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MICROSOMAL CYTOCHROME *P*450-DEPENDENT STEROID METABOLISM IN MALE SHEEP LIVER. QUANTITATIVE IMPORTANCE OF 6β-HYDROXYLATION AND EVIDENCE FOR THE INVOLVEMENT OF A *P*450 FROM THE IIIA SUBFAMILY IN THE PATHWAY

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Summary—The metabolism of testosterone (TEST), androstenedione (AD) and progesterone (PROG) was assessed in hepatic microsomal fractions from male sheep. Rates of total hydroxylation of each steroid were lower in sheep liver than in microsomes isolated from untreated male rat, guinea pig or human liver. 6β-Hydroxylation was the most important pathway of biotransfromation of each of the three steroids (0.80, 0.89 and 0.43 nmol/min/mg protein for TEST, AD and PROG, respectively). Significant minor metabolites from TEST were the 2β-, 15β- and 15α-alcohols (0.19, 0.22 and 0.17 nmol/min/mg microsomal protein, respectively). Apart from the 6β-hydroxysteroid, only the 21-hydroxy derivative was formed from PROG at a significant rate (0.27 nmol/min/mg protein). The 6β-alcohol was the only metabolite formed from AD at a rate >0.1 nmol/min/mg protein. Antisera raised in rabbits to several rat hepatic microsomal *P*450s were assessed for their capacity to modulate sheep microsomal TEST hydroxylation. Anti-*P*450 IIIA isolated from phenobarbital-induced rat liver effectively inhibited TEST hydroxylation at the 2β-, 6β-, 15α- and 15β-positions (by 31–56% when incubated with microsomes at a ratio of 5 mg IgG/mg protein). IgG raised against rat *P*450 IIC11 and IIB1 inhibited the formation of some of the minor hydroxysteroid metabolites but did not decrease the rate of TEST 6β-hydroxylation. Western immunoblot analysis confirmed the cross-reactivity of anti-rat *P*450 IIIA with an antigen in sheep hepatic microsomes; anti-IIC11 and anti-IIB1 exhibited only weak immunoreactivity with proteins in these fractions. Considered together, the present findings indicate that, as is the case in many mammalian species, 6β-hydroxylation is the principal steroid biotransformation pathway of male sheep liver. Evidence from immunoinhibition and Western immunoblot experiments strongly implicate the involvement of a *P*450 from the IIIA subfamily in ovine steroid 6β-hydroxylation.

INTRODUCTION

Hepatic steroid hydroxylation catalysed by multiple forms of microsomal cytochrome *P*450 (*P*450) [1–3] is an important process in the termination of steroid action [4–6]. It has been proposed that steroids may be the prototype substrates of the hepatic microsomal *P*450 monooxygenase system as these enzymes generally exhibit much higher affinities (micromolar K_m values) for steroids than for lipophilic foreign compounds (millimolar K_m values) [7]. Further, microsomal *P*450s usually catalyse steroid oxidation with greater regio- and stereospecificity than foreign compound oxidation [8–10]. Multiple hydroxylated metabolites are formed from steroids in hepatic microsomal fractions from certain species, such as the rat; these reflect the involvement of a number of *P*450s [8–10]. In contrast, fewer hydroxysteroid metabolites are produced in *in vitro* incubations with hepatic microsomal fractions from other mammalian species, such as the rabbit, dog and man [11–13]. The 6β-hydroxylation of C_{19} and C_{21} steroid substrates is a major microsomal oxidative biotransformation pathway and the IIIA subfamily of proteins catalyse 6β-hydroxylation in most species. Thus, *P*450 IIIA2 in untreated male rat liver, *P*450 IIIA1 in dexamethasone-induced rat liver, *P*450 IIIA6 in the rabbit and the analogous human *P*450s

IIIA3–5 contribute extensively to this reaction [11, 14, 15]. In addition, there is at least one *P*450 from dog liver that participates in the 6β-hydroxylation of androst-4-ene-3,17-dione (AD) and testosterone (TEST); this protein also appears to be from the *P*450 IIIA gene family [12].

Little precise information concerning *P*450 and its associated monooxygenase activities is available in mammalian species other than those already mentioned. Recently, however, a triacetyloleandomycin-inducible *P*450 IIIA protein was isolated from sheep liver and was shown to play a major role in the *N*-dealkylation of several drug substrates [16]. An IgG fraction raised in rabbits against this inducible IIIA protein was found to recognize an antigen in untreated sheep liver. The present study was undertaken to investigate the importance of steroid 6β-hydroxylation in untreated sheep liver and whether *P*450(s) from the IIIA subfamily may be involved in this pathway.

MATERIALS AND METHODS

Chemicals

[4-^{14}C]Testosterone (TEST), [4-^{14}C]progesterone (PROG) and [4-^{14}C]androst-4-ene-3,17-dione (AD) were obtained from Amersham Australia Pty Ltd, (North Ryde, NSW, Australia). The specific activities of the steroids were 56–59 mCi/mmol. Steroid reference standards were purchased from the MRC Steroid Collection (Queen Mary's College, London, U.K.), Steraloids (Wilton, NH, U.S.A.) or Sigma Chemical Co. (St Louis, MO, U.S.A.). 15β-HydroxyTEST was generously supplied by G. D. Searle & Co. (Skokie, IL, U.S.A.). Biochemicals were from Sigma or Boehringer–Mannheim (North Ryde, NSW, Australia). Solvents and other analytical reagent grade chemicals were obtained from Ajax Chemicals (Sydney, NSW, Australia).

Miconazole nitrate was purchased from Sigma, thiabendazole was provided by Dr E. Lacey (CSIRO McMaster Lab., Glebe, NSW, Australia), triacetyloleandomycin was a gift from Pfizer Research (West Ryde, NSW, Australia) and SKF 525-A was from Smith, Kline & French Pty Ltd (Frenchs Forest, NSW, Australia). Reagents for electrophoresis were purchased from Bio-rad Labs (Richmond, CA, U.S.A.) or Sigma and nitrocellulose was from Hoefer Scientific Instruments (San Francisco, CA, U.S.A.).

Livers and the preparation of microsomal fractions

Livers from adult male sheep were obtained from the McMaster Lab. (CSIRO Animal Health, Glebe, NSW, Australia), immediately after the animals had been killed. Male Wistar rats (*ca* 300 g) and adult male guinea pigs (*ca* 600 g) were provided by the Westmead Hospital animal facility. In some experiments rats received either phenobarbital (100 mg/kg i.p. in saline for 3 days) or dexamethasone (300 mg/kg i.p. in corn oil for 3 days) and were sacrificed 48 h after the final dose of inducer. Human liver samples were obtained as surgical waste from individuals undergoing resection for hepatic cysts or carcinomas. The material was taken from areas that appeared pathologically normal.

Microsomal fractions were obtained as the post-105,000***g*** pellet from standard differential centrifugation procedures. All microsomal pellets were resuspended in 10 mM potassium phosphate buffer containing 250 mM sucrose and 1 mM EDTA (pH 7.4) and were centrifuged again at 105,000***g*** for 30 min [17]. The final washed pellets were suspended in 50 mM potassium phosphate, 20% glycerol and 1 mM EDTA (pH 7.4) and stored at −70°C until required. Protein was estimated according to Lowry *et al.* [18] and cytochrome *P*450 by the method of Omura and Sato [19].

Microsomal steroid hydroxylation

Microsomal hydroxylation of the three ^{14}C-labelled steroids was performed essentially as described previously [20, 21]. Incubations (0.4 ml) contained hepatic microsomes (0.3 mg), steroid substrate (final concentration 50 μM, 4×10^5 dpm) and an NADPH-generating system composed of NADP (1 mM), glucose 6-phosphate (4 mM) and glucose 6-phosphate dehydrogenase (1 U). Reactions were initiated with NADP and terminated after 2.5 min by the addition of chloroform (10 ml). The organic phase was separated, evaporated and reconstituted in a small volume of chloroform for application to TLC plates (Merck silica 60 with F_{254} indicator, heated to 100°C for 15 min before use).

Products of TEST metabolism were resolved by chromatography in dichloromethane–acetone (4:1) followed by chloroform–ethyl acetate–ethanol (4:1:0.7) [8]. R_f values for the hydroxyTEST metabolites were found

to be AD 0.79, TEST 0.66, 2α-hydroxyTEST 0.59, 2β-hydroxyTEST 0.51, 6β-hydroxy TEST 0.44, 15β-hydroxyTEST 0.34, 7α-hydroxyTEST 0.32, 16α-hydroxyTEST 0.28 and 15α-hydroxyTEST 0.23. Metabolites of PROG were separated by three developments in the solvent system toluene–ethyl acetate–acetone (10:1:1) [21]. R_f values of the hydroxyPROG derivatives in this system were: PROG 0.60, 2α-hydroxyPROG 0.42, 21-hydroxyPROG 0.34, 6β-hydroxyPROG 0.20 and 16α-hydroxyPROG 0.10. Metabolites of AD were resolved by development (twice) in the system chloroform–ethyl acetate (4:1) [8]. R_f values of AD metabolites were: AD 0.64, TEST 0.55, 16α-hydroxyAD 0.44, 16β-hydroxyAD 0.40, 6β-hydroxyAD 0.34 and 7α-hydroxyAD 0.25. Radioactive metabolites were located on TLC plates following autoradiography (Hyperfilm, Amersham) for 48 h and were quantified by liquid scintillation spectrometry (ACS II, Amersham).

In other experiments the Michaelis constant (K_m) and maximal reaction velocities (V_{max}) of the TEST 6β-hydroxylase in sheep hepatic microsomes were determined by varying the substrate concentration over the range 5–200 μM.

Purification of rat hepatic P450s and preparation of antisera

*P*450s IIC11 and IIB1 were isolated from untreated and phenobarbital-induced rat liver microsomes, respectively [22, 23]. *P*450 IIIA1 was isolated from phenobarbital-treated rat liver [24] and was the generous gift of Dr Anders Åstrom (University of Stockholm, Sweden). Rabbits were immunized with the purified antigens according to established procedures [22]. The IgG fractions that were subsequently isolated from rabbit serum were used in the immunoinhibition of sheep and rat microsomal steroid hydroxylases and in Western immunoblot analysis [25].

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting

Hepatic microsomes (20 μg) were electrophoresed on 7.5% polyacrylamide gels as described by Laemmli [26] except that the Tris and glycine concentrations of all buffers were increased 2-fold. This modification has been found previously to enhance the resolution of proteins in the 40–60 kDa region [27]. Gels were electrophoresed overnight at constant voltage (25 V) and some were stained with Coomassie Blue R-250, followed by destaining in aqueous acetic acid (7%) and methanol (5%).

Proteins in analogous gels were subjected to electrophoretic transfer to nitrocellulose as described by Towbin *et al.* [25]. The concentrations of anti-*P*450 IgGs employed were: anti-*P*450 IIC11 (0.04 mg/ml), anti-*P*450 IIB1 (0.06 mg/ml) and anti-*P*450 IIIA (0.11 mg/ml).

Horseradish peroxidase-labelled donkey anti-rabbit IgG (Amersham) was used as the secondary antibody and the reagent H_2O_2/4-chloro-1-naphthol (Sigma) was used to detect peroxidase activity bound to the nitrocellulose filters.

RESULTS AND DISCUSSION

P450-Dependent steroid hydroxylation in hepatic microsomes from sheep and other species

Total microsomal *P*450 content in untreated male sheep liver was 0.81 ± 0.04 nmol/mg protein (mean ± SEM, $n = 3$) compared with 1.04 ± 0.11 and 1.24 ± 0.13 nmol/mg protein in male rat and guinea pig liver, respectively.

Activities of sheep hepatic microsomal *P*450 steroid hydroxylases were lower than in other species tested. The rate of total hydroxylation of TEST (Table 1) was 1.56 ± 0.42 nmol/min/mg protein ($n = 3$), which was similar to that observed in guinea pig liver (1.82 ± 0.12, $n = 3$). By comparison, total TEST hydroxylation proceeded 2.7-fold and 1.9 times more rapidly in

Table 1. Testosterone hydroxylation in microsomes from sheep, rat, guinea pig and human liver

Species	Hydroxytestosterone metabolite (nmol product/min/mg protein)							Total hydroxylated metabolites[a]
	2α	2β	6β	15β	7α	16α	15α	
Sheep	0.07 ± 0.01	0.19 ± 0.05	0.80 ± 0.27	0.22 ± 0.08	0.07 ± 0.01	0.05 ± 0.01	0.17 ± 0.02	1.56 ± 0.42
Rat	0.99 ± 0.07	0.09 ± 0.02	1.05 ± 0.14	0.02 ± 0.01	0.20 ± 0.04	1.49 ± 0.13	0.08 ± 0.01	4.16 ± 0.12
Guinea pig	0.12 ± 0.01	0.20 ± 0.01	0.57 ± 0.06	0.10 ± 0.03	0.25 ± 0.01	0.07 ± 0.01	0.04 ± 0.01	1.82 ± 0.12
Human	0.17 ± 0.06	0.10 ± 0.01	2.58 ± 0.19	0.09 ± 0.02	0.04 ± 0.02	ND[b]	ND	3.04 ± 0.36

Values are means ± SEM from determinations in 3 individual microsomal fractions.
[a] Determined as the sum of hydroxytestosterone metabolites formed.
[b] Not detected.

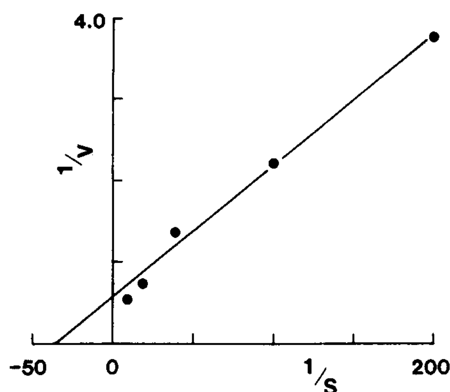

Fig. 1. Double reciprocal plot of TEST 6β-hydroxylase activity in male sheep hepatic microsomes. Units are S (substrate concentration), millimolar and V (reaction velocity), nmol/min/mg protein. Data were derived from a typical preparation of sheep liver.

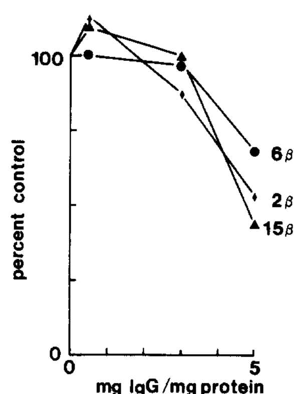

Fig. 2. Immunoinhibition of sheep hepatic microsomal TEST 2β (◆)-, 6β (●)- and 15β (▲)-hydroxylases by rabbit anti-rat anti-P450 IIIA IgG. Each point represents the mean of duplicate determinations that differed by <8%.

male rat liver and human liver, respectively, than in sheep liver. Although 8 identified metabolites were produced from TEST (AD and the 2α-, 2β-, 6β-, 15β-, 7α-, 16α- and 15α-alcohols), it was evident that the 6β-hydroxysteroid was the principal product ($K_m = 27\,\mu$M, $V_{max} = 1.8$ nmol/min/mg protein, Fig. 1; by comparison, in male rat liver, the K_m and V_{max} of the analogous reaction were 31 μM and 5.4 nmol/min/mg protein, respectively). This pathway constituted 40–56% of total hydroxylation which is a larger proportion than in guinea pig liver (29–34% of total) or male rat liver (20–33%) but was substantially smaller than in human liver (79–90% of total).

In the case of microsomal AD hydroxylation (Table 2), the principal metabolite formed was again the 6β-alcohol although, as expected, the 16α-alcohol was also produced extensively in rat liver. 6β-Hydroxylation was also the major route of PROG metabolism (Table 3) but it is noteworthy that the 21-hydroxylation of this substrate represented a substantial proportion (33%) of total hydroxylation in sheep liver microsomes. In rat liver progesterone 21-hydroxylation appears to be catalysed extensively by P450 IIC6 [10]. It is possible that sheep liver contains a protein that is similar to P450 IIC6 and that is also active in this reaction but further studies are required to assess this possibility.

Inhibition of hepatic microsomal TEST hydroxylases in sheep liver

IgG fractions, isolated from rabbits that had been immunized with different P450 antigens

Table 2. Androstenedione metabolism in microsomes from male sheep, rat, guinea pig and human liver

		Hydroxyandrostenedione metabolite (nmol product/min/mg protein)				Total
Species	Testosterone	16α	16β	6β	7α	hydroxylated metabolites[a]
Sheep	0.42 ± 0.02	0.09 ± 0.01	0.07 ± 0.01	0.89 ± 0.15	0.07 ± 0.01	1.13 ± 0.17
Rat	1.31 ± 0.17	2.01 ± 0.11	0.29 ± 0.01	1.18 ± 0.17	0.13 ± 0.02	3.61 ± 0.11
Guinea pig	5.16 ± 0.34	0.05 ± 0.01	0.27 ± 0.02	0.92 ± 0.08	0.18 ± 0.02	1.42 ± 0.08
Human	0.62 ± 0.09	0.05 ± 0.01	0.12 ± 0.02	1.66 ± 0.21	0.03 ± 0.01	1.85 ± 0.24

Values are means ± SEM from determinations in 3 individual microsomal fractions.
[a]Determined as the sum of total hydroxyandrostenedione metabolites formed (excludes testosterone).

Table 3. Progesterone hydroxylation in hepatic microsomes from male sheep, rat, guinea pig and human liver

	Hydroxyprogesterone metabolite (nmol product/min/mg protein)				Total
Species	2α	21	6β	16α	hydroxylated metabolites[a]
Sheep	0.03 ± 0.01	0.27 ± 0.03	0.43 ± 0.15	0.08 ± 0.01	0.82 ± 0.14
Rat	0.84 ± 0.03	0.62 ± 0.08	0.98 ± 0.06	1.01 ± 0.08	3.45 ± 0.13
Guinea pig	0.06 ± 0.01	0.03 ± 0.02	0.68 ± 0.07	0.06 ± 0.01	1.18 ± 0.09
Human	ND[b]	0.14 ± 0.01	1.24 ± 0.11	0.21 ± 0.01	1.59 ± 0.11

Values are means ± SEM from determinations in 3 individual microsomal fractions.
[a]Determined as the sums of hydroxyprogesterone metabolites found.
[b]Not detected.

Table 4. *In vitro* inhibition of testosterone hydroxylation in sheep liver microsomes by IgG fractions raised against rat $P450$s and by various chemicals

Addition	Formation of hydroxytestosterone metabolite (% of control)			
	2β	6β	15β	15α
Preimmune IgG (5 mg/mg)	100 (0.28)[a]	100 (0.91)	100 (0.40)	100 (0.19)
Anti-$P450$ IIC11 (5 mg/mg)	76[b]	95	60	60
Anti-$P450$ IIB1 (5 mg/mg)	104	106	75	76
Anti-$P450$ IIIA1 (5 mg/mg)	44	69	53	60
SKF 525-A				
10 μM	115	96	98	104
100 μM	60	46	50	52
Triacetyloleandomycin (100 μM)	55	40	50	68
Miconazole				
10 nM	51	26	31	50
100 nM	18	21	14	34
Thiabendazole (100 μM)	87	89	110	113

[a] Values in parentheses indicated actual rates of metabolite formation (nmol/min/mg protein).
[b] Replicates (duplicate or triplicate) that deviated from the stated mean values by <8%.

from male rat liver, were found to inhibit the positional hydroxylation of testosterone in sheep liver. Thus, anti-rat $P450$ IIIA1, when incorporated into sheep liver microsomes at a ratio of 5 mg IgG/mg protein, inhibited 5 pathways of TEST hydroxylation (the formation of the 2β-, 6β-, 15β- and 15α-alcohols) to 44–69% of control (Table 4 and Fig. 2). An important observation in the context of the significance of steroid 6β-hydroxylation is that $P450$s from the IIIA subfamily have been isolated from several species and all appear to be involved in microsomal steroid 6β-hydroxylation. It has been noted that the IgG raised against $P450$ IIIA proteins from one species often effectively inhibit 6β-hydroxylase activity in microsomes from other species [12, 13, 16]. A similar approach was taken in the present study where the anti-rat $P450$ IIIA1 IgG was found to inhibit TEST hydroxylation in sheep hepatic microsomes at the 2β-, 6β- and 15β-positions (normally produced by $P450$ IIIA subfamily members [28]) as well as at the 15α-position (produced in the adult male rat by $P450$ IIA2 [29]).

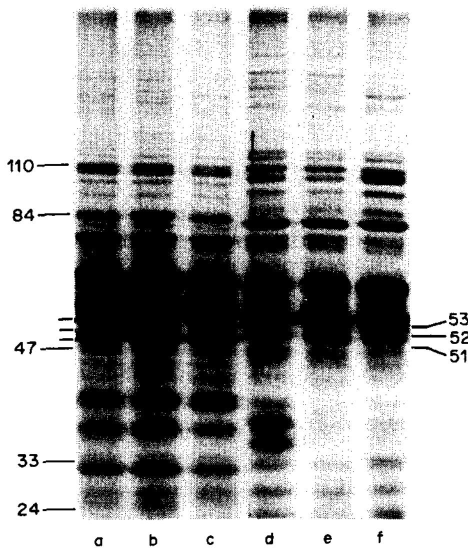

Fig. 3. SDS–PAGE of hepatic microsomes (20 μg/lane) from sheep (lanes a–c, from individual livers) and rats. Lanes d–f are hepatic microsomes from untreated, dexamethasone-induced and phenobarbital-induced rats, respectively. Molecular weights of standard marker proteins are indicated on the left of the figure and molecular weights of major proteins in the $P450$ region are on the right.

Anti-rat $P450$ IIC11 inhibited the microsomal formation of 2β-, 15β- and 15α-hydroxy-TEST to 76, 60 and 69% of respective control activities and anti-rat $P450$ IIB1 slightly decreased 15β- and 15α-hydroxylation (by about 25%).

In the light of the immunoinhibition findings that suggested that similarities may exist between $P450$s in sheep and rat liver, SDS–PAGE and Western immunoblot analyses were undertaken (Figs 3 and 4). Several proteins with molecular weights in the usual molecular weight range for $P450$s were observed on stained polyacrylamide gels and strong cross-reactivity was seen between anti-rat $P450$ IIIA IgG and sheep hepatic proteins (an immunoblot of $P450$ IIIA1 in dexamethasone-induced male rat liver microsomes is shown for comparison). The IgG directed against rat $P450$ IIC11 recognized at least one protein present in sheep liver although the molecular weight of the major protein appeared slightly lower ($\sim$1 kDa) than that of $P450$ IIC11 in control rat liver (Fig. 4). Finally, anti-rat $P450$ IIB1 exhibited only weak cross-reactivity with an antigen present in sheep hepatic microsomes compared with that observed in phenobarbital-induced rat microsomes. These findings suggest that sheep liver microsomes may contain low concentrations of proteins that may be similar to, or share a degree of sequence similarity with, rat hepatic $P450$s IIC11 and IIB1.

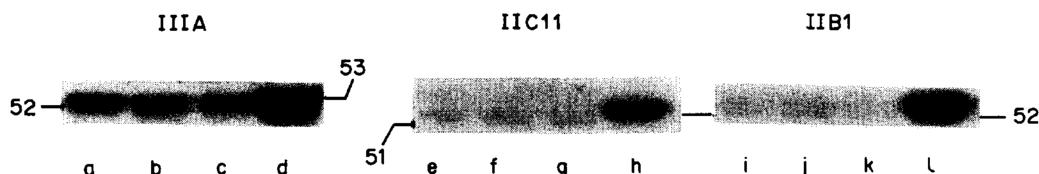

Fig. 4. Western immunoblots of anti-P450 IIIA (sheep in lanes a–c and dexamethasone-induced rat in lane d), anti-P450 IIC11 (sheep in lanes e–g and untreated rat in lane h) and anti-P450 IIB1 (sheep in lanes i–k and phenobarbital-induced rat in lane l) in hepatic microsomal fractions from sheep and rat.

Chemical inhibition studies were also undertaken in sheep hepatic microsomal fractions. The classical P450 inhibitor SKF 525-A (*N*,*N*-diethylaminoethyl 2,2-diphenylvalerate hydrochloride) inhibited TEST 2β-, 6β-, 15β- and 15α-hydroxylation quite effectively (40–54% inhibition observed at 100 μM SKF 525-A; Table 4). Triacetyloleandomycin (100 μM), which has been described as a preferential inhibitor of P450 IIIA-mediated activities [30], also inhibited these steroid hydroxylases effectively. The antimycotic agent, miconazole, was an extremely potent inhibitor of TEST hydroxylation *in vitro* (Table 4). Substantial inhibition (49–74%) was observed when miconazole was incorporated into microsomal incubations at a concentration of 10 nM and 100 nM drug elicited 66–86% inhibition of the various pathways. Miconazole also interacted with ferric P450 in sheep hepatic microsomes to produce a characteristic type II optical difference spectrum. Spectral dissociation constants (K_s values) of 0.3 and 0.8 μM and maximal binding interactions (ΔA_{max} values) of 0.063 and 0.058 absorbance units/nmol P450 were calculated from double reciprocal plots of the binding data derived using microsomes from individual sheep livers (data not shown). Thus, it is apparent that miconazole is a high affinity ligand for the heme iron of ferric P450. This property has been associated with potent inhibition of P450 action due to interference with oxygen activation and substrate binding [31]. It is evident from Table 4 that miconazole is a relatively uniform and potent inhibitor of steroid hydroxylase activities in sheep liver. This is consistent with previous findings in rat liver [32].

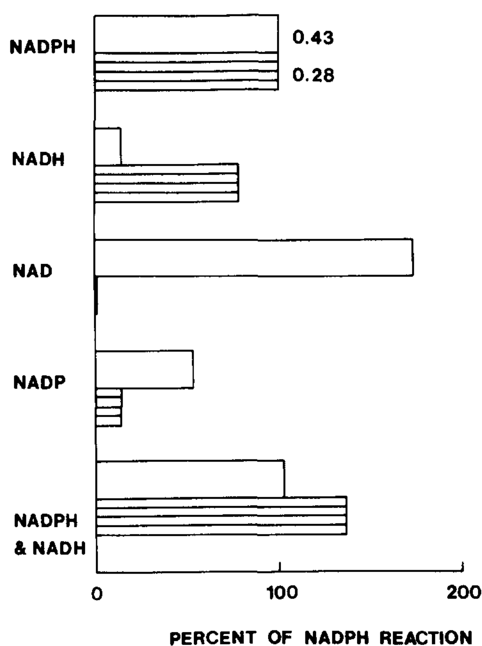

Fig. 5. Effects of pyridine nucleotides on sheep hepatic microsomal conversion of TEST to AD (□) and 5α-dihydroTEST (▤). Values are means of duplicate determinations that varied by <8%.

Non-P450-dependent pathways of TEST metabolism in sheep liver microsomes

Apart from P450-dependent hydroxylation, TEST also undergoes 17β-dehydrogenation to produce AD (catalysed by microsomal 17β-hydroxysteroid oxidoreductase [33]) and 5α-reduction of the double bond at C_4 to form 5α-dihydrotestosterone (catalysed by the microsomal 4-ene-3-ketosteroid 5α-oxidoreductase [34]). Studies were undertaken to measure the formation of these products from TEST in sheep microsomal fractions in the presence of different pyridine nucleotides. NADPH (1 mM) supported the conversion of TEST to AD and 5α-dihydroTEST at rates of 0.46 and 0.28 nmol/min/mg protein, respectively (Fig. 5). Since AD is also produced by P450-mediated biotransformation, the effect of NADH (1 mM) on microsomal TEST metabolism was assessed. As expected, NADH did not support steroid hydroxylation but AD and 5α-dihydroTEST were formed at 14 and 79% of the NADPH-catalysed rate. Thus, in sheep hepatic microsomes, reduced NAD supports the hydrogenation of TEST across the C_4—C_5 double bond but does not support the dehydrogenation of TEST to AD. NAD (1 mM), which functions as a cofactor for the microsomal 17β-hydroxysteroid oxidoreductase, actually

catalysed the conversion of TEST to AD at 173% of the NADPH-supported rate but 5α-dihydroTEST was not produced. In contrast, NADP (1 mM) sustained a somewhat lower rate of TEST 17β-dehydrogenation (53% of NADPH-reaction). Consequently, it is possible that the *P*450-dependent and *P*450-independent pathways in sheep liver catalyse AD formation from TEST at similar rates. The combination of NADPH and NADH (both 1 mM) was found to exert a synergistic effect on the activity of the 5α-reduction of TEST (to 137% of the NADPH-mediated pathway) but had little effect on AD formation (Fig. 4).

The reverse reaction (the NADPH-mediated reduction of AD to TEST) is also catalysed by the microsomal 17β-hydroxysteroid oxidoreductase [33]). In sheep liver this reaction proceeded at 0.42 ± 0.02 nmol/min/mg protein (Table 2) but in guinea pig, rat and human liver was 12, 3 and 1.5 times, respectively, greater than the sheep microsomal activity. Indeed, in guinea pig liver, 17β-reduction represented 78% of the total microsomal metabolism of AD, whereas this pathway constituted only 27% of overall AD metabolism in sheep microsomes. Thus, the present observations suggest that interesting species differences may exist in the hepatic concentration or turnover capacities of the microsomal 17β-hydroxysteroid oxidoreductase enzyme.

GENERAL DISCUSSION

There is increasing evidence that 6β-hydroxylation represents a significant pathway for the termination of steroid action in liver from many species. Other studies have reported that this pathway of steroid biotransformation is quantitatively important in hepatic microsomal fractions from rat, mouse, rabbit, dog, hamster, guinea pig and man [11–13, 35]. The present study establishes that extensive steroid 6β-hydroxylation also occurs in sheep liver and that it represents the dominant pathway of microsomal steroid hydroxylation.

A IIIA-type *P*450 was isolated recently from triacetyloleandomycin-induced sheep liver [16]. Cross-reactivity was observed between the rabbit IgG raised against the purified ovine *P*450 and an antigen present in rabbit hepatic microsomes. The *N*-terminal sequences of *P*450 IIIA members from man, sheep, rabbit and rat were noted in that study to be quite similar, with 10 of the first 20 amino acids from the *N*-terminal showing similarity between the sheep and rat enzymes. The report of Pineau *et al.* [16] quantified a *P*450 in untreated sheep hepatic microsomes that was immunoreactive with the antibody raised against the triacetyloleandomycin-inducible ovine *P*450 IIIA. The *P*450 IIIA protein in untreated sheep liver represented 0.21 nmol/mg protein (spectrally determined *P*450 was 0.37 nmol/mg protein) and triacetyloleandomycin treatment induced the hepatic content of this protein 2.5-fold [16]. It remains to be investigated whether the same protein is expressed in untreated and induced liver (like the rabbit) or whether the sheep has undergone a gene duplication in the IIIA subfamily (like the rat) [2]. the findings from Western immunoblotting experiments in the present study strongly suggest that a protein similar to rat *P*450 IIIA is present in untreated sheep liver. Certainly, the inhibition by anti-rat *P*450 IIIA IgG of steroid hydroxylations usually associated with *P*450 IIIA enzymes is consistent with this assertion.

The triacetyloleandomycin-inducible sheep *P*450 IIIA protein apparently catalyses the *N*-demethylation of agents such as erythromycin, chlorpromazine, chlorpheniramine and bromhexine but is not active in certain other drug oxidase pathways. The likely interpretation is that other *P*450s are present in sheep liver that may be active in such pathways. From the immunoblot experiments conducted in the present study it appears possible that a protein similar to the rat *P*450 IIC11 exists in untreated sheep liver. The anti-*P*450 IIC11 IgG employed in these experiments had been cross-adsorbed against isosafrole-induced female rat liver coupled to Sepharose 4B and does not recognize any antigens in female rat liver [23]. Thus, simple non-specificity of the polyclonal preparation may be largely excluded. Indeed, the finding that the IgG influenced the positional hydroxylations of TEST in sheep liver in a similar, but different, fashion to anti-rat *P*450 IIIA IgG is of interest in this regard. Whereas anti-IIIA IgG inhibited the metabolism of TEST to the 2β-, 6β-, 15β- and 15α-alcohols, the anti-IIC11 preparation inhibited 2β-, 15β- and 15α-hydroxylation slightly but, importantly, 6β-hydroxylation was uninhibited. However, a *P*450 in sheep liver that is functionally related to the male-specific rat *P*450 IIC11 is yet to be identified. An IgG raised against the major phenobarbital-inducible rat *P*450 IIB1 displayed only weak cross-reactivity with sheep

microsomal proteins on Western blots and had little effect on TEST positional hydroxylations.

Sheep are exposed to a wide variety of toxic lipophilic agents in the environment. Very few studies have investigated drug and steroid metabolizing enzymes in this species and there is almost no information relating to $P450$ multiplicity. The findings of this study as well as those of Pineau *et al.* [16] strongly suggest that a major $P450$ in sheep liver belongs to the IIIA subfamily of proteins. It now appears likely that this protein is involved in steroid 6β-hydroxylation, which is quantitatively significant in sheep liver. The importance of this enzyme in the oxidative metabolism of a range of therapeutic agents of veterinary importance has been established [16]. An earlier study reported that the activity of oxidative drug metabolizing enzymes was decreased in hepatic microsomes from sheep that had been infected with *Fasciola hepatica* [32]. If hepatic $P450$-mediated steroid deactivation is decreased similarly in sheep with fascioliasis then infection has the potential to produce perturbations in endocrine status. The extent to which the down-regulation of $P450$s involved in steroid disposition in infected sheep liver contributes to such a process remains to be clarified.

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