.6	14.0	5.8	10.6
									Total	40.2				
3	Male	2.5	82.5	13.38	1	7.8	156.7	2.0	0.936	7.0	50.0	23.0	1.7	25.3
					1	7.1	140.3	2.0	0.980	7.3	41.5	17.9	4.0	36.5
					1	9.4	154.8	1.6	0.455	3.4	35.0	17.4	5.6	42.0
					1	8.2	136.2	1.7	0.256	2.0	27.4	19.0	6.5	47.1
					1	7.3	121.7	1.7	0.203	1.5	23.6	24.0	6.5	45.9
					1	6.1	100.5	1.7	0.179	1.3	24.0	30.0	3.6	42.4
									Total	22.5				
4	Male	2.4	82.6	13.49	1	3.9	82.7	2.1	1.460	10.8	50.0	14.0	3.3	32.7
					1	3.5	71.1	2.0	0.885	6.6	61.0	16.7	6.5	15.8
					1	3.9	67.3	1.7	0.507	3.8	78.0	14.0	4.7	3.3
					1	3.6	74.2	2.0	0.303	2.2	38.1	9.5	4.0	48.4
					1	4.7	74.2	1.6	0.169	1.3	42.3	17.9	3.4	36.4
					1	4.7	73.3	1.6	0.116	0.9	34.4	25.0	6.4	34.2
									Total	25.6				

5	Female	1.8	82.7	13.52	1	8.3	130.0	1.6	1.670	12.3	86.0	13.5	1.5	0
						7.7	118.5	1.5	1.645	12.1	49.1	11.4	8.0	31.5
						9.4	137.1	1.5	0.455	3.4	50.0	11.9	6.6	31.5
						10.7	174.8	1.7	0.174	1.3	57.9	12.7	5.0	24.4
						10.3	165.3	1.6	0.127	0.9	46.4	9.3	5.3	39.0
						8.7	127.1	1.2	0.094	0.7	39.2	9.1	3.8	47.9
									Total	30.7				
6	Female	2.8	91.4	16.34	1	6.3	143.6	2.3	1.180	7.3	68.0	17.5	2.4	12.1
						6.6	136.3	2.1	1.071	6.6	71.5	11.0	2.7	14.8
						5.6	111.2	2.0	0.682	4.1	38.8	7.4	3.5	49.3
						5.2	99.4	1.9	0.396	2.4	38.4	5.6	4.9	52.1
						5.0	91.5	1.8	0.280	1.7	42.5	6.4	2.9	48.2
						5.1	89.9	1.7	0.300	1.8	37.4	5.9	4.0	52.7
									Total	23.9				

the 34 samples of bile collected was 1.8 ± 0.2 g./100ml. The mean volume of bile collected from the six animals in the first hour was 6.5 ± 1.4 ml. and 6.1 ± 1.4 ml. in the last hour. Thus, under these experimental conditions, the rabbit produces a much larger volume of bile than the cat, and there is no significant decrease in the hourly output of bile by the rabbit after 5 or 6 hr. This is in marked contrast with the cat, in which there is always a decrease in bile flow over 5–6 hr. (cf. Taylor & Scratcherd, 1961, 1963; Archer *et al.* 1965; Scratcherd, 1965).

Table 1 shows that after the administration of progesterone by a single injection (animals 1 and 2) about a quarter of the dose was excreted in the bile in the first hour, with gradually decreasing amounts of metabolites in later samples. When the steroid was administered over 45–60 min. (animals 3–6) the first two hourly samples contained approximately equal amounts of radioactivity and the amount of metabolites in subsequent samples decreased. There was no detectable difference in the rates of excretion of metabolites by male (animals 3 and 4) and female (animals 5 and 6) rabbits. However, the total amount of radioactivity recovered from bile after infusion (22.5–30.7%) was less than after a single injection (35.3 and 40.2%).

Table 1 also shows the distribution of neutral ether-soluble radioactivity after successive hydrolysis by β -glucuronidase, cold acid and hot acid and the amount of radioactivity not recovered from alkaline solutions of the samples after hydrolysis and extraction. This unaccounted-for radioactivity represents water-soluble metabolites, possibly unhydrolysed conjugates and phenolic or acidic substances, of unknown constitution.

Table 2 gives the corresponding results for the urine samples obtained from the same six rabbits. Because of the difficulty of maintaining urine flow in some animals the bladder was washed out with 0.15M-sodium chloride as described in the Materials and Methods section. The volumes of collections made in this way, indicated by an asterisk in Table 2, therefore represent urine volume plus 2 ml. of 0.15M-sodium chloride. No spontaneous urine flow could be established in rabbit 2 and therefore no attempt was made to record the volumes or total solids in any of the samples from this animal. In spite of this poor urine production by the animals a considerable amount of radioactivity appeared in the urine and saline washings. The radioactivity appears to be excreted more slowly in the urine than in the bile, but this may be due to the low rate of urine production and the larger 'dead space' of the renal excretory apparatus. In all animals a greater proportion of the dose was excreted in the bile than in the urine, the mean ratio of urinary to biliary metabolites being 0.62 ± 0.14 (range 0.44–0.89).

Table 2. *Excretion of radioactivity in urine of male and female rabbits after intravenous injection of [4-¹⁴C]progesterone*

The animals are those shown in Table 1.

Animal no.	Collection period (hr.)	Vol. of urine (ml.)	Urine				Distribution of radioactivity (% of total in sample)			
			Total solids		Radioactivity		Neutral ether-soluble radioactivity			Radio-activity un-accounted for
					(10 ⁻⁶ × counts/min.)	(% of dose)	Glucuronide	Cold-acid hydro-lysed	Hot-acid hydro-lysed	
			(mg.)	(g./100 ml.)						
1	1.5	1.0	43.0	4.3	0.001	0.01	42.8	2.4	7.1	47.7
	1	0.4	22.2	5.3	0.013	0.2				
	2.5	1.9	293.4	15.4	1.421	23.7	23.1	4.2	6.3	66.3
	Total					23.91				
2	1*	—	—	—	0.368	6.9	31.6	3.8	7.6	57.0
	1.5*	—	—	—	0.318	6.0	32.8	8.2	9.1	49.8
	1.5*	—	—	—	0.217	4.1	16.6	2.3	6.5	74.6
	1*	—	—	—	0.171	3.2	22.2	1.8	6.4	69.6
	1*	—	—	—	0.084	1.6	21.4	2.4	9.6	66.6
Total					21.8					
3	2	1.8	146.3	8.1	0.002	0.02	16.5	2.0	3.5	78.0
	2	2.0	364.8	18.2	1.930	14.4	14.9	1.9	2.5	80.7
	2*	5.8	561.4	9.6	0.745	5.6	28.6	3.4	5.5	62.4
Total					20.02					
4	2	1.9	105.8	5.7	0.206	1.5	13.8	2.9	7.8	75.5
	2	4.3	313.0	7.3	0.950	7.0	14.0	7.1	7.5	71.4
	2*	11.7	282.0	2.4	0.368	2.7	18.0	9.8	5.2	67.0
Total					11.2					
5	2	8.7	364.6	4.2	1.030	7.6	21.0	4.9	8.3	65.8
	2	4.4	399.3	9.2	1.115	8.3	19.0	6.2	3.8	71.0
	2*	8.4	586.2	7.0	0.304	2.2	17.0	4.5	7.4	71.0
Total					18.1					
6	2	9.0	399.2	4.4	0.740	4.5	34.0	2.0	4.9	59.1
	2	1.0	75.1	7.3	0.495	3.0	20.8	2.0	5.4	71.8
	2	3.7	138.1	3.8	1.040	6.3	23.4	4.4	11.4	60.8
Total					13.8					

* Bladder washed out with 0.15 M-NaCl (see the text).

Table 2 also shows the proportions in the samples of neutral ether-soluble metabolites obtained after successive hydrolysis and also the amount of radioactivity not accounted for after the hydrolyses and extractions.

DISCUSSION

Previous work on the metabolism of progesterone in the rabbit has been mainly concerned with the detection and assay of urinary pregnanediol (5 β -pregnane-3 α ,20 α -diol). This metabolite is found in female-rabbit urine after mating and during pregnancy (Verly, Sommerville & Marrian, 1950) and after administration of progesterone (Heard,

Bauld & Hoffman, 1941; Hoffman, 1942; Westphal, 1942), and in the faeces after oral but not subcutaneous administration of the hormone (Hoffman & Browne, 1942). Thomas (1962) isolated 20 α -hydroxypregn-4-en-3-one from rabbit urine after the administration of 50 or 500 mg. of progesterone to male and female animals. Using the more sensitive and specific method of gas-liquid chromatography Chamberlain, Knights & Thomas (1964) detected pregnanediol and its 5 α - and 5 α ,3 β -epimers in the urine of male and female rabbits after the administration of progesterone and in rabbit pregnancy urine.

The availability of ³H- and ¹⁴C-labelled pro-

gesterone now permits more precise investigation of the metabolism and excretion of this steroid. Cooke, Rogers & Thomas (1963) studied the metabolism of labelled progesterone in the rabbit, but administered large doses (380 μ g.-50 mg.) of the hormone to the animals and investigated metabolites in the urine only.

Under the experimental conditions employed in the present work, urethane-anaesthetized rabbits convert intravenously administered progesterone into water-soluble metabolites that are excreted in both bile and urine. A greater proportion of the dose is excreted in bile than in urine. Cooke *et al.* (1963) recovered only 14.7-37.1% of subcutaneously administered labelled progesterone as conjugated metabolites from the urine of intact rabbits, but they did not determine the proportion of the dose excreted in the faeces of the animals. The present work indicates that biliary and, thereby, faecal excretion of progesterone metabolites probably occur to a significant extent in the rabbit: the proportion of the dose present in the urine of anaesthetized rabbits (11-23.9%) is of the same order as that observed by Cooke *et al.* (1963).

Pregnanediol is a major metabolite of progesterone in the urine in man and the rabbit but not in the rat (Atherden & Grant, 1957). However, rabbits more closely resemble rodents than man in their excretion of progesterone metabolites. Intact rats excrete about 22% of a small dose of labelled progesterone in the urine; 66% of the dose is recoverable from the faeces. Rats with bile fistulae excrete 22% of the dose in the urine and 73% in the bile (Grady, Elliott, Doisy, Bocklage & Doisy, 1952). In intact human subjects, however, 46-59% of a dose of progesterone is excreted in the urine and 8-10% in the faeces, and patients with biliary fistulae excrete 38-70% in the urine and 20-35% in the bile (Sandberg & Slaunwhite, 1958). Pregnanediol has been isolated from the glucuronide fraction of rabbit bile after the administration of labelled progesterone (W. Taylor, unpublished work).

Under experimental conditions similar to those employed in the present work, Taylor & Scratcherd (1961) found that in cats virtually all of the metabolites of progesterone excreted in 6 hr. were present in the bile: less than 1% appeared in the urine. The anaesthetized cat produces smaller volumes of bile but larger spontaneous urine volumes than the rabbit (Taylor & Scratcherd, 1961, 1963; Archer *et al.* 1965). In spite of the large spontaneous bile flow and small urine volumes in the rabbit a high proportion of the dose of progesterone was found in the urine of the rabbits. Therefore the degree of bile and urine flow do not appear to determine the excretory route taken by progesterone metabolites in the cat and rabbit. Further, these results support

Table 3. Comparison of excretion of metabolites of [4-¹⁴C]progesterone in bile and urine of rabbits and cats

	Percentage of dose recovered					
	Rabbit			Cat*		
	Bile	Urine	Total	Bile	Urine	Total
Single dose	35.3	23.9	59.2	67.1	0.5	67.6
	40.2	21.8	62.0	65.6	0.5	66.1
Infusion	22.5	20.0	42.5	35.2	0.6	35.8
	25.6	11.2	36.8	26.2	0.4	26.6
	30.7	18.2	48.8			
	23.9	13.8	37.7			

* From Taylor & Scratcherd (1961).

the view that the anaesthetized animal is a suitable preparation for investigating the fate of steroid hormones (Taylor, 1965; Taylor & Scratcherd, 1962). The results obtained with cats and rabbits are summarized in Table 3. In rabbits there was no apparent sex difference in the total amount of the dose excreted as metabolites in the urine and bile.

Another striking difference between cats and rabbits is in the proportion of biliary excretion of progesterone metabolites as glucuronides. In male cats the highest proportion of biliary glucuronides was about 45% (Taylor & Scratcherd, 1961), whereas in the rabbit values up to 86% are found (Table 1). In cat urine the highest proportion of glucuronides was about 7%, whereas in rabbit urine values as high as 42.8% are reached (Table 2). These findings support the view of Dutton & Grieg (1957) that cat liver has a lower glucuronyltransferase activity than rabbit liver *in vitro* when *o*-aminophenol is used as the aglycone. Rao & Taylor (1965) have shown that glucuronides are the major conjugates formed during the metabolism of progesterone by rabbit liver *in vitro*.

In rabbit urine (Table 2) most of the radioactivity excreted is not extractable by ether from alkaline solutions. Cooke *et al.* (1962) found that after acid hydrolysis 41-77% of the radioactivity present in rabbit urine after the administration of labelled progesterone could be extracted from ether by alkali. Those authors consider that these alkali-extractable substances are not artifacts of hydrolysis, and that the steroid nucleus is essentially intact. These metabolites probably correspond to the unaccounted-for radioactivity in the present work (last column in Tables 1 and 2).

The present work indicates that the anaesthetized rabbit is a suitable preparation for the study of steroid metabolism in this species, since the results obtained correlate reasonably well with those obtained with intact animals and also with experiments with liver *in vitro*.

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