The Metabolism of Progesterone by Animal Tissues *in vitro*

SEX AND SPECIES DIFFERENCES IN CONJUGATE FORMATION DURING THE METABOLISM OF [4-¹⁴C]PROGESTERONE BY LIVER HOMOGENATES

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(Received 4 September 1964)

1. Sex and species differences during the metabolism of [4-¹⁴C]progesterone by liver homogenates from rat, rabbit, guinea pig and hamster have been investigated.
2. Liver homogenate from male rat formed 'water-soluble' metabolites faster and in significantly larger amounts than did liver homogenate from female rat. About 65–70% of the added progesterone was conjugated as glucuronide by liver homogenate from male rat and about 45–50% by that from female rat. Liver homogenate from male rat also formed glucuronides faster than did liver homogenate from female rat. Sulphate formation was low (8–16%) in liver homogenates from both male and female rats.
3. Hamster-liver homogenate did not show any sex difference in the rate of formation of 'water-soluble' metabolites, but a sex difference was observed in the amount of free steroids recovered at low tissue:steroid ratios. Liver homogenate from female hamster formed glucuronides faster and in significantly larger amounts than did liver homogenate from male hamster, the reverse of what was found in rat liver.
4. Liver homogenates from male and female rabbits and guinea pigs formed 'water-soluble' metabolites that were almost entirely glucuronides.
5. Neither rabbit liver nor guinea-pig liver showed any significant sex difference in the rate or amount of formation of total 'water-soluble' metabolites or glucuronides, but guinea-pig liver was considerably less active than rabbit liver.
6. Glucuronides were quantitatively the major type of conjugate formed by the liver homogenates from both sexes of all species except the male hamster.

Sex and species differences have been observed in several enzymic stages of steroid metabolism and of conjugate formation of steroids and phenols by livers of a variety of animal species *in vitro* (Table 1). Cooke & Taylor (1962) reported briefly on a sex difference in conjugate formation during the metabolism of progesterone by rat-liver homogenate. Conjugate formation during the metabolism of [4-¹⁴C]progesterone has now been studied in more detail with liver homogenates from both sexes of the rat and also of the rabbit, hamster and guinea pig. A preliminary account of part of this work has been published (Rao & Taylor, 1964).

MATERIALS AND METHODS

The trivial names of steroids used in the text are: aldosterone (11 β ,21-dihydroxypregn-4-ene-3,20-dione-18-al); corticosterone (11 β ,21-dihydroxypregn-4-ene-3,20-dione); cortisone (17 α ,21-dihydroxypregn-4-ene-3,11,20-trione); dehydroepiandrosterone (3 β -hydroxyandrost-5-en-17-one); deoxycorticosterone (21-hydroxypregn-4-ene-3,20-dione); 11-deoxycortisol (17 α ,21-dihydroxypregn-4-ene-3,20-dione); dihydrodeoxycorticosterone (21-hydroxy-5 α -

pregnane-3,20-dione or its 5 β -epimer); hydrocortisone (11 β ,17 α ,21-trihydroxypregn-4-ene-3,20-dione); oestriol (3,16 α ,17 β -trihydroxyoestra-1,3,5(10)-triene); pregnanediol (5 β -pregnane-3 α ,20 α -diol); pregnanolone (3 α -hydroxy-5 β -pregnan-20-one); progesterone (pregn-4-ene-3,20-dione); testosterone (17 β -hydroxyandrost-4-en-3-one); tetrahydrocortisone (3 α ,17 α ,21-trihydroxy-5 β -pregnane-11,20-dione).

The reagents used were as described by Cooke & Taylor (1963). The animals used were male and female Wistar rats, golden hamsters, large New Zealand White rabbits and guinea pigs, all about 3 months old. The oestrus state of female animals was not determined.

Homogenate preparation and incubation. Livers were chilled immediately after removal by placing them in 0.154 M-KCl-0.36 M-nicotinamide at 0°. Homogenate preparation and the composition of the incubation medium were as described by Cooke & Taylor (1963). Incubation vessels contained 2.5–20 μ g. of progesterone (depending on the tissue:steroid ratio required) and 250 or 300 mg. wet wt. of tissue as homogenate in a final volume of 3 ml. of phosphate-saline medium, pH 7.4. The incubation time was varied from 1 to 60 min. The gas phase was oxygen in all experiments.

Extraction, hydrolysis and assay of radioactivity. These

Table 1. *Sex differences in the metabolism of steroids and the conjugation of phenols and steroids*

Species and sex of animal showing higher activity	Reaction	Substrate	Reference
Rat			
Female	A-Ring reduction	Aldosterone, deoxycorticosterone, hydrocortisone, cortisone, corticosterone, progesterone, testosterone	Leybold & Staudinger (1959); Yates, Herbst & Urquhart (1958)
Female		11-Deoxycortiso	Forchielli, Brown-Grant & Dorfman (1958)
Hamster			
Male		Cortisone	Yates <i>et al.</i> (1958)
Rat			
Male	3-Oxo group reduction	Dihydrodeoxycorticosterone	Bell, Popoola & Taylor (1962)
Male		17-Oxo steroids	Rubin (1957); Rubin & Strecker (1961)
Male	11-Oxo group reduction	Cortisone	Hubener & Amelung (1953)
Male	20-Oxo group reduction	Cortisone	Hagen & Troop (1960)
Male	Glucuronide conjugation	<i>o</i> -Aminophenol	Inscoe & Axelrod (1960)
Rabbit			
Male		<i>o</i> -Aminophenol	Hartiala, Pulkkinen & Savola (1964)
Rat			
Female	Sulphate conjugation	Dehydroepiandrosterone	Roy (1955)

procedures were carried out as described by Cooke & Taylor (1963), with the following modifications. Proteins of the incubation mixture were precipitated by the addition of 15 ml. of acetone at approx. 50° and removed by centrifugation. The precipitate was resuspended in acetone twice more and removed by centrifugation as before. The extracts were combined and the acetone was removed in a stream of air below 50°. The aqueous residue was extracted three times with 8 ml. of ether to give the free-steroid fraction. This, and all subsequent ether extracts, except the hot-acid fraction, were washed with 0.1 vol. of 0.1 N-NaOH and then with 0.1 vol. of water until neutral. The residual aqueous phase was hydrolysed successively by β -glucuronidase from either limpet powder or female-rat preputial glands, 2N-H₂SO₄-ether (1:1, v/v) and finally by boiling at pH 1 for 1 hr. Extraction with 8 ml. of ether three times after each type of hydrolysis gave the glucuronide, cold-acid and hot-acid fractions respectively. Assay of radioactivity was carried out as described by Taylor & Scratcherd (1961).

Conjugate formation was studied by varying the time of incubation, at a constant tissue:steroid ratio, and also at varying tissue:steroid ratios by alteration of the amount of [4-¹⁴C]progesterone added to a constant amount (250 or 300 mg. wet wt.) of tissue and incubating for a constant time. The rate and amount of conjugate formation were therefore determined.

Statistical treatment of results. Student's *t* test was used as a test of significance where sex differences are claimed.

RESULTS

Fig. 1 shows the rate of disappearance of neutral free steroids extractable by ether from the incubation mixture at a tissue:steroid ratio of about 56 000:1 in liver homogenates from male and female

Fig. 1. Effect of varying the incubation time on the recovery of radioactivity as free steroids in liver homogenates from rat and hamster. Samples (5.4 and 3.34 μ g.) of [4-¹⁴C]-progesterone were incubated with: 300 and 250 mg. wet wt. of liver homogenate from rat and hamster respectively, phosphate buffer, pH 7.4 (120 μ moles), nicotinamide (36 μ moles), KCl (150 μ moles) and MgCl₂ (15 μ moles); the total volume was 3 ml. The gas phase was O₂. Each point represents a mean of duplicates of at least two experiments; standard deviations are shown as vertical bars. Δ , Male rat; $\circ$, female rat; $\blacktriangle$, male hamster; $\bullet$, female hamster. The incubation time for rat liver was up to 15 min., and that for hamster liver up to 60 min.

rats when the incubation time was varied from 1 to 15 min. After 15 min. only about 10% of the added radioactivity is recoverable as free steroids from livers of both male and female rats. However, after

1 min. about 26% of the free steroid is present in the medium after incubation with male-rat liver, but about 60% with female-rat liver. The radioactivity not extractable as free steroid is considered to have been converted into 'water-soluble' metabolites. The major portion of the radioactivity in this fraction was recovered by extraction with ether after successive hydrolysis by β -glucuronidase, cold acid and hot acid, and therefore presumably consists mainly of steroid conjugates of glucuronic acid and sulphuric acid. Such 'conjugate' formation is complete in 3 min. in male-rat liver, whereas 15 min. are required to reach the same degree of 'conjugation' in female-rat liver.

Fig. 1 also shows the results of similar experiments at a tissue:steroid ratio of 75 000 with liver homogenates from male and female hamsters. In contrast with rat liver, conversion of progesterone into 'water-soluble' metabolites requires approx. 60 min. to reach completion in hamster-liver homogenate from both sexes, and hamster liver does not show any significant sex difference in the rate of formation of these metabolites. Further, a greater amount of free steroid remains after incubation with hamster liver for 60 min. (30%) than with rat liver for 10 min. (10%).

A sex difference also occurs in the disappearance of free steroids from the medium when the tissue:steroid ratios of male-rat and female-rat liver are varied by increasing the amount of progesterone added and maintaining a constant incubation time of 15 min. Formation of 'water-soluble' metabolites increases with increase in tissue:steroid ratio in both sexes, but male-rat liver shows a higher degree of formation of these metabolites than female-rat liver at lower tissue:steroid ratios. For example, at a tissue:steroid ratio of about 12 500:1 about 60% of the added radioactivity is recovered as free steroids from female-rat liver whereas only about 25% is recovered from male-rat liver. This sex difference decreases with increase in tissue:steroid ratios and disappears at a tissue:steroid ratio of about 60 000:1.

The results of the same type of experiment carried out with liver homogenates from male and female hamsters at a constant incubation time of 60 min. also show a similar sex difference. Disappearance of free steroids increases with increasing tissue:steroid ratio and is maximal at a ratio of about 50 000:1. However, although there is no sex difference in the rate of formation of 'water-soluble' metabolites by hamster liver (Fig. 1), a significant sex difference is found in the amount of free steroid recovered in hamster-liver homogenate at low tissue:steroid ratios (e.g., at a tissue:steroid ratio of 12 500:1 about 50% of the free steroids is present in liver homogenate from female hamster but only about 30% in that from male hamster).

Table 2. Radioactivity in glucuronide, cold-acid- and hot-acid-hydrolysed fractions and total recovery of added steroid on incubation of [4- 14 C]progesterone with liver homogenates from male and female rats and hamsters

Incubation time (min.)	Rat	Radioactivity recovered (% of total counts/min. added)									
		Glucuronides		Cold-acid fraction		Hot-acid fraction		Total recovery		Experimental	
		Male	Female	Male	Female	Male	Female	Male	Female		
1		59.7 $\pm$ 0.5	23.3 $\pm$ 3.3*	5.4 $\pm$ 0.9	9.6 $\pm$ 2.1	8.5 $\pm$ 0.5	7.1 $\pm$ 2.0	99.7 $\pm$ 1.7	99.7 $\pm$ 0.3		
3		66.2 $\pm$ 2.1	39.8 $\pm$ 3.0*	5.7 $\pm$ 0.6	7.8 $\pm$ 0.8*	8.8 $\pm$ 0.3	10.9 $\pm$ 0.6	92.1 $\pm$ 2.8	99.3 $\pm$ 3.3		
6		65.3 $\pm$ 2.4	42.0 $\pm$ 2.0*	7.4 $\pm$ 1.7	11.2 $\pm$ 0.7	6.3 $\pm$ 1.9	11.8 $\pm$ 1.0	86.6 $\pm$ 2.9	90.0 $\pm$ 8.2		
10		67.3 $\pm$ 3.3	47.1 $\pm$ 5.5	5.1 $\pm$ 0.3	11.6 $\pm$ 0.8*	10.9 $\pm$ 3.6	12.8 $\pm$ 1.2	90.6 $\pm$ 1.2	87.1 $\pm$ 6.7		
15		69.9 $\pm$ 1.0	51.5 $\pm$ 2.2*	6.6 $\pm$ 0.9	12.3 $\pm$ 1.1*	9.0 $\pm$ 1.0	11.7 $\pm$ 1.8	94.4 $\pm$ 1.5	84.8 $\pm$ 1.6		
4	Hamster	11.1 $\pm$ 0.5	18.0 $\pm$ 0.7*	9.0 $\pm$ 0.3	9.3 $\pm$ 0.6	7.2 $\pm$ 0.3	0.9 $\pm$ 0.2*	88.9 $\pm$ 3.8	98.9 $\pm$ 0.3		
12		13.2 $\pm$ 1.0	26.5 $\pm$ 0.5*	14.3 $\pm$ 0.4	10.2 $\pm$ 0.8*	15.1 $\pm$ 0.3	12.1 $\pm$ 1.1	85.3 $\pm$ 1.8	97.2 $\pm$ 2.1*		
24		14.4 $\pm$ 1.5	29.9 $\pm$ 0.5*	15.3 $\pm$ 0.7	10.5 $\pm$ 0.1*	21.3 $\pm$ 0.3	18.9 $\pm$ 0.5*	82.8 $\pm$ 2.2	94.8 $\pm$ 1.0*		
40		15.0 $\pm$ 0.9	32.6 $\pm$ 1.1*	16.4 $\pm$ 0.8	12.0 $\pm$ 0.2*	24.6 $\pm$ 0.5	21.0 $\pm$ 1.0*	85.2 $\pm$ 2.3	96.0 $\pm$ 2.3		
60		13.6 $\pm$ 1.1	31.2 $\pm$ 1.1*	15.3 $\pm$ 0.6	14.0 $\pm$ 0.3	26.5 $\pm$ 1.2	24.0 $\pm$ 0.2	82.4 $\pm$ 2.3	98.7 $\pm$ 1.4*		

Each value is the mean $\pm$ s.d. of at least four determinations. The tissue:steroid ratio was 56 000:1 for rat liver and 75 000:1 for hamster liver. Experimental details are given in the text.

* Significant differences: $P < 0.01$.

This sex difference disappears at a tissue:steroid ratio of about 50000:1.

Sex and species differences in the rate or amount of formation of particular types of conjugate by liver homogenates from both rat and hamster were also investigated. Table 2 shows the rate of glucuronide formation in liver homogenates from male and female rats at a tissue:steroid ratio of about 56000:1. In 1 min. about 60% of the added progesterone is metabolized and conjugated as glucuronide in male-rat liver, whereas in the same time glucuronide formation in female-rat liver is less than 25%. After 15 min., when formation of the total 'water-soluble' fraction reaches the same level in both sexes (cf. Fig. 1), the amount of glucuronide formed by female-rat liver (about 50%) does not reach the same level as that by male-rat liver (about 70%).

Table 2 also shows the rate of glucuronide formation in liver homogenates from male and female hamsters at a tissue:steroid ratio of about 75000:1. Glucuronide formation increases with increasing incubation time up to 40 min. in female-hamster liver, but in male-hamster liver there is no appreciable increase after 12 min. The amount of glucuronide formed is significantly less in male-hamster liver than in female-hamster liver at all incubation times studied, but the rate of formation of total 'water-soluble' metabolites is the same in both sexes (cf. Fig. 1). After 60 min. incubation, when the formation of 'water-soluble' metabolites has reached its maximal level, the amount of glucuronide formed by female-hamster liver ($31.2 \pm 1.1\%$) is markedly higher than that by male-hamster liver ($13.6 \pm 1.1\%$).

Fig. 2 shows the effect of varying the tissue:steroid ratio on the amount of glucuronide formation in male-rat and female-rat liver homogenates at a constant incubation time of 15 min. Male-rat liver forms significantly more glucuronide than does

female-rat liver at all tissue:steroid ratios studied; this sex difference is most marked at low tissue:steroid ratios.

Fig. 2 also shows the results of the same type of experiment on glucuronide formation in hamster liver at a constant incubation time of 60 min. Glucuronide formation reaches a maximum at a tissue:steroid ratio of about 25000:1, and there is a significant sex difference at all tissue:steroid ratios studied. In contrast with the rat, female-hamster liver forms more glucuronides than does male-hamster liver.

Table 2 also shows the amount of radioactivity recovered as cold-acid- and hot-acid-hydrolysed fractions and the total recovery. Sex differences in the amounts of radioactivity in the cold-acid-hydrolysed fractions from rat-liver homogenate were not so consistent as the sex difference in glucuronide formation, and there was no significant difference in the amount of hot-acid-hydrolysed metabolites formed. Total recovery of added radioactivity is slightly lower from female-rat liver than from male-rat liver, and this sex difference is also more apparent over longer incubation periods. However, these differences are not significant at the $P < 0.01$ level.

The hamster forms larger quantities of cold-acid- and hot-acid-hydrolysable conjugates than the rat (Table 2). There is an increase in the hot-acid but not in the cold-acid fraction with increase in incubation time in hamster liver of both sexes. There is a significant sex difference in the formation of cold-acid fractions at incubation times of 12, 24 and 40 min. Male-hamster liver is significantly more active than female-hamster liver in forming hot-acid-hydrolysable conjugates over 4, 24 and 40 min. incubation periods. Total recovery of added radioactivity is lower from male-hamster liver than from female-hamster liver (significant at $P < 0.01$ after 12, 24 and 60 min.), the reverse of what is found with rat liver.

Table 3 shows the rate of disappearance of free steroid from the incubation mixture at a constant tissue:steroid ratio of about 50000:1 in liver homogenates from male and female rabbits when the incubation time was varied from 1 to 15 min. After 15 min. only about 20% of the added radioactivity is recoverable as free steroids from male-rabbit and female-rabbit liver, but there is no significant difference between the sexes in these experiments. Table 3 also shows the results of similar experiments carried out with guinea-pig liver homogenate. Because of the lower metabolic activity the incubation period was varied from 4 to 60 min. The formation of 'water-soluble' metabolites in guinea-pig liver increases with increasing time up to 40 min., but there is no significant sex difference in the rate of formation of these metabolites.

Fig. 2. Effect of varying the tissue:steroid ratio on glucuronide formation in liver homogenates from rat and hamster. Incubation conditions and experimental points were as given in Fig. 1, except that the incubation time for rat liver was 15 min. and that for hamster liver was 60 min. The tissue:steroid ratio was varied by varying the amount of [$4\text{-}^{14}\text{C}$]progesterone added.

Table 3. Radioactivity recovered as free steroids and as glucuronides on incubation of [$4\text{-}^{14}\text{C}$]progesterone with liver homogenates from male and female rabbits and guinea pigs

Each value is the mean $\pm$ S.D. of at least four determinations. The tissue:steroid ratio was 50000:1 for rabbit liver and 75000:1 for guinea-pig liver. Experimental details are given in the text.

Species	Incubation time (min.)	Radioactivity recovered (% of total counts/min. added)			
		Free steroids		Glucuronides	
		Male	Female	Male	Female
Rabbit	1	82.1 $\pm$ 7.3	71.3 $\pm$ 5.2	12.2 $\pm$ 1.4	9.25 $\pm$ 2.9
	3	56.8 $\pm$ 3.2	45.3 $\pm$ 5.0	26.8 $\pm$ 2.8	32.0 $\pm$ 2.4*
	6	38.2 $\pm$ 3.9	31.5 $\pm$ 2.4	41.4 $\pm$ 3.9	47.3 $\pm$ 2.4
	10	29.8 $\pm$ 1.0	24.6 $\pm$ 3.3	46.9 $\pm$ 3.0	52.7 $\pm$ 2.6
	15	22.2 $\pm$ 2.5	19.3 $\pm$ 2.7	50.7 $\pm$ 2.2	50.2 $\pm$ 4.4
Guinea pig	4	78.2 $\pm$ 3.9	75.6 $\pm$ 1.3	8.9 $\pm$ 1.1	5.9 $\pm$ 1.6
	12	58.8 $\pm$ 2.6	57.9 $\pm$ 5.8	20.9 $\pm$ 1.5	16.5 $\pm$ 1.5
	24	52.9 $\pm$ 3.1	50.9 $\pm$ 9.0	26.7 $\pm$ 0.8	26.3 $\pm$ 5.7
	40	45.5 $\pm$ 2.4	48.1 $\pm$ 5.0	32.6 $\pm$ 1.7	28.9 $\pm$ 4.7
	60	43.5 $\pm$ 1.9	45.4 $\pm$ 3.6	32.6 $\pm$ 2.6	33.1 $\pm$ 1.6

* Significant difference: $P < 0.01$.

Fig. 3. Effect of varying the tissue:steroid ratio on the recovery of radioactivity as free steroids in liver homogenates from rabbit and guinea pig. Experimental conditions and points were as given in Fig. 1. The incubation time was 15 min. for rabbit liver and 60 min. for guinea-pig liver; 250 mg. wet wt. of liver was used in each experiment. Δ , Male guinea pig; $\circ$, female guinea pig; $\blacktriangle$, male rabbit; $\bullet$, female rabbit.

Fig. 3 shows the effect on the formation of 'water-soluble' metabolites when the tissue:steroid ratio is varied from 12500 to 75000:1 in liver homogenates from male and female rabbits at a constant incubation time of 15 min. Free steroids decrease with increase in tissue:steroid ratio up to 50000:1. Fig. 3 also shows the results of similar experiments with guinea-pig liver. Because of the low activity of this tissue a constant incubation time of 60 min. was used. There is no sex difference in formation of 'water-soluble' metabolites, and at all tissue:

Fig. 4. Effect of varying the tissue:steroid ratio on the glucuronide formation in liver homogenates from rabbit and guinea pig. Incubation conditions and experimental points were as given in Fig. 3.

steroid ratios guinea-pig liver is considerably less active than rabbit liver. At the longest incubation times (Table 3) and highest tissue:steroid ratios (Fig. 3) used, the total amount of 'water-soluble' metabolites was always higher in rabbit liver (about 80%) than in guinea-pig liver (55%).

Table 3 shows the rate of glucuronide formation at a tissue:steroid ratio of about 50000:1 in male-rabbit and female-rabbit liver. Female-rabbit liver is slightly more active than male-rabbit liver in forming glucuronides, but the difference is significant ($P < 0.01$) only at an incubation time of 3 min. Table 3 also shows the rate of glucuronide formation at a tissue:steroid ratio of about 75000:1 in liver homogenates from male and female guinea pigs. No significant sex difference is seen in glucuronide formation, but both the rate and amount of

glucuronide formation are less in guinea-pig liver (approx. 33% in 60min.) than in rabbit liver (approx. 50% in 15min.).

Fig. 4 shows the effect on glucuronide formation of varying the tissue:steroid ratio in male-rabbit and female-rabbit liver while maintaining a constant incubation period of 15min. No significant sex difference is apparent in the amount of glucuronide formed. Fig. 4 also shows the results with guinea-pig liver over the same range of tissue:steroid ratios but with an incubation period of 60min. There is no sex difference in glucuronide formation, confirming the results shown in Table 3. Rabbit liver conjugates metabolites of progesterone as glucuronides at a higher rate (Table 3), and also to a greater extent (50%), than guinea-pig liver (33%) (Fig. 4), in spite of the longer incubation period used with guinea-pig liver.

As the recovery of the free-steroid and glucuronide fractions from rabbit liver and guinea-pig liver accounted for about 90 and 80% respectively of the added radioactivity, further hydrolytic procedures were not carried out on the aqueous residues after the hydrolysis of glucuronides.

DISCUSSION

Sex differences in conjugate formation in rat and hamster. It has been shown that rat liver and hamster liver exhibit sex and species differences in the metabolism of progesterone *in vitro*. Liver homogenate from male rat has the ability to form a significantly greater overall amount of 'water-soluble' metabolites and at a faster rate than has female-rat liver, but this sex difference is marked only at low tissue:steroid ratios and over short incubation periods (Fig. 1). Rat liver also exhibits a significant sex difference both in the rate and total amount of glucuronide formation. This difference also is marked only at low tissue:steroid ratios and short incubation periods (Fig. 2 and Table 1).

In contrast with the rat, hamster liver does not show any sex difference in the rate of formation of 'water-soluble' metabolites under the conditions employed (Fig. 1), and the total amount of 'water-soluble' metabolites formed is lower in hamster liver (65–70%) than in rat liver (90%) with the highest tissue:steroid ratio and the longest incubation period studied (Fig. 1). Also, the rate of total 'conjugate' formation is much lower in hamster liver than in rat liver. Hamster liver shows a significant sex difference in the amount of 'water-soluble' metabolites formed only at low tissue:steroid ratios at a constant incubation time, but not in the rate. The sex difference in glucuronide formation in hamster liver is the reverse of that found in the rat; female-hamster liver forms more glucuronides than

that of the male at all the incubation times and tissue:steroid ratios studied (Fig. 2). However, the two species are similar in that the sex difference in glucuronide formation does not disappear at longer incubation periods and at high tissue:steroid ratios.

In the present investigation glucuronides, quantitatively the major type of conjugate formed in liver homogenates from both male and female rats, were formed in much larger amounts than reported by Cooke & Taylor (1963) for liver homogenate from female rat. Those authors also found a wide variation (5–30%) in glucuronide formation from animal to animal, but no such variation was observed in the present work, in which the maximal values for female-rat liver (45–50%) and for male-rat liver (65–70%) were also higher than reported by Cooke & Taylor (1963). This more consistent and higher degree of glucuronide formation was probably due to the greater attention paid to the preparation of the homogenate under standardized cold conditions. This may have diminished the destruction of UDP-glucuronic acid, which is very labile and is rapidly destroyed after the death of the animal (Dutton & Storey, 1954). However, this cannot be the only explanation, since the homogenate prepared by the procedure used in the present investigation was found to retain its full power for steroid glucuronide formation when kept for 1 hr. at 0–4°.

The cold-acid-ether solvolysis method of Burstein & Lieberman (1958) is an accepted method for hydrolysis of steroid sulphates, and Cooke & Taylor (1963) presented evidence that this procedure hydrolysed at least some progesterone metabolites conjugated with sulphuric acid. In the present investigation sulphate formation in rat liver was found to be consistently low (8–16%) compared with the more variable and larger amounts (6–47%) reported by Cooke & Taylor (1963). The ratio of glucuronide to sulphate formation found in the present work is in agreement with that found *in vivo*: more than 3 when phenols are administered to intact rats (Bray, Humphries, Thorpe, White & Wood, 1952a,b). As glucuronide to sulphate formation *in vitro* has been reported to be mutually competitive (Storey, 1950), the variable and high sulphate formation found by Cooke & Taylor (1963) could be related to the low and variable glucuronide formation found by these authors. In the present work, some sex difference was observed in sulphate formation in rat liver (Table 2). This agrees with the report of Roy (1955) that female-rat liver has a greater ability to form dehydroepiandrosterone sulphate than that of male-rat liver. Female-rat liver appeared to form more cold-acid-hydrolysable metabolites than male liver, but the difference was not significant at the $P < 0.01$ level at all incubation times (Table 2). In hamster liver large quantities of cold-acid- and hot-acid-hydrolysable conjugates

were formed, and male-hamster liver is slightly more active than that of female in forming these conjugates (Table 2), in contrast with the greater ability of female-hamster liver to form glucuronide (Fig. 2).

The total recovery of radioactivity after hydrolysis and extraction has been found to decrease with increase in total 'conjugate' formation in liver homogenates from female rat and male hamster. It therefore seems that as conjugation increases other substances are formed that are not extractable from neutral aqueous solution by ether after all the methods of hydrolysis used. The nature of these non-extractable substances is not known.

Sex differences in conjugate formation may be due to several factors: the difference in the activity or amount of conjugate-forming enzymes, the concentration of substrates available, the presence or absence of inhibitors or activators or the nature of the steroid metabolites formed. Some of these factors may be responsible for the sex and species differences observed in the present work.

Glucuronide formation is catalysed by the microsomal enzyme, glucuronyltransferase (Dutton & Storey, 1954). Male-rat-liver microsomes are about four times as active as those of female-rat liver in forming glucuronides with *o*-aminophenol as aglycone (Inscoc & Axelrod, 1960). These authors have shown that this sex difference is not due to the presence of inhibitors in female-rat liver or to hydrolysis by hepatic β -glucuronidase of the glucuronides formed. They also found that the administration of testosterone propionate to female rats increased the activity of glucuronyltransferase, whereas the administration of oestrogens to males decreased this activity. However, this effect could not be demonstrated *in vitro* by the addition of particular hormones to the incubation medium. They also showed that mice and guinea pigs did not exhibit this sex difference. Progesterone and pregnanediol at concentrations of 0.6 and 0.4 mM respectively caused 50% inhibition of glucuronide conjugation of *o*-aminophenol, bilirubin and phenolphthalein by rat-liver homogenate (Hsia, Dowben, Shaw & Grossman, 1960). Lathe & Walker (1958) found that pregnanediol inhibited bilirubin glucuronide formation in rat-liver slices but not in rabbit-liver or monkey-liver slices. Therefore it is possible that the sex difference in progesterone metabolism and conjugation in some species reported here may also be due to the inhibition of glucuronyltransferase activity by unmetabolized progesterone and by some of the metabolites of progesterone. For example, pregnanolone inhibits bilirubin glucuronide formation in rat-liver homogenate (Holton & Lathe, 1964).

Another possible explanation of the sex and species differences observed in the present work is

that the steroid metabolites available for conjugate formation produced during the metabolism of progesterone are formed at different rates and amounts in liver homogenates from male and female rats and hamsters. As conjugation with glucuronic acid or sulphuric acid almost certainly requires the presence of a stable alcoholic or carboxylic hydroxyl group, conjugate formation should be preceded by the reduction of one or both of the 3-oxo and 20-oxo groups of progesterone and possibly by the introduction of one or more hydroxyl groups at other positions. There are several reports of such sex differences in the rate of reduction of steroids (see Table 1).

3β -Hydroxy steroid dehydrogenase from male-rat liver is about 20 times more active than that from female-rat liver (Rubin & Strecker, 1961). If such a sex difference were found for the reduction of the 3-oxo group of progesterone or its metabolites in rat liver, and if conjugation with glucuronic acid or sulphuric acid occurred at the 3β -hydroxyl position, then male-rat liver would be supplied with substrates for conjugate formation about 20 times faster than female-rat liver, and this would partly account for the faster rate of conjugate formation observed.

In contrast with the enzymes mentioned above, female-rat liver has a higher activity than male-rat liver in the reduction of the A-ring of several steroids including progesterone (Yates *et al.* 1958; Forchielli *et al.* 1958; Leybold & Staudinger, 1959). The Δ^4 -5 α -steroid hydrogenase, found in the microsomes, is 3–10 times as active in female-rat liver as in male-rat liver. Although the rate of reduction of the Δ^4 -bond of most steroids is faster in female-rat liver than in male-rat liver, female-rat liver cannot reduce the 3-oxo group of the 5 α -isomer of 21-hydroxypregnane-3,20-dione as readily as that of the 5 β -isomer, whereas the male can reduce the 3-oxo group of both isomers to the same extent (Bell *et al.* 1962). Therefore the more rapid formation of the 3-oxo-5 α -isomer in the female may be offset by the lower rate of the further metabolism of the 3-oxo group (and oxo groups at other positions) of the 5 α -isomer. This suggestion is supported by the results of the present investigation, which show that A-ring reduction is not rate-limiting for conjugate formation during the metabolism of progesterone by rat-liver homogenate, because conjugate formation occurs at a faster rate in male-rat liver than in female-rat liver, in spite of the considerably lower activity of A-ring reduction in male-rat liver. In contrast with the rat, male-hamster liver has higher activity in A-ring reduction than has female-hamster liver (Yates *et al.* 1958; Brown-Grant, Forchielli & Dorfman, 1962). This difference could account for the greater amount of conjugate formation that occurs in male-hamster liver at low tissue:steroid ratios.

The female rat has a larger adrenal than the male in relation to body weight, whereas the female hamster has a smaller adrenal than the male (Peczenik, 1944). The activity of hepatic A-ring reduction of steroids by the rat and hamster also shows such a reversed sex difference (Yates *et al.* 1958). The sex differences in glucuronide formation observed in the present work in the rat and hamster also show such a reversal. It therefore appears that there is an inverse correlation between A-ring reduction and glucuronide formation, but it is not known whether glucuronide conjugation occurs to a smaller extent with steroids of the 5 α -pregnane series as aglycones than with those of the 5 β -pregnane series.

Conjugation formation in rabbit and guinea pig and species differences. It has been shown that extensive conjugate formation takes place during the metabolism of [4-¹⁴C]progesterone by rabbit liver and guinea-pig liver at a tissue:steroid ratio of about 50 000:1. These results are in general agreement with those for rat liver and hamster liver. However, there are some overall similarities and differences in the way these four species convert progesterone into 'water-soluble' conjugates. Rabbit liver is comparable with rat liver in both the rate and total amount of formation of 'water-soluble' metabolites, but the marked sex difference observed in rat liver was not found in rabbit liver. Hamster liver resembles guinea-pig liver in requiring an incubation period of about 60 min. for conjugate formation to proceed to completion, but guinea-pig liver forms much less total 'water-soluble' metabolites than do the livers of the other species. Guinea-pig liver is also similar to that of hamster in that there is little or no sex difference in the rate of formation of 'water-soluble' metabolites. In guinea-pig liver (Fig. 3), however, a decrease in the tissue:steroid ratio does not reveal any marked sex difference, whereas, in the same type of experiment with hamsters, male-hamster liver is the more active in forming 'water-soluble' metabolites at tissue:steroid ratios less than 50 000:1.

The relatively low rate and lower total amount of formation of 'water-soluble' metabolites in guinea-pig liver (Table 3) is reflected in the ability of the liver of these animals to form glucuronide. Rabbit liver forms more glucuronide and at a faster rate than does guinea-pig liver (Table 3), and even at the highest tissue:steroid ratios studied guinea-pig liver produces much less glucuronide than rabbit liver (Fig. 4). There was only a slight sex difference in the glucuronide-forming activity of rabbit liver and no difference in this activity between livers from male and female guinea pigs (Fig. 4). This is in marked contrast with the results obtained with rat and hamster livers.

Species differences have been observed between rabbit and guinea pig in glucuronide conjugation

with *o*-aminophenol as aglycone. Dutton & Greig (1957) found that guinea-pig liver has greater glucuronyltransferase activity than has rabbit liver when *o*-aminophenol is used as aglycone *in vitro*. However, in the present work, rabbit liver formed glucuronides from progesterone metabolites to a greater extent and at a faster rate than did guinea-pig liver (Table 3). The glucuronyltransferase of guinea-pig liver, but not that of rabbit liver, is inhibited by progestational agents and their metabolites (Lathe & Walker, 1958; Hsia *et al.* 1960; Holton & Lathe, 1964). Further, guinea-pig liver is less active than rabbit liver in the reduction of ring A of a variety of steroids (Yates *et al.* 1958; Brown-Grant *et al.* 1962). Therefore the low rate and low overall amount of 'water-soluble' metabolites and glucuronide formation might be due to a combination of these factors. However, such an interpretation of these results must be treated with caution because of the different experimental conditions and aglycones used in this and other work. For instance, Hartiala *et al.* (1964) reported that male-rabbit liver has a much greater glucuronyltransferase activity than female-rat liver when *o*-aminophenol is used as substrate, yet no such marked sex difference in glucuronide formation was found when progesterone was metabolized by rabbit liver (Fig. 4). The lack of any marked sex difference in rabbit liver may be due to some difference in the metabolism of the steroid itself masking a sex difference in glucuronide formation. However, no sex difference has been observed in the rate of A-ring reduction of progesterone by rabbit liver *in vitro* (Brown-Grant *et al.* 1962). Inscoe & Axelrod (1960) found no sex difference in the ability of guinea-pig liver to conjugate *o*-aminophenol with glucuronic acid, and this is supported by the present results (Fig. 4).

Glucuronide formation from other steroid aglycones by rabbit liver and guinea-pig liver *in vitro* has been reported: Crepy (1947), natural and synthetic oestrogens; Isselbacher & Axelrod (1955), tetrahydrocortisone; Fishman & Sie (1956), testosterone; Smith & Breuer (1963), oestriol (rabbit liver only).

The metabolism of progesterone in the intact guinea pig and hamster have not been adequately investigated. Chamberlain, Knights & Thomas (1964) found traces of pregnanediol in the urine of guinea pigs after the administration of progesterone. However, it is known that the guinea pig differs from other animals in some aspects of steroid metabolism. Thus endogenous and administered corticosteroids are excreted unconjugated and with the Δ^4 -3-oxo group intact in the urine of this animal, whereas the urinary C₁₉ steroids are A-ring-reduced and conjugated (Nadel, Young, Hilgar & Burstein, 1958; Peron & Dorfman, 1958). However, guinea-

pig liver *in vitro* exhibits considerable Δ^4 -steroid-hydrogenase activity towards corticosteroids and high glucuronyltransferase activity with *o*-aminophenol (Dutton & Greig, 1957) and metabolites of progesterone (the present work) as aglycones.

These results have an important bearing on the physiological significance of the work *in vitro*. For instance, female-rat liver is as active as male-rat liver in the formation of 'water-soluble' metabolites at tissue:steroid ratios of about 56 000:1 and at longer incubation periods, but not at lower tissue:steroid ratios and at shorter incubation periods. This lack of sex difference at high tissue:steroid ratios and longer incubation periods indicates that caution must be observed in assigning physiological significance to results obtained from studies with isolated enzymes or in certain types of work *in vitro*, particularly if low tissue:steroid ratios are used to demonstrate sex differences, as applies to many of the investigations listed in Table 1. However, the marked sex difference observed in the present work in the rate of glucuronide formation in the rat and hamster at high tissue:steroid ratios much more nearly physiological than those used in most investigations of steroid metabolism *in vitro* may have some physiological significance.

The financial support of the Medical Research Council is gratefully acknowledged.

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