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A study of the expression of the xenobiotic-metabolising cytochrome P450 proteins and of testosterone metabolism in bovine liver

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Abstract

The expression of xenobiotic-metabolising cytochrome P450 proteins in the liver of cattle was determined using substrate probes and immunologically by Western blot analysis. Compared to the rat, cattle displayed much higher coumarin 7-hydroxylase (CYP2A) and ethoxyresorufin *O*-deethylase (CYP1) activity but, in contrast, it exhibited much lower debrisoquine 4-hydroxylase (CYP2D) and lauric acid hydroxylase activities (CYP4A). The ethoxyresorufin *O*-deethylase activity was markedly inhibited by furafylline and α-naphthoflavone, and coumarin 7-hydroxylase by 8-methoxypsoralen. Immunoblot analysis employing antibodies to rat CYP1A1 recognised two immunorelated proteins in bovine liver whose expression appeared to be higher compared with rat. Kinetic studies indicated that a single enzyme is likely to be responsible for the *O*-deethylation of 7-ethoxyresorufin in bovine liver. When bovine microsomes were probed with antibodies to rat CYP2A2, a single protein was detected in cattle liver. Kinetic analysis followed by construction of Eadie–Hofstee plots indicated that more than one enzyme contributes to the 7-hydroxylation of coumarin. Immunoblot analysis employing antibodies to human CYP2D6 and rat CYP4A1 revealed in both cases a single, poorly expressed immunoreacting band in bovine microsomes. Similar immunoblot studies detected proteins in cattle liver immunorelated to the CYP2B, CYP2C, CYP2E, and CYP3A subfamilies. Bovine microsomes metabolised testosterone but, in contrast to the rat, failed to produce 2α- and 16α-hydroxytestosterone. On the other hand, bovine microsomes produced levels of another hydroxylated metabolite, possibly 12-hydroxytestosterone. In conclusion, results emanating from this study indicate the presence of proteins in the cattle liver belonging to all the xenobiotic-metabolising families of cytochrome P450. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Cytochrome P450; Cattle; Coumarin 7-hydroxylase; Ethoxyresorufin *O*-deethylase; Testosterone

1. Introduction

Cytochromes P450 are undoubtedly one of the most important enzyme systems in the metabolism of xenobiotics by virtue of their ability to catalyse the biotransformation of structurally diverse chemicals, this characteristic being a consequence of their existence as a superfamily of proteins, each having its own substrate specificity [1]. Although cytochromes P450 was initially considered to be a deactivation system, enabling the excretion of lipophilic compounds, it is now clear that it can also carry out the reverse, i.e. convert innocuous chemicals to reactive electrophiles. Such products can interact covalently with proteins to give rise to cytotoxicity [2] or function as haptens generating neoantigens leading to immunotoxicity [3], or with DNA to cause genotoxicity leading to mutations and malignancy [4].

The cytochrome P450 proteins responsible for the metabolism of xenobiotics have been extensively studied in laboratory animals such as the rat, and subsequently in humans. It has become evident that some isoforms have been well conserved within the phylogenetic tree, whereas others have evolved with consequential alteration in substrate specificity [5]. For example, the CYP1A and CYP2E1 subfamilies have been conserved, and the rat and human proteins display very similar substrate specificity [6,7]. In contrast, the rat CYP2A proteins differ substantially from the corresponding hu-

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Abbreviations: CYP, Cytochrome P450; CID, Collision Induced Dissociation; APCI, Atmospheric Pressure Chemical Ionization; AGC, Automatic Gain Control

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man proteins in their substrate specificity [8,9]. Generally, cytochrome P450 proteins, even those belonging to the same subfamily, display species differences in their substrate specificity [10].

The expression of cytochrome P450 proteins in domestic, food-producing animals has received relatively very little attention. The few studies devoted to domestic animals have been carried out in piecemeal fashion with no systematic approach to fully define their cytochrome P450 composition in such animals. An appreciation of the cytochrome P450 profile in the liver of domestic animals will facilitate an appreciation of potential species differences in biotransformation, and thereby aid provision of data, for instance in support of extending the use of veterinary medicines licences for major species to minor or exotic food-producing species. Moreover, this would support the evaluation of the risk associated with the presence of drugs and other contaminant residues in edible tissues and milk that reach the consumer, a matter of current major concern.

In the present paper, we report the results of a comprehensive study concerned with a comparison of the cytochrome P450 profile in cattle liver with that of the rat, used as a reference species. Cytochrome P450 isoforms have been monitored using diagnostic substrates and/or immunologically by employing antibodies raised to rat and/or human proteins. Furthermore, the metabolism of testosterone to various hydroxylated products and androstenedione, all catalysed by a number of cytochrome P450 enzymes, has been investigated in bovine microsomes in comparison with the rat.

2. Materials and methods

Resorufin and alkoxyresorufins, lauric acid, coumarin and 7-hydroxycoumarin, erythromycin, chlorzoxazone, debrisoquine sulphate, α-naphthoflavone, furafylline, 8-methoxypsoralen, 2α-, 2β-, 6α-, 6β-, 11β-, 15α, 15β, 16α-hydroxytestosterone and androstenedione (Sigma Chemical Co.), all cofactors (Melford Labs), 6-hydroxychlorzoxazone, 4-hydroxydebrisoquine, 7α- and 16β-hydroxytestosterone (Ultrafine Chemicals) and [^{14}C]lauric acid, [4-^{14}C]-testosterone, [^{14}C]-chlorzoxazone, and [^{14}C]debrisoquine (Amersham International plc) were all purchased, as were polyclonal antibodies to rat CYP2B, CYP3A, CYP2C11 and CYP4A1, all raised in goat (Gentest Corporation), antibodies to rat CYP2E1 raised in rabbit (Amersham Life Sciences) and antibodies to human CYP2D6, and CYP2C10 raised in rabbit (Panvera Corporation). Sheep antibodies to rat CYP1A1, recognising both CYP1A1 and CYP1A2, were produced as previously described [11]. Polyclonal antibodies to rat CYP2A2 raised in rabbits and recognising both CYP2A1 and CYP2A2 were a generous gift from Y. Funae, Osaka City University Medical School, Japan. Peroxidase-linked donkey anti-sheep IgG, sheep anti-goat IgG, and goat anti-rabbit IgG were purchased from Sigma Co.

Male Wistar albino rats (200 g) were purchased from the Experimental Breeding Unit, University of Surrey, and male cattle liver, from 11- to 12-month-old animals (416–474 Kg) from Chitty's abattoir, Guildford, Surrey UK. The cattle received no medication for at least six weeks prior to killing. Liver microsomal preparations were prepared as previously described [12], and in the case of cattle always using the caudal lobe. The following determinations were carried out using microsomal preparations: the dealkylations of ethoxyresorufin [13], methoxyresorufin [14] and pentoxyresorufin [15], debrisoquine 4-hydroxylation [16], coumarin 7-hydroxylation [17], chlorzoxazone 6-hydroxylation [18], erythromycin *N*-demethylation [19], lauric acid hydroxylation measuring the combined products of 11- and 12-hydroxylation [20], total cytochrome P450 [21], and protein [22].

For the inhibition experiments, α-naphthoflavone and furafylline were dissolved in DMSO and 8-methoxypsoralen in methanol. In the case of furafylline and 8-methoxypsoralen, the inhibitors were preincubated with microsomes and NADPH for 10 minutes at 37°, and reaction was initiated by the addition of the appropriate substrate.

For the kinetic studies, coumarin 7-hydroxylase activity was determined over the substrate concentration range 0.25–10 μM and ethoxyresorufin *O*-deethylase at the concentration range 0.05–1.0 μM. Michaelis–Menten parameters (K_m and V_{max} values) were calculated from Lineweaver–Burk plots. Further analysis using Eadie–Hofstee plots enabled the enzyme kinetics to be categorised as mono- or bi-phasic, i.e. whether one or more enzymes participate in the reaction.

Immunoblot analysis of solubilised microsomes following resolution by electrophoresis [23] was performed as described by Towbin [24]. The precise conditions employed are described in the appropriate legends. To serve as positive controls in the Western blot analysis and for use in the kinetic analysis, groups of rats were treated with appropriate inducing agents. Induction of the CYP1 family and CYP2B subfamily was achieved by administration of a single intraperitoneal dose of Aroclor 1254 (500 mg/kg), the animals being killed on the 5th day following administration. Induction of the CYP2E1, CYP3A, CYP4A, and CYP1A (for kinetic studies) proteins was achieved by daily intragastric administration of isoniazid (100 mg/kg) and dexamethasone (100 mg/kg) and intraperitoneal administration of clofibrate (200 mg/kg) and β-naphthoflavone (25 mg/kg), respectively, all treatments being given for 3 days with the animals being killed 24 hr after the last administration.

Metabolism of testosterone was determined essentially as described on the Amersham information sheet; briefly, the incubation mixture comprised testosterone (250 μM) containing 100 nCi of the radiolabelled substrate, magnesium chloride (5 mM), NADPH (0.8 mM), hepatic microsomes (200 μL) and potassium phosphate buffer, pH 7.25 (50 mM) in a total volume of 1 mL. Testosterone and its metabolites were extracted into ethyl acetate, which was

evaporated off and the residue dissolved in 50% methanol; the metabolites were separated by a previously described HPLC procedure [25]. The effluent from the column was connected to an A525 radiodetector (Canberra Packard). [^{14}C]Testosterone metabolites were chromatographed with a HP1050 HPLC system (Hewlett Packard), on a 150 × 4.6 mm Hypersil Elite (Hypersil; 5 μ particle size) C18 column at a flow rate of 1.0 mL min^{-1} using a binary mobile phase with an initial gradient ranging from water: methanol (45: 55) to 70% methanol over 15 min followed by an isocratic elution at 70% methanol for 3 min.

The structure of testosterone metabolites was investigated using an ion-trap mass spectrometer (LCQ, Finnigan), with positive-ion APCI providing greatest sensitivity for the analysis of parent compound. Spectra were collected over the mass ranges m/z 100–350 and parent ions of testosterone metabolites selected for MS^2 analysis by matching peaks of putative $[M + H]^+$ selected ions with those from radio-HPLC chromatograms. MS^n experiments on testosterone and metabolites were performed with CID energies of 25% and mass isolation width of 1.5 atomic mass units. APCI mass spectral analysis was performed using the following settings; heated capillary temperature of 200°C, source voltage and current 6.0 kV and 5 μA respectively, sheath gas flow rate of 80 mL min^{-1} and a capillary voltage of 10 V. The maximum automatic gain control (AGC) ion storage time was 200 msec and 3 microscans were collected per spectrum. The product ion mass spectrum of [^{14}C]testosterone was determined by direct infusion of a 1 μg/mL standard at 10 μL min^{-1}. Identification of all testosterone metabolites was established on the basis of product ion (MS^2) mass spectra and comparison of spectral and chromatographic characteristics with authentic standards, where available.

Statistical analysis was carried out by the Student's *t*-test.

3. Results

Cytochrome P450 activity in the liver of cattle was assessed using a number of substrates (Table 1). Bovine microsomes were manyfold more effective in catalysing the *O*-deethylation of ethoxyresorufin and the 7-hydroxylation of coumarin than those of the rat. In contrast, the cattle were very poor in catalysing the hydroxylation of lauric acid, 4-hydroxylation of debrisoquine, and 6-hydroxylation of chlorzoxazone, compared with the rat (Table 1). In the case of chlorzoxazone, all bovine samples produced an additional minor metabolite which was not generated by rat liver microsomes (Fig. 1); its identity was not investigated.

Immunoblot analysis using antibodies raised to rat CYP1A1 and recognising both members of the CYP1A subfamily showed the presence of a single immunoreacting protein, within the cytochrome P450 region, in the rat (Fig. 2). In cattle two, more intense, bands were observed. When the hepatic microsomes were probed with antibodies to rat CYP2A2, two proteins were recognised in the rat but only a single protein in the cattle (Fig. 2). The same degree of CYP2A expression appears to be present in both species. When antibodies to the rat CYP2B subfamily were used, two closely migrating bands were revealed in the rat (Fig. 2). In the cattle, however, a single band of less intensity was revealed, despite the fact that double the amount of the protein was loaded and the cytochrome P450 content in the cattle is twice that of the rat. Probing of solubilised microsomes with antibodies to human CYP2C10 revealed a single band of similar molecular weight in both rat and cattle; the intensity was somewhat lower in the cattle microsomes despite the higher cytochrome P450 content. When the antibodies used were raised against rat CYP2C11, once again a single band of similar molecular weight was detected in both species; however, the band was much more intense in the rat (Fig. 2). Immunoblotting performed using antibodies to human CYP2D6, detected a single band in both species, with a higher degree of intensity being observed in the rat, despite the fact that half the amount of protein was loaded compared to the cattle (Fig. 2). Immunoblot analysis using antibodies to rat CYP2E1 revealed the presence of a single protein in both species, being somewhat more intense in cattle. The rat protein appears to have a larger molecular weight compared with its bovine counterpart (Fig. 2). When the solubilised microsomal proteins were probed with antibodies to rat CYP3A2, a single protein immunoreacted with the rat and bovine microsomal protein

Table 1
Metabolism of cytochrome P450 substrates by bovine and rat liver microsomes

Parameter	Rat	Cattle
Methoxyresorufin *O*-demethylase (pmol/min per mg protein)	7.6 ± 1.4	9.9 ± 0.6*
Ethoxyresorufin *O*-deethylase (pmol/min per mg protein)	13.6 ± 1.4	328 ± 28**
Pentoxyresorufin *O*-depentylase (pmol/min per mg protein)	0.53 ± 0.08	1.31 ± 0.17*
Coumarin 7-hydroxylase (pmol/min per mg protein)	0.64 ± 0.21	307 ± 35**
Debrisoquine 4-hydroxylase (pmol/min per mg protein)	83.3 ± 9.97	6.8 ± 2.3**
Chlorzoxazone 6-hydroxylase (pmol/min per mg protein)	604 ± 85	324 ± 76**
Erythromycin *N*-demethylase (nmol/min per mg protein)	1.00 ± 0.05	0.82 ± 0.13
Lauric acid hydroxylase (nmol/min per mg protein)	1.37 ± 0.23	0.14 ± 0.06**
Cytochrome P450 (nmol/mg protein)	0.30 ± 0.02	0.75 ± 0.07**
Microsomal protein (mg/g liver)	39.2 ± 3.9	45.4 ± 3.8

Results are expressed as means ± SEM for five animals.
* $P < 0.05$, ** $P < 0.001$.

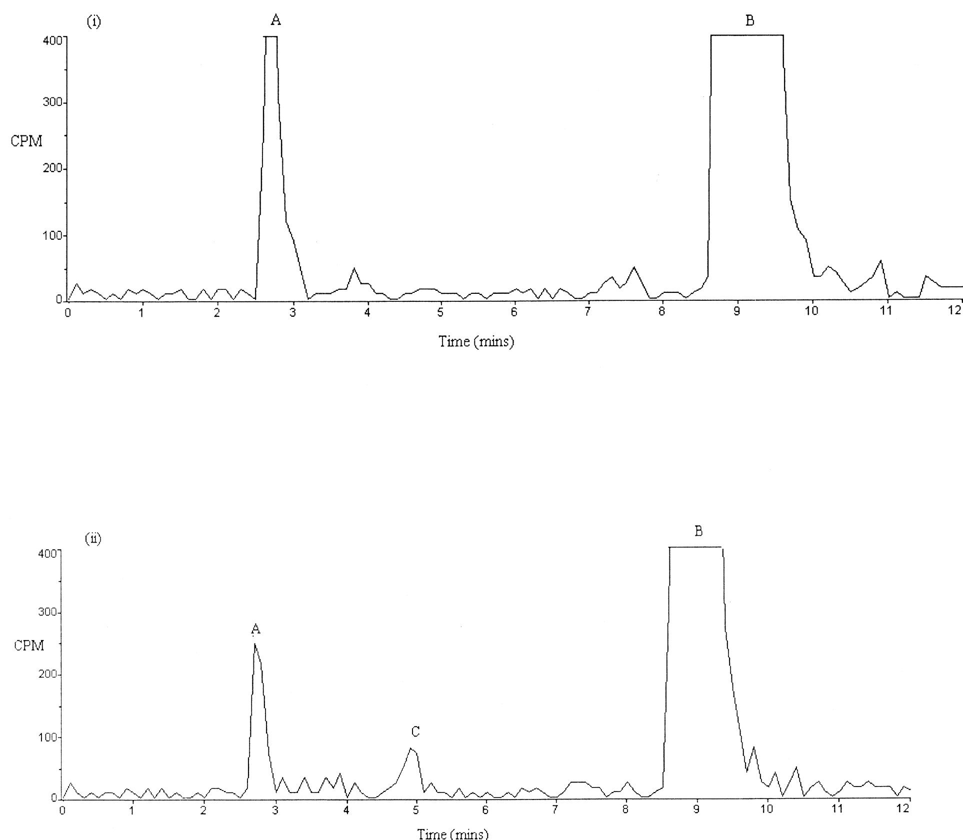

Fig. 1. Metabolism of chlorzoxazone by bovine and rat hepatic microsomes. Rat (i) and bovine (ii) hepatic microsomes were incubated with chlorzoxazone (0.5 mM) for 20 min (A), 6-hydroxychlorzoxazone (B), chlorzoxazone, and (C) unidentified metabolite.

within the cytochrome P450 region (Fig. 2). In the bovine microsomes, two additional bands of higher molecular weight are clearly visible. Finally, a single protein was immunodetected in both species with antibodies to CYP4A1 but appeared to be expressed at much higher levels in the rat (Fig. 2).

Following kinetic analysis, the K_m value, determined from Lineweaver–Burk plots, of ethoxyresorufin in the cattle was of the same order to that observed in the untreated rat (Table 2, Fig. 3). Eadie–Hoffstee plots gave a straight line within the concentration range studied (Fig. 3). The K_m value for coumarin was also calculated in cattle (Table 2); no activity was detectable in the rat. Eadie–Hofstee plots for coumarin revealed a biphasic response in cattle with K_m values of 0.6 and 9.5 μM (Fig. 4).

The *O*-dealkylations of methoxy- and ethoxyresorufin in bovine microsomes were both markedly inhibited by furafylline and α-naphthoflavone in a concentration-dependent fashion (Table 3). The 7-hydroxylation of coumarin was inhibited in a concentration-dependent way by 8-methoxypsoralen (Table 4).

Both rat and bovine hepatic microsomes readily metabolised testosterone to generate androstenedione and a number of hydroxylated metabolites (Fig. 5). However, quantitative and qualitative differences exist between the two species. Bovine hepatic microsomes, in contrast to the rat, did not produce the 2α- and 16α-hydroxylated metabolites of testosterone (Fig. 5). Similarly, quantitative differences were evident, in that the rat produced nearly 7 times more of the 6β-hydroxytestosterone and androstenedione (Table 5), whereas cattle produced more of an unidentified metabolite, designated as F (Fig. 5).

Mass spectrometry of metabolite F (retention time 7.5 min), produced by bovine microsomal preparations following incubation with testosterone, generated a molecular ion $[M + H]^+$ of m/z 305 which yielded a product ion mass spectrum similar to that of other hydroxylated metabolites of the parent compound. Product ion mass spectra of parent compound and hydroxylated metabolites (Table 6) provided relatively little structural information, since the predominant route of collision-induced dissociation was successive dehydration from their respective molecular ions. Comparison of the product ion mass spectra of testosterone with [4-^{14}C]testosterone indicated that the ions at m/z 97 and 109 originated from the A ring since M^{+2} ions of m/z 99 and 111 were evident in that of the latter (Fig. 6). These ions were also evident in the product ion spectra of the D-ring hydroxylated metabolites and metabolite F but not in those of the other hydroxylated metabolites. This would suggest

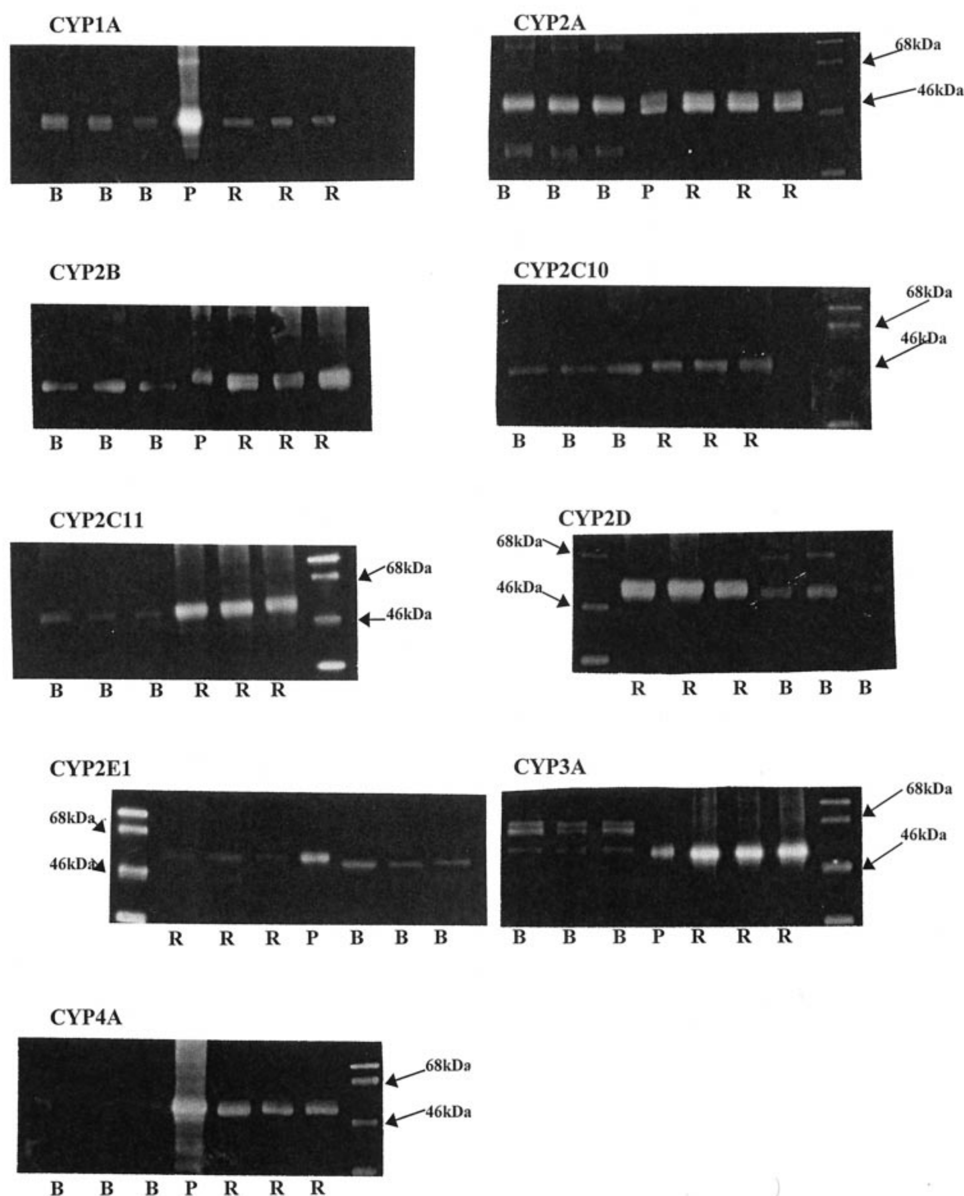

Fig. 2. Immunoblot analysis of cytochrome P450 expression in bovine liver. Hepatic microsomal samples from cattle and rats were resolved by 10% (w/v) SDS–PAGE and transferred electrophoretically onto nitrocellulose paper. The following primary antibodies were used: sheep anti-rat-CYP1A1 (1:10,000), rabbit anti-rat-CYP2A (1:1000), goat anti-rat-CYP2B (1:500), rabbit anti-human-CYP2C10 (1:1000), goat anti-rat-CYP2C11 (1:1000), rabbit anti-human-CYP2D6 (1:1000), rabbit anti-rat-CYP2E1 (1:200), goat anti-rat-CYP3A2, and goat anti-rat-CYP4A1 (1:500). The secondary antibodies were donkey anti-sheep (1:2000), goat anti-rabbit (1:2000), and sheep anti-goat (1:5000). The following amounts of hepatic protein were loaded per lane: for CYP1A, 7.5 μg for rat and bovine samples and 2.5 μg for the positive control; for CYP2A, 5 μg for all samples; for CYP2B, 10 μg for bovine samples, 5 μg for rat samples, and 2.5 μg for the positive control; for CYP2C10, 10 μg for rat and 20 μg for bovine samples; for CYP2C11, 10 μg for all samples; for CYP2D6, 10 μg for rat and 20 μg for bovine samples; for CYP2E1, 10 μg for all samples; for CYP3A4, 10 μg for bovine and 2.5 μg for rat and positive control samples, and for CYP4A1, 10 μg for all samples. The positive controls were derived from Aroclor 1254-treated rats (CYP1A, CYP2A, and CYP2B), isoniazid-treated rats (CYP2E1), dexamethasone-treated rats (CYP3A4), and clofibrate-induced rats (CYP4A1). B, bovine, R, rat and P, positive control.

that the site of hydroxylation of metabolite F was distal to the A ring. This contention is further supported by the increased abundance of product ion of *m/z* 251 (Fig. 6 and Table 6) which was more abundant in the D ring metabolites than the products hydroxylated on the other rings. The molecular ions at *m/z* 287, 269, and 251 represent the sequential loss of three water molecules from the $M + H^+$ of *m/z* 305, common to the hydroxylated metabolites of testosterone studied.

4. Discussion

A comprehensive study was undertaken to define the expression of cytochrome P450 proteins in the liver of cattle in comparison with the rat. This was achieved by using diagnostic probes for the various isoforms, and immunologically using antibodies to the rat or human proteins. Having taken this approach, it must be emphasised that in doing so

Table 2
Kinetic parameters for the ethoxyresorufin O-deethylase and coumarin 7-hydroxylase activities in bovine hepatic microsomes

Enzyme activity	Animal species	K_m (μM)	V_{max} (pmol/min/mg pr)	V_{max}/K_m (mL/mg pr/min)
Ethoxyresorufin O-deethylase	Cattle	0.12 ± 0.04	177 ± 24	1.48 ± 0.20
	Rat	0.04 ± 0.01	8.66 ± 3.6	0.22 ± 0.09
	Rat treated with β-naphthoflavone	0.75 ± 0.02	3788 ± 205	5.05 ± 0.27
Coumarin 7-hydroxylase	Cattle	1.49 ± 0.18	74.5 ± 16.4	0.05 ± 0.01
	Rat	ND	ND	ND

All values were determined from Lineweaver–Burk plots. Results are presented as means ± SD for four animals. ND, not determined.

caution must be observed in that the substrate probes employed in the current studies were established for the rat or human proteins; the assumption is made that they are suitable probes for the corresponding bovine proteins. However, this cannot be confirmed until the bovine proteins have been purified and their substrate specificity established. Similarly, the antibodies were raised to the human or rat antigens which may recognise epitopes of the bovine proteins, but do not constitute proof that the cattle and rat proteins are structurally identical or that quantitative comparisons are always valid. In order to minimise variation due to different inter-lobular cytochrome P450 distribution, the caudal lobe from bovine liver was always used in the present studies.

The CYP1A subfamily comprises two isoforms, A1 and A2, that can be monitored using the O-dealkylation of ethoxy- and methoxyresorufin respectively, although some overlap exists, i.e. to some extent rodent CYP1A2 can also O-deethylate ethoxyresorufin, albeit much less efficiently

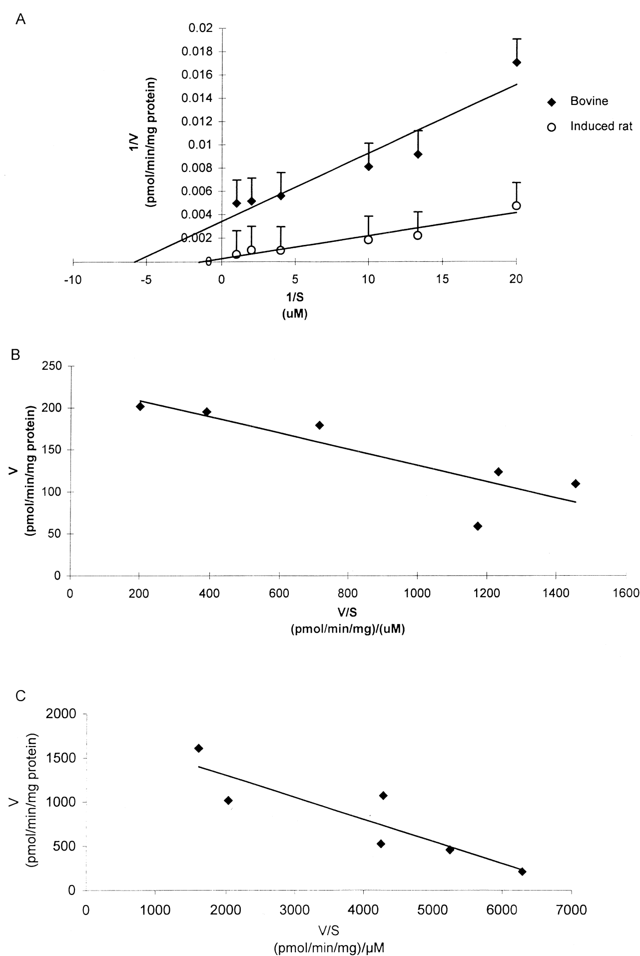

Fig. 3. Lineweaver–Burk plot and Eadie–Hofstee plots of ethoxyresorufin O-deethylase by bovine and β-naphthoflavone-induced rat liver microsomes. Ethoxyresorufin O-deethylase activity was measured over a series of substrate concentrations, ranging from 0.05 to 1 μM, in the presence of bovine hepatic microsomes and microsomes derived from β-naphthoflavone-treated rats. Results are presented as means ± SD for four animals. (A) Lineweaver–Burk plot; (B) Eadie–Hofstee plot of bovine activity, and (C) Eadie–Hofstee plot of activity in Aroclor 1254-induced rat.

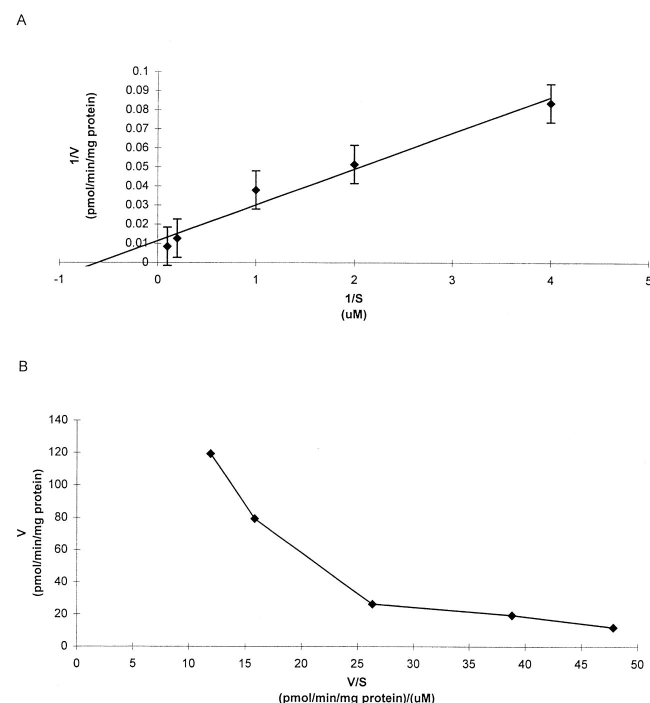

Fig. 4. Lineweaver–Burk plot and Eadie–Hofstee plot for coumarin 7-hydroxylase by bovine hepatic microsomes. Coumarin 7-hydroxylase activity was measured over a series of substrate concentrations, ranging from 0.25 to 10 μM, in the presence of bovine hepatic microsomes. Results are presented as means ± SD for four animals.

Table 3
Inhibition of alkoxyresorufin *O*-dealkylation by furafylline and α-naphthoflavone

Inhibitor and concentration (μM)	Methoxyresorufin *O*-demethylase (pmol/min/mg protein)	Ethoxyresorufin *O*-deethylase (pmol/min/mg protein)
No addition	6.36 ± 0.91* (100)	276 ± 83* (100)
Furafylline 10	3.97 ± 0.44 (62)	101 ± 13 (37)
Furafylline 25	3.10 ± 0.52 (49)	64 ± 4 (23)
Furafylline 100	1.52 ± 0.45 (24)	51 ± 5 (18)
No addition	26 ± 2	540 ± 47 (100)
α-Naphthoflavone 1	ND	15.5 ± 2.55 (3)
α-Naphthoflavone 10	ND	3.51 ± 1.00 (0.7)
α-Naphthoflavone 25	ND	2.31 ± 0.28 (0.4)

* These are the activities following a 10-min preincubation of microsomes, NADPH, and DMSO, the solvent for furafylline. The activities without preincubation were 15.1 ± 2.0 and 601 ± 102 pmol/min per mg protein for the *O*-dealkylations of methoxyresorufin and ethoxyresorufin, respectively.

Results are expressed as means ± SEM for three animals. Numbers in parentheses represent the percentage of control activity.

ND; Not detectable.

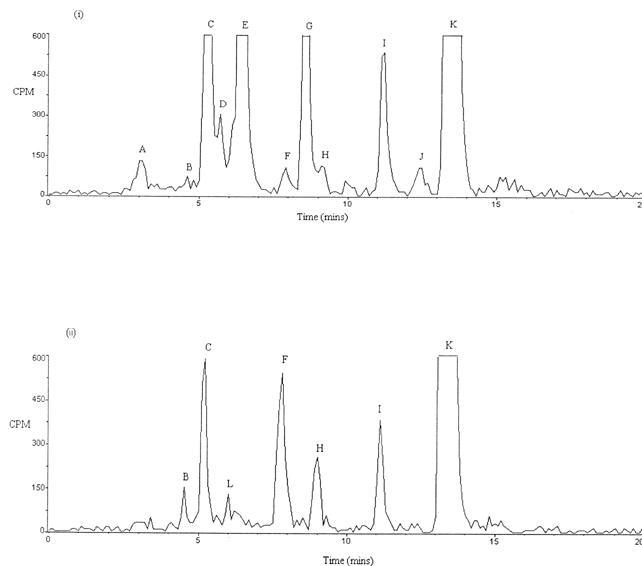

Fig. 5. Testosterone metabolism by rat and bovine hepatic microsomes. Rat (i) and bovine (ii) hepatic microsomes were incubated with [^{14}C]testosterone for 30 min. (A) unidentified metabolite, (B) 15β-hydroxytestosterone, (C) 6β-hydroxytestosterone, (D) unidentified metabolite, (E) 16α-hydroxytestosterone, (F) unidentified metabolite, (G) 2α-hydroxytestosterone, (H) 2β-hydroxytestosterone, (I) androstenedione, (K) testosterone, and (L) unidentified metabolite.

when compared with CYP1A1 [26]. Ethoxyresorufin *O*-deethylase activity was markedly higher in the liver of cattle when compared with the rat, implying increased expression of CYP1A1, an isoform that is not normally expressed in the liver at a significant level but which is highly inducible in this tissue [6]. As the *O*-demethylation of methoxyresorufin, an indicator of CYP1A2 activity [26], was similar in the two species, it may perhaps be inferred that the higher ethoxyresorufin *O*-deethylase activity in the bovine liver may be due to the presence of a protein related to CYP1A1. Other workers have also reported high *O*-deethylation of ethoxyresorufin in bovine liver but low *O*-dealkylation rates for other alkoxyresorufins [27]. It is relevant to point out that the CYP1A1 protein derived from a number of species including man has been extensively studied; it is a highly conserved protein [28], and ethoxyresorufin has been used successfully to monitor CYP1A1 in many animal species. The increased activity observed in cattle may be due to either the higher expression of CYP1A1 in this animal or the presence of an isoform with high catalytic activity towards ethoxyresorufin. The higher immunoreactivity in the cattle liver microsomes compared with the rat would suggest the presence of a higher concentration of CYP1A-related proteins; it is noteworthy that there may be two isoforms present in cattle (Fig. 2). Studies using furafylline, considered to be a specific CYP1A2 inhibitor [29], showed that the compound markedly inhibited the *O*-dealkylation of both methoxy- and ethoxyresorufin in a concentration-dependent fashion. When α-naphthoflavone, an inhibitor of CYP1A1 and CYP1A2 [30] was used, once again both activities were inhibited, the effect being more pronounced compared with that of furafylline. All the above observations suggest that the two proteins in bovine microsomes may be related to CYP1A1 and CYP1A2, but final conclu-

Table 4
Inhibition of coumarin 7-hydroxylase by 8-methoxypsoralen

8-Methoxypsoralen (μM)	Coumarin 7-hydroxylase (pmol/min per mg protein)
—	90 ± 11 (100)
0.1	79 ± 7 (88)
0.5	36 ± 5 (40)
1.0	24 ± 4 (27)

Results are expressed as means ± SEM for three animals. Numbers in parentheses represent the percentage of control activity.

* These are the activities following a 10-min preincubation of microsomes, NADPH, and methanol, the solvent for 8-methoxypsoralen. The activity without preincubation was 132 ± 11 pmol/min per mg protein.

Table 5
Metabolism of testosterone by bovine and rat hepatic microsomes

Site of testosterone hydroxylation	Rat (pmol/min per mg protein)	Bovine (pmol/min per mg protein)
2α	573 ± 76	ND
2β	66.6 ± 5.3	81.8 ± 23.0
6β	878 ± 58	133 ± 31**
15β	36.1 ± 3.5	34.0 ± 8.8
16α	1030 ± 112	ND
Androstenedione	382 ± 37	83.0 ± 18.9**

Results are expressed as means ± SEM for five animals. ND, none detected.

* $P < 0.05$, ** $P < 0.001$.

Table 6
Summary of product ion mass spectra of testosterone and its hydroxylated products

Analyte	Rt (min)	$[M + H]^+$	Product ions
Testosterone	13.0	289	271*, 253, 243, 213, 189, 171, 157, 109, 97
[4-^{14}C]Testosterone	13.0	291	273*, 255, 245, 213, 189, 175, 159, 111, 99
2α-Hydroxytestosterone	8.1	291	287*, 269, 251 (14%), 241, 217, 173
2β-Hydroxytestosterone	8.6	305	287*, 269, 251 (14%), 241, 217, 173
6α-Hydroxytestosterone	3.8	305	287, 269*, 259, 251 (23%), 241, 173, 121
6β-Hydroxytestosterone	4.9	305	287, 269*, 259, 251 (22%), 241, 211, 173
11β-Hydroxytestosterone	8.6	305	287, 269*, 251 (22%), 241, 173, 121
15α-Hydroxytestosterone	4.7	305	287, 269*, 251 (28%), 251, 211, 205, 187, 173, 109, 97
15β-Hydroxytestosterone	4.3	305	287, 269*, 251 (41%), 241, 211, 203, 187, 173, 109, 97
16α-Hydroxytestosterone	6.0	305	287, 269*, 251 (36%), 241, 211, 189, 109, 97
16β-Hydroxytestosterone	7.0	305	287, 269*, 251 (40%), 241, 211, 189, 109, 97
Metabolite F	7.5	305	287, 269*, 251 (28%), 241, 211, 173, 109, 97

* Denotes the most abundant (base) product ion.
Rt, Retention time.

sions must await their isolation and characterisation. The K_m for the O-deethylation of ethoxyresorufin determined in the cattle was lower than that in the β-naphthoflavone-treated rat, where more of the hepatic cytochrome P450 is in the form of CYP1A. In the untreated rat, the low level of ethoxyresorufin O-deethylation is largely catalysed by CYP2C6 [31,32]. Eadie–Hoffstee plots revealed a straight line indicating that, within the ethoxyresorufin concentration range studied, a single enzyme, or enzymes with similar K_m values, is responsible for the O-deethylation of ethoxyresorufin. Collectively, the above observations indicate that the higher ethoxyresorufin O-deethylase activity in the cattle is probably the consequence of the higher levels of expression of CYP1A rather than the presence of an isoform with high catalytic activity towards ethoxyresorufin. Whether the high expression of CYP1A in the bovine liver is genetically determined or reflects exposure to xenobiotics, e.g. through the diet, remains to be established. It is pertinent to point out that the CYP1A subfamily is inducible by many anutrients present in dietary plant components [33]. The CYP1A subfamily is one of the most active cytochrome P450 subfamilies in the bioactivation of chemicals such as polycyclic aromatic hydrocarbons, a ubiquitous class of environmental pollutants [6]. The high expression of this subfamily in bovine liver may predispose these animals to the toxicity and carcinogenicity to these chemicals.

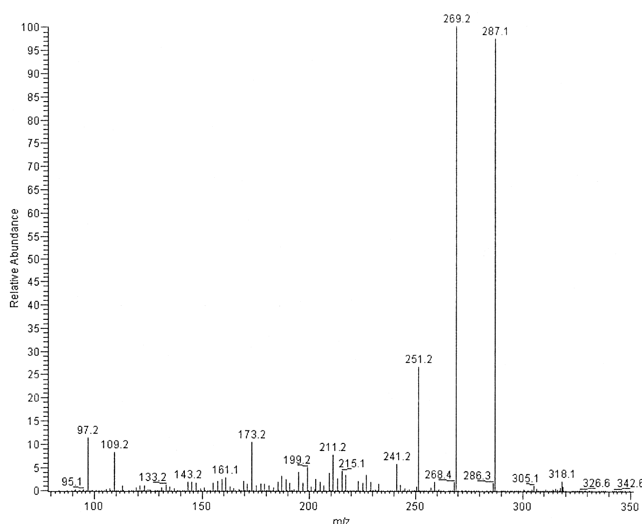

Fig. 6. Mass spectrometry analysis of unknown metabolite F of [^{14}C]testosterone. Full-scan (m/z 100–350) product ion mass of the unknown metabolite F (retention time 7.5 min) generated during the incubation of bovine hepatic microsomes with [^{14}C]testosterone for 30 min.

Cattle also displayed markedly higher coumarin 7-hydroxylase activity compared with the rat, in agreement with previous studies [34]. The rat CYP2A proteins are unable to catalyse the 7-hydroxylation of coumarin efficiently, in contrast to the human and mouse proteins [8,35–38]. High coumarin 7-hydroxylase activity was noted in cattle, suggesting that the CYP2A proteins in this species behave more like the human rather than the rat forms, although this requires confirmation using the purified protein(s). Coumarin hydroxylase activity in cattle liver was inhibited in a concentration-dependent way by the established CYP2A inhibitor 8-methoxypsoralen [39]. 8-Methoxypsoralen also inhibits CYP1A1 and CYP1A2 activity to some extent [40], raising the possibility that the CYP1A subfamily may contribute to the hydroxylation of coumarin. However, this is very unlikely since neither furafylline (100 μM) nor α-naphthoflavone (1 μM) inhibited the 7-hydroxylation of coumarin (results not shown). It should be emphasised that immunologically, a similar degree of expression was observed in the two species and no major difference between the two species was evident that could explain the marked difference in the 7-hydroxylation of coumarin. However, the antibodies recognised two proteins in the rat, the higher molecular weight protein corresponding to CYP2A2 and the lower molecular weight to CYP2A1 [41], but only one in the cattle. In studies employing precision-cut liver slices, it was observed that calf slices, in contrast to rat slices, could

readily hydroxylate coumarin at the 7-position [42]. Eadie–Hofstee plots are non-linear, indicating that at least two enzymes, a high affinity protein with a K_m of 0.6 μM and a low affinity protein with a K_m of 9.5 μM, contribute to the 7-hydroxylation of coumarin over the concentration range studied. In human liver, K_m values for coumarin have been reported to range between 0.4–2.5 μM [8,35,43]. The K_m for coumarin 7-hydroxylation in reconstituted systems containing purified mouse CYP2A5 was reported to be 14 μM, and in insect cell microsomes expressing recombinant human CYP2A6 the K_m value for the same reaction was 6 μM [44]. Whether the two enzymes catalysing this hydroxylation belong to the CYP2A subfamily cannot be determined on the basis of the available evidence. CYP2B activity may be monitored in the liver of rats using the *O*-depentylation of pentoxyresorufin as a probe [15], but this substrate is poorly metabolised by the human proteins [45]. Activity in cattle liver was double that of the rat, but this may reflect the higher cytochrome P450 concentration in the cattle liver as this difference was abolished when activity was expressed per nmol cytochrome P450. Western blot analysis showed that antibodies raised to rat CYP2B detected a single band in bovine liver microsomes, but two in the rat, the higher molecular weight being CYP2B2 and the lower molecular weight CYP2B1. These observations suggest that CYP2B-related proteins are expressed in the cattle liver. The expression of CYP2C proteins was only determined at the protein level using immunoblot analysis. Antibodies to human CYP2C10 immunoreacted with a single band in both animal species; similarly, antibodies to rat CYP2C11 also revealed a single band in both animal species which was, however, more intense in the rat. These observations suggest that CYP2C-related proteins are expressed in the liver of cattle. Similar analysis utilising antibodies to human CYP2D6 recognised a single immunorelated band in both rat and cattle, being much more intense in the former, compatible with the markedly higher debrisoquine 4-hydroxylase activity observed in the rat compared with cattle. Previous studies have shown the presence of debrisoquine 4-hydroxylase and bufurarol 1′-hydroxylase in bovine liver, two activities closely linked to CYP2D expression [16]. Cattle CYP2D proteins have been cloned which, at the nucleotide level, differed by less than 7% [46], in contrast to human where only a single protein belonging to this subfamily, CYP2D6, has been identified. Immunoblot analysis using antibodies to CYP2E1 detected a single protein in the cattle which appeared slightly more intense than the protein detected in the rat. The 6-hydroxylation of chlorzoxazone, a probe for CYP2E [18], however, was significantly higher in the rat. With the exception of the rabbit, where two proteins constitute the CYP2E subfamily [47], in all other animal species studied this subfamily comprises a single protein [48], and this also appears to be the situation in cattle liver.

The CYP3A, the most abundant subfamily in adult human liver [49], is responsible for the metabolism of many of the frequently used drugs [50] and may be monitored using the *N*-demethylation of erythromycin as a probe [19]. In the present study, no difference in microsomal erythromycin *N*-demethylase activity was noted between the rat and cattle liver, but when expressed in terms of nmol cytochrome P450, the activity was higher in the rat. The immunoblot analysis employing antibodies to rat CYP3A2, which also recognise human CYP3A4, revealed in the bovine liver the presence of a single protein in the cytochrome P450 region, similar to the rat liver. Polyclonal antibodies to CYP3A raised in one animal species generally cross-react with the proteins from another animal species, as a consequence of their high structural homology [50]. In recent studies [51], a bovine full-length cytochrome P450 cDNA has been expressed in V79 Chinese hamster cells; this protein could hydroxylate testosterone at a number of positions including 6β, and the activity was inhibited by the CYP3A inhibitor ketoconazole. Using monoclonal antibodies to rat CYP3A1, van't Klooster *et al*. [52] also detected only a single immunoreacting protein in postmitochondrial preparations of cultured cattle hepatocytes. A cytochrome P450 isoform, inducible by troleandomycin, has been isolated from the liver of sheep and shown to catalyse the *N*-demethylation of erythromycin [53].

CYP4A activity is most frequently monitored using the ω-hydroxylation of lauric acid [54]. The analytical method employed in the present study measures the combined ω- and ω-1 hydroxylation activity, the latter being catalysed predominately by CYP2E1 [55]. The hydroxylation of lauric acid was poorly catalysed by bovine liver microsomes, being about 10% of that observed in the rat. Since, at the protein level, CYP2E1 expression does not differ greatly between the two species and chlorzoxazone 6-hydroxylation in the cattle is only half of that of the rat, it may be inferred that the CYP4A-mediated lauric acid ω-hydroxylation is low in cattle liver. In accordance with these conclusions, immunological analysis utilising antibodies to CYP4A indicated a much lower degree of immunoreactivity in the cattle microsomes compared with the rat. This is the first study showing the presence of CYP4A-related proteins in the liver of cattle.

Both species readily metabolised testosterone to a number of hydroxylated products and androstenedione. The major bovine metabolites were androstenedione and 6β-hydroxytestosterone, in accordance with studies where testosterone metabolism was studied using isolated hepatocytes [52,56]. However, bovine microsomes failed to produce 2α- and 16α-hydroxytestosterone, two metabolites which in the rat are associated with CYP2C11 [57]. It is pertinent to point out that, following probing of hepatic microsomes with antibodies to CYP2C11, the immunoreacting band in the rat was far more intense than that in the cattle. The unknown metabolite F (Fig. 5) was a major metabolite in the cattle but not in the rat. The chromatographic retention time and product ion mass spectrum of metabolite F was not consistent with the reference D ring metabolites hydroxylated at the 15- or 16-positions. The

remaining sites for hydroxylation distal to the A ring include the 12- and 14-positions, and a 12-hydroxy testosterone metabolite has been reported to be produced by hepatocyte cultures from female cattle [52]. Further studies including either structural analysis by NMR or comparison of the chromatographic and spectral properties with reference standards are required for identification of metabolite F.

In conclusion, the present study demonstrates that proteins related to the CYP1A, CYP2A, CYP2B, CYP2C, CYP2E, and CYP4A subfamilies are expressed in the liver of cattle. In comparison with the rat, the bovine liver expresses CYP1A to a higher extent but, in contrast, the expression of CYP2D and CYP4A proteins is poor. Both species metabolised testosterone but, in contrast to the rat, bovine liver did not produce 2α- and 16α-hydroxytestosterone. On the other hand, bovine liver produced high levels of an unidentified metabolite, possibly 12-hydroxytestosterone. The present findings allow an appreciation of the relative metabolic capability of cattle compared with the rat, and further enable our understanding of the evolution of cytochromes P450.

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