J. steroid Biochem. Vol. 36, No. 6, pp. 569–574, 1990
Printed in Great Britain.

0022-4731/90 $3.00 + 0.00

17β-HYDROXYSTEROID OXIDOREDUCTASE ACTIVITY: AGE-DEPENDENT PROFILE IN RAT LIVER AND KINETIC PROPERTIES OF THE HEPATIC MICROSOMAL ENZYME IN RELATION TO CYTOCHROME *P*450-DEPENDENT STEROID HYDROXYLATION

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(*Received* 8 *January* 1990)

Summary—The functional relationship between the microsomal cytochrome *P*450 and 17β-hydroxysteroid oxidoreductase (HSOR) enzymes involved in steroid metabolism was investigated in rat liver. In male and female rat hepatic microsomes the NADPH-dependent conversion of androstenedione (AD) to testosterone (T) was approx. 4-fold greater at 6 weeks of age than in 1 week old animals. In hepatic microsomes from 15 week old rats the activity of the HSOR pathway was greater in males than in females (1.51 compared to 0.80 nmol T formed/min/mg protein). However, oestradiol administration to intact adult male rats did not decrease HSOR activity. Thus, androgen is not essential for maintenance of HSOR enzymes. Instead, it is likely that irreversible androgen imprinting of the HSOR enzyme occurs during the prepubertal period.

The *in vitro* characteristics of HSOR activity were also assessed. The K_m for NADH-dependent reduction of AD to T was 9.2 μM and the V_{max} was 3.0 nmol/min/mg protein but the NAD-mediated formation of AD from T did not follow Michaelis–Menton kinetics. pH markedly influenced HSOR-mediated AD/T interconversion with 17-ketosteroid reduction facilitated at low pH, and 17β-hydroxysteroid dehydrogenation about 2-fold more efficient at pH 8.0 than at pH 5.5. Product steroid activation of HSOR activity was noted. 17β-Hydroxysteroids, including T and oestradiol, activated the rate of conversion of AD to T and 17-ketosteroids such as oestrone and AD activated the NAD-dependent dehydrogenation of T. Activation was not observed at low steroid substrate concentrations so that it was not possible to analyse this phenomenon by a conventional kinetic approach.

INTRODUCTION

The liver is an important site of steroid metabolism. The hepatic endoplasmic reticulum contains the cytochrome *P*450 enzyme family that catalyses the position-specific hydroxylation of a range of steroid substrates, including androgens [1, 2], oestrogens [3, 4] and progestins [5, 6]. Other hepatic enzymes that are also involved in steroid metabolism include the cytosolic 3α-hydroxysteroid oxidoreductase [7], the microsomal 4-ene-3-ketosteroid 5α-oxidoreductase [8, 9], the cytosolic 4-ene-3-ketosteroid 5β-oxidoreductase and the 17β-hydroxysteroid oxidoreductases (HSOR; EC 1.1.1.62) that catalyse the interconversion of 17-keto and 17β-hydroxysteroids such as androstenedione (AD) and testosterone (T) [10]. Hepatic HSOR activity is present in the microsomal and cytosolic fractions [10, 11] and utilises either NADH or NADPH as cofactor. Although several cytosolic HSOR enzymes have been isolated and characterised, little information is available concerning the function of the microsomal HSOR enzyme, especially in relation to other enzymes of steroid metabolism. Steroids are not the only substrates for hepatic HSORs and it has been proposed that the majority of the hepatic benzene dihydrodiol dehydrogenase activity is catalysed by an NADP-dependent T dehydrogenase [12]. It therefore appears likely that the hepatic HSOR enzyme is involved in the metabolism of foreign compounds as well as steroid hormones in the intact animal.

The relationships between sex, age and the activity of various steroid metabolising enzymes have been investigated in numerous studies, particularly in the case of the microsomal steroid hydroxylases (*P*450 enzymes) [13, 14]. Gonadal and pituitary hormones clearly play an important role in the expression and maintenance of a number of hepatic enzymes of steroid metabolism. At this time, however, there is a deficiency of data concerning the relationship between hormonal factors and the microsomal expression of HSOR activity. The present study was

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undertaken to determine the significance of the age and sex of the animal in the capacity of rat liver to catalyse the reduction of AD to T in relation to *P*450-dependent androstenedione hydroxylation.

The present study also includes a characterisation of the kinetics of microsomal HSOR activity with AD and T as substrates. A series of steroids was investigated for the capacity to modulate the activity of the enzyme *in vitro*. The phenomenon of product activation was noted in connection with HSOR-mediated interconversion of T and AD.

EXPERIMENTAL

Chemicals

[4-^{14}C]AD (sp. act. 59 mCi/mmol) and [4-^{14}C]T (sp. act. 59 m/Ci/mmol) were obtained from Amersham Australia, Sydney, NSW. 7α-HydroxyAD was obtained from Professor D. N. Kirk of the MRC steroid reference collection, Queen Mary's College, London, England. All biochemicals, 6β- and 16α-hydroxyAD and all other steroids were purchased from Sigma Chemical Co., St Louis, Mo. 16β-HydroxyAD was prepared by the action of 3β-hydroxysteroid dehydrogenase (Sigma) on 3β,16β-dihydroxyandrost-5-ene-17-one (supplied by the MRC collection) [15].

Solvents and other chemicals were from Ajax Pty Ltd, Sydney, Australia, and were analytical reagent grade quality.

Animal treatment

Male or female Wistar rats (approx. 250 g and 7 weeks of age, except in the developmental profile study) were obtained from the Westmead Hospital animal house and were held in wire cages under conditions of constant temperature, humidity and lighting. In another experiment male rats (7 weeks of age) were given oestradiol (20 mg/kg/day i.p. on days 1–5 and 8–11 and sacrificed on day 12). Control rats weighed 346 ± 7 and 376 ± 7 g at the commencement and conclusion of this experiment, respectively. In contrast, the oestradiol-treated group weighed 317 ± 18 g at the commencement and 272 ± 18 g at the conclusion of the experiment. Thus, the control group increased to 109% of their starting weight whereas the treated group were, on average, only 86% of their initial weight. Microsomal fractions were prepared from individual livers by ultracentrifugation [16]. Microsomal protein was determined by the Lowry procedure with bovine serum albumin as standard [17].

Steroid metabolism in hepatic microsomes

Microsomal AD metabolism was conducted essentially as described previously [18]. NADPH- and NADH-dependent AD metabolism and NAD-dependent T metabolism were determined in separate experiments. Briefly, incubations contained AD or T (400 nmol, 4 × 10^5 dpm), either NADPH, NADH or NAD (4 μmol) as cofactor and microsomal protein (1 mg when AD was the substrate and 0.25 mg when T was substrate). The steroids were extracted with chloroform, the organic phase evaporated to dryness and the residue dissolved in a small volume of chloroform prior to application to TLC plates (silica gel 60 F_{254} activated at 100°C for 15 min prior to use, 20 × 20 cm × 0.25 mm thickness; Merck, Darmstadt, F.R.G.). Plates were developed twice in chloroform:ethyl acetate (1:2, v/v) in the case of AD metabolism [19]. When T was used as the substrate metabolites were resolved by TLC using the solvent systems dichloromethane:acetone (4:1, v/v) followed by chloroform:ethyl acetate:ethanol (4:1:0.7, v/v/v). Radioactive zones corresponding to steroid standards were visualised under u.v. light and scraped into vials for scintillation spectrometry (Aquasol scintillant, New England Nuclear Corp., Boston, Mass). Counting efficiency and counting time in these experiments were 94% and 5 min, respectively.

In experiments dealing with HSOR activation and inhibition the 17-keto- or 17β-hydroxysteroids were incorporated into the reaction medium in ethanol (100 μl; final solvent concentration 2.5%); solvent was added to control incubations.

Cytochrome P450

Hepatic microsomal cytochrome *P*450 content was measured by the spectrophotometric assay of Omura and Sato [20].

Data analysis

Mean values of data in different groups of animals in the age/sex-dependence study were compared using the Student–Newman–Keuls test, following single factor analysis of variance. In the oestradiol experiment differences between means were compared by the Student's *t*-test.

RESULTS AND DISCUSSION

Kinetic properties of rat hepatic microsomal NADH-dependent HSOR activity

The kinetic properties of T formation from AD by the microsomal HSOR enzyme were investigated in male and female rat liver. In male rat liver a value of 9.2 μM was estimated for the K_m for NADH-dependent T production with a V_{max} of 3.0 nmol/min/mg protein. The reverse reaction (NAD-supported AD formation from T) was also investigated in this study but these experiments produced data that, when subjected to double-reciprocal analysis, did not yield estimable kinetic parameters. It was shown recently that, due to hysteresis, the prostatic 4-ene-3-ketosteroid 5α-oxidoreductase does not follow Michaelian kinetics [21]. This was attributed to the enzyme adopting a sub-optimal conformation for catalytic activity following substrate binding. Kinetic analysis of the prostatic 5α-reductase was not possible as the apparent kinetics were dependent upon

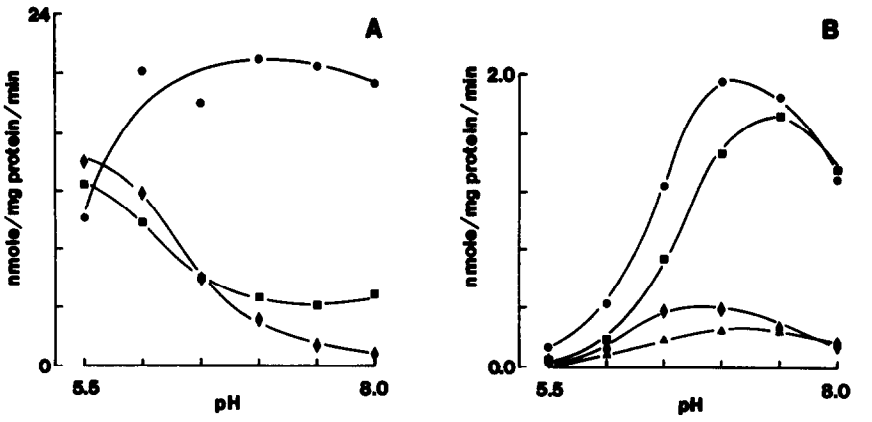

Fig. 1. Effect of incubation pH on HSOR and *P*450-dependent hydroxylase activities in male rat hepatic microsomes. Panel A: (●) NAD-mediated formation of androstenedione from testosterone, (■) NADH-mediated formation of testosterone from androstenedione and (◆) NADPH-mediated formation of testosterone from androstenedione. Panel B: NADPH-mediated formation of hydroxyandrostenedione metabolites. (●) 16α-hydroxylation, (■) 6β-hydroxylation, (▲) 16β-hydroxylation and (◆) 7α-hydroxylation.

incubation time [21]. In the present study, excessive dehydrogenation of the 17β-hydroxy group occurred at low T concentrations whereas similar rates of conversion occurred at higher substrate concentrations. Attempts to decrease the overall rate of T dehydrogenation by reducing the amount of microsomal protein in the assays were also unsuccessful. Thus, non-uniform substrate utilisation was apparent and may also reflect a hysteretic mechanism.

The effects of incubation pH on NADH-dependent conversion of AD to T, the NAD-dependent reverse reaction and NADPH-mediated AD metabolism were assessed in this study (Fig. 1A and B). At pH 5.5 the rates of NADH- and NADPH-catalysed T formation were 2.7- and 4.6-fold the rates observed at pH 7.0; a further decrease in activity was seen at slightly alkaline pH (8.0) in the NADPH-mediated reaction (Fig. 1A). The opposite effect was seen for AD formation from T (Fig. 1B) where, at pH 5.5, the reaction rate was approx. 50% of that seen at higher pH. Similar points to these have been noted by Kochakian, who examined HSORs in hamster liver [22], and Renwick *et al.*, who found that the pH optimum of the chicken liver oestradiol 17β-dehydrogenase was around 10 [23]. The inference that could be drawn from these observations is that the mechanism of the HSOR-catalysed conversion of AD to T involves protonation of the reaction intermediate. Presumably the reverse process involved abstraction of a proton from the reaction intermediate and this is facilitated at relatively basic pH. An alternate explanation is that an amino acid residue in the active site of the enzyme and which is involved in the catalytic process may be susceptible to protonation at low pH and deprotonation at high pH. These processes may enhance the observed rates of catalysis.

The *P*450-dependent steroid hydroxylations typically exhibited optimal reaction velocities around pH 7.0–7.5, thus further distinguishing these enzymes from the HSOR enzyme (Fig. 1B). Rates of AD hydroxylation were clearly suboptimal at the extremes of incubation pH investigated in the present study.

In vitro modulation of HSOR activity by steroids

In initial experiments it was noted that the 17β-hydroxysteroids T and oestradiol effected 4.2- and 6.2-fold enhancement, respectively, of NADPH-supported HSOR activity (Table 1). In contrast, several

Table 1. *In vitro* modulation of androstenedione metabolism in NADPH-supplemented microsomes from male rat liver

	Androstenedione metabolite formation (% control)				
Modulatory steroid	Testosterone	16α-Hydroxy	16β-Hydroxy	6β-Hydroxy	7α-Hydroxy
Testosterone	620	50	82	63	52
Epiandrosterone	36	48	370	5	65
Etiocholan-3α-ol-17-one	16	40	36	43	46
Etiocholan-3β-ol-17-one	67	23	29	16	23
Androsterone	54	58	49	66	72
Progesterone	64	19	22	24	59
17α-Hydroxyprogesterone	85	88	110	76	50
Oestradiol	420	45	48	20	33
Oestrone	86	83	66	51	80
Pregnenolone	84	31	70	78	44
17α-Hydroxypregnenolone	82	75	100	87	46

Data are presented as mean values derived from at least duplicate incubations. Individual values varied by less than 8% from the stated mean values. In these experiments the substrate (androstenedione) concentration was 175 μM, the protein concentration was 0.75 mg/ml and modulatory steroids were tested at 175 μM.

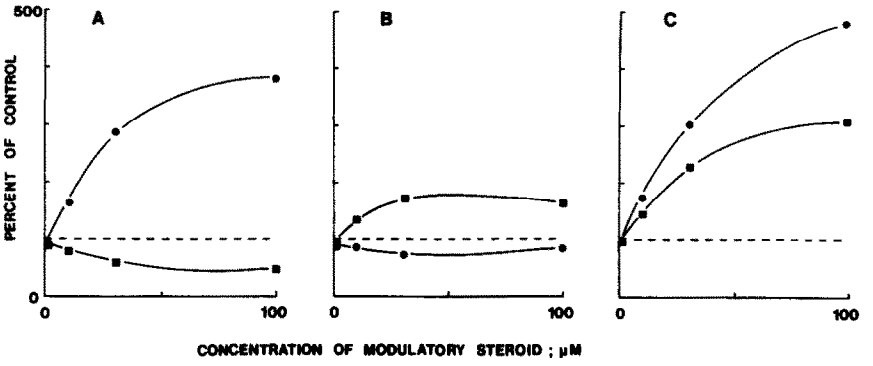

Fig. 2. Concentration dependence of the modulation of HSOR activity by steroids *in vitro*. Panel A: Effect of oestradiol on (●) NADH-mediated testosterone formation from androstenedione and on (■) NAD-mediated androstenedione formation from testosterone. Panel B: Effect of oestrone on (●) NAD-mediated androstenedione formation from testosterone and on (■) NADH-mediated testosterone formation from androstenedione. Panel C: (●) Effect of testosterone on testosterone formation form androstenedione and (■) effect of androstenedione on androstenedione formation from testosterone.

17-ketosteroids, including epiandrosterone, androsterone and etiocholan-3α-ol-17-one, decreased the rate of T production from AD *in vitro* (Table 1). The C21-steroids pregnenolone, 17α-hydroxypregnenolone and 17α-hydroxyprogesterone exerted minimal effects on HSOR activity although progesterone (when included at a concentration of 175 μM in the incubation) decreased the rate of T production to 64% of control. Enhancement of HSOR activity appeared to be independent of the effects of the modulatory steroids on *P*450-mediated AD hydroxylase activities *in vitro*. The data in Fig. 2 demonstrate the concentration dependence of steroid modulation of HSOR activity and confirm that this phenomenon is also observed with NADH as cofactor. The principal finding to emerge from these experiments is that HSOR reaction products are themselves stimulatory to the HSOR reaction *in vitro*. Thus, 17β-hydroxysteroids, including oestradiol and T, markedly enhanced the reduction of AD to T (100 μM T, for example, effected an increase in the rate of NADH-dependent AD reduction to 480% of control; Fig. 2C) and the 17-ketosteroids oestrone and AD enhanced the rate of T oxidation by HSOR when NAD was the cofactor. Attempts to characterise the kinetics of enhancement were largely unsuccessful. No enhancement or, in some instances, slight inhibition was noted when the substrate (AD) concentration was low (4 or 10 μM). Significant rates of enhancement of AD 17-keto reduction were only observed with higher substrate concentrations (>25 μM) and higher T concentrations (10 μM or greater). Thus, the substrate range over which a kinetic analysis would have been possible would have been too narrow to have yielded meaningful constants without extrapolation. Similar behaviour has been reported previously in the case of solvent-mediated enhancement of certain hepatic microsomal *P*450-dependent drug oxidations [24]. It is possible that the product steroids act as allosteric modifiers of HSOR activity. The nature of the HSOR active site has not been clearly delineated although in the case of the hepatic cytosolic HSORS there appear to be two sites: one a substrate binding site and the other a site for pyridine nucleotide cofactor binding [25]. Since potent product activation has been observed for the microsomal enzyme in the present study an additional site may also exist on the enzyme. It is possible that there are separate binding sites on the enzyme for 17-keto- and 17β-hydroxysteroids that are in close proximity to the cofactor binding region. Further studies are required to assess these possibilities.

Age- and sex-dependence of hepatic microsomal HSOR activity

The data in Table 2 indicate the influence of age and sex on the activity of the rat hepatic microsomal HSOR enzyme. In male rats the NADPH-dependent activity increased approx. 4-fold from 0.55 nmol T formed/min/mg protein at 1 week of age to 2.00 ± 0.05 at 6 weeks of age. By 15 weeks of age the observed rate of T production had decreased to 76% of the optimal rate (at 6 weeks of age). A similar trend was observed in female rat liver microsomes. Optimal rates of conversion of AD to T were noted at 4–6 weeks of age but the decline at 10 and 15 weeks of age was more striking than that in male rats. Thus, following puberty, a sexual dimorphism in microsomal HSOR activity was observed that reached statistical significance. At 10 and 15 weeks of age the rate of T production from AD in female rat liver was about 54% of that observed in hepatic fractions from male rats. Despite this finding it was also apparent that, following puberty, the ratio of total steroid hydroxylation to T formation was greater in males than females. Thus, HSOR activity is proportionately more significant than *P*450 activity in hepatic microsomes from post-pubertal female rats. Furthermore, steroid 5α-reduction is considerably more active in female than male rat liver [10]. In the experiments undertaken for the present study we noted that AD

Table 2. Age- and sex-dependence of microsomal hydroxysteroid 17β-oxidoreductase activity in rat liver

Age (weeks)	Testosterone formation (nmol/min/mg protein)	Hydroxyandrostenedione formation 6β	7α	16α	16β	Total hydroxylation	Total hydroxylation : testosterone
		(nmol/min/mg protein)					
				Male rat liver			
1	0.55	0.58	0.24	0.10	0.06	0.98	1.78
2	0.98 ± 0.10	1.19 ± 0.01	0.34 ± 0.01	0.09 ± 0.00	0.08 ± 0.01	1.70 ± 0.02	1.85 ± 0.22
4	1.78 ± 0.10	3.17 ± 0.40	0.80 ± 0.06	0.30 ± 0.01	0.28 ± 0.02	4.55 ± 0.46	2.58 ± 0.29
6	2.00 ± 0.05	2.34 ± 0.09	0.34 ± 0.02	1.14 ± 0.14	0.21 ± 0.00	4.03 ± 0.19	2.01 ± 0.09
10	1.27 ± 0.14*	1.84 ± 0.08	0.19 ± 0.01	1.66 ± 0.12	0.23 ± 0.02	3.91 ± 0.11	3.23 ± 0.44
15	1.51 ± 0.08†	1.85 ± 0.18	0.29 ± 0.02	2.41 ± 0.12	0.28 ± 0.02	4.82 ± 0.29	3.27 ± 0.31
				Female rat liver			
1	0.40	0.33	0.13	0.05	0.04	0.55	1.38
2	0.97 ± 0.12	1.10 ± 0.10	0.35 ± 0.03	0.06 ± 0.01	0.06 ± 0.01	1.57 ± 0.11	1.67 ± 0.14
4	1.93 ± 0.14	2.62 ± 0.44	0.86 ± 0.01	0.35 ± 0.09	0.30 ± 0.03	4.11 ± 0.53	2.15 ± 0.26
6	1.81 ± 0.13	0.12 ± 0.02	0.57 ± 0.02	0.12 ± 0.01	0.14 ± 0.01	0.95 ± 0.06	0.53 ± 0.02
10	0.69 ± 0.16*	0.09 ± 0.01	0.45 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.75 ± 0.03	1.32 ± 0.34
15	0.80 ± 0.06†	0.17 ± 0.01	0.43 ± 0.03	0.11 ± 0.01	0.16 ± 0.01	0.87 ± 0.05	1.11 ± 0.06

Data are expressed as mean ± SEM derived using 3–8 animals in each group, with the exception of the 1 week groups which are mean values of triplicate estimates obtained from pooled samples of 6 animals.
Hydroxylation : testosterone ratios were calculated for individual livers and were then converted to mean ± SEM values. Significant difference between means: *$P < 0.005$ and †$P < 0.001$.

reduction to 5α-androstanedione proceeded at a similar rate to T formation in female rat liver (not shown). However, it remains that total AD metabolism is greater in post-pubertal male rat liver microsomes due to more extensive *P*450-dependent AD hydroxylation.

In order to assess the possible role of androgen in the maintenance of microsomal HSOR activity oestradiol was administered to intact male rats. As shown in Table 3, although the male specific *P*450 pathways of steroid hydroxylation (AD 6β- and 16α-hydroxylation) were markedly reduced to less than 10% of control, T formation was unchanged. In view of this finding it would appear that androgen is not crucial for maintenance of HSOR activity in adult male rats. One explanation that could account for these observations is that imprinting in the neonatal or prepubertal period is irreversible so that androgen has a non-essential role.

General discussion

It is now clear that a number of hepatic enzymes are active in the conversion of steroids to less active metabolites. The *P*450 system is especially important in steroid deactivation by hydroxylation since the hydroxylated metabolites are more readily eliminated from the organism as water-soluble conjugates. The involvement of non-*P*450 enzymes and the significance of pathways of steroid metabolism other than hydroxylation are less apparent. For example, products of reactions catalysed by HSOR would still require hydroxylation (by *P*450s) for complete deactivation. Nevertheless, HSOR-dependent conversion of T to AD may be considered as a catabolic process since the latter possesses only about 30% of the androgenic potency of the former [26].

The hepatic HSOR enzymes are unlikely to contribute to circulating T concentrations since castration of male rats results in the loss of the hormone from the serum. Instead, the available evidence supports the assertion that the enzymes have a catabolic function. It has also been established that the cytosolic forms of the enzymes catalyse the interconversion of aromatic dihydrodiols and *ortho*-quinones. Blomquist *et al.* have suggested that the hepatic microsomal HSOR activity is quantitatively more important than its cytosolic counterpart [10]. Accordingly, it would seem appropriate to determine the capacity of the microsomal enzyme to catabolise dihydrodiols and related metabolites of foreign compounds. Since steroid-mediated enhancement of HSOR activity was noted in the present study it would be of considerable interest to assess the effect of such steroids on NADPH-dependent dihydrodiol metabolism.

Hepatic microsomal HSOR activity, like a number of other steroid metabolising enzyme systems in rat liver, appears to be subject to sex-dependence. It is notable that the dimorphism observed is distinct from that relating to oxidative pathways where specific *P*450 enzymes involved in steroid 16α- and 6β-hydroxylation are virtually absent from female rat

Table 3. Effect of oestradiol administration to intact male rats on microsomal hydroxysteroid 17β-oxidoreductase activity

Treatment group	Testosterone formation (nmol/min/mg protein)	Hydroxyandrostenedione formation 6β	7α	16α	16β
		(nmol/min/mg protein)			
None (control)	1.48 ± 0.07	2.06 ± 0.09	0.20 ± 0.04	1.48 ± 0.07	0.20 ± 0.02
Oestradiol	1.50 ± 0.11	0.13 ± 0.01	0.25 ± 0.04	0.12 ± 0.01	0.22 ± 0.02
Percentage of control	101	6.3	125	8.1	110
P	NS	<0.001	NS	<0.001	NS

Data are mean ± SEM derived using 7 animals in each group. Animals were treated as described in the Experimental Section. NS, difference between mean values not significant.

liver. Instead, HSOR activity is relatively lower in female rat liver. The precise hormonal factors involved in regulation of the microsomal HSOR enzyme in rat liver have not been evaluated in this study, although it now seems clear that androgen and oestrogen are not responsible for the observed sexual dimorphism. It also remains to be investigated whether androgens elicit enhancement of HSOR-dependent activities in the intact animal.

Acknowledgements—This work was supported in part by the Australian National Health and Medical Research Council (NH & MRC). M.M. is an NH & MRC Senior Research Fellow.

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