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ONTOGENIC DEVELOPMENT OF LIVER PROGESTERONE METABOLISM IN FEMALE SHEEP. CONTRIBUTION OF CYTOCHROME *P*4502B AND *P*4503A SUBFAMILIES

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Summary—Age-related changes in progesterone hepatic metabolism were measured in Lacaune ewes in the foetal, neonatal (1 and 4 weeks), growing (7 months), pregnant (11 months) and adult (6 years) stages. 6β-Hydroxylation and 20α-reduction were found to be the most efficient metabolic process in ovine microsomes. These activities were detected in 3-month-old foetuses and they increased rapidly during the first month of life, in a similar manner to the developmental expression of the cytochrome *P*4503A subfamily. 16α- and 21-hydroxylation of progesterone were characterized by low, constant turn over in sheep liver microsomes during development. The hepatic ovine *P*4502B isozyme was purified to electrophoretic homogeneity by means of successive DEAE cellulose, hydroxylapatite and CM cellulose chromatographic separations. This hemoprotein had an apparent molecular weight of 51 kDa and was characterized by spectral data, NH_2-terminal amino-acid sequence, immunological and catalytic properties. The relative contribution of this form and of the previously purified ovine *P*4503A subfamily was investigated in liver progesterone metabolism by immunoinhibition studies using polyclonal antibodies raised in rabbits and from the existence of induction and of significant correlations between microsomal activity and specific *P*450 content. In sheep liver microsomes, it would appear that cytochrome *P*4502B is involved in progesterone 21-hydroxylation whereas *P*4503A participates in the 6β- and 16α-hydroxylation and possibly in the reductive conversion of progesterone in its 20α-hydroxy derivative.

INTRODUCTION

Age-related changes in hepatic drug-metabolizing activity have previously been described in Lacaune ewes from the foetal to the adult stages [1]. Cytochrome *P*450 and certain *P*450-dependent monooxygenases increased regularly from 1-week- to 11-month-old animals whereas hepatic transferases including UDP glucuronyltransferase, glutathione *S*-transferase and *N*-acetyltransferase showed different developmental patterns in comparison to monooxygenases. Because of the lack of information concerning liver biotransformation of steroids in ruminant species, the objective of the present study was to measure the development of progesterone metabolism in female sheep with particular attention being paid to the involvement of microsomal cytochrome *P*450. These hemoproteins are the major catalytic components of the liver mixed-function oxidase system which catalyses the biotransformation of exogenous substrates such as drugs and chemicals and endogenous substrates including lipids and steroids. It is generally accepted that the broad substrate specificity of this system is due to the existence of several isozymes of cytochrome *P*450. They would be involved in hepatic steroid hydroxylation [2–4] which constitutes an important step in the termination of steroid action. In the rat, progesterone would be most efficiently metabolized by cytochromes *P*4502B1, 2C11 and 2C13 which principally hydroxylate at the positions 2α, 6β, 15α and 16α [5]. In the rabbit, progesterone has also been reported to be hydroxylated at the 6β and 16α positions by cytochrome *P*4502C3 [6].

Little information concerning cytochrome *P*450 associated monooxygenase activities is available in mammalian species other than in the rat, dog and man. In sheep, only one troleandomycin inducible liver cytochrome *P*450 from the *P*4503A gene subfamily was isolated [7]. Recently, however, the importance

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of steroid 6β-hydroxylation was measured in untreated male sheep liver and the involvement of a cytochrome *P*450 from the 3A subfamily in this pathway was proposed [8].

In the present study, liver microsomal fractions were prepared from foetal, neonatal (1- and 4-week-old), growing (7-month-old), pregnant (11-month-old) and adult (6-year-old) female sheep selected from the same flock. The development of progesterone metabolism was studied by measuring both the 6β-, 16α-, and 21-hydroxylations, and the reduction at the 20 position leading to 20α-hydroxyprogesterone, which has previously been shown to be a major circulating metabolite of progesterone in ewes [9]. This work also includes the purification and the characterization of a cytochrome *P*4502B isolated from sheep liver microsomes. In order to assess its contribution to progesterone metabolism and to compare it with that of the previously purified sheep liver *P*4503A, immunoinhibition studies were carried out by using specific polyclonal antibodies raised in the rabbit.

MATERIALS AND METHODS

Chemicals

Progesterone, 16α-, 17α-, 20α-, 20β-hydroxyprogesterone, deoxycorticosterone (21-hydroxyprogesterone), tergitol NP-10, sodium cholate, complete and incomplete Freund's adjuvant were purchased from Sigma Chimie (La Verpillière, France). 6β–Hydroxyprogesterone was acquired from Steraloids, Inc. (Wilton, U.S.A.). DE52 or CM52 celluloses and hydroxylapatite were respectively obtained from Whatman (Maidstone, England) and IBF Biotechnics (Villeneuve-La-Garenne, France). All other chemicals or biochemicals used were of the highest quality available and distilled deionized water was used in all studies.

Animals

Five groups of six Lacaune female sheep each were selected from the same flock (CASAM, Saint-Affrique, France). The animals had been reared parasite free from birth. These groups corresponded to lambs 1- (6.0 ± 0.7 kg) and 4- (11.9 ± 0.4 kg) week-old and to ewes 7- (40.7 ± 2.0 kg) and 11- (54.7 ± 1.9 kg) month-old and to adult ewes 6-year-old (61.3 ± 3.2 kg). The 11-month-old ewes were all pregnant and provided 6 foetuses approx. 3 months old (1.1 ± 0.1 kg). Except for the 1-week-old lambs, which received milk, all groups of animals were provided with hay, concentrates and water *ad libitum*. After an overnight fast animals were stunned and immediately exsanguinated.

Preparation of liver microsomes

The liver was removed, freed of extrahepatic tissue, weighed, washed with an ice-cold saline solution, and blotted free of excess moisture. All subsequent operations were carried out at 0–4°C. Samples (8 g) were homogenized in 24 ml of ice-cold 0.1 M potassium phosphate buffer (pH 7.4; 0.1 M tris-acetate; 0.1 M KCl, 1 mM EDTA; 0.02 mM butyled hydroxytoluene) in a glass–Teflon Potter homogenizer. The homogenate was centrifuged at 10,000 ***g*** for 30 min. The supernatant fraction was centrifuged for 1 h at 105,000 ***g***, suspended in 0.1 M pyrophosphate HCl buffer (pH 7.5) and re-centrifuged for 30 min in order to obtain a cleaner microsomal fraction. The microsomal pellets were suspended in a solution consisting of 0.1 M EDTA, 20% glycerol potassium phosphate buffer (0.1 M, pH 7.4) and stored at −80°C until required. Protein was estimated according to Lowry *et al.* [10], cytochrome *P*450 by the method of Omura and Sato [11] and specific contents in *P*4502B and *P*4503A by immunoblotting revealed with specific IgG fractions prepared from rabbit antisera.

Measurement of microsomal progesterone metabolism

Typically, incubations with hepatic microsomes contained cytochrome *P*450 (0.6 nmol), NADPH (1 μmol), potassium phosphate buffer (0.1 M, pH 7.4), and progesterone (320 nmol in 10 μl of ethanol) in a total volume of 1 ml and were agitated for 10 min at 37°C following addition of substrate. This reaction showed a linear dependence on protein and substrate concentration. Progesterone and its metabolites were extracted quantitatively by 10 ml of methylene chloride. Following evaporation of solvent under nitrogen, the sample residues were dissolved in the elution solvant and analysed by HPLC. Whilst conventional HPLC methods use gradient elution and reversed phase chromatography [12, 13], a sensitive and highly specific HPLC assay procedure was developed by using normal phase chromatography. The separation of progesterone and its hydroxymetabolites was achieved by using a normal phase Partisil 5 μm packing column (Whatman

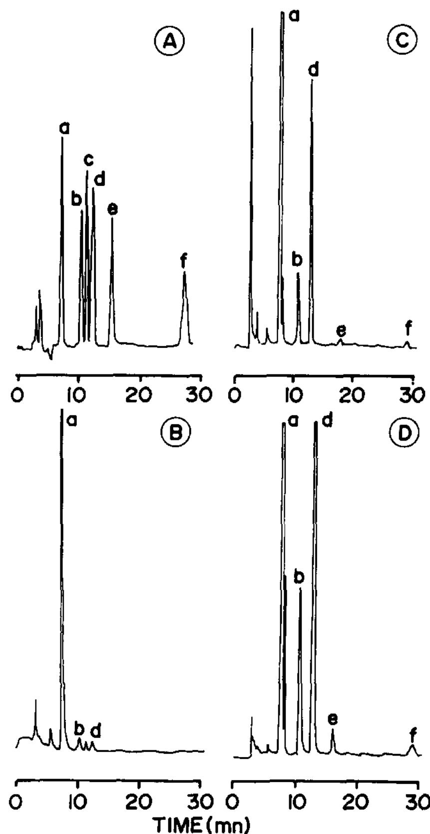

Fig. 1. Chromatographic profiles of progesterone metabolism. Resolution of reference progesterone and its hydroxylated metabolites (A) by HPLC on a normal phase Partisil column in hexane–ethanol (500:30, v/v). Chromatograms of progesterone metabolites formed by liver microsomes from foetus (B), 4-week-old (C) and troleandomycin-treated (D) female sheep. a, progesterone; b, 20α-hydroxyprogesterone; c, 17α-hydroxyprogesterone; d, 6β-hydroxyprogesterone; e, 21-hydroxyprogesterone; and f, 16α-hydroxyprogesterone.

S.A.). The chromatographic system consisted of a M45 Waters pump and a Kontron 360 automatic injector. The flow rate of the mobile phase (hexane–ethanol, 500:30, v/r) was maintained at 1 ml/min and the eluent was monitored at 254 nm using a model M440 Waters UV detector and a D2500 Merck integrator calculator. Retention times of the various compounds were determined by injecting a methanolic solution (10 μl) containing 100 μg of each authentic standard. The retention times were 7.7, 11.4, 12.4, 13.5, 17.0 and 29.5 min for progesterone, 20α-hydroxyprogesterone, 17α-hydroxyprogesterone, 6β-hydroxyprogesterone, 21-hydroxyprogesterone and 16α-hydroxyprogesterone. The recovery of progesterone and its metabolites was around 80%. Figure 1 shows typical chromatograms obtained with analytical standards and after incubation of progesterone with sheep liver microsomes. In all cases, the HPLC procedure permits an efficient separation of *P*450 from its major metabolites during a one-step chromatography assay of 30 min.

Purification of sheep liver P4502B

Three female adult Lacaune sheep weighing 49 ± 9 kg were used. The animals received daily an oral administration of 60 mg/kg body weight of sodium phenobarbital as aqueous solution for six consecutive days. Sheep were killed 24 h after the last administration. Ovine liver microsomes were prepared as described above.

The purification was carried out at 4°C. Microsomal protein was resuspended in buffer A (0.01 M tris acetate pH 7.4, 1 mM EDTA, 0.1 mM dithiothreitol, 20% glycerol) to a final concentration of 15 mg/ml, solubilized with 0.5 and 0.2%, respectively of (cholamidopropyl) dimethylammoniopropane sulphonate (CHAPS) and tergitol NP 10. Aprotinin was added (0.25 mg %) and the solubilization was conducted by gentle stirring for 90 min. Insoluble material was removed by ultracentrifugation at 105,000 *g* for 60 min. The clear supernatant was precipitated with polyethylene glycol at successive concentrations of 0–6, 6–8, 8–10 and 10–13%. The 6–8% precipitate was centrifuged at 10,000 *g* for 10 min and the pellet was resuspended in buffer A, resolubilized as described previously, and finally dialysed overnight against buffer B (5 mM potassium phosphate pH 6.5, 0.01 mM EDTA, 0.01 mM dithiothreitol, 0.2% tergitol, 0.01% NaN_3, 25% glycerol). This solution was applied to the first column (3 × 30 cm) of DEAE-cellulose (DE52) previously equilibrated in buffer B. After a wash with buffer B (1 ml/mg protein), fractions containing isozyme *P*4502B were eluted from a linear gradient of 0.100 mM K_2SO_4, as confirmed by SDS–polyacrylamide gel electrophoresis and Western blotting analysis. This fraction was dialysed overnight against buffer C (5 mM potassium phosphate pH 6.8, 0.01 mM EDTA, 0.01 mM dithiothreitol, 0.2% tergitol, 0.01% NaN_3, 25% glycerol). This solution was then applied to a column of hydroxylapatite (1.6 × 10 cm) previously equilibrated against the same buffer. Several fractions were eluted with buffer C containing 10 to 200 mM phosphate. The isozyme *P*4502B was eluted in the 70 mM fraction. This eluate was dialysed overnight against buffer B and then chromatographed in a CM cellulose (CM52) column (2.2 × 20 cm) previously equilibrated with buffer B. After a wash with buffer B, fractions containing

$P4502B$ were eluted at around 30 mM from a linear gradient 10–100 mM phosphate. Elimination of tergitol and concentration of the fraction were simultaneously performed on a column of hydroxylapatite (1.6 × 10 cm). This column had previously been equilibrated against buffer D (10 mM potassium phosphate pH 6.8, 0.01 mM EDTA, 25% glycerol). After $P450$ had been applied, the column was washed extensively with buffer D. This step allowed the elimination of tergitol whereas the purified hemoprotein was eluted as a sharp peak with buffer D containing 400 mM phosphate, 0.1 mM EDTA and 0.2% sodium cholate. This final fraction was dialysed overnight against buffer D containing 0.05 mM EDTA to eliminate cholate and stored at −75°C.

Characterization of P4502B

Throughout the purification procedure, polyacrylamide gel electrophoresis was carried out at room temperature in the presence of SDS according to the procedure of Kerckaert [14]. The slab gel (14 × 11 × 0.15 cm) contained 9% acrylamide with 0.2% bis-acrylamide as the linking reagent and 0.1% SDS. After incubation of microsomes, partly purified fractions or proteins with SDS mercaptoethanol (5 min at 100°C), the samples were applied and electrophoresis was performed for 3 h at 60 mA (200 V). The gel was fixed, stained with Coomassie Blue and destained with a mixture of water–acetic acid–methanol (80:10:10, by vol).

The absolute and difference spectra of $P450$ in fractions and purified preparation were recorded, sodium dithionite was used as reducing agent. Cytochrome $P450$ concentrations were determined with a Uvikon 860 Kontron spectrophotometer using an extinction coefficient of $91\ mM^{-1}\ cm^{-1}$.

The NH_2-terminal sequence of the purified $P4502B$ was determined by Edman degradation on an Applied Biosystem model 470A gas-phase sequencer coupled with a model 120A phenylthiohydantoin analyser.

Measurement of N-demethylase activities of the purified enzyme were carried out in 100 mM potassium phosphate buffer (pH 7.4) at 37°C in a reconstituted system [15]. Cytochrome $P4502B$ isozyme (0.1 μM) was incubated for 5 min at room temperature with 30 μg/ml of sonicated lauroylphosphatidylcholine and a 3-fold molar excess of cytochrome $P450$ reductase previously isolated [7]. This mixture was diluted to a final volume of 0.05 ml with buffer and substrate (1 mM) and the reaction was initiated by the addition of NADPH to a final concentration of 0.5 mM. Incubations were carried out for 10 min. Demethylation of the substrates (benzphetamine, aminopyrine, erythromycin) was determined by the method of Nash [16, 17].

Immunochemical studies

Antibodies against sheep liver cytochrome $P4502B$ were raised in female rabbits by three multisite subcutaneous injections (1 ml) of 1 nmol of protein in 50% complete Freund adjuvant at 1 month intervals. Each week after the last injection, 20 ml of blood was drawn from a marginal ear vein and serum collected. IgG enriched fractions of sera were prepared by ammonium sulphate precipitation. In order to assess the rise of $P450$ antibodies in rabbit sera, classical Ouchterlony double diffusion analysis was carried out.

In order to determine the contribution of $P4502B$ and of previously isolated sheep liver $P4503A$ to progesterone metabolism, the two IgG fractions were compared with pre-immune fractions for their ability to inhibit the monooxygenase activities catalysed by liver microsomes from phenobarbital- or troleandomycin-treated sheep. Furthermore liver microsomes prepared from 24 sheep including control and treated animals were assayed for 6β- and 16α-hydroxylation of progesterone and for specific content of $P4502B$ and $P4503A$ as determined by immunoblotting. Each gel contained lanes with 1 to 10 pmol of purified sheep $P450$. The nitrocellulose sheets were treated with 1/100 dilution of rabbit anti-$P450$ and revealed by peroxidase–IgG conjugate. The amounts of $P450$ were estimated using the ratio of peaks obtained after densitometry.

Statistics

Data were analysed by analysis of variance including a complementary range test used to compare the means. Correlations between $P450$ contents and progesterone hydroxylation activity were assayed. In all cases, a probability of $P < 0.05$ was considered significant.

RESULTS

Development of sheep liver progesterone metabolism

Changes in liver cytochrome $P450$ and progesterone metabolism in female sheep with age are shown in Table 1. If total cytochrome

Table 1. Incidence of age on hepatic microsomal cytochrome $P450$ and progesterone metabolism in female sheep

Parameters	Age					
	Foetus	1 week	4 weeks	7 months	11 months	6 years
Cytochrome $P450$ (nmol/mg protein)						
Total $P450$	ND[a]	0.40 ± 0.07[b]	0.66 ± 0.05[b]	0.91 ± 0.03[b]	0.99 ± 0.05	1.10 ± 0.12
$P4502B$	ND	0.03 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.07 ± 0.03	0.05 ± 0.01
$P4503A$	0.06 ± 0.02[b]	0.33 ± 0.05[b]	0.32 ± 0.02	0.18 ± 0.04	0.23 ± 0.06	0.20 ± 0.03
Hydroxyprogesterone metabolite (nmol/min/mg)						
6β	0.04 ± 0.01[a]	0.77 ± 0.42	1.32 ± 0.29	1.07 ± 0.23	1.50 ± 0.28	1.13 ± 0.48
16α	ND	0.03 ± 0.02	0.06 ± 0.03[b]	0.04 ± 0.01	0.04 ± 0.02	0.02 ± 0.01
20α	0.03 ± 0.02[b]	0.15 ± 0.12[b]	0.57 ± 0.12	0.51 ± 0.13	0.62 ± 0.20	0.51 ± 0.25
21	ND	0.03 ± 0.01	0.05 ± 0.03	0.04 ± 0.02	0.04 ± 0.02	0.04 ± 0.02

Values are means ± SE from determinations in 6 individual microsomal fractions.
[a]Not detected or below 0.01 nmol/mg or 0.01 nmol/min/mg.
[b]Significantly different ($P < 0.05$) from the adult corresponding value (6 years of age).

$P450$ was not detected spectrophotometrically in foetal microsomes, this enzyme activity doubled during the first 7 months of life and remained constant thereafter. Cytochrome $P4502B$ was only present in foetal liver at a concentration below 0.01 nmol/mg of microsomal proteins. After birth, there were only low levels of $P4502B$, ranging from 0.03–0.07 nmol/mg. By contrast, isozyme $P4503A$ was detectable in sheep foetal liver and represented, respectively 82 and 48% of the total $P450$ in 1- and 4-week-old lambs. This percentage decreased in older animals in which it represented 18–23%.

Concerning hydroxyprogesterone metabolite formation 17α- and 20β–hydroxylation were generally below the detection limit of the analytical method. 6β-Hydroxylation was the major route of progesterone metabolism and represented 66% of the total biotransformation found in adult ewes. This activity was present in foetuses, increased rapidly during the first month of life and remained constant from 4 weeks to the adult stage (1.07–1.50 nmol/min/mg). 16α- and 21-Hydroxylation of progesterone appeared as low metabolic pathways in ovine microsomes (1.1 and 2.3% of the total conversion, respectively). During animal growth,

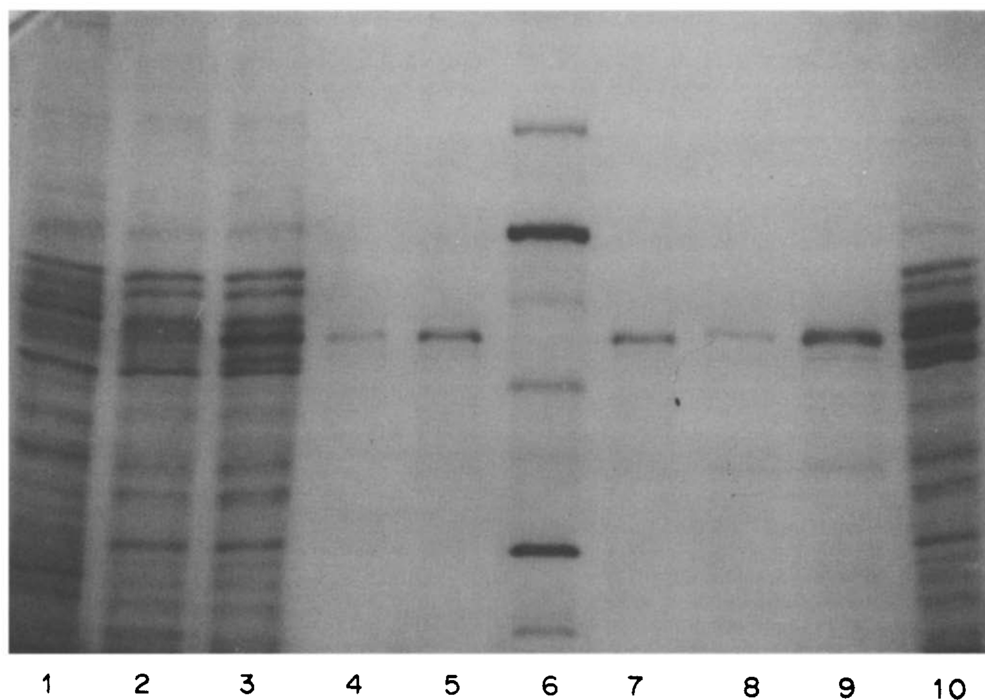

Fig. 2. SDS–polyacrylamide gel electrophoresis of microsomes from control rabbit (1), control sheep (2), phenobarbital-treated sheep (3, 10), of 5 and 10 pmol of purified ovine $P4502B$ (4, 5 and 7), and 5 pmol of purified ovine $P4503A$ (8) and of a mixture of 5 pmol of each sheep $P4502B$ and $P4503A$ (9). Lane 6 contained molecular weight standards (phosphorylase B, 92,500; bovine albumin, 67,000; egg albumin, 45,000; and carbonic anhydrase, 25,000) from Serva.

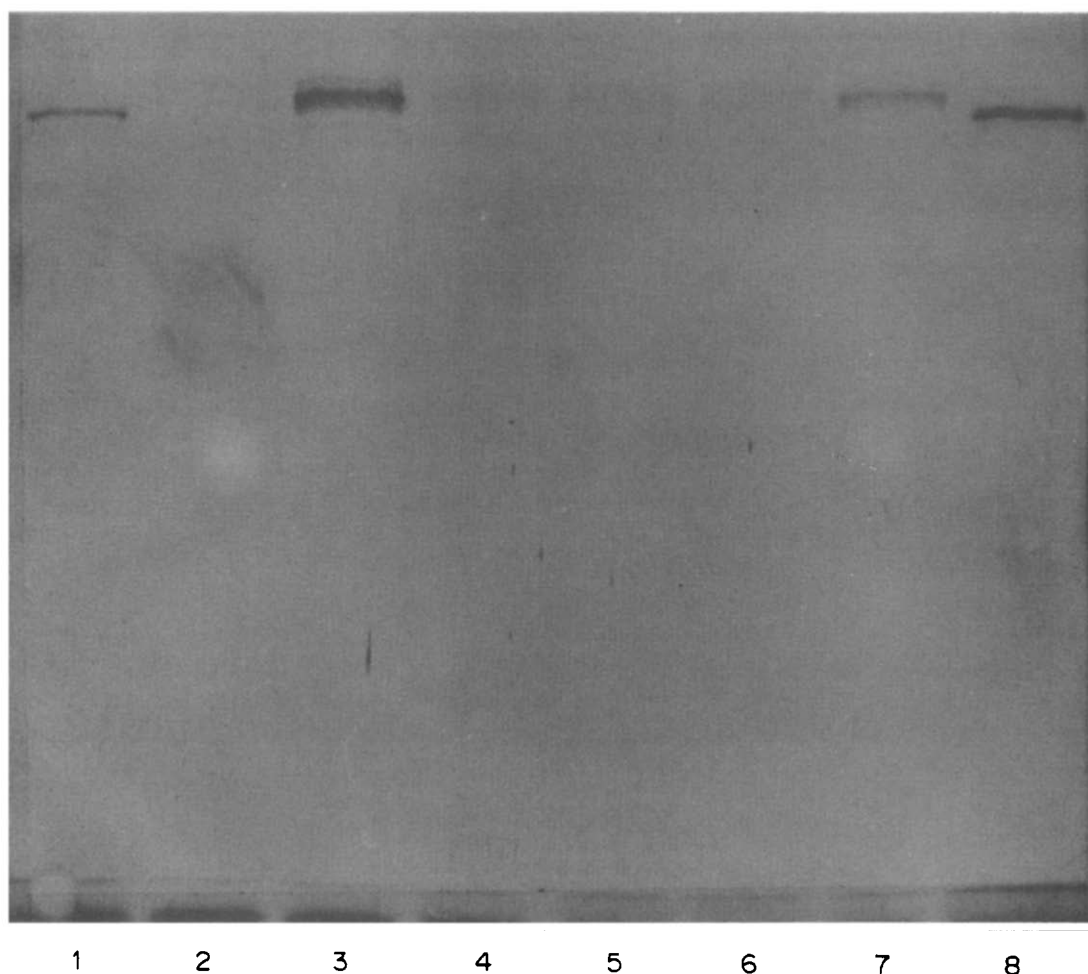

Fig. 3. Immunoblotting analysis of run gels of liver microsomes from control (2), phenobarbital- (3), rifampicin- (4), troleandomycin- (5), β-napthoflavone- (6) and isosafrole- (7) treated sheep. Microsomal proteins (5 μg) were assayed for cross reactivity towards antirabbit *P*4502B4 antibodies. Lanes 1 and 8 correspond to 0.5 and 1.0 pmol of purified rabbit *P*4502B4, respectively.

these activities were quite constant except for a significantly increased rate of progesterone 16α-hydroxylation in 4-week-old female lambs. The reductive conversion of progesterone to 20α-hydroxyprogesterone represented a substantial liver microsomal activity (30% of the total metabolization) which was measurable in foetal liver and increased rapidly during the first month of life to reach the adult level (0.51–0.62 nmol/min/mg).

Purification and characterization of sheep liver P4502B

Phenobarbital administration in ewes provoked a significant increase in liver microsomal cytochrome *P*450 from 0.82 ± 0.16 in controls to 2.80 ± 0.75 nmol/mg in treated animals. The comparative SDS–polyacrylamide gel electrophoretic analysis of liver microsomes (Fig. 2) revealed a strong band occurring in the zone located below the band of *P*4503A isozyme. On the basis of both its electrophoretic mobility and of its cross immunoreactivity with antibodies against rabbit cytochrome *P*4502B4 as demonstrated by Western blotting among microsomes from sheep receiving various treatments (Fig. 3), this band was tentatively

Table 2. Purification procedure for the preparation of cytochrome *P*4502B from phenobarbital-treated sheep liver microsomes

Fraction	Total (nmol)	Cytochrome *P*450 specific content (nmol/mg protein)	Yield (%)
(PB) microsomes	4569	2.50	100
PEG precipitate (6–8%)	1825	3.72	39.9
DE cellulose			
Fraction 0.05 M K_2SO_4	276	3.80	6.0
Hydroxylapatite (first)			
Fraction 0.07 M phosphate	132	4.86	2.9
CM cellulose			
Fraction 0.03 M phosphate	32	6.19	0.7
Hydroxylapatite (second)			
Fraction 0.40 M phosphate	10.4	13.23	0.3

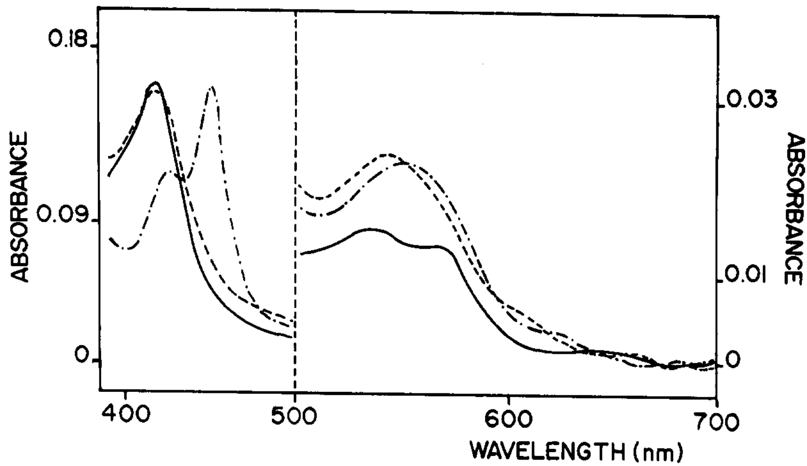

Fig. 4. Absolute spectra of sheep cytochrome *P*4502B isozyme. Electrophoretically homogenous isozyme (0.74 nmol) was diluted in 0.1 M phosphate buffer pH 7.4, volume 0.5 ml. The absolute spectrum of the oxidized form was recorded (—). Reduced spectrum (– –) was recorded after a small amount of sodium dithionite was added. Finally, carbon monoxide was bubbled into the cuvette and the absolute spectrum of the ferrous carbonyl complex obtained (– · –).

assigned to the sheep *P*4502B form [18] induced by phenobarbital.

The sheep liver *P*4502B isozyme was purified to 13.2 nmol/mg of protein (Table 2) following the successive chromatographic steps described above. The electrophoretic mobility was compared with that of the already isolated sheep liver *P*4503A subfamily [7]. A plot of mobility against standard proteins indicated a molecular weight around 51 kDa for sheep liver cytochrome *P*4502B.

Absolute spectra of ovine liver *P*4502B (Fig. 4) indicated that the oxidized cytochrome was essentially low spin with a soret maximum at 416 nm and α and β bands at 534 and 570 nm, respectively. In the ferrous state, the spectrum presented a maximum at 412 and at 543 nm. The ferrous carbonyl complex had maxima at 450 and 552 nm.

The comparison of the 20 NH_2-terminal amino acid sequence of sheep protein was compared (Fig. 5) with previously analysed rabbit [19], rat [20], mouse [21] and human [22] forms from the *P*4502B subfamily. This led to the observation of strong homologies in positions 1–8, 10–12 and 14–20. By contrast, there were only 6 amino acids common to the sheep liver *P*4502B and *P*4503A subfamilies.

N-demethylation of three substrates in reconstituted systems indicated a higher turnover rate in the case of benzphetamine (19.2 nmol/min/nmol *P*4502B) by comparison with aminopyrine or erythromycin (6.9 or 10.9 nmol/min/nmol, respectively).

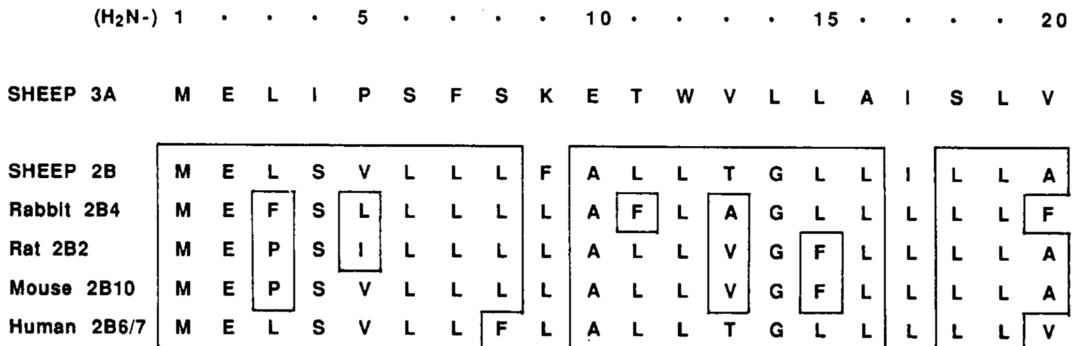

Fig. 5. Comparative *N*-terminal sequence of the previously purified sheep liver *P*4503A and of *P*4502B isoenzymes from different animal species. Data from rabbit, rat, mouse and human forms are from Heinemann and Ozols [19], Fujii-Kuriyama *et al.* [20], Noshiro *et al.* [21] and Yamano *et al.* [22]. Homologies in the ovine cytochrome *P*4502B sequence with other animal and human *P*4502B subfamilies are boxed.

Table 3. *In vitro* inhibition of progesterone metabolism by antisheep *P*4502B and *P*4503A in liver microsomes from phenobarbital- and troleandomycin-treated sheep

Addition	Formation of hydroxyprogesterone derivative (% control)			
	20α	6β	16α	21
Phenobarbital-treated liver microsomes				
Preimmune IgG	100 (0.88)[a]	100 (2.54)	100 (0.10)	100 (0.20)
Anti*P*4502B (1 mg/mg)	99 ± 4[b]	97 ± 6	101 ± 16	97 ± 9
Anti*P*4502B (2 mg/mg)	103 ± 5	99 ± 2	92 ± 20	96 ± 8
Anti*P*4502B (5 mg/mg)	87 ± 10	91 ± 6	89 ± 15	72 ± 7[c]
Anti*P*4502B (10 mg/mg)	95 ± 15	103 ± 9	87 ± 12	58 ± 19[c]
Troleandomycin-treated liver microsomes				
Preimmune IgG	100 (2.57)	100 (6.72)	100 (0.16)	100 (0.18)
Anti*P*4503A (1 mg/mg)	86 ± 19	97 ± 3	95 ± 10	87 ± 27
Anti*P*4503A (2 mg/mg)	102 ± 11	97 ± 8	115 ± 13	105 ± 16
Anti*P*4503A (5 mg/mg)	82 ± 5[c]	80 ± 11[c]	76 ± 4[c]	86 ± 20
Anti*P*4503A (10 mg/mg)	62 ± 4[c]	49 ± 12[c]	55 ± 16[c]	86 ± 14

[a]Values in parentheses indicated actual rates of metabolite formation (nmol/min/mg protein).
[b]Triplicates were obtained from three different animals.
[c]Rates were significantly different from corresponding preimmune values ($P < 0.05$).

Contribution of P4502B and P4503A to progesterone metabolism

Antibodies to sheep liver *P*4502B prepared in female rabbits gave a single band when reacting against the purified protein, as observed by means of an Ouchterlony double diffusion experiment. They also gave a single band on Western blots of sheep liver microsomes. These antibodies and the IgG fraction prepared from rabbit antisera against ovine *P*4503A [7] were examined for their ability to inhibit the formation of hydroxyprogesterone derivatives in microsomes from phenobarbital- or troleandomycin-treated sheep (Table 3). When compared with rabbit preimmune fraction, no immunoinhibition was observed with anti-*P*4502B except for 21-hydroxyprogesterone which was inhibited by 28–42% in phenobarbital-treated microsomes. By contrast, significant inhibition (18–51%) occurred with 6β-, 16α- and 20α-hydroxylation of progesterone by anti-*P*4503A in troleandomycin-treated microsomes, without any effect on 21-hydroxylation of the steroid.

The levels of *P*4502B and *P*4503A determined by Western blotting were plotted against the four hydroxylations of progesterone investigated in 24 different sheep liver microsomal preparations. Correlations were significant ($r = 0.56$ and 0.50) only when sheep *P*4503A was assayed against the formation of 6β- and 16α-hydroxyprogesterone derivatives (Fig. 6).

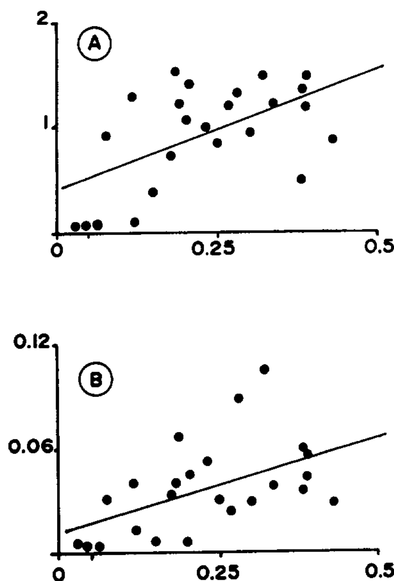

Fig. 6. Correlation of 6β- (A) and 16α- (B) progesterone hydroxylase activities with immunochemically determined *P*4503A level in liver microsomes from 24 control or troleandomycin or phenobarbital-treated sheep. The lines through the points were drawn using linear regression analysis. All correlation are significant ($P < 0.05$).

DISCUSSION

In this work, we have purified and characterized a sheep liver microsomal cytochrome *P*450 which belongs to the *P*4502B subfamily. This identification is based on its inducibility by phenobarbital, its electrophoretic motility around 51 kDa, its low spin spectral property, its strong homology of amino-acid sequence with other *P*4502B isoenzymes from other animal species including man, and its cross reactivity with antibodies raised against orthologous rabbit *P*4502B4. Moreover, this hepatic *P*450 isoenzyme corresponds to the sheep lung cytochrome which has been recently purified by Adali and Arinç [23] and sequenced by Williams *et al.* [24]. These authors described a similar *N*-terminal amino-acid sequence for this pulmonary hemoprotein and a high turnover of benzphetamine metabolism. Such similarities between corresponding liver and lung cytochrome *P*450 isoforms have previously been

reported in the case of closely related $P4502B4$ in the rabbit [25].

Among all investigated biotransformation pathways, 6β-hydroxylation appears as the major route of progesterone metabolism in sheep liver microsomes; this is in accordance with Murray [8] who measured an activity only half as great in male adult sheep liver. High activities were observed in young animals as already described in mice at postnatal age [26]. This early development could be related to the expression of cytochrome $P4503A$ which appears as a major constitutive form in sheep liver during the first month of life. The involvement of sheep liver cytochrome $P4503A$ in progesterone 6β-hydroxylation is revealed by the significant correlation between these parameters and by both the inhibition of this hydroxylation by antisheep $P4503A$ and its strong inducibility by troleandomycin (6.72 vs 1.13 nmol/min/mg in untreated controls), a specific inducer of the $P4503A$ subfamily [27]. These results emphasize the participation of cytochrome $P4503A$ in the 6β-hydroxylation of steroid as has been demonstrated in human liver microsomes [28] and proposed in sheep liver microsomes, by using chemical inhibitions and heterologous antibodies directed against rat $P450$ proteins [8].

16α-Hydroxylation appears as a low capacity metabolic pathway of progesterone. This finding is confirmed by Murray [8] who found only a turnover of 0.08 nmol/min/mg in male sheep liver whereas significantly higher values were obtained in human or rat liver microsomes (0.21 and 1.01 nmol/min/mg, respectively). During ontogenic development, this hydroxylation was at a maximum in 4-week-old animals. This pattern of evolution paralleled that of progesterone 6β-hydroxylation while there were positive correlations between $P4503A$ and 16α-hydroxylation and immunoinhibition by antisheep $P4503A$. For these reasons, the cytochrome $P4503A$ subfamily could also be involved in sheep liver 16α-hydroxylation of progesterone. Another argument for the participation of this isoenzyme is provided by the inducibility of progesterone 16α-hydroxylation by troleandomycin. A similar result was obtained by Schwab *et al.* [29] who described the simultaneous inducibility of $P4503A6$ and progesterone 16α- and 6β-hydroxylation in the liver of rabbits receiving rifampincin, another inducer of the $P4503A$ subfamily.

21-Hydroxylation of progesterone is fairly constant but of low hepatic activity in female sheep. Both the immunoinhibition study and the 5-fold increase of this activity in phenobarbital-treated sheep would indicate a possible contribution of cytochrome $P4502B$ subfamily to this activity in ovine liver. In the rat, the multiplicity of $P450$ isoenzymes involved in regiospecific 21-hydroxylation has been demonstrated, even if cytochrome $P4502C5$ is generally recognized as the isozyme most involved [30].

The reductive conversion of progesterone to 20-hydroxyprogesterone is characterized by a high turnover in sheep liver. This could be related to the importance of this derivative in the plasma of ewes receiving progesterone administration as already reported [9]. The ontogenic development of this reduction takes place during the first month of life and parallels those of 6β- and 16α-hydroxylation of progesterone. Both the immunoinhibition of 20α-hydroxylation by antisheep $P4503A$ IgG and the strong inducibility of this activity in troleandomycin-treated sheep (2.57 vs 0.51 nmol/min/mg in adult controls) lead us to propose a possible contribution of the $P4503A$ subfamily to this reductive conversion of progesterone in sheep.

In conclusion, progesterone metabolism is characterized by early development in the female sheep liver. This characteristic could be due to the postnatal expression of the cytochrome $P4503A$ subfamily which has previously been reported in rabbit liver [31]. In sheep liver, this isoenzyme would be involved in 6β-, 16α- and possibly 20α-hydroxylation of progesterone. By contrast, cytochrome $P4502B$ is a low expressed isozyme in ovine hepatic tissues while it has been described as a major pulmonary $P450$ in the same animal species [24]. Its participation would only be effective in 21-hydroxylation, a minor metabolic pathway of progesterone by comparison with the 6β-hydroxylation or the 20α-reduction of the steroid.

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