10

Human Cytochrome P450 Enzymes

F. Peter Guengerich

1. Background and History of Development of the Field

Much of P450 research has always been done with the view of application to humans, even when done with experimental animals and microorganisms. Research with the human P450s has been done in several stages, and clinical pharmacology has utilized the knowledge at each point in its development.

Aside from *in vivo* experiments with drugs, human P450 work in the late 1960s and 1970s was done with tissue samples, primarily biopsies. Some data on metabolic patterns and rates were collected[1]. In the late 1970s, several groups began to purify P450s from human liver microsomes. The first purified proteins, selected because of their abundance and ease of purification, were probably what are recognized now as P450 3A and 2C subfamily proteins[2–4]. Efforts were shifted to purifying individual P450s on the basis of catalytic activities with the evidence that in some cases a single P450 could be identified in this way; for example, the enzyme now known as P450 2D6 was found to be under monogenic control[5]. The approach is technically demanding because of the need to do separations in the presence of detergents and then remove them from individual chromatography fractions prior to analysis of catalytic activity. Nevertheless, human P450s 1A1 (ref. [6]), 1A2 (ref. [7]), 2A6 (ref. [8]), 2C9 (ref. [9]), 2D6 (refs [7], [10], [11]), and 3A4 (ref. [12]), were isolated in this general manner. Another general approach that was used was purification from tissue on the basis of immunochemical cross-reactivity with animal P450s[13–15].

With the development of recombinant DNA technology, cDNAs for many of the human P450s were cloned in the 1980s[16]. In the late 1980s, methods came into use for the heterologous expression of P450s, first in mammalian and yeast systems, and then in baculovirus and (by the mid-1990s) in bacterial systems[17–20]. In the late 1980s, the nomenclature system developed by Nebert[21] was applied and allowed individual human and other P450s to be discussed on the basis of their sequences. (For a guide to some of the earlier nomenclature, see ref. [22].)

By the early 1990s, much of the interest in P450 research had shifted to the human P450 enzymes because of the availability of systems for handling these. In particular, studies in the areas of drug metabolism and chemical toxicology/carcinogenesis were facilitated by the knowledge that a relatively small number of the P450s account for a large fraction of the metabolism of the drugs and other chemicals of interest. In the pharmaceutical industry, the roles of the major hepatic P450s are extensively studied in developing predictions

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about bioavailability, drug–drug interactions, and toxicity. Since the publication of the chapter on human P450s in the last edition of this book[23], apparently all of the remaining human P450 genes have been identified, and the number (57) appears to be complete because of more general knowledge about the human genome (html://drnelson.utmem.edu/CytochromeP450.html). Much of the progress since 1995 has involved extrahepatic P450s, many with roles in the processing of "endobiotic" chemicals, for example, sterols and vitamins.

The list of the 57 human P450s is presented in Table 10.1, along with available knowledge about

Table 10.1. Human P450s

P450	Tissue sites	Subcellular localization[a]	Typical reaction[b]
1A1	Lung, several extrahepatic sites, peripheral blood cells	ER	Benzo[*a*]pyrene 3-hydroxylation
1A2	Liver	ER	Caffeine N^3-demethylation
1B1	Many extrahepatic sites, including lung and kidney	ER	17β-Estradiol 4-hydroxylation
2A6	Liver, lung, and several extrahepatic sites	ER	Coumarin 7-hydroxylation
2A7		ER	
2A13	Nasal tissue	ER	Activation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)
2B6	Liver, lung	ER	(*S*)-Mephenytoin *N*-demethylation
2C8	Liver	ER	Taxol 6α-hydroxylation
2C9	Liver	ER	Tobutamine methyl hydroxylation
2C18	Liver	ER	
2C19	Liver	ER	(*S*)-Mephenytoin 4′-hydroxylation
2D6	Liver	ER[c]	Debrisoquine 4-hydroxylation
2E1	Liver, lung, other tissues	ER	Chlorzoxazone 6-hydroxylation
2F1	Lung	ER	3-Methylindole activation
2J2	Lung	ER	Arachidonic acid oxidations
2R1			
2S1	Lung	ER	
2U1			
2W1			
3A4	Liver, small intestine	ER	Testosterone 6β-hydroxylation
3A5	Liver, lung	ER	Testosterone 6 β-hydroxylation
3A7	Fetal liver	ER	Testosterone 6 β-hydroxylation
3A43	(mRNA detected in gonads)	(ER)	
4A11	Liver	ER	Fatty acid ω-hydroxylation
4A22		ER	
4B1	Lung	ER	Lauric acid ω-hydroxylation
4F2	Liver	ER	Leukotriene B_4 ω-hydroxylation
4F3	Neutrophils	ER	Leukotriene B_4 ω-hydroxylation
4F8	Seminal vesicles	ER	Prostaglandin ω-2 hydroxylation

Table 10.1. Continued

P450	Tissue sites	Subcellular localization[a]	Typical reaction[b]
4F11	Liver	ER	
4F12	Liver	ER	Arachidonic acid ω-,ω-2-hydroxylation
4F22			
4V2			
4X1			
4Z1			
5A1	Platelets	ER	Thromboxane A_2 synthase reaction
7A1	Liver	ER	Cholesterol 7α-hydroxylation
7B1	Brain	ER	Dehydroepiandrosterone 7α-hydroxylation
8A1	Aorta, others	ER	Prostacyclin synthase reaction
8B1	Liver	ER	7α-hydroxyprogesterone 12-hydroxylation (?)
11A1	Adrenals, other steroidogenic tissues	Mit	Cholesterol side-chain cleavage
11B1	Adrenals	Mit	11-Deoxycortisol 11-hydroxylation
11B2	Adrenals	Mit	Corticosterone 18-hydroxylation
17A1	Steroidogenic tissues	ER	Steroid 17α-hydroxylation
19A1	Steroidogenic tissues, adipose, brain	ER	Androgen aromatization
20A1			
21A2	Steroidogenic tissues	ER	17-Hydroxyprogesterone 21-hydroxylation
24A1	Kidney	Mit	25-Hydroxyvitamin D_3 24-hydroxylation
26A1	Several	ER	Retinoic acid 4-hydroxylation
26B1	Brain	ER	Retinoic acid 4-hydroxylation
26C1		(ER?)	
27A1	Liver	Mit	Sterol 27-hydroxylation
27B1	Kidney	Mit	Vitamin D_3 1-hydroxylation
27C1			
39A1	Liver (?)	ER	24-Hydroxycholesterol 7-hydroxylase
46A1	Brain	ER	Cholesterol 24-hydroxylation (?)
51A1	Liver, testes	ER	Lanosterol 14α-demethylation

[a]ER = endoplasmic reticulum (microsomal), Mit = mitochondria.
[b]If known.
[c]Mainly ER, some detected in mitochondria.

sites of tissue expression, subcellular localization, and a typical reaction. In the 1995 edition[23], the list included 31 human P450s, and 2 of these have been dropped as apparent cloning artifacts (2C10, 2C17), leaving 29. Thus, the list was only half complete at that time. It should be emphasized that we still have little knowledge about the roles of some of these P450s beyond the genomic information. Of the 57, most that have been examined appear to be expressed primarily in the endoplasmic reticulum and only 6 are located exclusively in mitochondria. (However, work in animal models by Avadhani has clearly demonstrated the import of what have been generally considered microsomal P450s into mitochondria[24, 25]. The basis of this transport appears to be signals in the N-terminal region[26]. Limited information is available about this phenomenon with human P450s;

recent collaborative experiments with Avadhani's group indicate that many human liver samples contain immunochemically detectable and catalytically active P450 2D6 in mitochondrial as well as microsomal fractions [N. Avadhani and F.P. Guengerich, unpublished results].) In many cases, no direct information is available because protein studies have not been done, although most of these "orphan" P450s are predicted to reside primarily in the endoplasmic reticulum.

Of the P450s with known catalytic activities, 14 are clearly involved in steroidogenesis, 4 are involved in what appear to be important aspects of metabolism of vitamins (vitamins A and D), 5 are involved in eicosanoid metabolism, 4 appear to have fatty acids as their substrates, and 15 catalyze transformation of xenobiotic chemicals (Table 10.2). Some of these categories should be considered tentative. This classification accounts for only 42 of the 57 P450s. Many of the xenobiotic-metabolizing P450s can also catalyze oxidation of steroids and fatty acids, but these functions do not appear to be critical to homeostasis (e.g., testosterone 6β-hydroxylation by P450 3A4, lauric acid 11-hydroxylation by P450 2E1, possibly 17β-estradiol 4-hydroxylation by P450 1B1). Most of the steroid-oxidizing enzymes are critical, and the levels of these P450s are relatively invariable among individuals, in contrast to the xenobiotic-metabolizing P450s, which vary considerably (Figures 10.1 and 10.2). Deficiencies in the expression or catalytic activities of most of the P450s involved in steroid metabolism lead to serious diseases (Table 10.3) (included in this list[29] are P450s 1B1 [true function unknown] and 24A1 [vitamin D hydroxylation]). In one case, a high level of P450 activity (P450 19) can be a problem (estrogen formation in estrogen-dependent tumors) and this P450 is a target for therapeutic attenuation[30]. The 15 identified xenobiotic-metabolizing P450 are mainly in the families 1–3, and levels of these vary considerably in humans (Figures 10.1 and 10.2). Studies with transgenic (knockout) mice do not indicate critical function associated with the apparent orthologs in the absence of xenobiotic challenge[31]. Analysis of the lists of drugs in which individual human P450s are involved[32] indicates that ~75% of the drugs can be oxidized by 3 P450s (3A4, 2D6, 2C9), and a set of 6–7 P450s will account for 90–95% of all drug metabolism (Figure 10.3)[33]. Similar numbers of P450s are involved in the metabolism of chemical carcinogens, although the pattern shifts from that of Figure 10.3, with P450s 2C19 and 2D6 being replaced by P450s 1A1, 1B1, 2A6, and 2E1 (Table 10.4). The relative contributions of these xenobiotic-metabolizing P450s are, to some extent, a function of the relative abundance (Table 10.5, Figure 10.4), although there are some important exceptions. Further, the

Table 10.2. Classification of Human P450s based on Major Substrate Class[a]

Sterols	Xenobiotics	Fatty acids	Eicosanoids	Vitamins	Unknown
1B1	1A1	2J2	4F2	24	2A7
7A1	1A2	4A11	4F3	26A1	2R1
7B1	2A6	4B1	4F8	26B1	2S1
8B1	2A13	4F12	5A1	27B1	2U1
11A1	2B6		8A1		2W1
11B1	2C8				3A43
11B2	2C9				4A22
17	2C18				4F11
19	2C19				4F22
21A2	2D6				4V2
27A1	2E1				4X1
39	2F1				4Z1
46	3A4				20
51	3A5				26C1
	3A7				27C1

[a]As pointed out in the text, this classification is somewhat arbitrary, for example, P450s 1B1 and 27A1 could be grouped in two different categories.

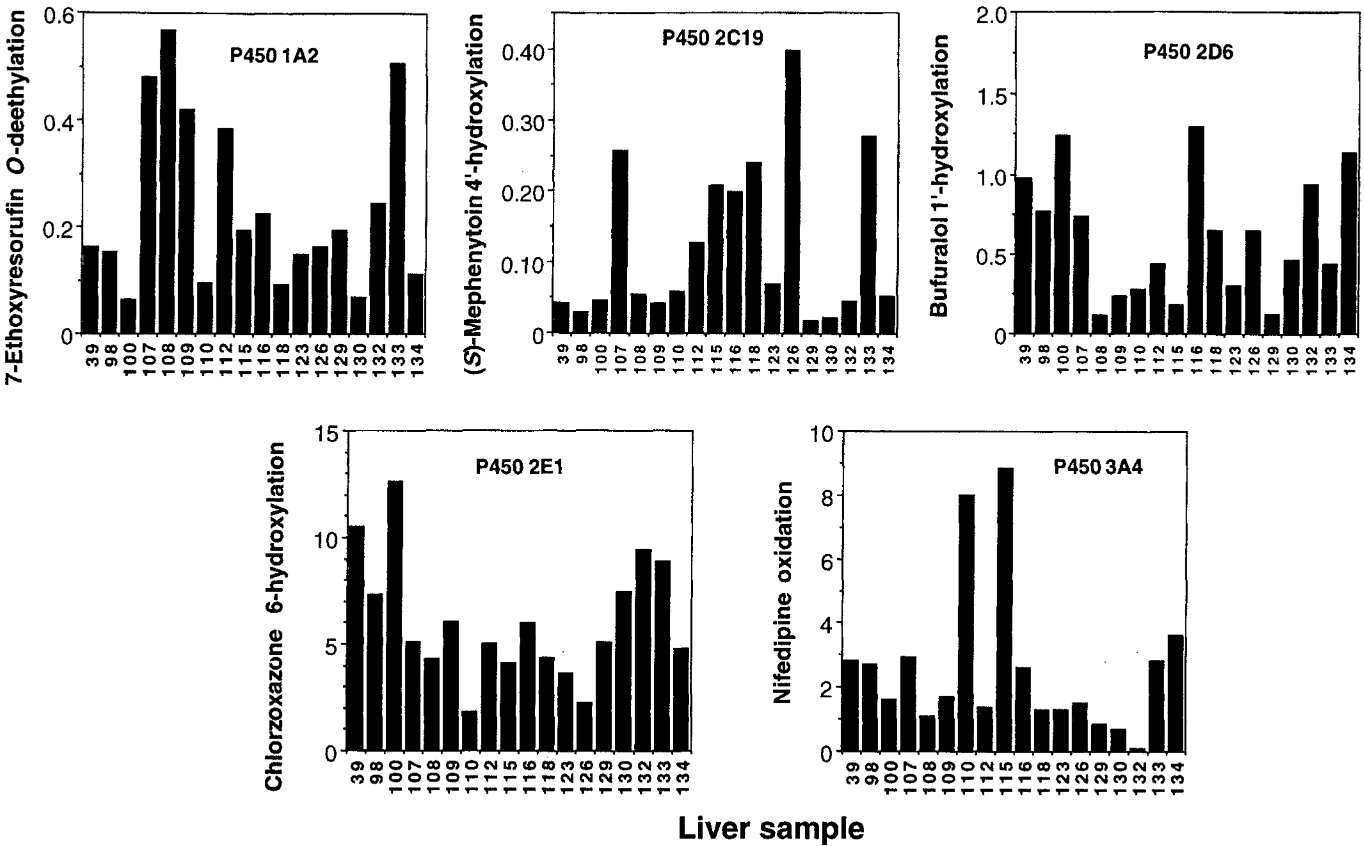

Figure 10.1. Variation in levels of five P450s in 18 human liver samples. Individual P450s and catalytic activities are indicated on each chart[27]. Sample number refers to a code from this laboratory.

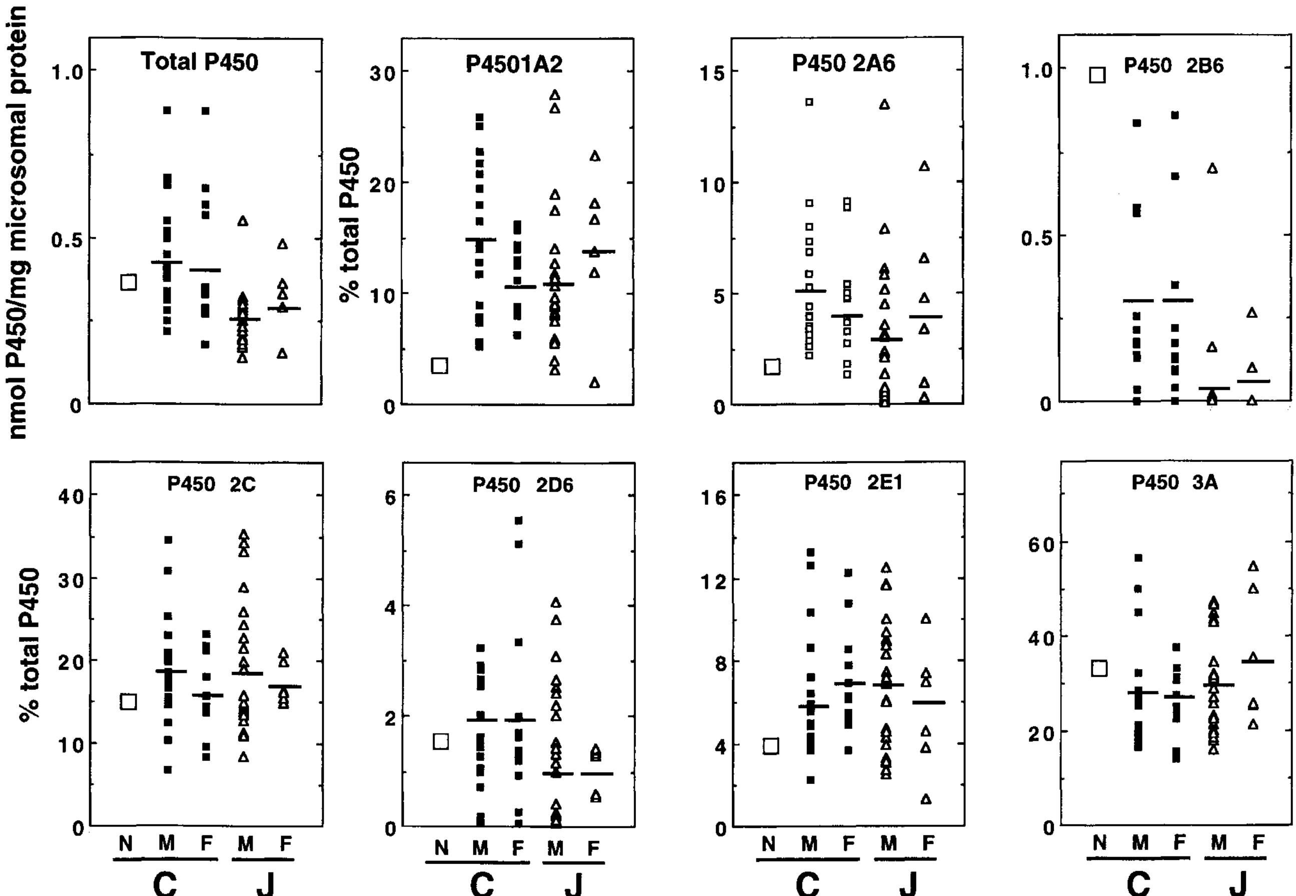

Figure 10.2. Comparison of some immunochemically determined levels of individual P450s and catalytic activities in human liver microsomes. Results from samples obtained from Caucasian (C) and Japanese (J) males (M), females (F), and a single neonatal (Japanese) sample (N) are shown[28]. The vertical axis is nmol P450/mg protein in the "Total P450" chart and percentage of total P450 in all other cases. Horizontal bars indicate mean values.

Table 10.3. Diseases Associated with Mutations in *CYP* Genes[29]

Gene	Disorder
CYP1B1	Primary congenital glaucoma (buphthalmos)
CYP4A, 4B[a]	Defects in salt metabolism, water balance leading to arterial hypertension
CYP5A1, 8A1	Defects leading to clotting and inflammatory disorders, coronary artery disease, and pulmonary hypertension
CYP7A1	Hypercholesterolemia, resistance to statin drugs
CYP7B1	Severe hyperoxysterolemia and neonatal liver disease
CYP11A1	Lipoid adrenal hyperplasia; occasional congenital adrenal hyperplasia (CAH)
CYP11B1	Occasional CAH
CYP11B2	Corticosterone methyloxidase deficiency type I, or type II; occasional CAH
CYP11B1, 11B2	Chimeric enzymes causing glucocorticoid-remediable aldosteronism; occasional CAH
CYP17A1	Mineralocorticoid excess syndromes, glucocorticoid, and sex hormone deficiencies; association with increased risk of prostate cancer and benign prostatic hypertrophy; occasional CAH
CYP19A1	Loss of function: virilization of females, hypervirilization of males, occasional CAH; gain of function: gynecomastia in young males
CYP21A2	>90% of all CAH
CYP24A1[a]	Hypervitaminosis D
CYP27A1	Cerebrotendinous xanthomatosis
CYP27B1	Vitamin D-dependent rickets type I

[a]Strong evidence of disease in animal models but not yet in clinical studies.

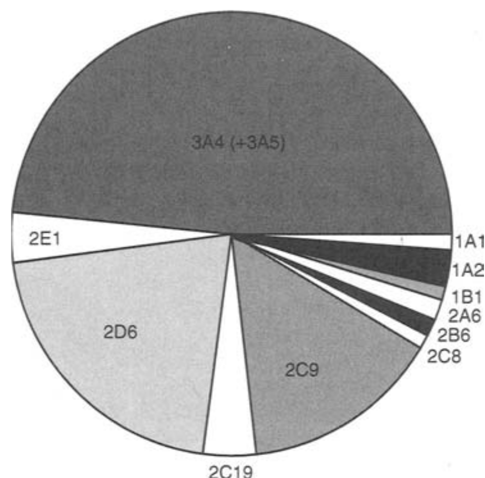

Figure 10.3. Estimated contributions of individual human P450s to the metabolism of all drugs, based upon literature available (adapted from Evans and Relling)[33].

patterns reported for human liver (Table 10.5, Figures 10.1, 10.2, and 10.4) do not necessarily apply in other tissues, some of which may be targets for carcinogens and other toxicants[37].

2. General Issues of Variability and Polymorphism

Variability in patterns of drug metabolism has been recognized for some time, even before the discovery of P450s. For instance, the phenomenon of pharmacogenetic variation had been identified by the 1950s[38, 39] and the early work of Remmer[40] showed the influence of barbiturates upon drug metabolism. Further, a number of congenital defects in steroid metabolism were known and some could be attributed to alterations in specific hydroxylations[41]. Much of the subsequent work on inducibility has been done in experimental animal models[42] and later, cell culture.

In the 1960s and 1970s, a number of accounts appeared describing variations in rates of metabolism of drugs in human liver biopsy samples[1]. The first characterization of a monogenic variability in a human drug-metabolizing P450 was the work of Smith with debrisoquine[5], which was paralleled by the work of Dengler and Eichelbaum on sparteine[43]. This polymorphism was first described in the context of extensive metabolizers (EMs) and poor metabolizers (PMs) (Figure 10.5). These

Table 10.4. Some Human P450 Enzymes involved in the Activation of Carcinogens[34] (See also Table 10.8)

P450 1A1	P450 1A2	P450 2A6	P450 2E1	P450 3A4
Benzo[*a*]pyrene and other polycyclic hydrocarbons	PhIP	*N,N*-Dimethylnitrosamine (DMN)	Benzene	Aflatoxin B_1
2-Amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP)	2-Amino-6-methyl-dipyrido[1,2-*a*:3,2′-*d*]-imidazole (Glu P-1)	*N,N*-Diethylnitrosamine (DEN)	Styrene	Aflatoxin G_1
	2-Aminodipyrido- [1,2-*a*:3,2′-*d*] imidazole (Glu P-2)	NNK	Acrylonitrile	Sterigmatocystin
	2-Amino-3-methylimidazo-[4,5-*f*] quinoline (IQ)	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butano (NNAL)	Vinyl carbamate	7,8-Dihydroxy-7,8-dihydrobenzo(*a*)pyrene and some other polycyclic hydrocarbons
	2-Amino-3,5-dimethyl-imidazo[4,5-*f*] quinoline (MeIQ) 2-Amino-3,8-dimethyl-imidazo[4,5-*f*]quinoline (MeIQx)	Nornitrosonicotine (NNN)	Vinyl chloride	17β-Estradiol
	3-Amino-1-methyl-5*H*-pyrido [4,3-*b*]indole (Trp P-2)	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	Vinyl bromide	6-Aminochrysene
	4-Aminobiphenyl		Ethyl carbamate	Senecionine
	2-Naphthylamine		Trichlorethylene	4,4′-Methylene-*bis* (2-chloroaniline) (MOCA)
	2-Aminofluorene		Carbon tetrachloride	*tris*(2,3-Dibromopropyl) phosphate
	2-Acetylaminofluorene		Chloroform	
			DMN	
			DEN	
			NNK	
			NNAL	
			NNN	
			Butadiene	

Table 10.5. Contents of Liver Microsomal P450 Enzymes in Japanese and Caucasian Populations[28]

	n	Total P450 (spectral assay)	P450 (pmol P450/mg protein [% of total P450])							Total of these 7 P450s (immunochemical sum)
			1A2	2A6	2B6	2C9	2D6	2E1	3A4	
Total	72	309 ± 175 (100)	37 ± 24 (13)	13 ± 13 (4.0)	0.68 ± 1.4 (0.15)	55 ± 28 (20)	4.5 ± 2.9 (1.7)	20 ± 13 (6.6)	87 ± 53 (29)	217 ± 107 (73)
Japanese	40	233 ± 102 (100)	26 ± 20 (12)	6.5 ± 7.3 (2.8)	0.14 ± 0.62 (0.03)	46 ± 23 (21)	3.0 ± 1.9 (1.4)	15 ± 9 (6.4)	72 ± 44 (30)	168 ± 80 (74)
Caucasian	32	406 ± 199 (100)	50 ± 22 (14)	21 ± 14 (5.6)	1.4 ± 1.8 (0.29)	68 ± 29 (18)	6.4 ± 2.8 (1.9)	26 ± 14 (6.9)	106 ± 58 (27)	277 ± 106 (73)

Total P450 contents in liver microsomes were determined spectrally and individual forms of P450 were assayed immunochemically. All values are the means and standard deviations. Parentheses indicate relative contents (% of total P450) of individual P450 forms.

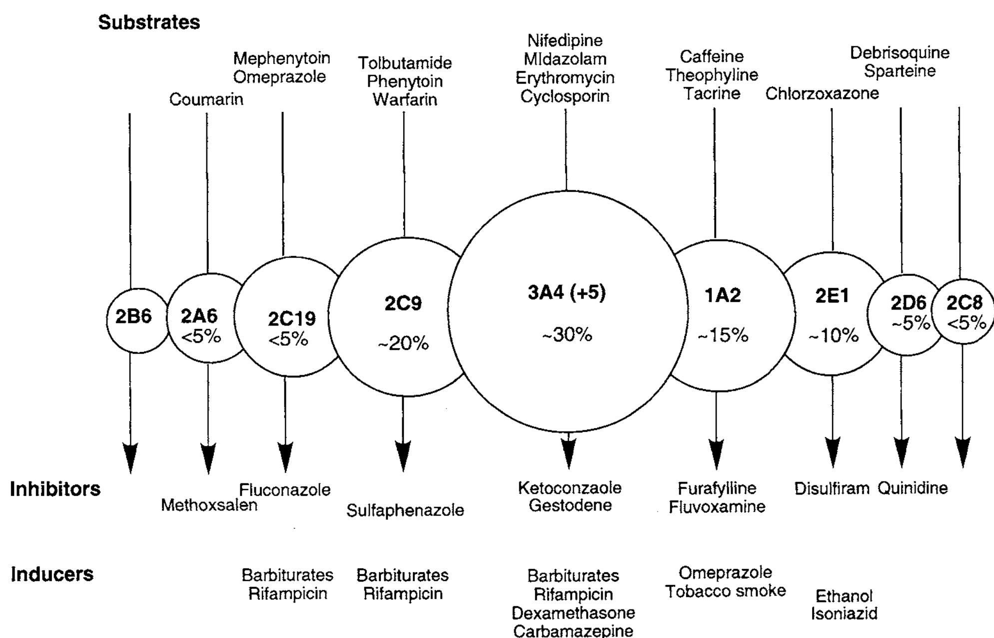

Figure 10.4. A summary of major human P450s involved in drug metabolism, including major substrates, inhibitors, and inducers (adapted from Breimer)[35, 36]. The sizes of the circles indicate the approximate mean percentages of the total hepatic P450 attributed to each P450 (See also Figure 10.1 and Table 10.5). The overlap of the circles is to make the point that overlap of catalytic action is often observed, although the overlap does not necessarily refer to the indicated substrates (or inhibitors).

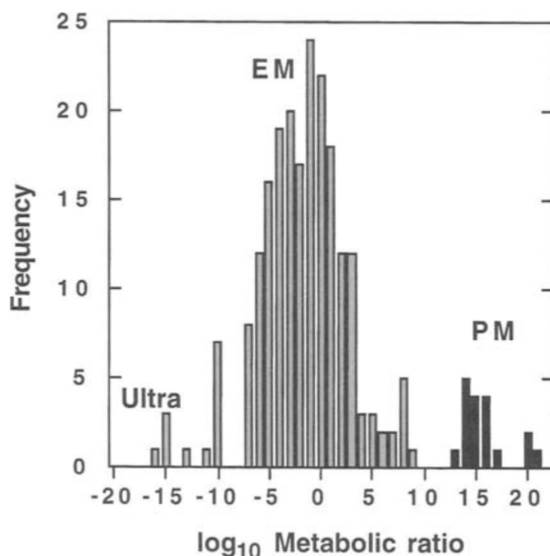

Figure 10.5. Frequency distribution histogram of (*in vivo*) debrisoquine 4-hydroxylation in a Caucasian population[44]. The metabolic ratio is the ratio of debrisoquine/4-hydroxydebrisoquine in the urine of individuals who were administered debrisoquine (10 mg free base) 8 hr previously. The groups are designated PM (poor metabolizers, solid bars) and EM (extensive metabolizers, gray bars). The group labeled "Ultra" is from retrospective research[45] and probably represents gene duplication.

polymorphisms were first studied at the level of phenotype, that is, pharmacokinetics and in some cases unusual responses to drugs due to reduced metabolism[46]. The area of pharmacogenetics (now also known as—or expanded to—"pharmacogenomics") was facilitated by the identification of the P450 enzymes involved in the drug metabolism phenotypes, and particularly by the development of molecular biology, which allows the precise characterization of genetic differences between individuals. The majority of the allelic differences are single nucleotide polymorphisms (SNPs), or single base changes. As anticipated from previous knowledge of pharmacoethnicity, many of these SNPs and polymorphisms show racial linkage. (A polymorphism is generally defined as a 1% frequency of an allelic variant in a population; below this frequency, the terms "rare genetic trait" or "rare allele" are applied or, in the case of a very detrimental allele, a mutant or "inborn error of metabolism.")

The debrisoquine polymorphism is now understood in terms of P450 2D6 and has been a prototype for research in this area. The characterization of the gene[47] led to a basic understanding of the PM phenotype. The incidence of the PM phenotype is about 7% in most Northern European populations, with different phenotypic incidence (and SNPs) in other racial groups[44, 48-50]. More than 70 allelic variants are now known, and 98% of the PMs in Northern European populations can be accounted for by four variant alleles[49, 51]. A nomenclature system has been set up for P450 alleles (using the suffixes *1, *2, *3, ...) and is maintained by Oscarson at http://www.imm.ki.se/Cypalleles/.

Several allelic variants clearly lead to the PM phenotype, for a variety of reasons. A relatively rare case is a gene deletion (*5)[52]. The most common (Caucasian) PM phenotype is an SNP that leads to aberrant RNA splicing (i.e., in splice site) and no mRNA or protein. Other alleles involve partial deletions, frameshifts, and coding for protein with either intrinsically low catalytic activity or instability (reduced halflife). These general patterns have been seen in other P450s (and other genes). In addition to the EM and PM phenotypes, there is also an "ultrarapid metabolizer" phenotype, due to gene duplication. A Swedish family has been identified with 13 gene copies, leading to 13 times more enzyme[45]. The level of hepatic P450 2D6 and a parameter of *in vivo* debrisoquine metabolism (the urinary metabolic ratio = urinary debrisoquine/4-hydroxydebrisoquine) vary $\sim 10^4$ fold among people (Figure 10.5). With P450 2D6, and several other P450s, the alleles describing the high and low levels of metabolism have been described, but the kinetic parameters for many of the alleles have not been determined by heterologous expression and measurement of catalytic activity. This is still the general case with most of the human P450s. P450 2D6 is regulated by a hepatic nuclear factor (HNF) element[53], but is not considered to be inducible by xenobiotics. With many other P450s, there is regulation and variability due to noncoding region SNPs, levels of inducers consumed, and interactions between P450s and transporters, such as *P*-glycoprotein[54, 55], may influence the phenotype.

Although the level of P450 2D6 may have a dramatic effect on the metabolism of certain drugs

Table 10.6. Some Major Inducers of Human P450 Enzymes

Class of inducers	Some sources	Example	P450s induced[a]
Ah ligands	Tobacco, broiled meat, accidental exposures	Polychlorinated biphenyls	1A1, 1A2
Barbiturates and similar compounds	Drugs, some polyhalogenated biphenyls, DDT	Diphenylhydantoin	2C, 3A4
PXR ligands	Some steroids and antibiotics, other drugs	Rifampicin	3A4
P450 2E1 inducers	Ethanol, isomiazid	Ethanol	2E1

[a]Based on *in vivo* responses.

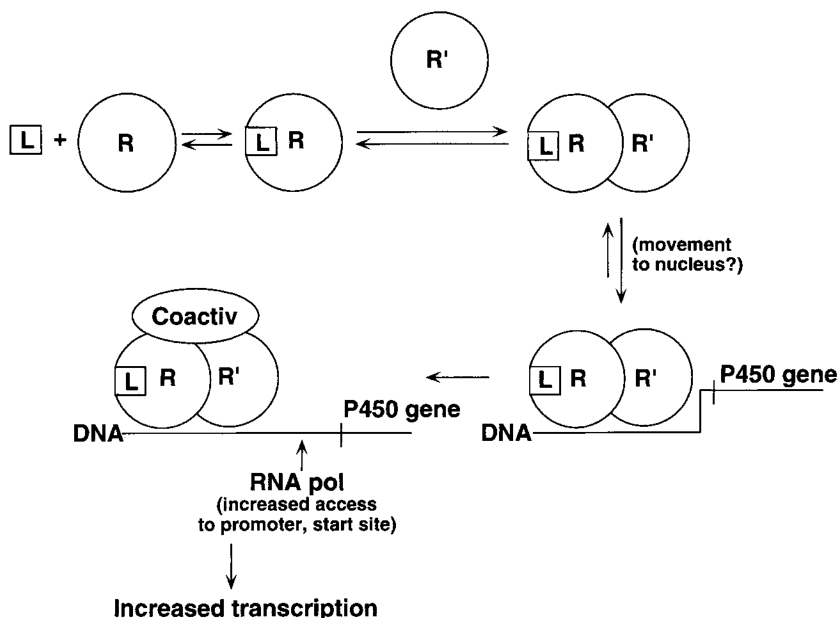

Figure 10.6. Generalized model for regulation of P450 genes by induction. L = ligand, R = receptor, R′ = partner protein for heterodimer of R, Coactiv = coactivator, RNA pol = RNA polymerase.

(Figure 10.5), no other biological changes have been reported in PMs. This appears to be the general case for many of the hepatic P450s primarily involved in the metabolism of xenobiotics, and few observable physiological effects have been reported in transgenic mice in which these genes have been deleted[31]. As pointed out earlier, however, deficiencies in some of the steroid-hydroxylating P450s can be very debilitating or lethal[41]. In general, the variation in the levels of these "more critical" P450s is limited in most of the population, compared to the xenobiotic-metabolizing P450s in which an order of magnitude variation is not unusual[28].

The influence of inducers on the expression of each P450 will be mentioned later (Table 10.6, Figure 10.6). It should be pointed out that several of the P450s can be downregulated by cytokines, and the result has practical significance in the impairment of drug metabolism in individuals with colds or flu, or who have received vaccinations[56]. Another general point to make is that, in contrast to some animal models (see Chapter 8)[57], human P450 expression shows little if any gender differences. When developmental differences are seen in humans, they tend to be relatively soon after birth (e.g., P450 3A4, 3A7, see refs [58], [59]), and changes in expression seen in the elderly have not been very dramatic[60–62].

3. Approaches to Defining Catalytic Specificity of Human P450s

Knowledge of the roles of individual P450s in specific reactions is critical in the application of P450 biochemistry to practical issues in drug metabolism. Originally some of the P450s were purified on the basis of their catalytic activities toward certain specific drugs[7, 9, 11, 12], but even in those cases, there are the issues of the extent of contribution of that form and the involvement of that P450 in other reactions, particularly with new drugs. Identification of the individual P450s contributing to the metabolism of a new drug candidate is routinely done in the pharmaceutical industry. This information is usually required by the Food and Drug Administration at the time of

application. Identifying P450s involved in oxidation is important in predicting drug–drug interactions and the extent of variation in bioavailability. In general, it is desirable to develop drugs for which several P450s have a contribution to metabolism. Drug candidates that are metabolized exclusively by a highly polymorphic P450 (e.g., 2D6, 2C19) are usually dropped from further development.

A combination of methods involving the use of human tissues and recombinant human P450s is usually used to identify P450s involved in a particular reaction, using an approach outlined earlier[23, 34]. A combination of the following methods is usually done, not necessarily in a particular order. Lu[63] has recently reviewed these approaches.

3.1. Inhibitors

The reaction is demonstrated in NADPH-fortified human liver microsomes (if the reaction of interest is restricted to another tissue, then this tissue would be used instead). The effects of selective inhibitors on the reaction are examined. A list of some of the inhibitors that have been used is presented elsewhere in this volume by Correia (Chapter 7)[64, 65].

The choice of concentration parameters is important in this and some other approaches. Ideally the effect of the substrate concentration on the rate of catalytic activity should be determined in the absence of inhibitor to determine V_{max} and K_m parameters. If this information is available, the inhibition experiments are best done with a concentration of substrate at or below the K_m, in order to observe the effect of the inhibitor on the ratio V_{max}/K_m, which is the parameter usually most relevant to human drug metabolism. If the V_{max} and K_m information is not available, an alternative is to select a substrate concentration near that expected for the *in vivo* plasma concentration ($C_{p,max}$ or less).

With regard to inhibitor concentration, ideally a range of concentrations would be used. However, if a single concentration of the diagnostic inhibitor is used, it must be selected on the basis of previous literature because nonselective effects are often observed. For instance, α-naphthoflavone (αNF) (5,6-benzoflavone) can inhibit P450s other than 1A2 at high concentrations[66] and azoles inhibit many P450s at higher concentrations[64, 65]. Use of a titration approach (concentration dependence) has merit[63].

Another general issue is the selection of a protein concentration. Microsomal proteins can bind drugs in a nonselective manner and effectively lower the free concentration of substrate or inhibitor[67, 68], which can influence the interpretation of results. Another point is that the concentration of the P450 of interest should be less than that of the drug and the inhibitor, in order for the basic assumptions about steady-state kinetics to apply (and for the reaction to remain linear during the incubation time, although some of the inhibitors are mechanism-based and the loss of activity will be time dependent, requiring preincubation). A corollary of these latter points, which also apply to the other approaches that follow, is that having a very sensitive assay method is very desirable. Thus, methods such as HPLC/fluorescence and particularly HPLC/ mass spectrometry have gained popularity.

Finally, the choice of an organic solvent is an issue. Ideally the substrate should be dissolved in H_2O or very little organic solvent, but this may not be possible with many drugs. Several examinations of the effects of individual solvents on human P450s have been published[69, 70].

In principle, the extent of inhibition of a reaction by a P450-selective inhibitor indicates the fraction of that reaction attributable to that P450. For instance, if a 1 μM concentration of quinidine (a P450 2D6 inhibitor) inhibits 50% of a reaction, then 50% of that reaction may be attributed to P450 2D6. If one desires a more global view than within a single liver sample, then a pooled set of microsomes (e.g., from 10 samples, balanced on the basis of liver weight or protein) may be used for the inhibition assays. However, if one desires to examine the differences among individuals in terms of the contribution of a P450, then doing several experiments with individual liver samples is the approach to use.

3.2. Correlations

Another approach with a set of human tissue microsomal samples is to measure the new reaction of interest in each and attempt correlation with rates of marker activities (for individual P450s). Lists are also published in this volume in Chapter 7 by Correia[65] and elsewhere[71].

Correlation can be done by plotting the specific activity for the new reaction vs the marker reaction (Figure 10.7). In principle, the correlation coefficient r^2 estimates the fraction of the variance attributable to the relationship between the two activities, that is, the fraction of the activity catalyzed by the particular enzyme (assuming that all of the marker activity is catalyzed by this enzyme). In some cases, excellent correlations have been reported[72, 73]. An alternative method of analysis is the Spearman rank plot, which has some deficiencies but avoids the overweighting of unusually high or low values[74].

Although the approach works well when high correlation coefficients are generated, the method is less useful when several P450s contribute to a reaction, that is, $r^2 < 0.4$. The results should, in all cases, be considered in the context of results obtained with other approaches.

3.3. Antibody Inhibition

The points raised in the Section 3.1, Inhibitors, apply to antibodies as well. Antibodies are used to inhibit activities in human liver (or other tissue) microsomes and are of several general types: (a) polyclonal antibodies raised against purified animal P450s, (b) polyclonal antibodies raised against purified human P450s, (c) monoclonal antibodies raised against purified human P450s, (d) polyclonal antibodies raised against peptide fragments of P450s, and (e) antibody phage display library antibodies selected for recognition of individual P450s.

At this time, almost all antibodies raised against intact P450s have been generated using recombinant P450s (or against peptides), in contrast to early work in the field with P450s isolated from liver. Another point to make is that not all antibodies inhibit catalytic activity. Further, specificity in one immunochemical assay (e.g., electrophoretic/ immunoblotting) does not necessarily implicate specificity in another (immunoinhibition).

Three points should be made in designing immunoinhibition experiments. (a) The concentration of antibody should be varied and increased to the point where the extent of inhibition is constant. (b) A nonimmune antibody should be used as a control, using the same concentrations as with the antibody raised against the P450. (c) The antibody should be shown not to inhibit a reaction known to be attributable to other P450s. Immunoglobulin G fractions are generally preferred in that they produce less nonspecific inhibition than crude preparations such as sera. Polyclonal antibodies can vary in their specificity and titer from one animal to another and from one bleed to another, so constant properties cannot necessarily be assumed. In principle, monoclonal antibodies and antibodies eluted from phage display libraries should not vary, although this has not always been the case with monoclonals.

In general, antibodies are often selective for individual P450 subfamilies, for example, 1A vs 1B vs 2A vs 2B vs 2C, etc., but cross-reaction among families can be detected, and in some cases the (P450) sites of cross-reactivity have been identified[75]. Achieving selectivity among individual P450 subfamily members (e.g., P450 3A4 vs 3A5 vs 3A7) is more difficult. With polyclonal antibodies, this can be achieved by cross-adsorption[76]; with monoclonals and phage display libraries, this can be done by selection. The point should be made that any selectivity demonstrated among classes of animal P450s (e.g., rat P450 families) cannot be assumed to carry over to human P450s.

Antipeptide antibodies have become popular in recent years and have two major advantages: (a) peptides can be synthesized and readily purified by HPLC, avoiding the need to express and rigorously purify P450 proteins (although demonstration of purity by HPLC, capillary electrophoresis, and mass spectrometry is still in order), and (b) peptides can be selected for use as antigens by sequence comparisons, favoring specific regions.

Phage display antibody libraries are relatively new and have been used in a few P450 applications to date (D.S. Keeney personal communication). These have a number of advantages, including potential selectivity due to the large number of potential antibodies in libraries, the ability to avoid animal protocols, the immediate availability of libraries (as opposed to waiting on animals to develop antibodies), the consistency of reproduction of the proteins propagated in bacterial systems, and the ability to include a second "epitope tag" for recovery, etc.

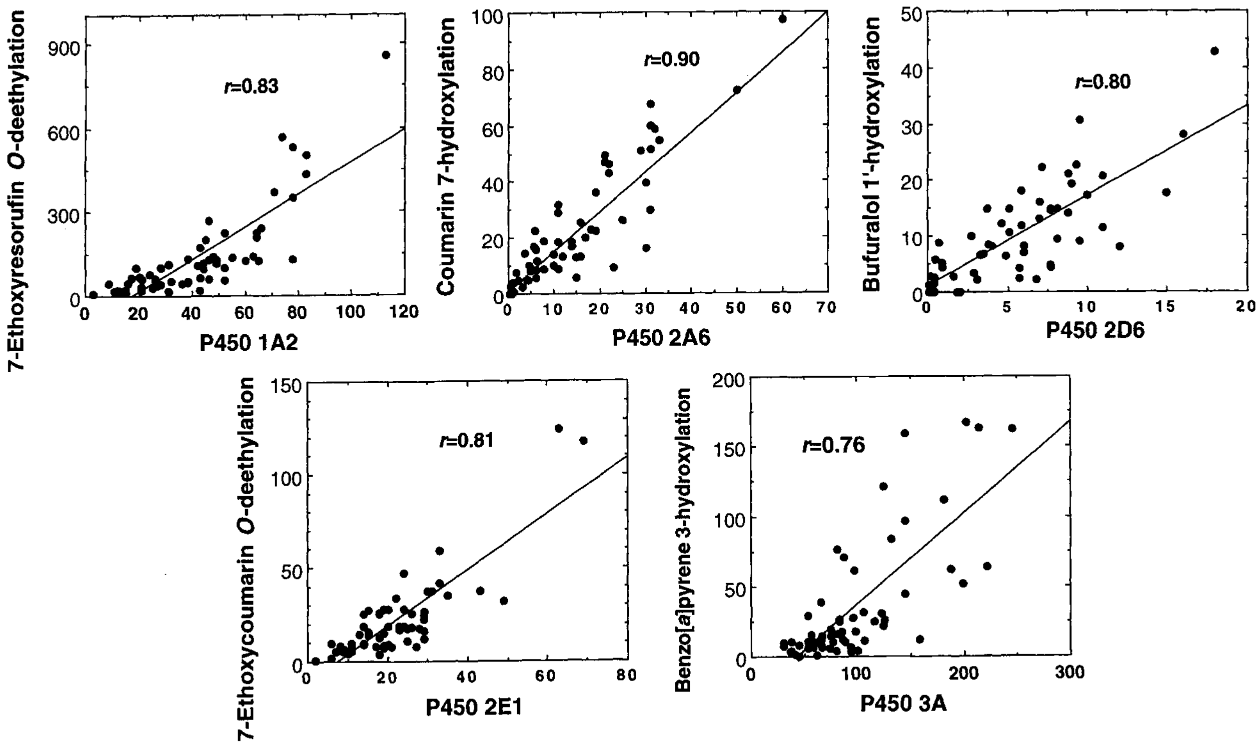

Figure 10.7. Correlation of catalytic activities with immunochemically determined levels of five P450s in human liver microsomes. The correlation coefficients (r) were determined using linear regression analysis[28].

3.4. Demonstration of Reaction with Recombinant P450

In early work in this field, this point would have been the demonstration of the reaction of interest with an enzyme purified from tissue. Today P450 proteins are generally produced in recombinant systems and seldom purified from tissue sources. In routine practice in the pharmaceutical industry, new reactions are examined with a battery of the major recombinant human (liver) P450s, many of which are available from commercial sources. Systems used for expression include bacteria, yeast, baculovirus (-infected insect cells), and mammalian cells. The P450s need not be purified for these comparisons but must have suitable provision for NADPH-P450 reductase in a crude system (and cytochrome b_5 [b_5] in certain cases).

Usually activity results obtained with several of the major P450s are compared to each other and to those obtained with tissue microsomes, in order to put the work in context. Ideally assays are done at several substrate concentrations and the parameters k_{cat} (V_{max}) and k_{cat}/K_m are obtained. These values should be normalized on the basis of P450 concentration, in that any values based on milligram protein for the expression system cannot be used for comparisons with tissue microsomes. In principle, the k_{cat} (total P450 basis) should be at least as high for the recombinant reaction as for the tissue microsomes. A more realistic way to make a comparison is to immunoquantify the amount of the particular P450 in the tissue microsomes and then use this value in correcting the microsomal k_{cat} for comparison with the recombinant system. The matter of scaling these parameters to generate predicted microsomal (or *in vivo*) rates from *in vitro* experiments with recombinant enzymes is not trivial, but a number of efforts have been made[77–80].

4. Relevance of P450s in *In Vivo* Drug Metabolism

P450s are the major enzymes involved in human drug metabolism. In looking at the fraction of the number of drugs processed by "Phase I" enzymes (Figure 10.3), P450s account for ≥80% (and the number is even higher if one moves the esterase and epoxide hydrolase reactions to the Phase II group because they are not involved in redox reactions). Constructing a figure of this type can be somewhat misleading in that the contribution of each P450 is more difficult to evaluate *in vivo* than *in vitro* (for a more original tabulation, see ref. [32]). The large contributions of P450s 3A(4) and 2C9 are driven to a large extent by the high levels of expression of these two enzymes in human liver (and small intestine) and to their broad substrate specificity. The charts do not necessarily reflect all drugs currently in development. A current tendency has been the development of larger molecules as drug candidates, in order to achieve target specificity and affinity, and a general axiom is that these are more readily accommodated by P450s 3A(4) and 2C9. In recent years, pharmaceutical companies have tried to avoid developing drug candidates that are substrates (or inhibitors) for the highly polymorphic P450s 2D6 and 2C19. With all of these caveats in hand, the allocation of the chart in Figure 10.3 is probably a good estimate and may not change considerably in the near future. However, a point to be made here is that the metabolism of many drugs is a function not only of P450s but also of other enzymes and, as recognized more in recent years, transporters that alter the concentrations of drugs within cells. A discussion of drug transporters is outside the scope of this chapter, and the reader is referred elsewhere[81].

The subjects of P450 regulation and polymorphism (or mutation in some cases) have already been mentioned, and will be treated again, with individual P450s. At this point, some general practical considerations will be discussed. If one considers the total concentration of P450 in liver samples from different healthy individuals (on a milligram protein basis), most individuals fall within a range of ~3-fold[1]. However, when individual "drug-metabolizing" P450s (e.g., families 1, 2, 3) are considered, the variation is considerable, with 5–10-fold being common and 40-fold not unusual, for example, P450 1A2 (ref. [73]). With P450 1A2, a similar variability (40-fold) is seen in *in vivo* caffeine pharmacokinetics[82]. With highly polymorphic enzymes, the variability in the same *in vivo* pharmacokinetic parameters can be as much as 10^4-fold (Figure 10.5).

Two examples of studies of the variability among individuals are presented in Figure 10.5

(Caucasians) and Figure 10.2 (Caucasian and Japanese). Gender has not been shown to have a major influence on levels of expression of the major xenobiotic-metabolizing P450s, and inter-gender pharmacokinetic differences are probably due to other influences on bioavailability or volume of distribution[55]. Racial differences exist due to allelic variations, which may influence either levels of expression or the inherent catalytic activity of the P450s [ref. [49]). Some apparent racial differences are seen here (Figure 10.2) and have also been reported in *in vivo* studies (e.g., 3A4 (ref. [83]), 2E1 (ref. [84])). Controlling diets is an issue in many *in vivo* studies of this type, and *in vitro* studies can also be affected. In general, the differences in activities of a given P450 between races are much less than within a race (e.g., Figure 10.2). Finally, the point made above should be noted that the levels of the P450s involved in steroid metabolism (e.g., families 11, 17, 19, 21) vary considerably less than do the xenobiotic-metabolizing P450s (families 1, 2, 3), probably due to their well-defined roles in regulation of physiological processes.

Many chemicals are capable of inducing P450s, as clearly demonstrated in animals and with cell culture systems[85]. *In vivo* induction experiments with humans are not as readily done as with animals, but ample evidence for P450 induction is available, going back to the barbiturate observations of Remmer in the 1950s[40]. A list of some established P450 inducers is presented in Table 10.6. This list is rather conservative in that only information is included from studies in which *in vivo* evidence has been obtained. Many of the studies have involved pharmacokinetics, but some "moderately invasive" studies have involved direct measurement of proteins, mRNA, or enzyme activities in peripheral blood cells or small intestinal biopsies; liver biopsy data is rare. Table 10.6 could probably be expanded considerably if all information from *in vitro* studies were included, for example, P450s 1B1 and 2S1 are probably inducible by Ah ligands[86, 87]. The major problem in demonstrating human P450 induction *in vivo* is the lack of diagnostic pharmacokinetic parameters for many of P450s.

The clinical influence of differences in P450 activity can be rationalized using the scheme of Figure 10.8. In this model example, the drug doses have been developed with the EMs as the general population of interest. The plasma concentration rises to a peak ($C_{p,max}$) following the first dose and then decreases to a lower level prior to the next dose. With subsequent doses, the plasma concentration remains within this region and yields the desired pharmacological effect. Without prior knowledge about a problem with this drug, the PM (lower panel of Figure 10.8) would be administered the same doses. Very limited metabolism would occur between doses, and the plasma concentration of the drug (and presumably the concentration of the drug in the target tissue) will rise to an unexpectedly high level, with an attendant increase in the area under the curve (AUC). The simplest effect would be an exaggerated (and probably undesirable) pharmacological response. One can also imagine a situation in which metabolism is more rapid than expected in the typical patient, for example, due to gene amplification or enzyme induction. In this case, $C_{p,max}$ and AUC would be smaller than in the case of the EM (Figure 10.8, upper panel), and decreased drug efficacy would be expected.

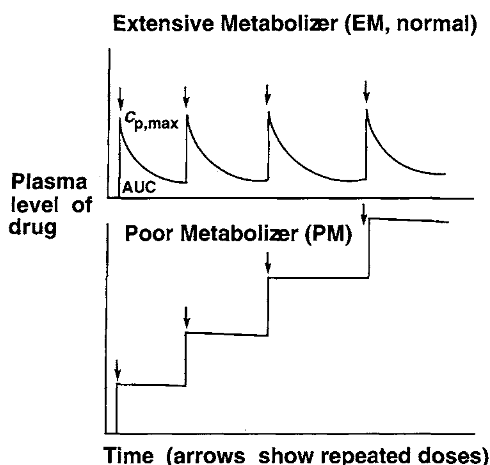

Figure 10.8. Significance of low metabolism of a drug by P450s (or other enzymes). A "typical" pattern is seen in the upper panel (EM), where the plasma level of the drug is maintained in a certain range when a particular repetitive dose is prescribed. Unusually slow metabolism (lower panel, PM) results in an elevated plasma level of the drug. $C_{p,max}$ = maximum plasma concentration; AUC = area under the curve.

Some practical situations follow. With regard to polymorphisms, several are known that can render some drugs dangerous due to toxicity

(e.g., perhexiline, leading to peripheral neuropathy due to lack of metabolism by P450 2D6 (ref. [88]) or can alter the recommended dose (e.g., warfarin/P450 2C9 (refs [89–91]) and omeprazole/P450 2C19 (refs [92], [93]). Drug interactions are a serious problem, and pharmacokinetic interactions have several molecular bases. One is enzyme induction, which usually results in decreased bioavailability. The decreased bioavailability of a drug can be the result of induction by that same drug or by another drug. A classic example is the decreased bioavailability of the oral contraceptive 17α-ethynylestradiol following treatment of individuals with rifampicin, barbiturates, or St. John's wort and consequent P450 3A4 induction[72, 94, 95]. Another aspect of drug–drug interactions involves P450 inhibition. The inhibition can be of a competitive nature, that is, two substrates competing for a limiting amount of a P450 or a *bona fide* inhibitor (no enzymatic transformation) competing with substrates. An example here is the antihistamine terfenadine, the metabolism of which is inhibited by the P450 3A4 inhibitors, erythromycin and ketoconazole. Another major type of P450 inhibition is "mechanism-based" (or "suicide") inactivation, in which oxidation of a substrate destroys the P450 (refs [64], [96]). An example here is the inactivation of P450 3A4 by bergamottin and other flavones found in grapefruit juice[97–100].

In the above cases, the effects have been discussed only in terms of altered bioavailability; that is, with increased clearance of 17α-ethynylestradiol, unexpected menstruation and pregnancies have resulted[95, 101, 102]. Some of the drug interaction problems can be more complex, even when the analysis is restricted to pharmacokinetic aspects. For instance, in the example mentioned above, terfenadine can be considered a prodrug[103]; in most individuals, the P450 oxidation (followed by further oxidation) yields fexofenadine, the circulating form of the drug. Low levels of P450 3A4 activity (due to inhibition or other reasons) cause the accumulation of the parent (prodrug) terfenadine to toxic levels that can cause arrhythmia[103, 104]. Another possibility is that blocking a primary route of metabolism of a drug may favor secondary pathways that lead to toxicity, for example, blocking phenacetin *O*-deethylation (P450 1A2) can lead to deacetylation, *N*-oxygenation, and methemoglobinemia[105]. Although a good example is not available, it is possible that blocking the oxidation of one drug by a P450 could cause it to accumulate and behave as an inhibitor toward another. A potential example would be decreasing the P450 3A4-catalyzed oxidation of quinidine and having the accumulated drug inhibit P450 2D6 (ref. [106]). P450 induction could result not only in decreased oral availability but also in the enhanced bioactivation of chemicals. This is a general concern with potential carcinogens, as discussed in the next section of this chapter, and one of the reasons why regulatory agencies have concerns about P450 1A inducers.

The phenomenon of P450 stimulation has been studied in some detail *in vitro*[107]. By stimulation we mean the enhancement of P450 catalytic activity by the direct addition of another compound, outside of a cellular environment in which gene regulation is involved. Some aspects of P450 stimulation will be treated under the topic of P450 3A4 (Section 6.20.4), with which much of the work has been done. An open question is whether such behavior occurs in humans. At least four pieces of evidence suggest that such behavior is possible: (a) cooperativity has been reported in hepatocyte cultures[108], (b) an early experiment with neonatal mice (individual P450s unknown) by Conney's group indicated the immediate enhancement of an activity by flavones[109]; (c) the work of Slattery and Nelson with rats showed an interaction between caffeine and acetaminophen that implies such behavior[110]; and (d) quinidine enhanced the *in vivo* oxidation of diclofenac in monkeys, in a manner consistent with *in vitro* human work[111]. If stimulation does occur *in vivo*, it is a phenomenon that has been very difficult to predict (even *in vitro*), and in the case of P450 3A4 substrates, the situation would probably be further complicated by issues involving *P*-glycoprotein behavior (and *P*-glycoprotein also shows cooperativity of its own[112]).

In the process of drug development, there are three guiding principles to dealing with P450 metabolism, aside from details of each specific case: (a) use *in vitro* screening to delete compounds that will have poor bioavailability (i.e., rapid *in vitro* oxidation); (b) use *in vitro* screens to avoid obvious problems of toxicity, induction, and inhibition; and (c) seek drug candidates in which the metabolism is the result of several different enzymes and not dependent upon a single one,

particularly a highly polymorphic P450 (or another highly polymorphic enzyme).

5. Relevance of P450s in Toxicology and Cancer Risk

Historically much of the attention given to P450s has come from the interest in cancer, going back to some of the first demonstrations of redox reactions in the metabolism of chemical carcinogens[113] and the inducibility of P450s by carcinogens[42]. The interest in P450s was also extended to chemical toxicities other than cancer with the demonstration of bioactivation of compounds such as the drug acetaminophen[114] and the insecticide parathion[115, 116]. Many studies have been done with P450 animal models, particularly using P450 inducers and inhibitors and genetically modified mice, either naturally occurring or transgenic. These studies provide strong evidence that alterations in the activities of P450s can modify the sensitivity of mice to various chemicals. For instance, the *Ah* locus (which controls P450s 1A1, 1A2, and 1B1 as well as some Phase II enzymes) can modify the sensitivity in Ah receptor-deficient mice, depending upon the chemical *and* the organ site[117]. Effects of specific P450 knockouts have been reported in transgenic mice as well, for example, prevention of acetaminophen toxicity by deleting P450 2E1 (ref. [118]) and of 7, 12-dimethylbenz[*a*]anthracene-induced lymphomas by deleting P450 1B1 (ref. [119]).

Despite the strong evidence for effects of variability of P450 on chemical toxicity and cancer risk in animals and the knowledge that human P450 levels vary considerably (Figures 10.1, 10.2, 10.5, and 10.7), demonstrating relationships with human disease has been difficult. In the 1960s, the demonstration of the inducibility of aryl hydrocarbon hydroxylase (thought to be what is now known as P450 1A1) by Nebert and Gelboin[120] led to more investigations with human samples, particularly peripheral blood cells. The work of Shaw and Kellerman[121, 122] suggested that the inducibility of aryl hydrocarbon hydroxylase (now recognized as P450 1A1 and 1B1 under these conditions) is correlated with susceptibility of smokers to lung cancer. In the early work, this apparently genetic variability was trimodal. Subsequently, this phenomenon has proven difficult to study, in part due to technical difficulties in the earlier phases of the work[123]. Many of the early problems have been circumvented with the ability to measure mRNA expression and the access to DNA sequences. While evidence for correlation of P450 1A1 mRNA expression with lung cancer incidence has been obtained[124], an unresolved issue is the nature of any genetic variability. In contrast to the situation seen in mouse models[125], the allelic variations in the human *Ah* receptor (which has apparently considerably lower affinity for many of the ligands of interest than the mouse receptor[126]) do not appear to account for interindividual levels of inducibility of P450 1A1 (refs [127], [128]). Kawajiri's laboratory has presented epidemiological evidence for association of lung cancer incidence with an *Msp*I polymorphism of P450 1A1 (ref. [129]). However, these results, from studies done with Japanese, have not been reproducible in Caucasians (refs [130–132]). Further, the heterologously expressed human P450 1A1 allelic variant (V462I) showed only a relatively small change in oxidation of the prototype polycyclic aromatic hydrocarbon carcinogen benzo[*a*]pyrene diolepoxide[133, 134]. A recent explanation to the quandary comes from the work of Kamataki's group, who have shown that P450 1B1, not P450 1A1, is the major P450 responsible for the aryl hydrocarbon hydroxylation activity in lymphocytes and that it is P450 1B1 expression that shows the classic trimodality, not P450 1A1, (ref. [135]).

Today the field is such that the search for roles of a particular P450 in human disease follows a route similar to that just discussed for P450 1A1, that is, the identification of SNPs is a basis for epidemiological associations with various maladies. This approach is commonly applied to the possible roles of P450s in cancers at various organ sites. The general concept is also utilized for other diseases and is the major basis for the Environmental Genome Project of the National Institute of Environmental Health Sciences (which includes many other gene candidates in addition to P450s)[136]. The positive aspects of this strategy are that we have an extensive knowledge base of allelic variations of P450s (e.g., http://www.imm.ki.se/CYPalleles/), sophisticated and very sensitive biological tools, and the potential to noninvasively analyze large populations, at least in the case of some diseases and P450s. On the negative side, the ability to rapidly screen for associations without serious thought about chemical exposure

levels has lead to many studies with little or only marginal biological plausibility. Many association studies have been difficult to repeat. An example in point is the reported association of attenuated lung cancer risk (of smokers) with the P450 2D6 PM phenotype. Although the initial reports were quite exciting[137], subsequent studies yielded variable results, and meta analysis has not supported an association[138]; moreover, no real experimental support for a biological association was ever found[139]. A recent review by Vineis[140] concludes that the risks of cancer due to genetics are considerably less than those associated with smoking or other environmental factors.

What associations of P450 have been adequately demonstrated? The list below is short and not intended to necessarily be totally inclusive, but emphasizes some of the more positive associations found to date. (The absence of several of the steroid-oxidizing P450s is known to be debilitating [Table 10.3], but these are not treated here; see the sections on individual P450s and ref. [29].) The possible association between P450 1A1 and lung cancer has already been discussed above; a confounding factor may be expression of P450 1B1. Truncation of P450 1B1 is associated with glaucoma, for unknown reasons[141]; this defect has not been seen in the P450 1B1-knockout mice[31, 119]. Allelic variants in P450 1B1 do not appear to have major effects in the oxidation of carcinogens[142]; some differences in cancer risk have been reported in the epidemiology literature[143, 144]. P450 1A2 activity has been reported to be associated with colon cancer incidence, when the factors of *N*-acetyltransferase and well-done meat intake are considered[145]; an association has plausibility in the activation of heterocyclic amines by P450 1A2 (ref. [146]). One of the strongest associations reported to date involves that of P450 2A6 with lung cancer; the association is driven by the data obtained with individuals with the gene deletion[147]. A relationship is plausible due to the demonstrated ability of P450 2A6 to activate *N*-nitrosamines (Table 10.4), and possibly via the decreased smoke intake of null-type individuals due to impaired metabolism of nicotine[148] (see Section 6.4.6). Although many epidemiological studies have been done with SNPs of P450 2E1, any putative changes in P450 2E1 phenotype have not been validated with *in vivo* assays and must be considered suspect[149].

In the process of drug development, the induction of P450 1 and P450 2B enzymes (in animals or in human cell or reporter assays) has often been considered an issue for potential toxicity[150, 151]. The concern about induction is that the rodents may be likely to develop liver or other tumors in cancer bioassays with these compounds, and any association between these inductions and human cancer is not established; for example, epileptics with long-term exposure to barbiturates and hydantoins have not been found to have more cancer[152]. Likewise, the induction of P450 4A is an indicator of peroxisomal proliferation, a phenomenon associated with rodent liver tumors but probably not human[153]. Thus, induction of rodent P450s has been shown to be a means of identifying types of potential rodent toxicity[154], some of which may be relevant to humans, but should not be used as evidence for adverse roles of these agents in humans.

6. Individual Human P450 Enzymes

Each of the 57 human P450s will be covered here. Clearly much more information is available about some than others. Points to be covered with each, when possible, include sites of expression and relative abundance, polymorphism and inducibility, substrates and reactions, knowledge of important residues and active site characteristics, inhibitors, and clinical issues.

6.1. P450 1A1

6.1.1. Sites of Expression and Abundance

The gene has seven exons, and the cDNA region is ~70% identical to that of the closest relative, P450 1A2. P450 1A1 is expressed in fetal liver but not at appreciable levels in adult liver[155–157]. P450 1A1 can be induced in primary human hepatocyte cultures[158]. The dominance of P450 1A2 over 1A1 *in vivo* may be due to preferential induction of P450 1A2 > 1A1 at low doses of inducers (a phenomenon established in rats[159]) or the presence of factors in liver that are not preserved in hepatocyte cultures.

P450 1A1 is expressed in human lung and has been partially purified[6]. A recent estimate of a median level of P450 in human lung is 6.5 pmol/mg microsomal protein ($n = 7$) and 16 pmol/mg microsomal protein for smokers ($n = 18$)[160]. The variation in levels of P450 1A1 is very high (>100-fold)[6, 160], as suggested from earlier work in which only benzo[*a*]pyrene hydroxylation was used as an indicator[161].

P450 1A1 is also expressed in placenta[162] and peripheral blood cells (lymphocytes, monocytes)[163], and these tissues have been used in many studies. Expression (at least at the mRNA level) has been reported in a number of other extrahepatic tissues including pancreas, thymus, prostate, small intestine, colon, uterus, and mammary gland[164].

6.1.2. Regulation and Polymorphism

Polymorphism in the inducibility of benzo[*a*] pyrene hydroxylation activity has attracted considerable interest following the reports of Shaw and Kellerman[121, 122] that the induction in lymphocytes of smokers can be associated with susceptibility to lung cancer. The link to lung cancer has been studied extensively but few general conclusions can be reached. Smoking clearly induces levels of lung P450 1A1 (refs [124], [160], [165]). Some epidemiological investigations link the *2A (*Msp*I) and *2B (I462V) polymorphisms to lung cancer incidence in Japanese[129], but this association has not been reproducible in other studies with Caucasians[130, 131]. These two alleles are in linkage disequilibrium[132]. Two studies with recombinant human P450 1A1 have not shown a major difference in any catalytic activities due to the substitution at codon 462 (refs [133], [134]). Although there is a general consensus that phenotypic variation in the inducibility of P450 1A1 is observed, extensive searches have not associated the inducibility with any known polymorphisms in the P450 1A1, Ah receptor, or arylhydrocarbon nuclear translocator (ARNT) genes[166, 167].

The induction of P450 1A1 has been studied extensively and is discussed elsewhere in this book[168]. Briefly, the Ah receptor resides in the cytosol and, when activated by binding of an appropriate agonist, loses the accessory protein Hsp90 and dimerizes with the ARNT protein, moving to the nucleus and interacting with an XRE element to initiate transcription (Figure 10.6, with R = Ah receptor), R^1 = ARNT, and L = TCDD or other inducers. A number of details regarding this scheme remain to be elucidated, such as roles of coactivators, whether an endogenous ligand exists and if so what it is, etc. The list of inducers reported from *in vitro* studies includes TCDD and is quite long. The list of compounds for which *in vivo* evidence of induction is more limited, but it is generally accepted that it includes cigarette smoke, heterocyclic amines, polychlorinated biphenyls[169], and some drugs (e.g., omeprazole[170]).

6.1.3. Substrates and Reactions

This enzyme was first explored in the context of an aryl hydrocarbon hydroxylase, using fluorescence assays that measured primarily the 3-hydroxylation of benzo[*a*]pyrene[120]. (It should be noted that the fluorescence assay also detects other fluorescent products, for example, 9-hydroxybenzo[*a*]pyrene, and that other P450s also catalyze the 3-hydroxylation reaction, for example, P450 2C9 in human liver[171].) Another classic model reaction used for P450 1A1 is 7-ethoxyresorufin *O*-deethylation[172, 173]. Human P450 1A1 oxidizes benzo[*a*]pyrene to a variety of products[174, 175]. Many other polycyclic hydrocarbons are substrates for P450 1A1 and have been studied extensively[176, 177]. Some heterocyclic and aromatic amines can also be activated by P450 1A1 (ref. [178]). P450 1A1 does not appear to play a major role in the metabolism of many drugs, possibly because of its locations of expression.

6.1.4. Knowledge about Active Site

Relatively little is known about the active site of P450 1A1. Early work on pharmacophore models for rat P450 1A1 was done by Jerina's group[179]. The early modeling of substrates and inhibitors suggested that P450 1A1 ligands were relatively planar. Some homology modeling has been done by Lewis[180], although little has been done with site-directed mutagenesis on the roles of individual amino acids. The lack of effect of interchanging Val and Leu at position 462 has already been mentioned[133, 134].

6.1.5. Inhibitors

Despite the long interest in this enzyme, the list of inhibitors is relatively short, and many inhibitors show overlap with P450s 1A2 and 1B1 (ref. [181]). For instance, αNF is often used as an inhibitor but is more effective against P450 1A2 (refs [181], [182]). Another inhibitor is ellipticine[64]. 1-(1′-Propynyl)pyrene and 2-(1-propynyl) phenanthrene were found to be selective P450 1A1 inhibitors when compared with human P450s 1A2 and 1B1 (ref. [181]).

6.1.6. Clinical Issues

Due to a rather limited role of P450 1A1 in drug metabolism, there are no real pharmacokinetic issues. The issue with P450 1A1 is induction and a possible role in chemical carcinogenesis. Work with animal models shows that P450 1A1 inducers can be co-carcinogens[85, 117]. Thus, regulatory agencies tend to look unfavorably at induction of P450 1A1 by potential drugs in animal models. However, the point should be made that there is presently little experimental or epidemiological evidence to support this hypothesis, and Ah inducers can afford protection from cancer in some animal models[117].

6.2. P450 1A2

6.2.1. Sites of Expression and Abundance

As mentioned earlier, human P450s 1A1 and 1A2 both have seven exons and 70% sequence identity in their coding regions. Both these genes show similar patterns of regulation by the Ah receptor system, but P450 1A2 is essentially expressed only in the liver[164], probably due to the involvement of HNF in its regulation (*vide infra*). Several lines of evidence indicate that the level of expression is substantial (Figures 10.1 and 10.2, Table 10.5), ~10–15% of the total P450, on the average, with levels varying ~40-fold among individuals (Figure 10.4).

Occasional reports cite mRNA expression in some extrahepatic tissues, for example, colon[183]. Extensive searches have not found expression in human lung[164].

6.2.2. Regulation and Polymorphism

The variability and inducibility of P450 1A2 have been recognized for some time, indirectly, going back to studies on phenacetin metabolism by Conney and his associates[184]. The characterization of P450 1A2 ("$P450_{PA}$") as the low K_m phenacetin *O*-deethylase[7] led to some interpretation of the earlier results. P450 1A2 was shown to be the caffeine N^3-demethylase[73], and the 40-fold variation in levels of liver P450 1A2 is reflected in the 40-fold variation in some *in vivo* parameters of caffeine metabolism[82], some of Vesells's earlier work on the metabolism of antipyrine in twins suggests a role for genetic polymorphism in P450 1A2 activity[185], and a more recent twin study confirms the strong genetic component of caffeine demethylation[186]. However, elucidating details of any functional polymorphism has been difficult.

At least 13 allelic variants of P450 1A2 are now known[50]. Of these, at least five have changes in the coding sequences that cause amino acid changes. Recent work in this laboratory with the expressed coding region variants indicates that most do not differ more than 2-fold in their kinetic parameters for several assays (phenacetin *O*-deethylation and *N*-hydroxylation of heterocyclic amines), although one of the variants (R431W) did not express holoprotein in *Escherichia coli*[186a]. Some (*1C and *1F) have been proposed to modify levels of expression[50]. However, the basis for a polymorphism is not clear. One view is that the variability is not genetic, based upon the lack of modality breaks in analysis of some *in vivo* parameters of caffeine metabolism[187].

One complication with genetic polymorphism, as with P450 1A1 (*vide supra*), is the inducibility. Because of the availability of markers of hepatic P450 1A2 function (phenacetin is no longer used but caffeine and theophylline are), demonstrating *in vivo* changes in P450 is relatively easy to do and the effects are consistently seen, at least quantitatively. The mechanism of induction appears to be similar to that of P450 1A1 (Figure 10.6), with expression restricted to the liver because of the need for HNF (ref. [188]). An interesting observation made recently in mice is that the inducer 3-methylcholanthrene causes a persistent induction (of P450 1A1) in liver, lasting beyond the time suggested by pharmacokinetic expectations[189].

One interpretation is that a P450 1A2-generated metabolite is involved. Further details and any relevance to humans remain to be established. With animal P450 1A2, one mechanism of induction involves protein stabilization, for example, by isosafrole-derived products[190]. Whether or not this mechanism is relevant in humans is unknown. Reported inducers include cigarette smoking, charbroiled food (presumably via polycyclic hydrocarbons and heterocyclic amines), cruciferous vegetables, vigorous exercise[191], and the drug omeprazole (actually a metabolite)[192].

6.2.3. Substrates and Reactions

P450 1A2 has been expressed in a number of systems and is used in analyses of catalytic selectivity. Of the P450s, this has one of the highest levels of expression in bacterial systems[193, 194].

The list of drug substrates is long[32], and only a few of the more well-known reactions are listed in Table 10.7.

Many carcinogens are substrates, particularly aromatic and heterocyclic amines (Table 10.4). Other carcinogens shown to be substrates include polycyclic hydrocarbons, nitropolycyclic hydrocarbons, and some *N*-nitrosamines[202].

The only major endogenous substrates are 17β-estradiol and estrone (2-hydroxylation). The physiological relevance of this reaction is unknown, particularly because of the wide variation in levels of P450 1A2 (this reaction is also catalyzed by other P450s, e.g., 3A4 (ref. [203])). Induction of P450 1A2 and 2-hydroxylation has been proposed as a means of preventing oxidation of 17β-estradiol to the potentially more reactive 4- and 16α-hydroxy products[204, 205].

Table 10.7. Some Drug Substrates for Human P450 1A2[a]

Drug[a]	References
Acetaminophen (3′)	195
Antipyrine (4,3-methyl)	196
Bufuralol (1,4)	197
Caffeine (3)	73
Clozapine	49
Olanzapine	49
Ondansetron (7,8)	198
Phenacetin	7
Tacrine	199, 200
Theophylline (1,3,8)	201

[a]See also Rendic[32].

6.2.4. Knowledge about Active Site

Considerable site-directed mutagenesis work has been done with rat P450 1A2 (refs [206–208]) but relatively little with human P450 1A2. Some pharmacophore[209] and homology[180, 210] modeling work has been reported.

An approach was developed in this laboratory for doing random mutagenesis of P450 1A2, utilizing changes in the rates of formation of mutagenic hydroxylamines[211]. Changing Glu226 (to Ile or Gln) had the effect of increasing rates of phenacetin *O*-deethylation 8-fold (effect on k_{cat})[212]. Also of interest is a 100-fold decrease of activity seen in mutation of the neighboring Phe225 (to Ile or Tyr)[212]. The mutation of Asp320 (to Ala) also decreased activity[213]. Kinetic deuterium isotope effect studies suggest little change in rate-limiting steps of the *O*-dealkylation reaction over a wide range of activities with these mutants, and the mutations do not affect ground-state substrate binding or rates of P450 reduction[213]. The effects of these substitutions have not been interpreted in terms of specific roles.

The issue of cooperativity will be discussed later under P450 3A4. Cooperativity has not been reported for human P450 1A2 but behavior of the rabbit ortholog has been interpreted in the context of multiple, overlapping binding sites[214].

6.2.5. Inhibitors

Several human P450 1A2 inhibitors are known from clinical work, including furafylline (mechanism based)[215] and fuvoxamine. αNF is a commercially available and strong inhibitor of human P450 1A2 (K_i ~6 nM)[181] for *in vitro* work. A number of polycyclic acetylenes are potent inhibitors of P450 1A2 (ref. [181]). With rat P450 1A2, 2,3,7,8-tetrachloro[*p*]dibenzodioxin and some polyhalogenated biphenyls are strong inhibitors, but these studies have not been extended to human P450 1A2 (ref. [216]).

6.2.6. Clinical Issues

Some drug interactions have been reported. An older example is that of low activity toward phenacetin favoring a potentially toxic secondary pathway, deacetylation followed by quinoneimine formation and methemoglobinemia[105]. Furafylline was a drug candidate but was never developed because of its strong P450 1A2 inhibition and interference with caffeine metabolism[217]. High levels of P450 1A2 activity have also been associated with ineffectiveness of theophylline therapy (for asthma)[218, 219].

The other concern about P450 1A2 is the same discussed earlier for P450 1A1, the co-carcinogenic effect. In this regard, there is some epidemiological evidence that high P450 1A2 activity (measured as *in vivo* caffeine metabolism) is associated with enhanced risk of colon cancer, although the effect was not seen in the absence of high *N*-acetyltransferase activity and high consumption of charbroiled meat[145].

6.3. P450 1B1

6.3.1. Sites of Expression and Abundance

P450 1B1 was originally discovered in keratinocyte cultures in a search for new dioxin-inducible genes[86] and in work on adrenals in animal models[220]. In contrast to P450 1A1 and 1A2 (seven exons), the P450 1B1 gene has only three exons and is located on chromosome 2 instead of 15 (ref. [221]). Although most of the detailed studies of tissue-specific expression have been done at the mRNA level and not protein, strong responses are seen in fetal kidney, heart, and brain, in that order[178]. In adults (human), there is little detectable expression in liver, but there is more detectable expression in kidney, spleen, thymus, prostate, lung, ovary, small intestine, colon, uterus, and mammary gland[178]. Many of these tissues are of particular interest because of the tumors that develop there. Immunochemical staining of P450 1B1 has been reported in a variety of malignant tumors[222].

The level of expression (of the protein) in human lung has been estimated to be at the level of ~1 pmol/mg microsomal protein in nonsmokers and 2–4 pmol/mg microsomal protein in smokers, the levels are of an order of magnitude lower than for P450 1A1 (ref. [160]). These low values may explain the lack of immunostaining in (nontumor) tissues reported by Murray *et al.*[222] Specific values for levels of expression in tissues other than lung have not been published. Recently Chang *et al.*[223] found traces of P450 1B1 mRNA in human liver using real-time PCR, but the protein was undetectable within the limit of sensitivity.

6.3.2. Regulation and Polymorphism

Levels of P450 1B1 in human lung vary by at least one order of magnitude[160]. An interesting observation is that a termination variant of P450 1B1 is strongly associated with glaucoma[141, 224]. A similar phenotype has not been seen in P450 1B1-knockout mice[119]. Other polymorphisms of (human) P450 1B1 are known and are predominant in a set of haplotypes involving four variations Arg/Gly 48, Ala/Ser 119, Val/Leu 432, and Asn/Ser 453. Assays involving the metabolism of 17β-estradiol and polycyclic hydrocarbons by recombinant P450 1B1 variants show some variations but have not been particularly dramatic (reviewed by Shimada *et al.*[142]).

In vitro experiments show the inducibility of P450 1B1 in patterns expected for an *Ah*-responsive gene, which is the way in which the gene was found[86]. Unlike P450 1A1 and particularly 1A2 (*vide supra*), there is limited direct evidence for inducibility of P450 1B1 *in vivo* because of the low, extrahepatic expression and the lack of a diagnostic probe drug. Although the expression of P450 1B1 is driven by the *Ah* system, additional factors must be involved because of the known tissue and cell line selectivity of expression. For instance, major differences are seen between HepG2, MCF-7, and ACHN cells (of liver, breast, and kidney tumor origins, respectively)[221]. With the information available today, one would expect the gene to be induced (in extrahepatic tissues) by the compounds that induce P450s 1A1 and 1A2.

6.3.3. Substrates and Reactions

Human P450 1B1, like P450 1A1, has never been purified from tissue and all of our information has come from protein expressed in heterologous systems. 7-Ethoxyresorufin *O*-deethylation

can be used as a model reaction[225]. The catalytic activity of P450 1B1 is intermediate between P450s 1A1 and 1A2 (ref. [181]). Some other model reactions can be used as well[225].

Much of the interest in P450 1B1 has been because of its ability to activate a broad spectrum of chemical carcinogens, including polycyclic hydrocarbons and their oxygenated derivatives, heterocyclic amines, aromatic amines, and nitropolycyclic hydrocarbons[178] (Table 10.8). Of particular interest is the observation that human P450 1B1 is at least as active as P450 1A1 in the conversion of the classic carcinogen benzo[*a*]pyrene to the 7,8-dihydrodiol, the first step in the formation of the diol epoxide[230]. In general, it would appear from the available information that the rodent P450 1B1 enzymes have similar catalytic specificity as human P450 toward carcinogens, from the available information[231]. If this is a valid view, then the observation that P450 1B1-knockout mice do not form tumors from 7,12-dimethylbenz[*a*]anthracene is of particular importance[119].

One of the interesting findings with human P450 1B1 is that this enzyme is an efficient catalyst of 17β-estradiol hydroxylation and that the pattern is for 4- > 2-hydroxylation[203, 228, 232]. This pattern is the opposite of that seen for P450s 1A2 and 3A4 (2- > 4-hydroxylation)[203, 233] and is of significance because 4-hydroxyestradiol is chemically more reactive with oxygen and more likely to oxidize (to *O*-quinone) and bind DNA[234]. Thus, 4-hydroxyestrogens are considered to be candidates for causing estrogen-dependent tumors[235]. The available information indicates that mouse P450 1B1 does not catalyze estrogen hydroxylation[221, 231], providing a potentially important difference with the human enzyme. This apparent lack of conservation of selectivity has relevance in the use of mouse (and rat) models in some of the biology, for example, the human glaucoma mentioned earlier[141, 224].

Table 10.8. Carcinogens Activated by Human P450 1B1

Substrate	References	Substrate	References
Polycyclic aromatic hydrocarbons		IQ	178
Benzo[*a*]pyrene	181	Trp-P1	178
Benzo[*a*]pyrene-4,5-diol	178	Trp-P2	178
(+) Benzo[*a*]pyrene-7,8-diol	178	PhIP	178
(−) Benzo[*a*]pyrene-7,8-diol	178	*Aromatic amines*	
Dibenzo[*a,l*]pyrene	226	2-Aminoanthracene	178
Dibenzo[*a,l*]pyrene-11,12-diol	178	2-Aminofluorene	178
Benz[*a*]anthracene	181	4-Aminobiphenyl	178
Benz[*a*]anthracene-1,2-diol	178	3-Methoxy-4-aminoazobenzene	178
Benz[*a*]anthracene-*cis*-5,6-diol	178	*O*-Aminoazotoluene	178
7,12-Dimethylbenz[*a*]anthracene	178	6-Aminochrysene	178
7,12-Dimethylbenz[*a*]anthracene-3,4-diol	178		
Benzo[*c*]phenanthrene-3,4-diol	178	*Nitropolycyclic hydrocarbons*	
Fluoranthene-2,3-diol	178	1-Nitropyrene	227
Benzo[*b*]fluoranthene-9,10-diol	178	2-Nitropyrene	178
Chrysene-1,2-diol	178	6-Nitrochrysene	178
5-Methylchrysene	226	2-Nitrofluoranthene	227
5-Methylchrysene-1,2-diol	178	3-Nitrofluoranthene	227
5,6-Dimethylchrysene-1,2-diol	178	6-Nitrobenzo[*a*]pyrene	227
Benzo[*g*]chrysene-11,12-diol	178	1,8-Dinitropyrene	227
6-Aminochrysene-1,2-diol	178	1-Aminopyrene	227
Heterocyclic amines		*Estrogens*	
MeIQ	178	17β-Estradiol	228
MeIQx	178	Estrone	229

6.3.4. Knowledge of Active Site

Very little knowledge about the active site is available. The general pattern of catalytic specificity, with similarity to P450 1A1 and 1A2, would argue for some similarity. The effects of the allelic variants are probably not strong enough to be of much use in understanding the effects of those residues[142]. Some homology modeling has been done[236].

6.3.5. Inhibitors

αNF is a strong inhibitor, as in the case of P450 1A2 (ref. [181]). Some acetylenes developed by Alworth's group have been found to selectively inhibit P450 1B1 (at least relative to P450s 1A1 and 1A2), including 2-ethynylpyrene[181]. A potential drawback to these compounds is that they are rapidly oxidized by P450 1B1.

Resveratrol is a polyphenol found in red grapes and has been of interest in the context of its potential to inhibit cancer[237]. This compound is a noncompetitive inhibitor of P450 1B1, with a K_i value of 23 μM in model systems[238] (with selectivity toward P450 1A1). Recently, Potter *et al.*[239] reported that P450 1B1 oxidizes resveratrol to the known anticancer agent piceatannol, a tyrosine kinase inhibitor. Further studies showed that the natural product rhapontigenin is a low K_i inhibitor of P450 1A1 (ref. [240]). A series of methoxy-substituted *trans* stilbene compounds of the resveratrol/rhapontigenin family were prepared and tested: of these, 2,4,3′,5′-tetramethoxystilbene was found to be a strong and selective competitive inhibitor of P450 1B1 (K_i = 3 nM) and resisted demethylation[240].

6.3.6. Clinical Issues

No issues regarding drug interactions have been raised. As with the P450 1A subfamily enzymes, an issue is that induction of P450 1B1 might increase the activation of procarcinogens. This issue may be real, although presently there is no epidemiological evidence to support such a relationship. Although the coding region polymorphisms have only indicated a limited potential for contribution to cancer (*vide supra*), the recent evidence for trimodal induction[135] is certainly of interest (see Section 5, *vide supra*), particularly in light of the number of carcinogens that P450 1B1 activates (Table 10.8). The issue of oxidation of estrogens to reactive products is one worth considering, in light of the experimental evidence supporting a link with cancer in estrogen-dependent tumors. Another matter that has not been addressed is the possible metabolism of the various estrogens in postmenopausal hormone treatments (e.g., Premarin® by P450 1B1, e.g., see refs 234 and 241 regarding DNA adducts formed by some of these estrogens).

6.4. P450 2A6

6.4.1. Sites of Expression and Abundance

P450 2A6 (formerly termed IIA3 and 2A3[22]) was purified from human liver microsomes[8] and a cDNA was isolated from a human liver library[242]. The protein is expressed at medium to low levels in liver (Table 10.5, Figure 10.2). In one study, the fraction of total human liver P450 attributed to P450 2A6 ranged from <0.2% to 13% among individual samples, with a mean of ~4%[28]. P450 2A6 was not found in placenta (full term)[243].

P450 2A6 is also expressed in other tissues, particularly in the nasopharyngeal region. Expression has been detected in nasal mucosa, trachea, lung[244], and esophageal mucosa[245]. These sites of expression are of interest regarding certain cancers. In liver cancers, overexpression of P450 2A6 protein was associated with chronic inflammation and cirrhosis[246].

6.4.2. Regulation and Polymorphism

The regulation of P450 2A6 expression has been studied in primary cultures of human hepatocytes. Expression (mRNA, protein) is inducible by rifampicin[247] and phenobarbital[248] and, to a lesser extent, clofibrate, cobalt, griseofulvin, and pyrazole[248]. The nuclear receptor HNF-4 is involved in the expression of cultured hepatocytes[249].

Many polymorphisms (≥11) are known for the *CYP2A6* gene[50]. These include a splice variant (*12) in which *CYP2A7* exons are included and the protein has lost catalytic activity[250, 251]. Another SNP polymorphism (*2), recognized earlier, is the L160H change which yields very low catalytic activity[252]. Also of interest is a gene deletion (*4).

The incidence of these polymorphisms is racially linked[50]. P450 2A6 is involved in nicotine oxidation, and in 1998, Tyndale and her associates reported that individuals with low P450 2A6 activity smoke less and might have lower cancer risk[148]. This proposal seems reasonable but the findings have been questioned. General agreement exists that defective P450 2A6 genes cause reduced nicotine metabolism (the presumed basis for reduced smoking)[253–255]. Several reports conclude that deficient P450 2A6 reduces smoking[256–259] and also lung cancer[147, 260, 261] in smokers. The latter hypothesis has biological plausibility because many carcinogens from tobacco are activated by P450 2A6 (Table 10.4 and *vide infra*). However, other studies have not revealed any relationship between *CYP2A6* genotype and smoking; cancer is also controversial[262–265]. Some of the discrepancies may be racial[266] but even this is unclear[267]. Some problems are attributed to technical shortcomings in genotype analyses[268] and a definite relationship is still lacking[268] in Caucasians, but is more likely in Asians[269], where the incidence of gene deletion is higher.

6.4.3. Substrates and Reactions

The most characteristic and specific reaction of P450 2A6 is coumarin 7-hydroxylation[8, 242]. Coumarin 7-hydroxylation has also been used as an *in vivo* diagnostic assay[270–272].

One issue with P450 2A6 is whether b_5 is required for optimal catalytic activity. Soucek[273] demonstrated that a 1:1 ratio of b_5 to P450 was optimal in coumarin 7-hydroxylation catalyzed by the purified recombinant enzyme. The effect of b_5 on catalytic selectivity has not been evaluated in all reports on P450 2A6.

Coumarin 7-hydroxylation can be used *in vivo* with humans as a phenotypic assay. An alternative procedure is to administer caffeine to individuals and determine the conversion of 1,7-dimethylxanthine to 1,7-dimethyluric acid, a reaction catalyzed by P450 2A6 (ref. [274]).

Some industrial chemicals are substrates for oxidation by P450 2A6, including alkoxyethers (used as fuel additives, e.g., *tert*-butyl methyl ether)[275] and the vinyl monomer 1,3-butadiene, a cancer suspect[276].

Some drugs are also substrates, including (+) *cis*-3,5-dimethyl-2-(3-pyridyl)thiazolidin-4-one (SM-12502)[277, 278] and tegafur[279, 280], which is converted to 5-fluorouracil. Halothane is reductively converted to a free radical by P450 2A6, which can yield at least two products and initiate lipid peroxidation[281].

Some of the catalytic selectivity of P450 2A6 overlaps with that of P450 2E1 (*vide infra*). One area in which the overlap has been noted is in the oxidation of nitrosamines. P450 2A6 preferentially catalyzes the oxidation (and activation) of *N*-nitro- sodiethylamine, in contrast to P450 2E1 which preferentially oxidizes *N*-nitrosodimethylamine[282, 283]. P450 2A6 is also involved in the oxidation of many tobacco-specific nitrosamines, including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)[283–286]. P450 2A6 appears to be the major human P450 involved in the activation of *N*-nitroso-benzylmethylamine[287], *N*-nitrosodipropylamine, *N*-nitrosobutylamine, *N*-nitrosophenyl- methylamine, and *N*-nitrosonornicotine[288]. Fujita and Kamataki[289] studied the bacterial mutagenicity of a number of tobacco-specific *N*-nitrosamines and concluded that P450 2A6 is the major human enzyme involved in the activation of all examined.

P450 2A6 is also involved in the metabolism of nicotine (*vide supra*). P450 2A6 is the main catalyst in the oxidation of nicotine to cotinine[290–292]. P450 2A6 is also involved in the 3′-hydroxylation of cotinine[293]. In addition, P450 2A6 catalyzes 2′-hydroxylation of nicotine, yielding a precursor of a lung carcinogen[294].

P450 2A6 can also *N*-demethylate hexamethylphosphoramide[295].

Several forms of human P450 catalyze the 3-hydroxylation of indole[296], and the product dimerizes to indigo. P450 2A6 was the most active human P450 identified for this activity and could also catalyze several oxidations of indole[296]. Mutants of P450 2A6 generated from a randomized library were shown to catalyze the oxidation of several substituted indoles to generate variously colored indigos and indirubins[297].

6.4.4. Knowledge about Active Site

Relatively little site-directed mutagenesis work has been done on P450 2A6 (although the rodent P450 2A enzymes were an early target for the approach)[298]. Some information has been gleaned

from naturally occurring SNPs. *In vivo* studies are consistent with a shift from coumarin 7-hydroxylation to 3-hydroxylation associated with the L160H allele[252], although this phenomenon has not been verified *in vitro*. The substitution R128Q yields a protein with half the content of heme, but the inability to bind carbon monoxide (to ferrous iron) (plus a loss of 98% of the coumarin 7-hydroxylation activity)[299].

Lewis has published several homology models of P450 2A6 (refs [300–302]) and also attempted to rationalize the pattern of nicotine oxidation using molecular orbital calculations[303].

6.4.5. Inhibitors

Several selective inhibitors of P450 2A6 are known. Diethyldithiocarbamate appears to be a mechanism-based inactivator, although the inactivation has not been extensively characterized[283]. Diethyldithiocarbamate and its oxidized form, disulfiram, also inhibit P450 2E1 (ref. [304]). *In vivo* single-dose treatment of people with disulfiram inhibits P450 2E1 but not P450 2A6 (ref. [305]). The vegetable watercress, a source of phenethyl isothiocyanate, did not inhibit P450 2A6 *in vivo*[306].

A number of chemicals have been tested as inhibitors of P450 2A6 in human liver microsomes[307]. Of these, the most selective and potent inhibitors appear to be 8-methoxypsoralen, tranylcypromine, and tryptamine, with K_i values $\sim 1\ \mu M$[307–309]. The inhibition by the natural product 8-methoxypsoralen (in many foods) is mechanism based[310]. 8-Methoxypsoralen (methoxysalen) inhibits P450 2A6 *in vivo*[305] and has also been reported to decrease nicotine metabolism in smokers[311]. Both of the inhibitors 8- and 5-methoxypsoralen were covalently bound to P450 2A6 during incubation with NADPH[312]. Menthofuran, another natural product, is also a mechanism-based inactivator of P450 2A6 (ref. [313]).

Isoniazid has been reported to be a weak mechanism-based inactivator of P450 2A6 (ref. [314]).

6.4.6. Clinical Issues

As indicated in Section 6.4.2, the major issue regarding P450 2A6 polymorphisms is the effects on lung and esophageal cancers and smoking habits, which have good epidemiology in Asians[260] but remain controversial in Caucasians[266, 268, 269, 315, 316].

Some drugs are P450 substrates, although the relative contribution of P450 2A6 is still so small (Figure 10.3) that P450 2A6 reactions are generally not included in screens.

P450 2A6 expression has been reported to be induced during infection by (carcinogenic) liver flukes[317] and downregulated during infection by hepatitis A virus[318].

6.5. P450 2A7

The situation involving the *CYP2A7* gene is complex, and sometimes this has even been erroneously referred to as a pseudogene[50]. Two pseudogenes (*CYP2A7PTX* and *CYP2A7PCX*) are known. The P450 2A7 mRNA transcript is produced in human liver, at roughly the same level as that for P450 2A6 (ref. [250], [319]). Gonzalez's laboratory had isolated cDNA clones now recognized as 2A6, the 2D6 variant L160H, and 2A7, and expressed all three in HepG2 cells[242]. Of the three, only the "wild type" P450 2A6 incorporated heme. Others have also expressed P450 2A7 in heterologous systems but not reported any evidence of a catalytically active P450 2A7 holoprotein[250]. Whether or not a functional P450 2A7 is transcribed from the mRNA in human tissues is still unclear, and nothing can be said about catalytic activity.

Gene conversion events between the *CYP2A6* and *CYP2A7* genes have been reported, yielding chimeric proteins in humans[250, 251, 320]. These proteins have some of the coumarin 7-hydroxylation conferred by the 2A6 component[251].

6.6. P450 2A13

6.6.1. Sites of Expression and Abundance

The *CYP2A13* gene has been recognized for some time[321]. The gene is expressed in human liver[295, 322] and several other extrahepatic tissues, including nasal mucosa, lung, trachea, brain, mammary gland, prostate, testis, and uterus[295]. The highest level seems to be in nasal mucosa[295], which is of interest in the context of tobacco-related cancers because of some of the catalytic activities toward nitrosamine substrates (*vide infra*).

6.6.2. Regulation and Polymorphism

Little is known about the regulation and inducibility of the *CYP2A13* gene. Several variant alleles have been identified, including one in the coding region (R257C) with somewhat less activity toward NNK[323].

6.6.3. Substrates and Reactions

Recombinant P450 2A13 has much lower coumarin 7-hydroxylation activity than does P450 2A6, but coumarin is also converted to the 3,4-epoxide[324]. P450 2A13 also catalyzes several reactions at rates as high or higher than P450 2A6, including 2,6-dichlorobenzonitrile activation, *N*-nitrosodiethylamine *N*-deethylation, hexamethylphosphoramide *N*-demethylation, *N,N*-dimethylaniline *N*-demethylation, 2′-methoxyacetophenone *O*-demethylation, *N*-nitrosomethylphenylamine *N*-demethylation, and the activation of NNK[295]. The latter reaction is of particular interest with regard to tobacco-related cancer because of the localization of expression of this P450 in nasal mucosa.

6.6.4. Knowledge about Active Site

No information is presently available beyond an effect of the R257C variant allele[323].

6.6.5. Inhibitors

No inhibitors have been reported.

6.6.6. Clinical Issues

P450 2A13 probably does not make a major contribution to the metabolism of drugs. The major interest in P450 2A13 involves a possible role in chemical carcinogenesis[295].

6.7. P450 2B6

6.7.1. Sites of Expression and Abundance

P450 2B6 is expressed primarily in liver, and the protein has been partially purified[325]. The protein has also been detected in human lung[326].

Much of the early work with P450s in experimental animals was focused on the phenobarbital-inducible enzymes now recognized to be in the 2B subfamily[327, 328] and a general expectation was that similar P450s would be prominent in human liver (and further suggested by immunochemical studies[3] and early cloning work[329]). However, the major P450 in human liver (and small intestine) proved to be P450 3A4 (Figures 10.2 and 10.3). The mean level of P450 2B6 in human liver has been somewhat controversial. One of the problems has been antibody specificity. Antibodies raised against rat P450 2B1 have not been very specific[325]; unfortunately many papers in this area show only limited sections of gels or actually show major cross-reactive material. The results tend to fall into two groups. One set reports levels very low to 80 pmol P450 2B6 per milligram protein[330–332]. Another set of reports range from near zero levels to 28 pmol P450 2B6/mg microsomal protein[325, 333–336]. However, the mean values differ considerably in both the former and latter groups. While some of the discrepancy may be attributable to the differences in liver samples, the main difference is probably with the antibodies used and cross-reactivity with other proteins, as well as error inherent in other aspects of immunochemical methods. Our own work is in line with the lower set of estimates of expression levels (mean ~1% of total P450, with values rarely exceeding 5% even in samples from individuals administered inducers)[336]. This level is an order of magnitude less than for P450 3A4 (Figures 10.2 and 10.4).

6.7.2. Regulation and Polymorphism

Until recently, the mechanisms of induction by barbiturates had been rather vague in humans and experimental animals. Studies with HepG2 cells (derived from hepatocytes) show the role of the constitutive androstane receptor (CAR), a member of the steroid receptor superfamily, and its interaction with the phenobarbital-responsive enhancer module (PBREM) in the region between −1733 and −1683 bp in the 5′ flanking region[337]. Other work with HepG2 cells has implicated the liver-selective transcription factor C/EBPα[338]. Kliewer's group[339] also demonstrated the involvement of another previously orphan receptor, pregnane X receptor (PXR), in binding to PBREM in primary

human hepatocytes to induce P450 2B6. PXR is active only when ligand-activated but CAR apparently acts without an added ligand; both CAR and PXR heterodimerize with (liganded) RXR[340]. "Cross-talk" also exists at the PBREM site with the vitamin D receptor as well as CAR and PXR[341, 342]. The levels of CAR and PXR mRNA in individual human livers are correlated with the level of P450 2B6 mRNA[343]. The regulation of P450 2B6 has considerable similarity to that of P450 3A4 (*vide infra*), with some differences. Several recent findings provide some further insight into the mechanism, although several questions persist. CAR does have ligand-activated effects and 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime has been identified as an agonist[344]. A novel distal enhancer regulated by PXR and CAR has been identified in the *CYP2B6* gene[345].

Alternative splicing in the *CYP2B6* gene was already identified in 1990[346], with use of a cryptic exon within introns 3 and a splice site acceptor within exon 4. Extensive polymorphism (mainly SNPs) has been identified in the *CYP2B6* gene[50, 347, 348]. Several of the mutants appear to yield attenuated levels of protein and catalytic activity[348]. Some evidence for enhanced catalytic activity of a P450 2B6 SNP variant (N172H) has been reported (~2-fold) and attributed to the homotropic activation seen in 7-ethoxycoumarin *O*-deethylation[349].

6.7.3. Substrates and Reactions

Many reactions have now been demonstrated to be catalyzed by recombinant P450 2B6, mirroring the early research in the P450 field with rat P450 2B1 and rabbit P450 2B4 (refs [350–353]). However, this information must be considered in the context of the amount of P450 2B6 present in liver and intestine, particularly in comparison with P450 3A4 (*vide supra*). One estimate has been made that P450 2B6 is involved in ~3% of drug metabolism reactions (Figure 10.3).

Lists of P450 2B6 substrates have been published elsewhere, for example, refs 32 and 354, and will not be reiterated here. One of the drugs to which P450 2B6 apparently makes a significant contribution is cyclophosphamide[355, 356]. Some other reactions attributed to P450 2B6 involve anesthetics, e.g., ketamine *N*-demethylation[357] and propofol hydroxylation[358]. The *N*-demethylation of (*S*)-mephenytoin has been used as a marker of P450 2B6 *in vitro* (microsomes)[336, 359, 360]. However, a valid *in vivo* probe for P450 2B6 is still lacking[354, 360, 361].

As with animal P450 2B enzymes, P450 2B6 can also oxidize some environmental pollutants[362].

Nonhyperbolic kinetics have been reported for some P450 2B6-catalyzed reactions but these have not been extensively characterized[354].

6.7.4. Knowledge about Active Site

Several homology models of P450 2B6 have been published[363, 364], including one using molecular dynamics[365].

Relatively little site-directed mutagenesis has been done with P450 2B6. Halpert's laboratory modified 10 residues and measured some activities, although most of the changes were ≤2-fold[366].

Recently, Halpert's laboratory has solved a crystal structure of a derivative of the related rabbit P450 2B4 (ref. [366a]), which should be of relevance in understanding P450 2B6.

6.7.5. Inhibitors

Lists of the reported inhibitors of P450 2B6 have been compiled by Rendic[32]. Orphenadine had been utilized in some work with microsomes but does not appear to be particularly selective[361, 367]. More recently 2-isopropenyl-2-methyladamantane and 3-isopropenyl-3-methyldiamantane have been reported as selective inhibitors of P450 2B6 (ref. [368]). Triethylenethiophosphoramide has also been reported to be a selective inhibitor of P450 2B6 (ref. [369]).

The oral contraceptive 17α-ethynylestradiol is a mechanism-based inactivator of P450 2B6 and modifies the (apo)protein[370], but the *in vivo* relevance of the inhibition has not been established.

6.7.6. Clinical Issues

No real clinical issues have been identified yet, primarily because of the difficulty in identifying reactions catalyzed *in vivo* due to the lack of specific inhibitors and the overlapping regulatory mechanisms with other enzymes. Some pharmaceutical companies have begun to include P450

2B6 in their *in vitro* screens for individual P450s with potential roles, however.

The phenomenon of barbiturate-like enzyme induction is still an issue in drug development, however. The point is not only drug interactions, but particularly the prospect of tumor promotion in rodent cancer bioassays, which is probably unrelated to the P450 induction[152].

6.8. P450 2C8

The P450s in the 2C subfamily have been of interest for some time. In retrospect, some of the first human P450 preparations purified were probably P450 2C9 (refs [3], [4]). A major impetus for research in this field was the observed genetic polymorphism in (*S*)-mephenytoin 4′-hydroxylation[371, 372], which led to efforts at purification. Purified proteins had some catalytic activity toward mephenytoin[9], but subsequent *in vivo* pharmacokinetic[373] and heterologous expression experiments[374] demonstrated a distinction between tolbutamide and (*S*)-mephenytoin hydroxylation. Genomic analysis indicated the complexity of the *CYP2C* gene subfamily[375]. Subsequently the subfamily was characterized in terms of four P450s: 2C8, 2C9, 2C18, and 2C19 (ref. [376]). P450 2C19 is the polymorphic (*S*)-mephenytoin 4′-hydroxylase[377, 378]; P450 2C9 is involved in a considerable number of drug oxidations (Figure 10.3). Two previous entries, 2C10 and 2C17, are considered allelic variants of other genes or other artifacts and have been deleted (Table 10.1)[379].

6.8.1. Sites of Expression and Abundance

P450 2C8 was first purified from human liver[9]; the enzyme is known to be expressed in liver and kidney[380]. The available data indicate that the level of expression of P450 2C8 is relatively low in liver but may be one of the more substantial P450s in the kidney. Other sites of P450 2C8 (mRNA) include adrenal gland, brain, uterus, mammary gland, ovary, and duodenum[381].

6.8.2. Regulation and Polymorphism

The level of P450 2C8 expression in human liver varies at least 20-fold[382]. Rifampicin induces P450 2C8 in hepatocyte culture[247]. The enzyme appears to be inducible by barbiturates[383] but a PBREM was not found in the 5′ untranslated region of the gene[381].

Several polymorphisms have been reported and studied[380, 382, 384]. Two coding region polymorphisms involve the amino acid substitutions I264M and K399R, with the latter appearing in a haplotype with R139K (ref. [382]). Polymorphisms upstream of the coding region are also known[382]. The metabolism of taxol (paclitaxel) is decreased with the *3 allele (K399R/R139K haplotype), but the extent of the decrease has been variable in different studies, ranging from 90%[380] to 25%[382, 384]. The *1C polymorphism appears to be associated with some attenuation of the mean level of expression[382].

6.8.3. Substrates and Reactions

P450 2C8 does not appear to have the general significance of P450 2C9 (or 2C19) in drug metabolism. An important substrate is taxol (paclitaxel)(6α-hydroxylation)[66, 385]. Another substrate for P450 2C8 is all-*trans* retinoic acid[386]. P450 2C8 also contributes to the oxidation of troglitazone[387] and verapamil, rosiglitazone, cerivastatin, amiodarone, dapsone, and amodiaquine (reviewed in refs [32, 382]).

In general, P450 2C8 has relatively low catalytic activity toward the known substrates of P450s 2C9 and 2C19. However, Mansuy's laboratory has recently synthesized a sulfaphenazole derivative toward which all of the human P450 2C subfamily P450s have activity[388].

6.8.4. Knowledge about Active Site

Some of the knowledge about the catalytic selectivity of various P450 2C enzymes can be interpreted in terms of the active site. The rabbit P450 2C5 structure provides some possible insight in this area. Recently, the groups of Johnson and Mansuy[389] have obtained a three-dimensional structure with a common P450 2C subfamily substrate[388] bound. Two binding modes were observed, one of which corresponds to the observed oxidation[389]. Very recently Johnson's group has obtained a crystal structure of a slightly modified P450 2C8 (ref. [389a]).

6.8.5. Inhibitors

In contrast to P450 2C9, sulfaphenazole is not a strong inhibitor of P450 2C8. Mansuy's group has synthesized some sulfaphenazole-based selective inhibitors of individual P450 2C enzymes, including P450 2C8 (refs [390], [391]). The early work on paclitaxel metabolism suggests that high concentrations of the natural flavonoids naringenin, quercitin, and kaempferol and the synthetic αNF inhibit[66], but little *in vivo* inhibition would be expected.

6.8.6. Clinical Issues

Induction and inhibition of P450 2C8 are not particular issues at this point. Although P450 2C8 may play a prominent role in the hepatic and renal oxidation of arachidonic acid and retinoic acid, no disease etiology has been implicated at this point. The most serious issue is probably any impact on the disposition of the cancer chemotherapeutic agent paclitaxel. Polymorphisms may have some effect on *in vivo* 6α-hydroxylation[380, 382], although any influence may be modulated in part by the contribution of P450 3A4 to other reactions[66].

6.9. P450 2C9

In retrospect, many of the observations regarding *in vivo* metabolism of barbiturates[40, 392] are some of the first reports on what is known as P450 2C9. P450 2C9 is one of the major enzymes involved in drug metabolism (Figure 10.3). Retrospectively some of the first purified human liver P450s can now be recognized as P450 2C9 (refs [3], [4]). The protein purified with some mephenytoin 4′-hydroxylation activity (MP-1) is also P450 2C9 (ref. [9]), and the cDNA corresponds[393]. Proteins were also purified from liver on the basis of their oxidation of tolbutamide[373] and hexobarbital[394, 395]. The human P450 2C subfamily is complex[375] and characterization of individual members was not achieved without heterologous expression and careful analysis of catalytic activities[374, 396]. A transcript designated as P450 2C10 from this laboratory had only two apparent coding region changes (Cys358 and Asp417) from the *CYP2C9*1* allele, one of which (reported initially as Cys358) was subsequently shown to result from a sequencing error[375]. This is now thought to be an allelic variant of P450 2C9, although since no evidence for the Asp417 mutation has been found in population studies, no allele designation has been made; the original assignment had been based on the now unexplained distinct 3′ noncoding sequence[375]. Most of the literature dealing with P450 2C10 can be interpreted as 2C9.

6.9.1. Sites of Expression and Abundance

P450 2C9 is primarily a hepatic P450. The level of expression is probably the highest, on the average, except for P450 3A4 (Figures 10.1 and 10.4; Table 10.5)[28]. All P450 2C enzymes are absent in fetal liver, including P450 2C9 (ref. [393]), and levels rise quickly in the first month after birth[397]. Pharmacokinetic experiments with accepted P450 2C9 substrates indicate that the level of hepatic P450 2C9 does not change with age, at least to 68 years[398].

P450 2C9 is also expressed in the small intestine[399].

6.9.2. Regulation and Polymorphism

Early work with human hepatocytes showed induction of P450 2C9 by barbiturates and rifampicin[400], consistent with earlier *in vivo* work on the induction of barbiturate metabolism[392]. Subsequent studies have shown that P450 2C9 is the only P450 2C subfamily enzyme expressed at a significant level in untreated hepatocytes and that expression is induced by rifampicin, dexamethasone, and phenobarbital[383, 401]. The induction involves a glucocorticoid receptor, CAR, and PXR, with CAR and PXR apparently competing at the same site[402].

Recently, evidence for action of CAR at an additional site has been presented[403]. It should be emphasized that the action of CAR is somewhat different than other receptors from the steroid receptor superfamily, in that it may be enhanced in the absence of a bound ligand and some of the control is at the level of nuclear translocation[404]. Other factors involved are HNF-4 (ref. [405]) and C/EBPα (ref. [338]), accounting at least in part for hepatic localization.

The genetic polymorphism of P450 2C9 has been studied extensively and has clinical

significance, although P450 2C9 probably does not have a critical function in normal physiology. Tolbutamide metabolism had been reported to display polymorphism[406], which was an impetus to purify the protein catalyzing the hydroxylation[373]. A 6-base deletion in the coding region lowered catalytic activity in a recombinant enzyme[407]. A number of P450 2C9 SNPs have been identified[408] and their racial linkage has been explored[409].

P450 2C9 polymorphism has been reviewed recently[410, 411] and the reader is referred to these reviews and to the website http://www.imm.ki.se/Cypalleles/ for more details. Of some interest, in addition to the *2 and *3 alleles with generally lower catalytic activity, is the *5 allele (of higher frequency in Africans) with lower catalytic activity[412]. Some of the SNPs occur in the 5′-flanking region and attenuate the expression of P450 2C9 (ref. [413]). Also of interest is an unusual phenomenon in which the *CYP2C18* exon 1-like locus is fused with combinations of exons and introns from *CYP2C9* to yield chimeric RNA transcripts[414]. Finally, linkage between *CYP2C8* and *CYP2C9* genetic polymorphisms has been reported[415].

6.9.3. Substrates and Reactions

P450 2C9 is one of the major P450s involved in drug metabolism (Figures 10.3 and 10.4). Some aspects of substrate specificity have been reviewed by Miners and Birkett[416]. A more extensive recent compendium of substrates has been developed by Rendic[32].

One of the early substrates examined was phenytoin, which undergoes 4-hydroxylation[9]. P450s 2C19 and 2C18 (R. Kinobe and E.M.J. Gillam, personal communication) can also catalyze this reaction but P450 2C9 is the major catalyst[417].

Recently, Mansuy's group has used the P450 2C9 inhibitor sulfaphenazole to build a substrate common to all four P450 2C subfamily enzymes[388].

Some compounds normally in body are oxidized by P450 2C9, including linoleic acid (epoxidation)[418] and vitamin A (all-*trans*-retinoic acid, 4-hydroxylation)[419], although the physiological significance is unknown.

Several reactions have been used as *in vivo* probes, including tolbutamide, warfarin, flurbiprofen, and losartan[420].

One substrate of recent interest is celocoxib, a cyclooxygenase (COX)-2 inhibitor (Celebrex®). P450 2C9 is the major catalyst of oxidation, and polymorphisms affect the *in vivo* pharmacokinetic parameters[421, 422].

Several aspects of P450 2C9 reactions are of concern regarding interpretation of results, at least in *in vivo* research. One issue is the effect of solvents on catalytic activity[423]. A concentration of 1% (v/v) CH_3CN markedly inhibited the catalytic activity of P450 2C9 (ref. [423]). Another issue is the enhancement of most reactions by b_5 (ref. [424]). Further work also showed that apo-b_5 (devoid of heme) was as effective as b_5 (ref. [425]), arguing against a need for electron transfer. Other work showed that even other P450s could enhance the rates of some P450 2C9 reactions, even though those P450s did not catalyze the reactions themselves[424]. These results are reminiscent of some of the interactions of rabbit P450s 1A2 and 2B4 reported by Backes[426] and are still unexplained.

Other work with P450 2C9 has provided evidence for cooperativity in some reactions, although the area has not been as developed as for P450 3A4 (*vide infra*). Dapsone and some analogs enhance the binding and 4-hydroxylation of diclofenac[427, 428]. However, the activity of P450 2C9 toward dapsone is unaffected by diclofenac, in a situation similar to that of P450 3A4, aflatoxin B_1, and αNF[429]. The interpretation that P450 2C9 uses two binding sites in these interactions is probably valid[428], although (as with P450 3A4) the mechanism remains to be elucidated (including the exact nature of the binding).

6.9.4. Knowledge about Active Site

The point should be made before detailed considerations of site-directed mutagenesis, etc., that changes in particular residues of P450 2C9 yield markedly different effects depending on the substrate and reaction under consideration. For instance, the polymorphism *3 (I359L), which appears to be very conservative, changed catalytic efficiencies of different reactions by factors of 3–27-fold (*in vitro*)[430]. Although the *2 and *3 polymorphisms cause considerable changes with some substrates, diclofenac metabolism is not altered[431], consistent with the *in vitro* findings.

With the above caveats, roles of a number of amino acids have been examined with several reactions, although extrapolation to more reactions

requires caution. Arg97 and Arg98 affected activity toward diclofenac in a yeast recombinant system[432]; in contrast, mutation of Arg97 ablated hemoprotein expression in a bacterial system (E.M.J. Gillam, personal communication). Mutation of Lys72 failed to affect affinity for ibuprofen or diclofenac (E.M.J. Gillam, personal communication). Asp293 has been shown to have a relatively general structural role, possibly by bonding to a partner amino acid or amide[433]. Studies with coumarins suggested two sites, one for Π-stacking of aromatic rings and an ionic binding site for organic anions[434]; many P450 2C9 ligands have an anionic charge[435, 436].

P450 2C9 was converted into an enzyme with (*S*)-mephenytoin 4′-hydroxylation activity (i.e., P450 2C19-like) with a relatively small number of changes (I99H, S220P, P221T, S286N, V292A, F295L). Comparisons with the crystal structure of rabbit P450 2C5 suggests that most of these residues are unlikely to directly contact the substrate but probably influence packing of substrate-binding sites and substrate-access channels[437]. Conversely, P450 2C19 could be transformed to an enzyme with warfarin hydroxylation activity similar to that of P450 2C9 (and also sulfaphenazole binding) with the changes N286S, I289N, and E241K (ref. [438]). Other work identified roles of residues 292, 295, and 399 plus residues 231–288 (substrate-binding sequence [SRS] 3) as important in P450 2C9 activities[439]. Mansuy's laboratory identified residues 476, 365, and 114 as being important in diclofenac and sulfaphenazole binding and in inactivation by tienilic acid[440]. Phe114 is proposed to be involved in Π-stacking[440] perhaps serving the role proposed in the coumarin studies mentioned earlier[434]. It might be speculated that Phe120 in P450 2D6 could serve a similar role in that enzyme (*vide infra*)[441].

Several models of P450 2C9 have been published[436, 442–445]. Some of these take experimental binding studies into consideration in their formulation while others are only based on homology. Of interest is the recent work with rabbit P450 2C5 using P450 2C9 ligands, showing multiple substrate-binding modes[389].

A crystal structure of P450 2C9 with bound warfarin has been published recently[445a]. Obviously no information is available regarding ligand interactions either. Very recently Johnson's group has also announced a P450 2C9 crystal structure[446].

6.9.5. Inhibitors

Sulfaphenazole has been recognized as a highly selective competitive inhibitor of P450 2C9 for some time[447] and has relatively poor affinity for other P450 2C subfamily enzymes[390]. Mansuy's group has examined some other similar compounds as ligands and inhibitors[389, 435].

Other inhibitors have been reported, although some have relatively poor affinity[448, 449], including several warfarin analogs[450]. For a more extensive compilation of inhibitors, see Rendic[32].

Tienilic acid is a mechanism-based inactivator of P450 2C9 (ref. [451]). The mechanism involves *S*-oxygenation, and the unstable product reacts with P450 2C9 (ref. [452]). Subsequently, autoimmune antibodies develop in some patients that recognize unmodified P450 2C9 (ref. [451]). Exactly how (or if) this process is related to the hepatitis seen in some individuals who used tienilic acid is still unclear[453], but the phenomenon has raised concerns about whether such processes might be associated with other drugs that covalently modify proteins and could lead to idiosyncratic drug reaction in patients, one of the major concerns today for safety assessment in drug development. Structure–activity relationships have been reported on thiophenes other than tienilic acid[436].

6.9.6. Clinical Issues

The major issue regarding P450 2C9 is its role in drug development because of the sizeable fraction of drugs oxidized by this enzyme (Figure 10.3)[32]. Although the polymorphism is not as dramatic as with P450 2C19 or P450 2D6 (*vide infra*), it can be an issue in drug interactions and safety.

A general issue with P450 2C9, because of its relatively high abundance (Figures 10.1 and 10.4), is its role in reducing bioavailability. However, estimating *in vivo* pharmacokinetic properties from *in vitro* data is still not trivial. Houston has reviewed the issue with P450 2C9 recently[454].

Goldstein[455] has reviewed the clinical relevance of genetic polymorphisms in the P450 2C subfamily. One of the most relevant involves warfarin,

which has a relatively low therapeutic index[455]. (*R*)-Warfarin is oxidized by P450 1A2 (6- and 8-hydroxy) and P450 3A4 (10-hydroxy), and (*S*)-warfarin is oxidized primarily by P450 2C9 (7-hydroxy)[456, 457]. The metabolism of (*S*)-warfarin is competitively inhibited by (*R*)-warfarin, but the converse is not the case[458]. The hydroxylation of (*S*)-warfarin by P450 2C9 (ref. [459]) is an issue because of reduced catalytic efficiency by the *2 and *3 variants[89, 460, 461]. The differences are manifested in altered toxicity of warfarin (hemorrhaging) at a given dose and in an altered optimal dose of warfarin[89–91, 462, 463]. The issue extends to the analog acenocoumarol[464]. The principles of physiologically based pharmacokinetic modeling have been applied to the variation of warfarin risk in individuals with different genotypes/phenotypes[465]; this effort may serve as a paradigm for other efforts to convert *in vitro* data on P450 variability into estimates of risk.

Tolbutamide hydroxylation is another example of a manifestation of *in vitro* knowledge about P450 2C9 in clinical pharmacology[466–468]. In one sense, this is rather logical because the *in vitro* work with tolbutamide[373] was developed from *in vivo* findings[406].

In other clinically relevant research involving P450 2C9, the genotype has been reported to predict the blood pressure response to the drug irbesartan[469], a relative to the P450 2C9 substrate (and the prodrug losartan)[470, 471]. Although P450 2C9 is involved in the metabolism of diclofenac, no relationship of the genotype with the cases of diclofenac-induced hepatitis was observed[472].

The final issue about P450 2C9 is possible relevance to cancer risk. Some carcinogens are substrates (e.g., benzo[*a*]pyrene[171]) although many of the reactions are probably detoxications. *CYP2C9* SNPs have been analyzed in relation to colorectal cancer. An association was found in one study[473], but not a subsequent one[474]. In another study, no association of *CYP2C9* SNPs was found with lung cancer[475].

6.10. P450 2C18

6.10.1. Sites of Expression and Abundance

Of the four human P450 2C subfamily members, the level of hepatic expression appears to be lowest for P450 2C18, at both the mRNA[376, 476] and protein[477] levels. However, expression in lung and skin appears to be significant[244, 478, 479].

6.10.2. Regulation and Polymorphism

The variability in levels of expression of P450 2C18 in human liver is difficult to assess because of the very low levels (<2.5 pmol/mg microsomal protein)[477]. The extent of variability in other tissues is not known.

Rae *et al.*[247] reported that P450 2C18 was not inducible by rifampicin in human hepatocytes, in contrast to P450s 2C8 and 2C9.

Polymorphisms in the *CYP2C18* gene have been reported[480], but the effects on expression and catalytic activities are not well characterized. One possible polymorphism has an exon 5 deletion[481].

6.10.3. Substrates and Reactions

P450 2C18 has low catalytic activity in tolbutamide methyl hydroxylation[481]. Limited activity toward drugs has been shown, and P450 2C18 probably does not make much contribution in general drug disposition, in part because of low expression levels. P450 2C18 is active in phenytoin metabolism, having an enzyme efficiency (k_{cat}/K_m) for 4-hydroxylation comparable to P450 2C9, and being more active in the bioactivation to a reactive intermediate (R. Kinobe and E.M.J. Gillam, personal communication).

Minoletti *et al.*[476] studied a series of derivatives of tienilic acid and characterized an aroylthiophene, 3-[2,3-dichloro-4-(2-thenoyl)phenoxy]propan-1-ol, as a selective substrate for 5-hydroxylation by P450 2C18 ($k_{cat} = 125\ min^{-1}$, $K_m = 9\ \mu M$).

6.10.4. Knowledge about Active Site

Information about the active site of P450 2C18 is relatively limited beyond the substrates cited above[476], the interaction of other P450 2C proteins with general 2C substrates[388] and inhibitors[390], and inferences from the rabbit P450 2C5 structures[389]. At least one homology model has been published[482].

6.10.5. Inhibitors

P450 2C18 is not appreciably inhibited by sulfaphenazole. Mansuy's group has published on

some synthetic inhibitors (sulfaphenazole derivatives) that can be used *in vitro*[390, 391].

6.10.6. Clinical Issues

The limited expression and repertoire of catalytic activity of P450 2C18 preclude consideration of clinical issues at this point in time.

6.11. P450 2C19

Interest in P450 2C19 developed from the discovery of the polymorphic metabolism of the *S*-isomer of mephenytoin, the first major polymorphism to be studied following P450 2D6 (refs [371], [372]). Initial work led to the purification of an enzyme with some (*S*)-mephenytoin 4′-hydroxylation activity[9]. Exactly how this and other gene products from the complex P450 2C family[375, 393] were involved was unclear[483, 484]. Although there were some indications that the hexobarbital 3′-hydroxylase (P450 2C9) was the enzyme of investigation[395, 485], expression of P450 2C9 cDNA[393] in yeast yielded a protein with activity towards tolbutamide but not (*S*)-mephenytoin[374, 396]. P450 2C18 had also been suggested to be the enzyme[376].

Wrighton[377] compared (*S*)-mephenytoin 4′-hydroxylation activity in different liver samples with a protein gel band recognized by anti-rat P450 2B1 and correlated this with P450 2C19, a sequence which had been reported earlier. Subsequently, Goldstein *et al.*[378] expressed several P450 2C subfamily cDNAs in yeast and identified P450 2C19 as having the highest activity.

6.11.1. Sites of Expression and Abundance

Apparently significant expression only occurs in the liver. As with all other P450s examined to date, there appears to be no gender difference[486]. P450 2C19 is a relatively minor P450 in its abundance, probably accounting for <5% of total P450 even in EM liver samples (Figure 10.4).

P450 2C19 and (*S*)-mephenytoin 4′-hydroxylation activity were not detected in fetal liver samples[393].

6.11.2. Regulation and Polymorphism

In vivo work had shown that the enzyme was inducible by rifampicin[487]. Thus, this P450 differed from P450 2D6 in that it was both polymorphic and inducible. Analysis of the regulatory system has not been extensive, but studies with human hepatocytes have demonstrated induction of P450 2C19 mRNA by rifampicin, dexamethasone, and phenobarbital[401].

The polymorphism is now relatively well understood. The incidence of the PM phenotype in Caucasians is generally 3–5% but the incidence in Asians is ~20%[48]. On some Pacific islands, the incidence is as high as 75%[488, 489]. The major defect in Caucasians and Japanese was first identified in an exon 5 mutation that leads to an aberrant splice site and yields a truncated protein[490]. Other polymorphisms are collected at the website http://www.imm.ki.se/CYPalleles/. These are rather diverse and include a mutation of the initiation codon[491] and altered enzymatic properties[488].

6.11.3. Substrates and Reactions

(*S*)-Mephenytoin 4′-hydroxylation is the classic reaction attributed to P450 2C19. Early studies on the basis of the polymorphism of tolbutamide hydroxylation suggested that the same enzyme might be responsible for both activities[373], but *in vivo* work[373] and heterologous expression studies[374] distinguished the two activities. Nevertheless, recombinant P450 2C19 has now been shown to have some tolbutamide hydroxylation activity[492].

Extensive lists of reports of P450 2C19 reactions have been published by Rendic[32] and only a few will be mentioned. The scope of P450 2C19 in drug metabolism is relatively restricted (Figure 10.3). One drug of particular interest is the ulcer drug omeprazole (and related compounds), because individuals with low enzyme activity show a better response to treatment for ulcers[92, 93]. Some of the early variations seen in warfarin metabolism[493] can be explained by the finding that P450 2C19 catalyzes the 8-hydroxylation of (*R*)-warfarin[494]. 18-Methoxycoronaridine is *O*-demethylated by P450 2C19[495]. P450 2C19 is responsible for the 5- and 5′-hydroxylation of thalidomide, an older drug notorious for teratogenic effects that has been "rediscovered"[496]. Whether the polymorphism was related to the birth defects is unclear.

P450 2C19 also oxidizes steroids, including progesterone 21-hydroxylation and testosterone 17-oxidation[497]. Finally, the organphosphate insecticide diazinon is activated in human liver by P450 2C19 (ref. [498]).

6.11.4. Knowledge about Active Site

As with other P450 2C subfamily enzymes, P450 2C19 activities are usually stimulated by b_5 (ref. [425]). In this case, stimulation is not dependent on heme in the b_5 so electron transfer cannot be involved[425].

Homology models of P450 2C19 have been published[302, 444].

Goldstein's group did chimeric analysis and then site-directed mutagenesis on P450 2C9 to convert it to a protein with P450 2C19-characteristic omeprazole hydroxylation activity[499]. Only three changes were needed to achieve the activity of wild-type P450 2C19: I99H, S200P, and P221T. However, at least three different mutations were needed to convert P450 2C9 to an enzyme with (*S*)-mephenytoin 4′-hydroxylation activity, even to a catalytic efficiency one third of wild-type P450 2C19 (ref. [437]). In an opposite experiment, P450 2C19 was converted to a P450 2C9-like warfarin hydroxylase with high sensitivity to sulfaphenazole[438]. Residues 286 and 289 appear to be important. However, these residues may exert an indirect influence by adjusting the active site or substrate-access channels[437].

6.11.5. Inhibitors

Relatively little has been published concerning P450 2C19 inhibitors, although screening may be done in some pharmaceutical companies. Recently, Mansuy's group has developed some P450-selective inhibitors for the 2C subfamily enzymes, including P450 2C19 (refs [390], [391]).

6.11.6. Clinical Issues

The issue is the polymorphism, particularly for drugs marketed in Asian populations. At least eight alleles have been associated with the PM phenotype[489]. Desta *et al.*[489] have reviewed some of the drugs for which the 2C19 phenotype is a problem.

Most pharmaceutical companies and regulatory agencies discourage development of a P450 2C19 substrate because of potential problems for PM individuals. However, several studies indicate that PM patients may have more effective therapy (for ulcers) with omeprazole and related compounds[489, 500–503].

As with many polymorphisms, epidemiology studies have been done to explore risks to diseases in the absence of information about etiology, substrates, etc. Some of the reports include suggestion of more hepatocellular cancer in PMs[504] and lack of association of leukemia with polymorphism[505]. Other possible relationships have been explored but evidence for any associations is limited at this time[489].

6.12. P450 2D6

P450 2D6 is one of the main enzymes involved in drug metabolism (Figure 10.3). It was the first "xenobiotic-metabolizing" P450 recognized to be under monogenic regulation[5].

6.12.1. Sites of Expression and Abundance

P450 2D6 is expressed mainly in liver and was first purified from liver microsomes[7, 10]. In the average person, P450 2D6 accounts for ~5% of total P450 (with wide variation)[28]. However, this enzyme is involved in the oxidation of ~25% of all drugs oxidized by P450s (Figure 10.3).

Developmental studies show little P450 2D6 in fetal liver and a rapid increase in protein shortly after birth, yielding a peak accumulation in newborns and decline in adulthood[506].

P450 2D6 is also expressed at low levels in lung (bronchial mucosa and lung parenchyma)[507].

Another site of P450 2D6 expression is brain, with localization in large principal neurons[508]. Higher levels of brain expression have been reported in alcoholics[509].

6.12.2. Regulation and Polymorphism

All information available indicates that P450 2D6 is not inducible. Some factors are known to be involved in constitutive expression, including C/EBPα[338] and HNF-4α[53].

The wide variability in the activity of P450 2D6 is attributed to genetic variability (Figure 10.5). Reduced ability to metabolize the drug debrisoquine was first noted (personally) by Smith in a drug trial. Subsequent work led to the report of polymorphic hydroxylation of debrisoquine[5], including a phenotypic hypotensive response[510]. Racial differences were first noted with Africans[44]. The phenomenon of polymorphic debrisoquine hydroxylation[511] was also reported for sparteine oxidation[43, 512]. Purification of the P450 2D6 enzyme[7, 10, 11] was followed by Gonzalez's cloning of the gene[47] and identification of some of the genetic defects as mRNA splicing variants[513].

Today more than 70 alleles of P450 are known and have been classified with a nomenclature system[51]. Systems for genotyping have become relatively powerful[514] and the "intermediate metabolizer" phenotype has been characterized[515]. The most significant decreases in activity for P450 2D6 alleles, aside from mRNA splicing problems and gene deletion[52], are considered to result from less stable proteins[516], although low activity P450 2D6 variant proteins have also been reported[517, 518]. Some of the allelic differences are present as haplotypes[519].

In addition to the "poor" and "intermediate" metabolizer phenotypes, an "ultrarapid" metabolizer phenotype was identified in early work (Figure 10.5). Ingelman-Sundberg's group identified the basis for this as a gene duplication, with up to 13 copies present in some individuals[45]. The main form of this phenomenon is a haplotype resulting from gene duplication[45, 520]. The amplification appears to result from unequal segregation and extrachromosomal replication of the acentric DNA[521]. As many as 7% of Caucasians show some of this effect, and the incidence is even higher in some Ethiopian and Middle Eastern populations[522].

6.12.3. Substrates and Reactions

Since the original work with debrisoquine[5], many substrates and reactions have been reported

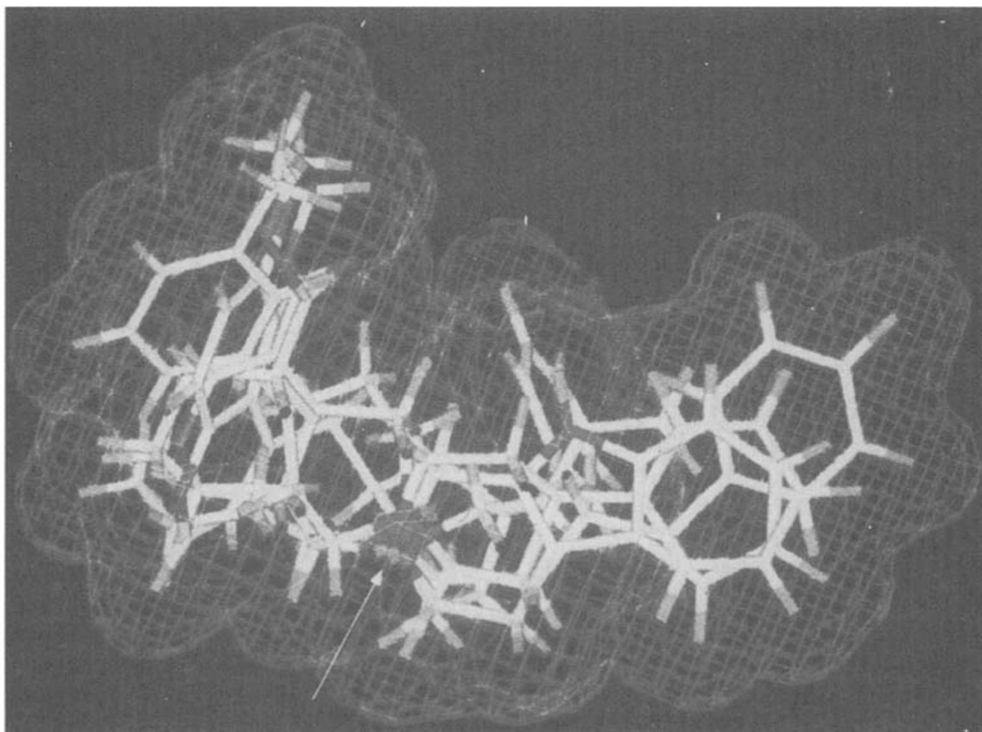

Figure 10.9. A pharmacophore model for the active site of P450 2D6 (ref. [527]). Inhibitors are overlaid to keep the nitrogen atoms (marked with arrow) in a fixed position.

for P450 2D6. In some cases, the role of P450 2D6 is very dominant *in vivo* and the clinical manifestations of genetic polymorphism are important and even deadly[510, 523]. An extensive list of P450 2D6 substrates has been published recently by Rendic[32].

P450 2D6 catalyzes many of the basic kinds of oxidative reactions of P450s, for example, aliphatic and aromatic hydroxylations, heteroatom dealkylations, etc[524]. In early work in this laboratory[525], the observation was made that most of the substrates contained a basic nitrogen atom situated ~5 Å away from the site of oxidation, possibly due to a specific anionic charge in P450 2D6. Subsequently, more detailed pharmacophore models have been developed[526–529] (Figure 10.9). All of these are based on the premise that a basic nitrogen atom in the molecule interacts (coulombic bond) with an acidic amino acid in P450 2D6, usually Asp301 in most studies. (Recent work shows a role for Glu216, however, *vide infra.*)

The use of these models requires some caveats. Although the pK_a of the substrate has been proposed to have a dominant influence[530], work in this laboratory has shown that the intrinsic pK_a of a substrate can be altered in the active site of P450 2D6 (ref. [531]). Another issue is that some compounds with a single amine nitrogen undergo *N*-dealkylation, for example, deprenyl[532], which cannot be rationalized with an amine-oxidation site interatomic distance of 5–7 Å. Some substrates devoid of basic nitrogen (and any nitrogen) have been reported, including steroids[533, 534]. Spirosulfonamide and several analogs are devoid of basic nitrogen and have been shown to be good substrates and ligands for P450 2D6 (ref. [535]) (Figure 10.10).

A large fraction of the population is devoid of active P450 2D6 but appears to function well. This

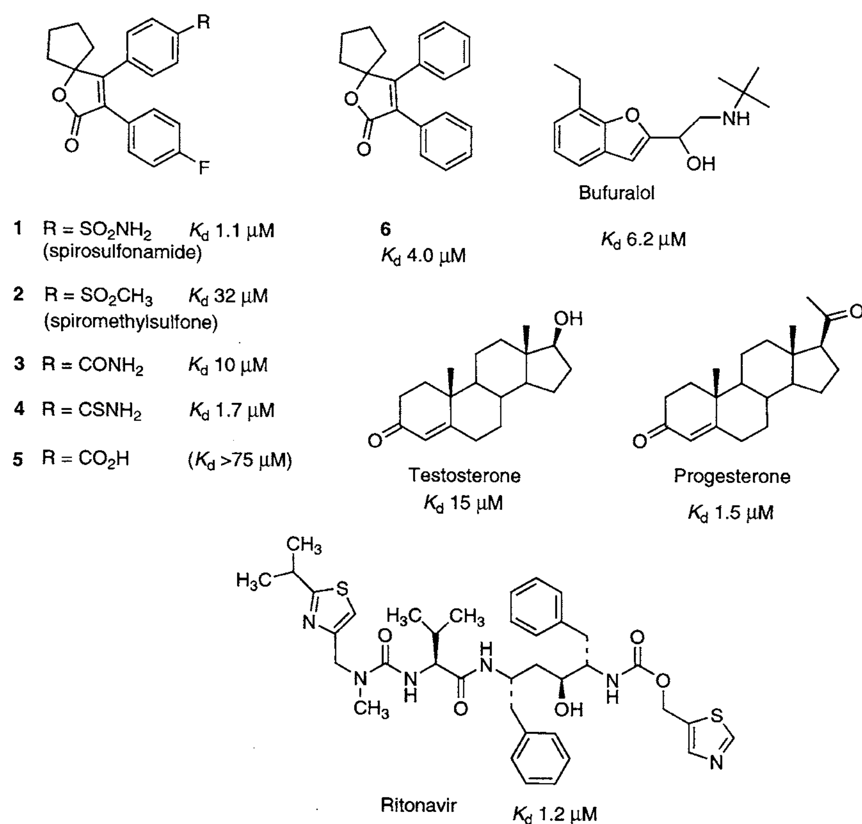

Figure 10.10. Analogs of spirosulfonamide and other P450 2D6 ligands. K_d values were estimated by spectral titrations[535].

information may be interpreted to mean that P450 2D6 has no "physiological" substrate. Nevertheless, some reactions may be catalyzed by P450 2D6 and yield physiological responses that yield less than obvious changes. For instance, overexpression of human P450 2D6 in transgenic mice produces a somewhat lethargic phenotype (F.J. Gonzalez, personal communication). Tryptamine has been proposed as a physiological substrate in one study[536] but discounted in another[537]. Proposed physiological reactions catalyzed by P450 2D6 are the *O*-demethylations of 5-methoxytryptamine, 5-methoxy-*N*,*N*-dimethyltryptamine, and pinoline (6-methoxy-1,2,3,4-tetrahydro-β-carboline)[537, 538]. Whether significant catalytic is seen at the low concentrations that occur *in vivo* and what the effect is remains to be established.

6.12.4. Knowledge about Active Site

The active site of P450 2D6 has been the subject of considerable interest, probably because of the relevance to issues in the pharmaceutical industry. Some residues have been identified as being important, and many homology and pharmacophore models have been published[526–529, 539–545] (Figure 10.9).

The original clone reported by Gonzalez[47] had Met at position 374 but this now appears to be an artifact and the correct residue is Val[546, 547]. This residue appears to be in the active site and affects activity.

In 1995, Ellis *et al.*[548] found that mutation of Asp301 to neutral residues reduced catalytic activity toward several substrates and concluded that this acidic residue was involved in docking amine substrates through coulombic interaction. Subsequently, all models published until recently have been based on this view. A caveat about the reduction in the catalytic activity of the Asp301 mutants is that heme incorporation is diminished (and is completely abolished when basic residues are substituted)[549]. Further, as indicated earlier, some P450 2D6 substrates (e.g., spirosulfonamide) are devoid of basic nitrogen but the hydroxylations are still attenuated by mutation at Asp301 (ref. [535]). Subsequent work in this laboratory showed that the oxidations of basic amine substrates (and their binding) are dependent upon Glu216 (Asp216 is also effective)[441], a result independently reported by Wolf's group[550].

A list of P450 2D6 residues postulated to form the active site includes at least Asp100, Trp316, Pro371 (ref. [539]), Pro103, Ile106, Thr107, Leu110, Pro114, Ser116, Ala122, Asp301, Ser304, Ala305, Thr309, Val370, Gly373, Val374, and Phe483 (refs [542], [551]), Phe120, Glu216 (refs [441], [541], [543], [544], [550], [552], [553]), and Gln117, Leu121, Leu213, Phe219, and Phe481 (ref. [543]). Only six of these residues have been examined experimentally to date. The effects of Asp301 have already been mentioned, with caveats about general changes in the protein[441, 549]. Changing Val374 to Met also has an effect[546, 547]. Mutation at Asp100 or Ser304 has been reported to have little effect, if any[548, 554]. Mutation of Phe483 to Ile produced some alteration of the pattern of testosterone oxidation by P450 2D6 (ref. [551]). A change in Phe481 yielded a 10-fold lower catalytic efficiency (k_{cat}/K_m) toward some substrates but not others[555]. The effects of Glu216 have already been mentioned[441, 550] and seem to be restricted largely to the basic amines[441]. Recent models of the P450 2D6 active site (Figure 10.11) suggest that both Asp301 and Glu216 are within bonding distance of amine substrates[441, 545]. Another suggestion from the more recent models[441, 544] is that one role of Asp301 is to use amide hydrogen bonds to establish the juxtaposition of Phe120, which may be involved in hydrophobic bonds with substrates. Site-directed mutagenesis experiments with this residue are currently in progress (F.P. Guengerich and E.M.J. Gillam, unpublished results).

The work cited above brings up the point that certain mutations may alter activity toward some substrates but not others (e.g., Phe481 (ref. [555]), Glu216 (ref. [441])). Similar behavior is seen with some of the natural allelic variants of P450 2D6 as well[556].

Modi *et al.*[557] reported differences in product profiles of P450 2D6 reactions supported with artificial oxygene surrogates and NADPH-P450 reductase, and interpreted these as evidence for an allosteric influence of the reductase. Subsequent experiments in this laboratory did not support this conclusion and are in accord with some differences in the chemical mechanisms for the oxygen surrogates[558].

Detailed experiments have been done on the *O*-demethylation of 3- and 4-methoxyphenethylamine by P450 2D6 (ref. [559]). Analysis of kinetic deuterium isotope effects, kinetic simulation, and

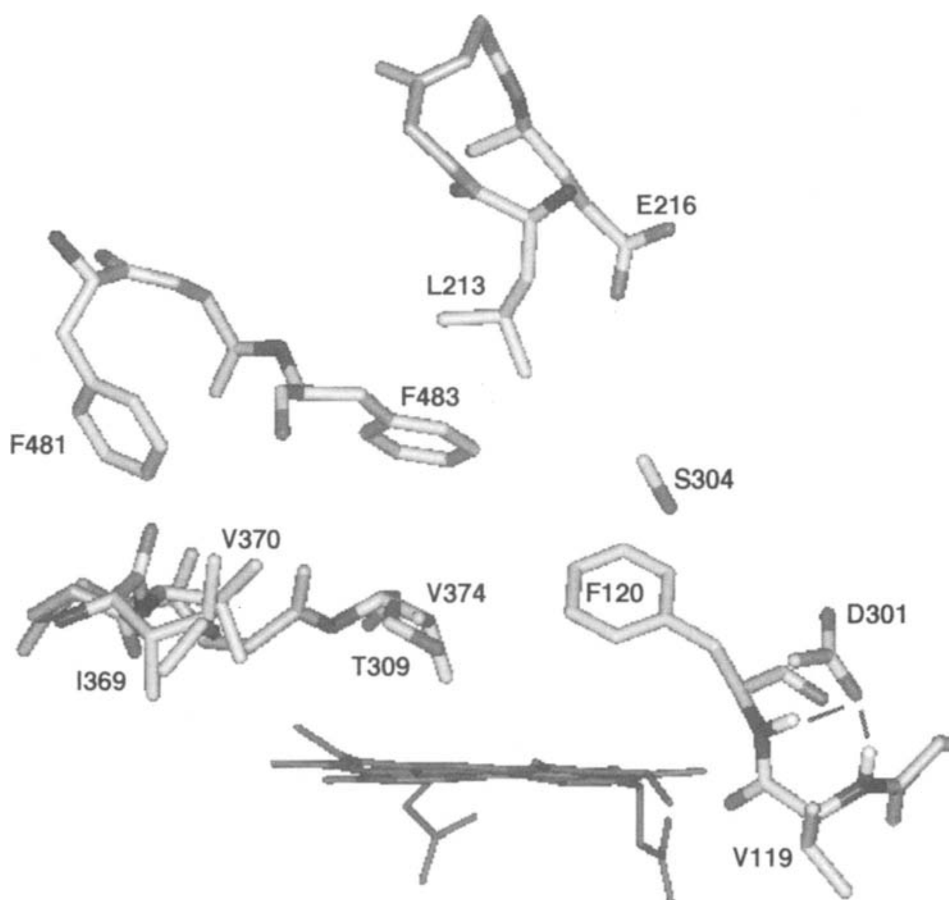

Figure 10.11. Model of some residues in the P450 2D6 active site[441]. Views of the substrate-binding cavity in the P450 2D6 homology model are shown with only the relevant residues, plus the heme, I-helix backbone, and other areas of peptide backbone shown. Hydrogens are not shown except for the amide hydrogens of residues 119 and 120 hypothesized to hydrogen bond to the carboxylate oxygen atoms of Asp301. Side view of the active site from the perspective of the I-helix: I-helix residues have been cut away excepting the side chains of residues 309, 301, and 313 shown at the front of the view.

other experiments yield evidence that both late steps in O_2 activation *and* C–H bond breaking contribute to k_{cat}. The exact meaning of K_m is still not defined with this and most P450 reactions. Some of the P450 2D6 allelic variants show no changes in k_{cat} for certain reactions but do show K_m differences[560]; these are probably more complex than simple "affinity" for the substrate.

6.12.5. Inhibitors

Many inhibitors of P450 2D6 have been reported; for a compilation of the literature, see Rendic[32]. Inhibition of P450 2D6 is an undesirable issue in drug development, and most pharmaceutical companies have screening programs in place.

The most established inhibitor of P450 2D6 is quinidine[561]. The K_i is ~50 nM and inhibition is competitive. Interestingly, quinidine is not a substrate for P450 2D6 (refs [106], [559]).

Mechanism-based inactivation of P450 2D6 is known, for example, 5-fluoro-2-[4-[(2-phenyl-1*H*-imidazoyl-5-yl)methyl]-1-piperazinyl]pyrimidine (SCH66712)[562]. In the case of this compound, covalent binding to protein was detected but the position of attachment has not been identified.

6.12.6. Clinical Issues

The clinical issues regarding P450 2D6 are considerable due to the large variation in the genetics in the population (Figures 10.2 and 10.5), and the contribution of P450 2D6 in the total scheme of drug metabolism (Figure 10.3). Individuals seem to be rather tolerant of the wide variability in expression with many marketed drugs, probably because of generally wide therapeutic windows selected for in the basic process of drug development. However, P450 2D6 PMs can be at considerable risk when they encounter certain drugs, as first observed by Smith[5, 510]. The problem is seen with drugs having a relatively narrow therapeutic index, for example, debrisoquine[5], phenformin[563], captopril[564]. The effects of P450 2D6 deficiency are seen not only in short-term treatments but also in long-term therapy[565]. The issue of ineffectiveness of drugs that are very rapidly metabolized by "ultrarapid" metabolizers is an issue (Figure 10.5). Modeling of the variability is still an issue[566] and may be a function of particular drugs. The issue of whether genotyping/phenotyping is economical has been considered, particularly in the case of neuroactive and antipsychotic drugs[567, 568]. The overlap between P450 2D6 substrates and neuroactive drugs is also an issue in drug development, largely due to the overlap of these two groups of compounds[569].

Another issue with P450 2D6 is the relevance of the polymorphism to cancer risks. In 1984, Idle[137] reported an association of lower risk of lung cancer (in smokers) with the P450 2D6 PM phenotype. These epidemiology results were reproduced in some studies[570], but not others[138]. Attempts were made to resolve the discrepancies on the basis of levels of smoking[571]. Although some expression of P450 2D6 is detectable in lung[507], no clear role for P450 2D6 in carcinogen activation could be established, even with crude tobacco smoke fractions[139]. The issue of whether lung cancer is associated with P450 2D6 was not resolved by changing analyses from phenotyping to genotyping. The generally accepted epidemiological conclusion today is that P450 2D6 is not related to lung cancer[138, 572–575].

Other epidemiology studies have suggested relationships of P450 2D6 with other cancers[576, 577], but these findings have not been scrutinized as much as the lung cancer hypothesis.

Another disease in which P450 2D6 has been proposed to play a role, on the basis of epidemiology, is Parkinson's disease[578]. Contradictory findings have been reported[579, 580]. Although a hypothesis has been raised that induction of P450 2D6 by smoking might explain some discrepancies[581], this proposal lacks biological plausibility in light of the known refractory response of P450 2D6 to induction.

A final issue is that of autoantigens. Autoantigens (LKM1) that recognize P450 2D6 have been known for some time[582, 583]. These antibodies are associated with some cases of hepatitis. The exact mechanism of how they arise is still unclear, as is the relationship with hepatitis. The antibodies may arise by molecular mimicry[584] or they may result from P450 2D6 translocation to the outer plasma membrane[585, 586]. These "LKM1" antibodies may serve as diagnostic tools for particular types of hepatitis[587, 588], but causal relationships have never been demonstrated.

6.13. P450 2E1

The mixed-function oxidation of ethanol was reported nearly 40 years ago[589]. The view that ethanol could be a P450 substrate was not readily accepted because of the hydrophilic nature of the molecule, but Lieber's group characterized the enzyme in rat liver[590, 591]. Collaborative work with Levin led to the isolation of the P450 ("j"), which was also found to be inducible by isoniazid[592]. Human P450 2E1 was purified by Wrighton *et al.*[593], and Gonzalez's group characterized the human gene[594].

6.13.1. Sites of Expression and Abundance

The greatest concentration is in the liver, and P450 2E1 is a moderately abundant P450 (Figure 10.4). The inter-individual variation is an order of magnitude (Figure 10.2)[28, 595]. A racial difference exists, with Japanese samples having mean expression levels less than Caucasians (Figure 10.2)[84].

P450 2E1 is reported not to be present in fetal liver but appears within a few hours after birth,

regardless of the gestational age[596]. The activity increases during the first year of childhood, and transcriptional regulation due to hypermethylation has been proposed.

P450 2E1 is expressed in many extrahepatic sites including lung[326], esophagus, small intestine[597], brain[598, 599], nasal mucosa[600], and pancreas[601] (some of the evidence is extrapolated from rat work but not necessarily extended to humans).

P450 2E1 is found mainly in the endoplasmic reticulum. With heterologous expression in bacteria, (rabbit) P450 2E1 is membrane bound and catalytically active even when amino acids 3–29 are deleted[17, 602]. The same bacterial localization was seen with human P450 2E1 from which 21 N-terminal residues were deleted[603]. However, P450 2E1 can show some unusual localization in mammalian systems. Ingelman-Sundberg's group deleted residues 2–29 of rat P450 2E1 and demonstrated the presence of a shattered fragment in the mitochondria of a mouse hepatoma cell line[604]. Avadhani's group found P450 2E1 intact in rat liver mitochondria and reported that it could couple these with adrenodoxin and adrenodoxin reductase there with full catalytic activity[605]. Subsequent work demonstrated a cryptic mitochondrial targeting signal at positions 21–31 that was activated by cyclic AMP-dependent phosphorylation of Ser129 (ref. [26]). Neve *et al.*[606] found that the charge of the *N*-terminus of (rat) P450 2E1 was such that a part is directed to either the lumen of the endoplasmic reticulum or the outside of the plasma membrane. The relevance of these localizations to human tissues is still unknown but likely.

6.13.2. Regulation and Polymorphism

Early work in experimental animals was focused on the induction of P450 2E1 in rat liver[590]. Subsequently, many other chemicals, including isoniazid and some solvents, were shown to induce P450 2E1 (ref. [607]). It is also of interest to note that some of the common polycyclic hydrocarbons and other inducers of P450 1 family enzymes attenuated the level of P450 2E1 (ref. [607]). The regulation of P450 2E1 has come to be recognized to be relatively complex, involving transcriptional activation, mRNA stabilization, increased mRNA translation efficiency, and decreased protein degradation[608].

HNF-1 is reported to regulate *CYP2E1* gene transcription[609]. Obesity and diabetes are known to modulate P450 2E1 in rat models. In rat hepatocyte cell culture, insulin attenuated mRNA levels and glucagon or dibutyryl cyclic AMP elevated mRNA, with the latter effect downregulated by a protein kinase A inhibitor[610]. mRNA levels are also selectively attenuated in mice or cell culture (relative to other P450s) by interleukin-6[611], interleukin-4[612], or interleukin-1β or tumor necrosis factor (TNF)α[613]. Multiple mechanisms have been invoked, including kinase pathways, control of HNF-1α function, and regulation of other transcription factors.

Evidence for control at the level of mRNA stability and enhanced translation efficiency has been presented by Novak[614, 615]. The 3′-region of the gene appears to be important in stability. The relevance of this rat model to human P450 2E1 is still unknown.

Another mechanism, generally well accepted although not completely understood, involves protein stabilization by substrate. Rat studies (*in vivo*) showed that ~1/2 of P450 2E1 was lost in 1 hr, and a ubiquitin-linked pathway was invoked[616]. Similar findings were also reported for human P450 2E1 in HepG2 cells[617]. An attempt has been made to estimate the halflife of P450 2E1 in humans *in vivo* using chlorzoxazone pharmacokinetics and a P450 2E1 inhibitor[618]. The halflife was estimated at 50 ± 19 hr, but this approach may not be sensitive enough to detect a short-lived P450 2E1 pool. The relevance of substrate stabilization of P450 2E1 to *in vivo* parameters has been addressed by Thummel and Slattery[619].

P450 2E1 is polymorphic and, because of the nature of many of the substrates, many efforts have been made to determine the relevance of SNPs and other polymorphisms to disease and risk of injury. For a current update on *CYP2E1* polymorphisms, see http://www.imm.ki.se/Cypalleles/. A polymorphism in the 5′ flanking region was suggested to be related to the binding of a transcription factor and related to alcohol intake[50, 620]. A number of other polymorphisms have been identified[50, 621, 622]. However, the evidence to date indicates that these polymorphisms do not seem to have much significance in terms of their effects on *in vitro* or *in vivo* activity of P450 2E1 (refs [84], [621], [623–625]).

6.13.3. Substrates and Reactions

P450 2E1 was originally characterized as an ethanol-oxidizing enzyme. P450 2E1 can oxidize some compounds that are present in the body, including acetone and possibly other ketones involved in certain physiological syndromes (fasting, diabetes)[626]. Transgenic P450 2E1-knockout mice appear to be relatively normal, although the blood acetone levels become much higher (than in wild-type mice) after fasting[627].

The role that P450 2E1 plays in ethanol metabolism has been debated for many years[628]. What seems to be the general consensus is that alcohol dehydrogenase is the main enzyme involved in ethanol oxidation. P450 2E1 may make a contribution at very high ethanol concentrations or in individuals with low levels of alcohol dehydrogenase activity. P450 2E1-knockout mice have blood ethanol levels not significantly different from wild-type animals after administration of ethanol[629]. Acetaldehyde, the product of ethanol oxidation, is also oxidized to acetic acid by rat and human P450 2E1 (ref. [630–632]).

The oxidation of 4-nitrophenol to 4-nitrocatechol has been used as an *in vitro* marker of human P450 2E1 (ref. [633]). Chlorzoxazone 6-hydroxylation was demonstrated to be a relatively specific reaction catalyzed by human P450 2E1; other enzymes (e.g., P450 1A1) can catalyze the reaction but with poor catalytic efficiency[634, 635]. Chlorzoxazone is a relatively innocuous muscle relaxant and the assay can be used *in vivo* to estimate hepatic P450 2E1 function noninvasively[84, 625].

One group of substrates of interest is *N*-nitrosamines, which are carcinogens at many sites and can be formed by chemical reactions within the body (e.g., stomach acid)[636]. Early research on the activation of *N*-nitrosodimethylamine (*N,N*-dimethylnitrosamine) indicated biphasic kinetics of the activating *N*-demethylation reaction and the possible contribution of multiple P450s and possibly other enzymes[637, 638]. The enzyme involved in the "low K_m" reaction was shown to be P450 2E1 in rat and human liver[639, 640]. An *in vivo* role of P450 2E1 has been confirmed in rats[641]. However, P450 2A6 has a significant share of the role of activation of some more complex nitrosamines, even *N*-nitrosodiethylamine[282, 283].

P450 2E1 has been shown to be a major P450 involved in the oxidation of a number of low molecular weight cancer suspects including not only nitrosamines but also benzene, styrene, CCl_4, $CHCl_3$, CH_2Cl_2, CH_3Cl, CH_3CCl_3, 1,2-dichloropropane, ethylene dichloride, ethylene dibromide, vinyl chloride, vinyl bromide, acrylonitrile, vinyl carbamate, ethyl carbamate, and trichloroethylene[304]. The oxidations by P450 2E1 all have relevance to the activation and detoxication of these compounds and their risk assessment[304, 642]. Another substrate is the gasoline additive methyl *tert*-butyl ether[643]. A role of P450 2E1 has been shown in the activation of some of these chemicals in knockout mice[644, 645].

Another substrate for human P450 2E1 is lauric acid, which undergoes 11-hydroxylation[646, 647]. The physiological relevance of this reaction is unknown. Indole is oxidized by P450 2E1 (3-hydroxylation, generating indigo) as well as by other P450s, particularly P450 2A6 and 2C19 (refs [296], [648]). The relevance of this reaction to the urinary excretion of indigoids[649] is still unclear.

Relatively few drugs are oxidized by P450 2E1 (Figure 10.3). Chlorzoxazone is one[634]. Halogenated anesthetics are often metabolized by P450 2E1, including halothane[650] and isoflurane[651].

For more lists of substrates, see Rendic[32].

6.13.4. Knowledge about Active Site

One of the issues in P450 2E1 reactions is the need for b_5, first demonstrated with the rat enzyme[639] and also the human enzyme[640, 652]; the involvement also exists in microsomes[653]. b_5 also augments P450 2E1 activity in bacterial expression systems[425, 654]. In contrast to several of the P450s, apo-b_5 (minus heme) does not function, arguing for a "classic" role of electron donation in enhancement of catalysis[425, 655].

A number of homology models of human P450 2E1 have been published, based upon bacterial P450s and rabbit P450 2C5 (refs [302], [656], [657]). One of the difficulties in dealing with models for the low molecular weight substrates is that many of these compounds have very little in the way of features to bond to, other than hydrophobic residues or halogens. Utilizing these models for both very small substrates (e.g., ethanol, CH_3Cl) and larger, more conventional ones (e.g., chlorzoxazone, lauric acid) is an issue, unless only parts of the larger substrates are inserted. One problem is

that inherent binding affinities are generally unknown and spin-state changes have not been very useful with P450 2E1 (refs [652], [658]).

Mathematical models have also been developed for rates of oxidation by P450 2E1 (refs [659], [660]). In essence, these are based on chemical reactivity at individual substrate atom sites. In both of the cited examples[659, 660], the models were used for relatively small sets of related compounds and may have some utility. An inherent problem in more extended sets is the difficulty in interpretation of the parameters k_{cat} and K_m. Thus, the rate-limiting step may not be related to hydrogen abstraction or a similar chemical step involving the substrate (*vide infra*).

Keefer *et al.*[661] reported a kinetic deuterium isotope effect on the carcinogenicity of *N*-nitrosodimethylamine in rat liver. Subsequent work with rat and liver microsomes indicated that the effect of the deuterium substitution was expressed in the parameter K_m but not k_{cat} (V_{max})[662], and that isotope effects on K_i were seen when deuterated *N*-nitrosodimethylamine was used as a competitive inhibitor of other P450 2E1 reactions[663]. These results were of interest in that deuterium substitution would not be expected to modify the affinity of a substrate for an enzyme. Studies with deuterated and tritiated ethanol in this laboratory also indicated an isotope effect on the oxidation of both ethanol and acetaldehyde by recombinant human P450 2E1, manifested mainly in K_m[632, 652]. The results are understood in the context of a reaction sequence where burst kinetics are observed, that is, the first reaction cycle is much faster (~400 min^{-1}) than the subsequent ones, which control k_{cat}. Pulse-chase experiments suggest that little of the acetaldehyde (or its hydrated form $CH_3CH(OH)_2$) dissociates, due to kinetic phenomena. Neither ethanol, acetaldehyde, nor acetic acid has much affinity for P450 2E1. The rate-limiting step occurs after product formation (for both ethanol and acetaldehyde oxidations), but is not product release *per se*. This view of the reaction sequence may apply to some P450 2E1 reactions but not others. Recent work in this laboratory with both P450s 2E1 and 2A6 has shown a kinetic isotope effect primarily on K_m, for the *N*-dealkylation of *N*-nitrosodimethylamine but not *N*-nitrosodiethylamine[664], and the kinetic mechanisms remain to be further elaborated. An interesting point of the deuterated ethanol work is that the intermolecular isotope effect is expressed in the K_m parameter, which includes the C–H bond-breaking step. k_{cat} is governed largely by an enzyme physical step after oxidation of substrate. In this system, the K_m term contains k_{cat} as a variable[632, 652].

A final point involves a report of the kinetics of CO binding to human P450 2E1 following flash photolysis[658]. The kinetics appeared to be monophasic and the rate was decreased in the presence of (400 mM) ethanol. One interpretation of the results is that binding of the substrate makes P450 2E1 more rigid[658].

6.13.5. Inhibitors

As mentioned earlier, many low molecular weight solvents are substrates for P450 2E1. These are also inhibitors of P450 2E1 (refs [69], [70]). Such inhibition is a problem in that historically many insoluble P450 substrates have been added to enzymes using final solvent concentrations of 1% (v/v), which is often ~100 mM. Thus, care is needed in analyses. It is possible to dilute many of the P450 2E1 low molecular weight substrates directly in water to add them to incubations, for example, methylene chloride has a solubility of ~100 mM in H_2O[665].

Some of the alcohol and aldehyde dehydrogenase inhibitors are also inhibitors of P450 2E1, making interpretations of *in vivo* ethanol metabolism studies difficult. 4-Methylpyrazole is an excellent inhibitor[202, 666] and probably one of the best choices for *in vitro* experiments at this time. 3-Amino-1,2,4-triazole[667] and diethyldithiocarbamate[304] are mechanism-based inactivators. The latter is of interest in that the oxidized form, disulfiram (Antabuse®), is an aldehyde dehydrogenase inhibitor used in patients in alcohol aversion therapy. Many of the early animal and human studies on interactions of ethanol and disulfiram with various chemicals can now be rationalized in the context of P450 2E1 (refs [668], [669]).

A number of compounds of natural origin have also been examined as P450 2E1 inhibitors, many of which are derived from vegetables such as onions, garlic, and cruciferous vegetables[670, 671].

6.13.6. Clinical Issues

The major clinical issues involve the role of P450 2E1 in the oxidation of certain drugs, alcoholism, oxidative stress, and risk from cancer.

As pointed out earlier, the most generally accepted noninvasive human assay involves 6-hydroxylation of the muscle relaxant chlorzoxazone[625, 634]. Studies with humans show little effect of diabetes[625, 672], but an effect of body weight/obesity[672, 673]. As mentioned before, genotype has shown little impact on the *in vivo* parameters to date[84, 673].

Another issue is drug metabolism and toxicity. Acetaminophen overdose remains a major cause of liver failure in the United States. Several P450s are involved in the oxidation to the reactive iminoquinone[195]. Studies with P450 2E1 knockout mice indicate that P450 2E1 is a major determinant of acetaminophen toxicity, because the toxicity was considerably attenuated in null animals[118].

P450 2E1-null mice have the same blood ethanol levels as wild-type animals after ethanol dosing[629] suggesting that P450 2E1 activity is not a major factor in ethanol metabolism, at least in mice. The situation regarding a role for P450 2E1 in alcohol-induced liver injury in other models is unclear, with some reports suggesting a link[674, 675] and others not[629, 676]. Autoantibodies against P450 2E1 have been reported in alcoholics[677] and attributed to hydroxyethyl radicals[678] (which may arise from lipid peroxidation processes rather than as intermediates in P450-catalyzed oxidation, *vide supra*). P450 2E1 is also a major autoantigen associated with halothane hepatitis, a rather idiosyncratic response[679]. As with other autoimmunities involving P450s, causal associations remain to be demonstrated[453].

Another issue is the contribution of P450 2E1 to oxidative stress. Ingleman-Sundberg reported that P450 2E1 contributed ~20% of the NADPH-dependent lipid peroxidation in rat liver microsomes (and 45% in microsomes prepared from rats treated with acetone to induce P450 2E1)[680]. Transfection of human P450 2E1 into a rat hepatic stellate cell culture system yielded elevated production of reactive species[681]. Cederbaum[682] has reviewed studies on the relationship of oxidative stress to P450 in liver cell models. The exact relevance to liver injury and alcohol-induced disease requires more investigation.

Many studies have been reported on the relationship of *CYP2E1* polymorphisms to risk of diseases. Benzene poisoning in Chinese workers showed some changes in risk with one genotype but only in smokers[683]. With regard to cancers, the results appear to be very mixed. An early report suggested a link of lung cancer with a polymorphism[684], but since then the results have been mixed for cancers of the lung[685–690], oral cavity[691, 692], and stomach[693]. In most of these cases, it should be emphasized that there is little information about exposure and the only relevant etiology is probably tobacco-derived nitrosamines. In a study of workers exposed to vinyl chloride (a P450 2E1 substrate[304]), some association was found between P450 2E1 polymorphisms and p53 mutations[694]. However, it should be emphasized again that the relevance of *CYP2E1* polymorphisms to known P450 2E1 reactions is unclear, particularly *in vivo*[673], and it is difficult to define roles of these genetic polymorphisms in cancer risk; overall P450 2E1 expression due to environmental influences may have a role but is more difficult to establish.

6.14. P450 2F1

This is primarily a lung P450. In 1990, Nhamburo *et al.*[695] cloned the cDNA from a human lung library. The level of expression appears to be relatively low, as judged by the mRNA abundance. The apparent orthologs 2F2 and 2F3 have been studied in mouse and goat lung, respectively.

P450 2F1 has been expressed in heterologous systems. Catalytic activity was observed for 7-ethoxy- and -propoxycoumarin *O*-dealkylation and 7-pentoxyresorufin *O*-depentylation. The enzyme showed modest activation of the lung toxin and (potential drug candidate) 4-ipomeanol[696]. However, the ability of P450 2F1 to activate the potential lung toxicants 3-methylindole, naphthalene[697], and styrene[698] is more impressive (the activation of 3-methylindole appears to involve initial desaturation[697]).

The basis for the selective expression of P450 2F1 in lung is unknown. Recently, Carr *et al.*[699] isolated the *CYP2F1* gene. Using luciferase-based constructs, they identified a specific promoter element that binds a protein in the −152 to −182 5′ region. This protein is termed a lung specific factor (LSF).

6.15. P450 2J2

The P450 2J2 cDNA was first isolated from a human liver library but was found to be most highly expressed in heart[700]. Expression (mRNA)

has also been found in kidney and muscle[700], lung[701], and the gastrointestinal tract[702].

Zeldin's group has done most of the work on this P450, including the initial cloning and analysis of tissue expression. Incubation of a recombinant P450 2J2 (plus reductase and NADPH) with arachidonic acid yielded all four epoxides, that is, epoxyeicosatetraenoic acids (EETs)[700]. These EETs were found in heart tissue, and the stereochemistry of the recombinant P450 2J2 products was found to match that of the compounds isolated from tissue. A number of physiological functions have been postulated for the EETs, reviewed elsewhere[703].

The extent of human variability of expression of P450 2J2 has not been reported. However, Zeldin's group has sequenced *CYP2J2* genes and found a number of SNPs[704]. One was in the promoter region, eight were exonic regions, five were in introns, and four were in the 3′-untranslated region. Only four of the SNPs resulted in amino acid changes. These allelic variants were expressed in a baculovirus system; all had activity toward arachidonic and linoleic acids within a 2-fold level of wild-type P450 2J2, with the N404Y variant showing only 10% catalytic activity (all assays only done at a 100 μM substrate concentration), although some qualitative changes in products were seen with the I192N substitution. The physiological relevance of these substitutions is presently unknown.

6.16. P450 2R1

The only information available is the presence of the *CYP2R1* gene in the human genome[705].

6.17. P450 2S1

This gene was identified by searching databases by Rylander *et al.*[706] mRNA and protein blotting work indicate highest expression in trachea, lung (and fetal lung), stomach, small intestine, and spleen. Expression was also relatively abundant (mRNA level) in colon, appendix, liver, kidney, thymus, substantia nigra, peripheral leukocytes, and placenta. Absolute levels of abundance are unknown.

Rivera *et al.*[87] demonstrated that both mouse and human P450 2S1 mRNA transcripts are inducible by TCDD in cell culture.

No other information is presently available about P450 2S1.

6.18. P450 2U1

As with some of the other human P450 genes, the only information presently available is the identification of the *CYP2U1* gene in the human genome[705].

6.19. P450 2W1

No information is available at this time except for the existence of the *CYP2W1* gene in the human genome[705].

6.20. P450 3A4

P450 3A4 is the most abundant P450 in the body (e.g., Figures 10.2 and 10.4) and has a dominant role in drug metabolism (Figure 10.3). Some of the earliest preparations of human P450 (refs [3], [4]) were retrospectively found to be P450 3A4. Two approaches led to an extensive characterization. Watkins *et al.*[13] isolated a P450 from human liver using the criterion of immunochemical cross-reactivity with what is now recognized as a rat 3A subfamily P450; this laboratory isolated an enzyme from human livers that catalyzed the oxidation of the hypotensive dihydropyridine drug nifedipine[12]. cDNA cloning yielded sequences corresponding to *CYP3A3* (ref. [707]) and *CYP3A4* (ref. [708]). (The former differed from *CYP3A4* at 14 sites and could be considered a rare allele, although it has not been reported again[709–711] and originally came from the same single-liver cDNA library as the *CYP3A4* clone; *CYP3A3* has been dropped from the nomenclature and earlier references to this should probably be considered to indicate P450 3A4.)

Subsequently, studies with microsomes, antibodies, and purified P450 3A4 quickly indicated that nifedipine was not the only substrate; other substrates included other dihydropyridines[712], steroids[12, 713], quinidine[106], the oral contraceptive 17α-ethynylestradiol[72], and the carcinogen aflatoxin B_1 (ref. [714]). With more studies and the application of recombinant systems, the repertoire of substrates expanded rapidly[715].

6.20.1. Sites of Expression and Abundance

P450 3A4 is the most abundant P450 in human liver and in the small intestine. The average fraction of the total P450 in liver accounted for by P450 3A4 is ~25–30%[28] (Figures 10.2 and 10.4); in the small intestine, the fraction attributed to P450 3A4 is even higher. A study with the selective inhibitor gestodene, which destroys P450 3A4, indicates that P450 3A4 can constitute 60% of the total hepatic P450 (ref. [716]).

P450 3A4 is also expressed in some extrahepatic tissues, including lung[244, 717], stomach, colon[244], and adrenal (weak)[718]. P450 3A4 does not appear to be expressed in kidney, prostate, testis, or thymus, but other 3A subfamily P450s are[718]. The literature is mixed on whether expression occurs in peripheral blood lymphocytes or not[718, 719].

A gender difference in P450 3A4 expression does not appear to occur[28] and apparent pharmacokinetic differences may be attributable to *P*-glycoprotein, not P450 3A4 (ref. [55]). In fetal liver, P450 3A7 is the most abundant form and P450 3A4 expression is very low[58, 720]. P450 3A4 expression increases rapidly after birth and reaches 50% of adult levels between 6 and 12 months of age[720]. Although many general regulatory concerns have been expressed about additional safety margins for children with drugs and other chemicals, the evidence in this case indicates that P450 3A4 activity levels in infants are slightly higher than in adults[720].

P450 3A4 is expressed in some tumors, although the literature is mixed as to reports of levels lower and higher than the surrounding tissue[721–723].

6.20.2. Regulation and Polymorphism

The *CYP3A4* gene is at chromosome 7q22.1 (ref. [724]). Although 3A subfamily enzymes were long known to be inducible in animals[725] and considerable literature existed on the *in vivo* induction of many activities by barbiturates and macrolide antibiotics (e.g., rifampicin)[40], early demonstrations of inducibility were indirect but some progress was made[13]. A general correlation between enzymes and mRNA levels could be shown in human livers[707, 709]. Defining the mechanism of regulation was difficult[726], to some extent because of difficulty in finding appropriately responsive cells to utilize the *CYP3A4* gene and vector constructs derived from it. Guzelian's laboratory reported that the source of liver cells was a greater issue than the *CYP3A* regulatory region in comparing interspecies differences in *CYP3A* gene regulation[727], and this result can now be rationalized in the context of new knowledge about receptors (*vide infra*).

Although most *CYP3A* subfamily genes are inducible by dexamethasone, the classic glucocorticoid receptor was shown not to be involved in rat liver[728]. In early 1998, Maurel reported that the macrolide antibiotic rifampicin acted as a nonsteroid ligand and agonist of the human glucocorticoid receptor, providing a possible mechanism for regulation and a difference with the rodent systems[729]. The interpretation of these conclusions was questioned by Ray *et al.*[730].

Shortly thereafter, Kliewer's group characterized the human homolog of a mouse receptor (PXR) that bound steroids and interacted with *CYP3A* subfamily genes in the manner expected for a major regulatory influence[731, 732] (some literature also refers to the human PXR as "SXR"). This member of the steroid receptor family "orphan" group interacted with barbiturates, steroids (including dexamethasone), statin drugs, macrolide antibiotics, and some organochlorine pesticides[732, 733].

Knowledge of the PXR and its cognate binding site has led to the development of PXR receptor and reporter assays to screen for P450 3A4 induction with new drug candidates[734–736]. The discovery of the PXR receptor suggested that alleles of this receptor might be responsible for the variable inducibility in different individuals. However, the SNPs found to date have not been found to control P450 3A4 induction[737]. The regulation of *CYP3A4* expression is more complicated than simple loading of activated PXR (e.g., Figure 10.6), as suggested by Kliewer's early work showing the roles of coactivators[731, 732]. However, the glucocorticoid-mediated induction of P450 3A4 is mediated by elements in addition to the now-canonical PXR site[738, 739]. Some compounds (e.g., ketoconazole) suppress *CYP3A4* gene expression, apparently via binding to the PXR and interaction with "co-repressors" (NCoR, SMRT)[740]. CAR (see Section 6.7.2) appears to interact with the *CYP3A4* gene at the PXR site and induce[741]. Further, there is

evidence that 1α,25-dihydroxyvitamin D_3 (see Section 6.53) also controls the transcription of P450 3A4 (ref. [742]). This effect is mediated through the vitamin D receptor[342], which has similarity to PXR and CAR in the steroid receptor superfamily. Kinases have been shown to modulate the induction of P450 3A4 via the vitamin D receptor in Caco-2 cells[743].

Other factors also contribute to P450 3A4 regulation. Among these are C/EPPα and DBP[744] and HNF-4α (ref. [745]). Interleukin-6 has been reported to downregulate P450 3A4 through translational induction of the repressive C/EBPβ-LIP protein[746]. Thus, the transcriptional regulation of P450 3A4 expression centers on PXR but involves many other aspects.

Another aspect of P450 3A4 regulation involves degradation. Troleandomycin, erthyromycin, and some related amine macrolide antibiotics form "metabolite complexes" (C-nitroso : iron, R–N=O : Fe) and inactive protein accumulates[747, 748]. These studies have relevance to *in vivo* P450 3A4 inhibition by these drugs.

P450 3A4 appears to be degraded by a ubiquitin-linked pathway[221]. Correia's group also reported that protein kinase C modified P450 3A4 at Thr264 and Ser420; the relevance of these phosphorylations to ubiquitin-linked degradation is yet unknown[749].

The issue of polymorphism is considered in the context of attempts to explain the population variability in P450 3A4 activity, which does not show true modality in its distribution[750]. A number of SNPs and other polymorphisms have been identified, but they have not shown much relationship to catalytic activities yet[751–757].

6.20.3. Substrates and Reactions

Analysis of the catalytic activity of P450 3A4 and other 3A subfamily enzymes is not always easy to assess because of nuances about the effects of the membranes and other proteins, as discussed in Section 6.20.4. Wrighton has examined P450s 3A4, 3A5, and 3A7 under identical conditions and concluded that P450 3A4 is generally more catalytically active than 3A4 or 3A7 toward all substrates examined[758].

P450 3A4 contributes to the metabolism of ~50% of the drugs on the market or under development (Figure 10.3). For an extensive list, see Rendic[32]. Many of these are important drugs such as lovastatin (Mevacor®) and other statins[759], the prostate hypertrophy inhibitor finasteride (Proscar®/Propecia®)[760], the immune suppressant cyclosporin[761, 762], protease inhibitors such as indinavir[763], and sildenafil (Viagra®)[764].

In the course of these reactions, P450 3A4 catalyzes examples of some atypical reactions[524] including desaturation[759], oxidative carboxylic acid ester cleavage[765], and oxidation of a nitrile to an amide[766]. An unexpected reaction encountered in this laboratory was the oxidation of alkylphenyl ether non-ionic detergents, which have been commonly used in enzyme purifications[328] and also have some medical and industrial applications[767]. Methylene hydroxylations yield hemiacetals, which break down to shorten the chains[767].

One of the classic (and fastest) reactions catalyzed by P450 3A4 is testosterone 6β-hydroxylation[12]. However, the physiological significance of this and other (P450 3A4-catalyzed) steroid hydroxylations[713] is unclear. The significance of P450 3A4 in physiology may be questioned, given its variability (Figure 10.1 and Table 10.5). However, some contributions are possible and may be suggested in recent work. Cholesterol is oxidized by P450 3A4 to 4β-hydroxycholesterol, a major circulating oxysterol[768, 769]. P450 3A4 also catalyzes the 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol[770, 771]. The product is a potent PXR agonist, and this system might function as an autoregulatory pathway (i.e., excess triol activates PXR and P450 3A4, which reduces the level of triol[772]).

P450 3A4 also functions in the metabolism of cancer chemotherapeutic drugs. In addition, attention has been given to activations of drugs and chemical carcinogens. P450 3A4 activates the estrogen receptor antagonist tamoxifen to produce DNA adducts[773]. Another example of carcinogen activation involves aflatoxin B_1, which undergoes both a detoxicating 3α-hydroxylation and formation of the highly mutagenic 8, 9-*exo*-epoxide[714, 774, 775]. Some other carcinogen substrates of P450 3A4 are listed in Table 10.4.

One of the issues with P450 3A4 is which reaction provides the most appropriate index of activity, both *in vitro* and *in vivo*. Historically nifedipine oxidation and testosterone 6β-hydroxylation were among the first activities identified[12] and are still used *in vitro*[71]. Midazolam 1′-hydroxylation has also been used[71], in part because of its acceptance for *in vivo* assays.

Some higher throughput fluorescence assays have also been developed and gained commercial appeal[776, 777]. One issue regarding these and also several other P450 3A4 reactions is that they show variable effects of added chemicals, that is, one compound may inhibit a certain P450 3A4 reaction but stimulate another. Chauret *et al.*[778] reported a fluorescence reaction that behaves in a very similar way to testosterone 6β-hydroxylation. Houston has examined the behavior of P450 3A4 probe substrates *in vitro* and grouped them into two categories. Although all of these reactions are catalyzed by P450 3A4, they are categorized into two groups by their behavior in the presence of other compounds, as mentioned above[779]. One group includes testosterone, cyclosporin, and erythromycin. The second includes midazolam, triazolam, dextromethorphan, and diazepam. Terfenadine fits in either group and nifedipine seemed to have properties unique from both groups[779].

The ambivalence about the variability of probe drugs is even worse for *in vivo* human experiments than *in vitro*, as one might expect. A number of reactions have been used including nifedipine oxidation[780], erythromycin *N*-demethylation[781], lidocaine oxidation[782], dapsone *N*-hydroxylation[783], midazolam 1′-hydroxylation[784], and quinine 3′-hydroxylation[785]. In most cases, the test drug is administered orally for convenience, except for some uses of erythromycin and midazolam (i.v.). The ratio of urinary 6β-hydroxycortisol to cortisol has also been used to assess P450 3A4 function[786]. Many of the assays reflect the activity of P450 3A4 in the small intestine, particularly with the drugs administered orally. The erythromycin breath test (exhaled CO_2 produced from the HCHO released in the reaction) is generally used to estimate hepatic P450 3A4 and has been used as an aid in selecting cyclosporin doses for liver transplant patients[787]. The lack of correlation of these indicators is still a problem in the practical analysis of drug interactions[788–790]. Some of the discrepancies are probably inherent in the nature of P450 3A4 itself (i.e., see *in vitro* assays, *vide supra*). Other issues involve the lack of coordinate regulation of hepatic and intestinal P450 3A4 (ref. [791]) and the activity of *P*-glycoprotein[792], which shows some overlap in regulation patterns with P450 3A4 (ref. [793]) and influence the availability of substrates to P450 3A4 in both small intestine and liver.

6.20.4. Knowledge about Active Site

Because of the importance of this enzyme in drug metabolism (Figure 10.3), many efforts have dealt with developing a better understanding of its function and there is hope that intelligent predictions can be made regarding activities toward new drug candidates. However, as already alluded to, P450 3A4 has some unusual properties and a number of important issues have not been resolved.

In the early purifications of P450 3A4 (ref. [12]), reconstitution conditions were difficult to optimize and showed some unexplained variations, which was also the case with recombinant enzyme[715, 794]. A major factor was the composition of the lipid/detergent environment[795, 796]. Another issue was the NADPH-P450 reductase. P450 3A4 expressed in yeast showed poor coupling with yeast NADPH-P450 reductase[715], although P450 2C9 had coupled well[374]. Pompon developed a yeast system in which yeast NADPH-P450 reductase and b_5 were eliminated and replaced by the human counterparts, enabling P450 3A4 function[797]. Studies with purified P450 3A4 and NADPH-P450 reductase have shown that P450 3A4 reduction is generally slow in the absence of substrate and greatly enhanced by substrate[798].

The role of b_5 in P450 3A4 reaction is a somewhat complex subject. Some reactions of purified P450 3A4 are stimulated by b_5 but others are not[799, 800]. With reactions that are influenced by b_5 (e.g., nifedipine oxidation and testosterone 6β-hydroxylation), a role for b_5 could be demonstrated in human liver microsomes using antibodies[801]; stimulation by b_5 can also be demonstrated with P450 3A4 heterologously expressed in bacterial membranes[425, 802], although the presence of b_5 does not seem as critical for function as with the purified systems[803, 804]. Other considerations suggest that the amount of b_5 in heterologous expression systems should not be an issue in the development of "relative activity factors" for extrapolations from hepatic systems[805]. The mechanism of stimulation of P450 3A4 activities by b_5 is still not clear. Pompon used a membrane system and interpreted the results in the context of a "classical"[655, 806] transfer of an electron to the FeO_2^{2+} complex[807]. However, the activities of purified P450 3A4 were stimulated by apo-b_5 (devoid of heme) as effectively as by b_5 (ref. [808]), and similar effects have now been

reported for several other P450s (refs [425], [809], [810]). Although an alternate mechanism involving rapid transfer of P450 3A4 heme to apo-b_5 has been proposed[811], evidence ruling out this mechanism has been presented[812].

A number of models of the P450 3A4 protein have been presented[301, 813–815], most of which are based on homology modeling. At the time of this proof (April 2004), the Astex company has publicly announced a crystal structure for P450 3A4, presumably in the absence of ligands, but the information is proprietary and no details have been released.

A number of site-directed mutagenesis studies on the possible roles of individual residues have been published. Phe304[816] and Ala305 (ref. [817]), in the putative I-helix, are proposed to control access to the catalytic center. Phe304 was also implicated in the partitioning of aflatoxin B_1 oxidation (between 3α-hydroxylation and 8,9-*exo*-epoxidation)[818]. A role for Asn206 was also proposed in the work with aflatoxin B_1 (ref. [818]). Leu211 is also postulated to control the size of the active site[819].

Another issue about considerations of predicting sites and rates of P450 3A4 reactions deals with models based on chemical reactivity. The concept has been proposed that P450 3A4 has a relatively open active site and that reactions are influenced largely by the chemical lability of C–H bonds[820], and some commercial software systems have utilized such concepts. This approach to P450 catalysis may have some utility, and substrate linear free energy relationships have been exploited in our own research with rat P450 2B1 (ref. [821]). However, there are some concerns about exactly how appropriate the predictions are beyond specific sets of substrates. Knowledge of rate-limiting steps in P450 3A4 reactions is still rather rudimentary, in part because of some of the various complications discussed here. The rate of transfer of the first electron from NADPH-P450 reductase is slow in the absence of substrate, but quite rapid in the presence of substrate and an equimolar concentration of NADPH-P450 reductase[798]. In liver microsomes, the testosterone-stimulated rate of P450 reduction appears to be faster than the overall rate of 6β-hydroxylation[798], but conclusions are complicated by the inability to observe only the P450 3A4 component of the reduction. Although some apparent (FeO) intermediate complexes do appear to accumulate in P450 3A4 reactions[822], these are not well characterized yet. One approach to analysis of rate-limiting components of P450 (and other enzyme) reactions is the use of kinetic deuterium isotope effects[823, 824]. Interpretation of kinetic isotope effects in enzymatic reactions is a complex subject, but a simple interpretation of a significant intermolecular noncompetitive hydrogen isotope effect is that C–H bond-breaking is at least partially rate limiting[825]. Despite the interest in P450 3A4, relatively few kinetic deuterium isotope effect studies have been reported. Obach[826] reported an isotope effect of only 1.3 on the hydroxylation of the drug ezlopitant, although details regarding k_{cat} and K_m are lacking and further interpretation of this result is difficult. Work in this laboratory with 6-d_2-testosterone has somewhat surprisingly shown a low competitive isotope effect ($^{D}V$and $^{D}(V/K)$ ~3, J.A. Krauser and F.P. Guengerich, unpublished results). Testosterone 6β-hydroxylation is one of the fastest reactions catalyzed by P450 3A4 and one might expect less masking of an isotope effect in a system in which other steps are known to be occurring efficiently.

In some of the previous sections, compounds have been mentioned that stimulate the catalytic activities of P450s after direct addition to the enzyme, as opposed to regulation of genes in cells or animals. This process is referred to as "stimulation" (as opposed to induction). The phenomenon has been recognized for some time with P450s (refs [107], [109], [827]). Conney's group demonstrated the activation of benzo[*a*]pyrene 3-hydroxylation and aflatoxin B_1 activation by αNF in human liver microsomes[828, 829]. Johnson also demonstrated the enhancement of human liver microsomal 17β-estradiol 2-hydroxylation by αNF[830]. Subsequent work in this laboratory provided evidence that P450 3A4 was the human liver P450 most stimulated by αNF[146, 714]. P450 3A4 is the P450 about which most of the discussion about cooperative behavior has been given, although some reports of cooperativity have appeared regarding P450s 1A2, 2B6, and 2C9 (Sections 6.7.4 and 6.9.4). In addition, P450 2C19 may show stimulation by some compounds (R. Kinobe, B.D. Hammock, and E.M.J. Gillam, personal communication).

In considering cooperativity, two types will be described, using a convention we[429, 831] have adapted from Kuby[824]. "Homotropic" cooperativity refers to nonhyperbolic phenomena (either

steady-state reaction kinetics or binding) seen with the addition of a single compound to an enzyme. The most typical type is a sigmoidal, or "S-shaped" curve, which when analyzed by a Hill plot ($v = V \bullet S^n/[S + S^{50}]$) yields a value for $n > 1$ (S^{50} is an approximation of the usual K_m), or "positive cooperativity." Examples of "negative cooperativity" are less common but documented ($n < 1$) as in a case with rabbit P450 1A2 (ref. [214]). The other phenomenon is "heterotropic cooperativity," described above as "stimulation," where two compounds are added to an enzyme and one enhances the catalytic action of the enzyme on the other. In some cases, both homotropic and heterotropic cooperativity can be operative[429].

Homotropic cooperativity was seen in the oxidation of aflatoxin B_1 by P450 3A4 (ref. [832]). The previously reported stimulation of P450 3A4 activities by αNF[714] was not seen for some reactions, and the *N*-oxygenation of 4, 4′-methylene-*bis*(2-chloroaniline) was inhibited[833]. Subsequently, studies with aflatoxin B_1 oxidation showed that 3α-hydroxylation was inhibited and 8,9-(*exo*)-epoxidation was stimulated by αNF[429, 774]. Aflatoxin B_1 (or an analog) did not modify the 5,6-epoxidation of αNF, however. Interestingly, the positive cooperativity seen in Hill plots for the oxidation of aflatoxin B_1 (for both reactions) was eliminated in the presence of αNF[429]. The values for n in the Hill plots (2.1–2.3) are probably the highest for any P450 apparent cooperativity reported to date. Most are much lower. One technical issue of particular note is that those reactions that proceed too far at low substrate concentrations will show apparently low rates (due to substrate depletion or product inhibition) and artificial sigmoidicity can be created.

Many seemingly unusual P450 3A4 reactions and patterns have been reported (*vide supra*). P450 3A4-catalyzed testosterone 6β-hydroxylation and erythromycin *N*-demethylation are not very competitive[834]. Hydroxylation of meloxicam is stimulated by another substrate, quinidine[835]. Lu and his group showed that inhibition patterns for several known P450 3A4 reactions were substrate dependent[836]. Similar discrepancies were reported by Weinkers' laboratory[837]. The (mechanism-based?) inactivation of P450 3A4 by diclofenac was stimulated by the substrate quinidine[838]. One interpretation of some of the results is that a single active site accommodates two (or more substrates), and many of the data can be fit to a model with this much freedom (basically Michaelis–Menten expression with two values each for k_{cat} and K_m, or insert of proportionality factors before the parameters)[839–843].

Halpert's laboratory has changed a number of the amino acids in P450 3A4, based mainly upon homology modeling, and found that several can alter the homotropic and the heterotropic cooperativity. These residues include Ala305 (ref. [844]), Leu211, Asp214 (ref. [845]), Ser119, Ala370, Ile301 (ref. [846]), and possibly some others as well[847]. A general conclusion from much of this work is that two or possibly three ligands co-occupy the binding site and alter each other's juxtaposition to generate some of the observed effects. One problem with much of the work in this field is that actual binding phenomena are not necessarily investigated. However, binding has been analyzed in some of the work[831, 845] and shown to exhibit homotropic and heterotropic cooperativity. Further, combinations of binding and inhibition results obtained with several ligands in this laboratory were consistent with a scheme in which three ligand subdomains exist in the overall binding site of P450 3A4 (ref. [831]), in agreement with the current hypotheses of Halpert[846]. More evidence consistent with such a model is available from a fluorescence study by Atkins and Weinkers[848], in which pyrene–pyrene stacking spectra were observed. This work provides some of the stronger evidence to support the "multiple-substrate site" model.

A crystal structure of bacterial P450 107A1 has been solved with two ligand molecules present[849]. The binding titration shows homotropic cooperativity[849] and also some heterotropic cooperativity[850]. Because the redox partner of P450 107A1 is not known, obtaining reasonable catalytic activity is difficult and the relevance to P450 3A4 is still not definite[850].

Another aspect and possibly another solution to the issue comes from work by Friedman using flash photolysis kinetics (of CO rebinding after photodissociation from ferrous P450 3A4). The kinetics were multiphasic and were selectively altered by the presence of different substrates[851]. Heterotropic effects were observed with benzo[*a*]-pyrene and αNF[852]. The interpretation of the results is that different substrates differentially modulate these kinetics by (a) changing the P450

conformation to alter the rate, and/or (b) steric effects (of ligands) that reduce rates[853]. Both effects are possible, although the enhancement of rates in some cases[851] argues against the generality of the latter explanation and in favor of multiple conformations for P450 3A4 bound to various ligands. The concept advanced is that some ligands act as allosteric factors to "switch" P450 3A4 conformations[854]. Some possibly relevant work has been done by Anzenbacherová *et al.*[855], who did pressure studies on P450 3A4 and found that the compressibility of P450 3A4 was less than that of bacterial P450 102; the compressibility was modified by the ligand troleandomycin (TAO). The concept of preexisting multiple conformers of P450 3A4 is an explanation for the flash photolysis work[851–854] and has support in newer nonclassical approaches to general protein chemistry[856–858]. This view differs from the more general static "lock-and-key" view of enzyme/substrate complexes and the induced-fit theory in which enzymes are "shaped" by their substrates. The basic concept is protein dynamics present an ensemble of structures of an enzyme in solution and different ligands bind to individual states depending upon their complementation[856–858]. Another consideration in this discussion, somewhat related, is that there is good evidence that P450 conformations change during the course of the catalytic cycle[859], and evidence has already been presented that different forms of P450 3A4 can differ in their binding of a ligand (e.g., ferric and ferrous)[831].

Where does all of the work in this area to date leave us? A recent review by Atkins *et al.*[860] summarizes much of the work in more detail and presents a cogent analysis. Summarizing and expanding on this, there are several major possibilities to explain the observed cooperativity of P450 (and the other P450s showing this behavior), which are not necessarily exclusive: (a) a "classic" allosteric model with binding of effectors at a site that then regulates the conformation of substrate binding, (b) a relatively rigid P450 with a large active site that can accommodate 2–3 ligands, with the results depending on the chemical interactions of the two ligands with each other and with P450 residues; and (c) a series of preexisting conformations of P450 3A4 that selectively interact with individual ligands[856–858]. A general concept of induced fit is related to the third possibility, as in the phenomena already mentioned that different protein conformations exist throughout the catalytic cycle, can differ in affinities and substrate orientation, and may not be in rapid equilibria. Many steady-state kinetic schemes have been proposed but, in considering the possible origins[824, 861], can never be considered unique and do not provide mechanistic answers. The availability of a series of three-dimensional X-ray structures of P450 3A4 with various ligands would provide insight into the conformational rigidity of P450 3A4 and the number of modes of binding. Another possible set of experiments involves restraining conformational changes through engineering (e.g., with reversible disulfide bonds) and examination of the effects. Another concern, already expressed here, is that most of the studies in this field have avoided measuring binding, with some exceptions[831, 845, 848]. Some attempts have been made to directly quantify P450 3A4-ligand interactions (e.g., dialysis and equivalent methods), but the technical problems associated with equilibrium binding (e.g., insolubility and nonspecific components) are not trivial. At this time *a priori* prediction of cooperativity is not really possible, except perhaps in extension of chemical classes already covered. The lack of ability to predict P450 3A4 cooperative ligand interactions indicates a deficiency in being able to predict all ligand interactions.

Is the cooperativity of P450 3A4 relevant to any practical issues? Atkins[862] has discussed the general implications of the issue to toxicology, although conclusions are speculative because the function of P450 3A4 can be good, bad, or not really selected for anyway. Some evidence for an interaction between caffeine and acetaminophen in rat models is suggestive of a heterotropic interaction[110, 863]. Dextromethorphan studies in primary hepatocytes also show cooperativity[108]. Cooperativity in human hepatocyte systems has also been reported for oxidation of midazolam and warfarin[864]. Cooperativity is a possible mechanism for a drug interaction between felbamate and carbamazepine[865]. One of the strongest cases involves an *in vivo* study on the enhancement of diclofenac in monkeys by quinidine[111, 864], which apparently cannot be attributed to induction. In summary, there is some evidence for *in vivo* P450 3A4 cooperativity, but at this time, the issue is generally considered to be less of a problem than enzyme induction or inhibition.

6.20.5. Inhibitors

Inhibition of P450 3A4 is a major issue in the pharmaceutical industry because of a number of important drug–drug interactions. One example of a problem leading to recall of a drug is that of terfenadine[103, 104, 866].

Erythromycin and ketoconazole are two of the most established inhibitors of P450 3A4, based on clinical experience. Ketoconazole, used at $\sim 1\ \mu M$, is probably the best established P450 3A4 inhibitor for *in vitro* use[64]. Another P450 inhibitor is TAO[867], which also has clinical implications. TAO has been used as a diagnostic *in vitro* inhibitor of P450 3A4, although its mode of action (activation to a nitroso that complexes P450 iron) requires time for the inhibition to occur.

A compendium of P450 3A4 inhibitors has been compiled by Rendic[32]. Only a few other specific examples of P450 inhibitors will be mentioned here.

One issue is the inhibition of P450 3A4 by grapefruit juice, first reported by Bailey[868]. The effect was rather specific for grapefruit and a few other citrus fruits (not orange), and warning labels now include this contraindication for many drugs[869]. Naringenin has some effect[870], but the most active principles appear to be the furanocoumarins bergamottin and 6′,7′-dihydroxybergamottin, which behave as mechanism-based inactivators to destroy intestinal P450 3A4 (refs [99], [100]). The magnitude of the effect of the interaction varies with drugs, with some of the statins, buspirone, terfenadine, astemizole, and amiodarone reported to show the greatest interactions[869].

Many of the HIV protease inhibitors are also potent inhibitors of P450 3A4 as well as substrates in some cases[871]. Because of the variety of drugs that AIDS patients use, the potential for interactions is considerable.

The effects of some herbal medicines on P450 3A4 have already been mentioned. In addition to P450 3A4 induction (e.g., St. John's wort), some of these materials also contain inhibitors. For instance, kava-kava extracts produce kavapyrones that inhibit P450 3A4 (ref. [872]).

Oral contraceptives contain acetylenes and can be mechanistic inactivators of P450 3A4. Inactivation has been demonstrated for 17α-ethynylestradiol, the major estrogenic component of oral contraceptives[72, 873], and several of the progestogenic components, particularly gestodene[716]. Because of the very low doses of these contraceptives that are used today, the effects might be expected to be small[874] although some *in vivo* effects have been reported[875, 876].

Finally, some chemicals and also oxidants have been shown to cause the covalent crosslinking of heme to apo-P450 (ref. [877]). Correia's group has characterized the products of the destruction of P450 3A4 with cumene hydroperoxide; the information is consistent with a dipyrrolic fragment of heme bound to a fragment of the protein[878].

6.20.6. Clinical Issues

The major issues involving P450 3A4 in drug development and clinical use are related to the role of the enzyme in drug disposition, particularly bioavailability and drug–drug interactions due to induction or inhibition[879, 880]. High enzyme activity toward a drug will reduce bioavailability, and variations in levels of P450 3A4 can cause clinical problems when the therapeutic window is narrow. For instance, low cyclosporin levels will not prevent organ rejection during transplant but high levels cause renal toxicity, so adjustment of the dose can be very useful[881]. Terfenadine has a relatively wide window for use but a few serious problems were encountered[104, 882]. Renwick has considered population models of P450 3A4 variability and concluded that there is more inter-individual variability from the oral route than i.v., which is not surprising in light of the previous discussion of the intestinal contribution to drug metabolism. A "default factor" for adults of 3.2-fold is presented, but a factor of 12(-fold) was calculated to be needed to cover 99% of the neonates as well[883].

The effect of disease on P450 3A4 has been considered. P450 3A4 expression appears to be decreased as a result of liver cirrhosis or cancer[595, 721, 884]. P450 3A4 levels were also decreased in celiac disease and reversed by a change in diet[885].

The interactions of herbal medicines with P450 3A4 have already been mentioned and are one of the worst problems with these mixtures[886]. One of the most studied issues is St. John's wort, which induces P450 3A4 as an agonist of the PXR receptor[887, 888]. The induction of P450 3A4 by St. John's wort has been responsible for the loss of the effectiveness of oral contraceptives[95, 889]. The resulting pregnancies

are the result of contraceptive failure due to more rapid elimination of 17α-ethynylestradiol, a phenomenon previously reported for P450 3A4 induction by rifampicin and barbiturates[72, 94, 101].

P450 3A4 is also of some interest regarding cancer, regarding exogenous carcinogens, drugs used to treat cancer, and metabolism of steroids or other compounds that may affect cancer risk or response to chemotherapy. Some chemical carcinogens activated by P450 3A4 are shown in Table 10.4. The activation and detoxication of aflatoxin B_1 have already been discussed in the context of 3α-hydroxylation (to aflatoxin Q_1) and formation of the highly reaction *exo*-8,9-epoxide[714, 774]. However, aflatoxin B_1 is a hepatocarcinogen and must reach the liver to cause damage. In a rat model, induction of rat P450 led to an increase in small intestinal DNA adducts, suggesting that activation of aflatoxin B_1 at this site constitutes a detoxication process, in that these cells are rapidly sloughed and do not progress to tumors[890].

CYP3A4 genotypes have been reported to be related to leukemias caused by prior treatment with epipodophyllotoxin[891]. P450 3A4 expression, measured at the mRNA level, has shown an inverse correlation with response of breast cancer patients to docetaxel, presumably due to changes in bioavailability[892]. However, no relationships were found for any *CYP3A4* genotypes in therapy-related myeloid malignancies[893]. One of the more controversial issues involves whether *CYP3A4* genotypes are linked with prostate cancer, with reports for and against an association[894–899]. The point should be made that strong evidence for a change in an accepted P450 3A4 phenotype has not been made in many of these cases.

6.21. P450 3A5

P450 3A5 has 85% sequence identity with P450 3A4 and, although generally accepted to have less importance than P450 3A4, is of interest because of its polymorphic and racial distribution and possible relevance to clinical issues with P450 3A subfamily reactions.

6.21.1. Sites of Expression and Abundance

P450 3A5 ("Hlp3") was first purified from human adult liver and found to be polymorphically expressed[900]. Gonzalez found a liver sample apparently expressing only P450 3A5 and not 3A4, and used this to clone the cDNA[901].

P450 3A5 expression has been reported in liver, small intestine, kidney, lung prostate, adrenal gland, and pituitary[718, 902–904]. Some researchers have reported expression of P450 3A5 in peripheral blood cells (and *not* P450 3A4)[905] but others have not[718].

P450 3A5 is expressed in fetal liver, in contrast to P450 3A4, but in a polymorphic manner[906]. The overall expression of P450 3A5 (mRNA) as a part of all P450 3A subfamily transcripts has been estimated at 2%[718]. However, only about 25% of Caucasians express P450 3A5, and when it is present, the level is usually less than that of P450 3A4. However, a few individuals have been identified in which P450 3A5 is the predominant P450 3A subfamily enzyme. The variability in expression levels has been linked to a polymorphism (*vide infra*).

6.21.2. Regulation and Polymorphism

The regulation of *CYP3A5* gene seems to be similar to that of *CYP3A4*, although P450 3A5 does not seem as inducible. The fetal/adult selectivity of P450 3A4/3A7 is not seen with P450 3A5 (ref. [906]).

Maurel[907] reported genomic clones and found a CATA box (not TATA) in the promoter. The responses to glucocorticoids are probably explained by the PXR system[908]. A general conclusion has been reached that P450s 3A4 and 3A5 are co-regulated in the liver and intestine, in terms of transcriptional control[909], although other factors may alter the expression[791].

The polymorphic variation of P450 3A5 has been studied and several variants have now been identified (http://www.imm.ki.se/Cypalleles/)[757]. Alternate splicing is a very common phenomenon with *CYP3A5*, with ~50% of liver samples showing this (E.G. Schuetz, personal communication). Most Caucasians with low P450 3A5 protein expression have the *3 allele with an inserted intron[757, 910]. Interesting, the representation of the *1 allele is much higher in Africans and they express active P450 3A5. Other alleles are known, including changes in the 5′-regulatory region where transcription factors bind[911].

The *in vivo* consequences of 3A5 polymorphism are not clear. For instance, Huang found no

significant effect of the *3 polymorphism on midazolam pharmacokinetics[912].

6.21.3. Substrates and Reactions

Since the discovery of P450 3A5, the catalytic selectivity has been known to be similar to that of P450 3A4 (ref. [900]), and subsequent comparisons with P450 3A4 confirmed this view[913]. However, a general problem with P450 3A subfamily enzymes is that they are sensitive to the membrane environment and many reactions of P450 3A5 (but not all) are stimulated by b_5 (refs [425], [800]). In a few cases, the selectivity of P450 3A5 for different oxidation sites appears to differ from that of P450 3A4, for example, aflatoxin B_1 3α-hydroxylation vs 8,9-epoxidation[800, 818].

Recently, as noted above, Wrighton reported an extensive comparison of many reactions by recombinant P450s 3A4, 3A5, 3A7 under identical reconstitution conditions and concluded that P450 3A5 had equal or reduced activity compared to P450 3A4 in all cases[758].

6.21.4. Knowledge about Active Site

Because of the similarity of reactions of P450s 3A4 and 3A5, homology models for P450 3A4 are probably about as valid for P450 3A5. The availability of the Astex P450 3A4 three-dimensional structure should improve the understanding of P450 3A5 as well. Relatively little site-directed mutagenesis of P450 3A5 has been done, but one study of note is the effort by Correia and Halpert to utilize the differences in reactions with aflatoxin B_1 (refs [774], [800]) to probe the effects of changing residues in the putative active site[818].

6.21.5. Inhibitors

In general, the P450 3A4 inhibitors also inhibit P450 3A5. For instance, ketoconazole and fluconazole inhibited both P450s 3A4 and 3A5 (ref. [914]). The mechanism-based inactivator gestodene[716] also inhibits P450 3A5 (ref. [913]).

6.21.6. Clinical Issues

At this point, the significance of the wide variability in P450 3A5 is difficult to assess. As mentioned previously, Huang[912] found no significant effect of the *3 allele on midazolam pharmacokinetics in Chinese individuals. However, it is possible that the extrahepatic expression[718] may influence the course of particular drugs and other chemicals.

6.22. P450 3A7

Early work in the field of human P450 research was done by Kamataki and his associates with fetal samples which led to the purification of a P450 termed HFLa, now known as P450 3A7 (refs [915], [916]). Early research established that this was a major P450 in fetal liver (not in adult liver), and that the enzyme could catalyze several reactions[916].

6.22.1. Sites of Expression and Abundance

Early work established that P450 3A7 is the major P450 present in fetal liver[916] and is also present in other fetal tissues including kidney, adrenal, and lung[917]. Further work by Kamataki's group showed the existence of some immunochemically detectable P450 3A7 in gynecologic tumors and in human placenta, but interestingly not in cynomologous monkey placenta[918]. Guzelian's group also reported P450 3A7 protein in human placenta and endometrium, with elevation in the latter site during pregnancy or during the secretory phase of the menstrual cycle[919]. Subsequently, Sarkar *et al.*[920] reported 10-fold greater expression of P450 3A7 in endometrium in the proliferative rather than the secretory phase. Hakkola *et al.*[921] reported some expression of P450 3A7 mRNA in some first trimester placentas but not in full-term placenta[243].

With regard to development in fetal tissues, Juchau's group found expression of P450 3A7 in early fetal tissue (50–60 days)[922]. Schuetz *et al.*[923] found P450 3A7 mRNA in all fetal liver samples analyzed and also reported its presence in one half of adult liver samples. However, the issue may be the level of expression because Kamataki's group[58] had reported the fetal > adult selectivity. De Wildt *et al.*[720] also found fetal specificity and only very low levels of P450 3A7 in adults. P450 3A7 expression was high during embryonic and

fetal life, and decreased rapidly during the first week of life. Similar findings were reported by Hakkola *et al.*[906] Also, the variability of P450 3A7 expression was 5-fold in fetal tissue (and 77-fold in mRNA). In another report[924], P450 3A7 also disappeared rapidly after infancy.

6.22.2. Regulation and Polymorphism

As in the case of P450 3A4, relatively little solid evidence is available regarding the functional relevance of coding region SNPs. However, the regulation of this gene is complex, as one might expect after considering the temporal patterns of expression during development that were discussed earlier.

Kamataki's group published the cDNA[925] and genomic[926] sequences, which are similar to those of P450 3A4. However, more identity (~90%) is seen in the coding region than elsewhere[907, 926]. Recent work by Koch *et al.*[718] re-established that P450 3A7 only accounted for <2% of all P450 expression in adult human liver; a bimodality of P450 3A7 expression was seen, however. P450 3A4 and 3A7 constructs were expressed in various cell lines by Ourlin *et al.*[744], who showed differential responses to C/EBPα and DBP. As in the case with P450 3A4, P450 3A7 was inducible by rifampicin in cell culture[927]. P450 3A7 has a functional PXR element[928], as does P450 3A4 (*vide supra*), explaining the rifampicin response. Thus, one would expect fetal P450 3A7 induction by the usual P450 3A4 inducers.

Bertilsson *et al.*[929] have also reported a distal xenobiotic response enhancer module (XREM) in the *CYP3A7* gene. An $3A7_{\kappa}B$ element in *CYP3A7* is inactive in *CYP3A4* (ref. [930]), and this element has recently been shown to respond to p53 (T. Kamataki, personal communication). *CYP3A7* expression is regulated by Sp1, Sp3, HNF-3β, and upstream stimulatory factor (USF) 1. Far upstream (~11 kb) there are HNF-1 and HNF-4 and USF1 elements, which differ from the *CYP3A4* gene. Exactly how these and other sequence differences are involved in the rapid onset of P450 3A4, and decrease in P450 3A7 shortly after birth[59] is still not totally clear. Recent work in Kamataki's group suggests that after birth, CEB/Pα recruits an uncharacterized protein factor to squelch the $3A7_{\kappa}B$ site (T. Kamataki, personal communication).

Some interesting variants of *CYP3A7* genes have been reported. An mRNA species was found that contains exons 2 and 13 of a nearby *CYP3A* pseudogene spliced at the 3 end[931]. The *CYP3A7*1C* allele is unusual in the sense that a part of the *CYP3A4* promoter replaces the corresponding region of *CYP3A7* (ER6 motif) and thus confers high levels of expression to *CYP3A7*1C* (ref. [932]).

6.22.3. Substrates and Reactions

Early studies with P450 3A7 purified from fetal liver established that testosterone 6β-hydroxylation is catalyzed by this enzyme[933]. Another early study indicated 16α-hydroxylation of dehydroepiandrosterone (DHEA) 3-sulfate[934]. These activities were later verified with the use of recombinant P450 3A7 (ref. [935]).

In general, P450 3A7 has catalytic activities rather similar to P450 3A4 and 3A5 (refs [936], 937]). Activation of aflatoxin B_1 (refs [938–940]) and heterocyclic amines[938] has been observed in various recombinant and transgenic systems, including transgenic mice[941]. Retinoic acid 4-hydroxylation by P450 3A7 has also been reported[942]. Wrighton's laboratory has done an extensive comparison of catalytic activities and concluded that rates for P450 3A7 are generally considerably lower for P450 3A7 than for P450 3A4 or 3A5 under similar conditions[758].

6.22.4. Knowledge about Active Site

Much less has been done with P450 3A7 than with P450s 3A4 and 3A5. Because the catalytic selectivity of P450 3A7 is similar to P450s 3A4 and 3A5, those models are probably about as applicable. One point of interest is the work of Kamataki's group showing that the substitution T485P improved holoprotein expression in *E. coli*[943].

6.22.5. Inhibitors

Inhibitors have not been studied extensively, but presumably all inhibitors of P450 3A4 are effective with P450 3A7, for example, ketoconazole, troleandomycin, etc.

6.22.6. Clinical Issues

The general point has already been made that P450 3A7 is the major human fetal P450 and, therefore, makes a major contribution to drug metabolism in the fetus. Thus, many, if not most, of the considerations regarding drug interactions etc. with P450 3A4 should be considered with respect to the fetus during pregnancy.

Another potentially important aspect is a report that P450 3A7 expression increases in hepatocellular carcinoma[944], possibly as a part of dedifferentiation.

6.23. P450 3A43

In 2001, three groups reported the characterization of a fourth member of the 3A subfamily, P450 3A43 (refs [945–947]). The sequence identity with the other 3A subfamily proteins is 71–76%. Expression could be detected in liver, kidney, pancreas, and prostate. Rifampicin was reported to induce P450 3A43 in human liver[945]. The level of expression in liver was generally agreed to be very low (~0.1% of P450 3A4).

Heterologous expression was achieved in bacteria[946] but not any of several eukaryotic systems[947]. The recombinant protein had only very low testosterone 6β-hydroxylation activity[946].

No information is available about polymorphisms, although the transcripts appear very prone to splicing[945]. There is a general agreement that P450 3A43 makes little contribution to drug metabolism, but specialized roles in extrahepatic tissues may be possible.

6.24. P450 4A11

6.24.1. Sites of Expression and Abundance

P450 4A11 cDNAs were first cloned from human kidney cDNA libraries using rodent P450 4A probes[948–950]. P450 4A11 is now known to be the major lauric acid ω-hydroxylase in human liver and kidney[951, 952], a fact of some historical interest in the sense that this was the activity first utilized in the separation and reconstitution of (rabbit) P450 (ref. [953]). The exact level of expression in these tissues is unknown; P450 4A11 expression has also been reported in human keratinocytes[954]. In cell cultures, P450 4A11 expression has been observed in primary cultures of human kidney proximal tubular cells[955] and HepG2 cells[956].

A gene originally assigned as *CYP4A11* by Kawashima's group[957] has now been assigned as that corresponding to *CYP4A22* and replaced by the *CYP4A11* gene corresponding to the P450 4A11 cDNA[952].

6.24.2. Regulation and Polymorphism

The levels of hepatic P450 4A11 vary ~10-fold among humans[951, 956]. To date no polymorphisms have been reported (April 2004; http://www.imm.ki.se/CYPalleles/). The regulation of P450 4A11 expression is not well understood but has relevance in consideration of the peroxisome proliferation system and any relevance to cancer. Induction of P450 4A11 by peroxisome proliferators has not been seen in primary human hepatocytes cultures, although P450 4A11 expression is readily detected[958]. In confluent HepG2 cell cultures, P450 4A11 is induced (independently) by peroxisome proliferators (e.g., Wy 14643) and dexamethasone[956]. The relevance of these results to the induction *in vivo* and the observed variability in expression is unknown.

6.24.3. Substrates and Reactions

P450 4A11 is the major lauric acid ω-hydroxylase[950, 951, 957, 959, 960]. The enzyme also catalyzes ω- and some ω-1 hydroxylation of myristic and palmitic acids[957, 960]. Some papers have reported arachidonic acid hydroxylation[952, 961, 962], but most studies have not associated this activity with P450 4A11 at any appreciable level[950, 957, 960]. Oliw's group has reported some hydroxylation of prostaglandin H_2 and analogs by P450 4A11 (ref. [963]).

6.24.4. Knowledge about Active Site

Some information is available about the active site from the catalytic selectivity among fatty acid substrates[960]. Some homology models have been presented[301, 964].

The interesting observation was made that the L131F mutant catalyzes only ω-1 hydroxylation and not ω-hydroxylation of lauric acid[959]. Residue 131 also controlled access to substituted imidazole inhibitors. Interestingly, some of the results on binding of imidazoles provide evidence that the ferric enzyme undergoes a conformational change that depends on both reduction of the iron and the presence of both NADPH-P450 reductase and NADPH[959].

Another interesting observation is that P450 4A enzymes, including P450 4A11, show at least partial covalent heme attachment[965]. Covalent heme binding involves a conserved I-helix glutamic acid (apparently unique in the P450 4A subfamily) and covalent heme binding occurs via an ester bond to the heme 5-methyl group, mediated by an autocatalytic process[965]. The extent of effect of this modification on catalytic activity is difficult to define, although with animal P450 4A enzymes, there appears to be some effect.

6.24.5. Inhibitors

Substituted imidazoles have been used as inhibitors *in vitro*[959]. Presumably some acetylenic fatty acids might be inhibitors but no studies have been reported.

6.24.6. Clinical Relevance

The significance of P450 4A11 is not very clear. Apparently individuals can vary in their expression levels by an order of magnitude[956]. Further, the ω-hydroxylation of medium chain fatty acids occurs, but its relevance is generally considered not to be as important as in the case of long chain fatty acids.

6.25. P450 4A22

Relatively little is known about P450 4A22. The originally reported *CYP4A11* gene[957] was subsequently shown to be *CYP4A22* (ref. [952]), but the cDNA and protein have not been reported. The similarity of the two genes is 95%.

Johnson's laboratory[956] has reported that P450 4A22 is expressed at lower levels than P450 4A11 in human liver, as well as kidney[952]. There was no correlation of expression levels of P450 4A11 and 4A22 in human liver[956]. P450 4A22 expression could not be observed in HepG2 cells or PPARα-overexpressing cells[956].

6.26. P450 4B1

6.26.1. Sites of Expression and Abundance

P450 4B1 was cloned by Nhamburo *et al.*[966] from a human lung cDNA library. P450 4B1 expression has also been reported (in addition to lung) in kidney, bladder[967], breast[968], and prostate[969]. Expression has also been reported in bladder and breast tumors[967]. Definitive information about the level of expression of P450 4B1 is not available.

6.26.2. Regulation and Polymorphism

The extent of variability of P450 4B1 expression is considerable, at least in bladder where the variation is two orders of magnitude[967]. Several SNPs have been reported, including one resulting in a premature truncation[970].

No evidence for the inducibility of human 4B1 has been presented.

6.26.3. Substrates and Reactions

Direct information about the catalytic specificity of human P450 4B1 has been difficult to obtain because of problems in heterologous expression. Following the initial cDNA cloning, expression in a baculovirus system was unsuccessful and only yielded inactive cytochrome P420 (ref. [971]). The substitution S427P allowed for expression, and ω-hydroxylation of lauric acid could be demonstrated. However, information about the native human enzyme has not been available (the S427P mutant does not occur naturally[970]).

Imaoka *et al.*[972] found that functional P450 4B1could be successfully expressed as a fusion protein with NADPH-P450 reductase. They were also successful in developing transgenic mice in which functional P450 was expressed in liver. The authors postulate that expression in the presence of auxiliary proteins (NADPH-P450 reductase, b_5) may stabilize P450 4B1 (ref. [972]). With these systems, it was possible to demonstrate that

P450 4B1 catalyzes the *N*-hydroxylation of 2-aminofluorene and ω-hydroxylation of lauric acid, as expected from studies with rabbit P450 4B1. If other results from work with animal P450 4B1 enzymes also carry over to the human enzymes, one might expect the reaction 4-ipomeanol activation (epoxidation?), 3-methoxy-4-aminoazobenzene *N*-hydroxylation, 2-aminoanthracene *N*-hydroxylation, valproic acid hydroxylation (and desaturation?), and dehydrogenation of 3-methylindole[970].

6.26.4. Knowledge about Active Site

Because of the paucity of information about catalytic selectivity (*vide supra*), little can be said about the active site of human P450 4B1. Some kinetic hydrogen isotope effect work with *rabbit* P450 4B1 suggests that the active site is more restricted than that of P450 2B1 (ref. [973]). Another interesting observation with rabbit P450 4B1 is the covalent linking of the heme to the protein[974], a phenomenon observed with several of the P450 4 family proteins[965, 975]. Whether this binding is seen in human P450 4B1 is unknown.

6.26.5. Inhibitors

No inhibitors of human P450 4B1 have been reported.

6.26.6. Clinical Issues

There are two issues with P450 4B1. First, the enzyme has been shown to activate carcinogens, for example, 2-aminofluorene, and could be a risk factor in bladder cancer[967]. The level of P450 4B1 in tumorous tissue was not higher than in the surrounding tissue, levels of bladder P450 4B1 were higher in tumor patients than in controls[967].

The other aspect is the use of P450 4B1 as a means of drug delivery. Rabbit P450 4B1 has been utilized as an experimental transgenic activation system in the activation of 4-ipomeanol and 2-aminoanthracene, to date only in cell culture models[976].

6.27. P450 4F2

The Kusunose laboratory reported the cloning of a human liver cDNA corresponding to the leukotriene B_4 ω-hydroxylase[977]. The site of expression was distinct from P450 4F3, which is restricted to polymorphonuclear leukocytes. P450 4F2 is found not only in liver but in several extrahepatic tissues, however[978], including kidney (S2 and S3 segments of proximal tubules, in cortex and outer medulla). The extent of variation of P450 4F2 in human liver was ~5-fold[979].

P450 4F2 catalyzes ω-hydroxylation of several lipids, including leukotriene B_4 (refs [979], [980]), arachidonic acid[962], 6-*trans*-leukotriene B_4, lipoxin A_4, 8-hydroxyeicosatetraenoic acid, 12-hydroxyeicosatetraenoic acid, and 12-hydroxystearic acid[981]. The physiological relevance of some of these reactions is of interest but the effects of variability of P450 4F2 have not been demonstrated. Part of the interest lies in the fact that leukotriene B_4 is a potent proinflammatory agent[978, 979].

6.28. P450 4F3

In 1993, Kikuta *et al.*[982] cloned a P450 now known as P450 4F3 from a human leukocyte cDNA library. The protein was expressed in a yeast vector system and was shown to catalyze leukotriene B_4 ω-hydroxylation. The K_m (0.7 μM) was much lower than that reported for P450 4F2 for this reaction (although the k_{cat} and k_{cat}/K_m values have not been carefully compared)[978].

The gene was cloned in 1998[983]. Interestingly, the *CYP4F3* gene has been shown to use tissue-specific splicing and alternate promoters. A 4F3A form contains exon 4 (but not 3) and is expressed in neutrophils; a 4F3B form contains exon 3 (but not 4) and is expressed in fetal and adult liver and kidney, trachea, and gastrointestinal tract[984, 985]. The K_m of the 4F3B (liver) form was 26-fold higher than for the 4F3A (neutrophil) form[984], although the significance of this report is qualified by the absence of k_{cat} or k_{cat}/K_m parameters. Further studies by Soberman's group have shown that the substitution of exon 3 changes the catalytic selectivity from leukotriene B_4 to arachidonic acid (ω-hydroxylation in both cases)[985]. Again, the usefulness of the observation is limited by the lack of kinetic parameters. The relevance of the preferential localization[985] and altered catalytic selectivity are presently unknown but there is potential clinical relevance in light of the known physiological action of both leukotriene B_4 and 20-hydroxyeicosatetraenoic acid.

6.29. P450 4F8

Bylund *et al.*[986] used degenerate PCR primers and isolated a cDNA from human seminal vesicles, denoted P450 4F8. This P450 was shown to be a 19-hydroxylase with prostaglandin endoperoxides[986, 987] Recombinant P450 4F8 catalyzed the ω-2 hydroxylation of arachidonic acid and three stable prostaglandin H_2 analogs but prostaglandins D_2, E_1, E_2, and $F_{2\alpha}$ and leukotriene B_4 were poor substrates[987]. (19*R*)-Hydroxy prostaglandins E_1 and E_2 are the main prostaglandins of human seminal fluid. Bylund *et al.*[987] propose that ω-2 hydroxylation of prostaglandins H_1 and H_2 by P450 4F8 occurs in seminal vesicles, and that isomerization to (19*R*)-hydroxy prostaglandin E is the result of action of prostaglandin E synthase.

Further investigations by Bylund and Oliw[988] have demonstrated the expression of P450 4F8 protein in human epidermis, hair follicles, sweat glands, corneal epithelium, proximal renal tubules, and epithelial linings of the gut and urinary tract. P450 4F8 was shown to be upregulated (mRNA and protein) in the epidermis in psoriasis[988]. The exact physiological role of P450 4F8 is unclear, although 19-hydroxy prostaglandins do have several biological activities[988].

6.30. P450 4F11

P450 4F11 is another member of the *CYP4A* gene cluster found on chromosome 19[989]. Expression has been demonstrated primarily in liver, with some expression also in kidney, heart, and skeletal muscle. No other information is presently available, although it might be expected to be capable of leukotriene hydroxylation based upon its similarity to other P450 4F enzymes.

6.31. P450 4F12

P450 4F12 was originally cloned from human liver[990] and small intestine[991] cDNA libraries. Expression has been demonstrated in liver, kidney, colon, small intestine, and heart[990, 991]. Actual levels of abundance are unknown, although this would appear to be a minor P450.

Two groups have expressed P450 4A12 in yeast. Catalytic activities include the hydroxylation of arachidonic acid at carbons 18 (ref. [990]) and 20 (ref. [991]), hydroxylation of the antihistamine ebastine[992], and ω-oxidation of leukotriene B_4 (refs [990], [991]), and ω-hydroxylation of some prostaglandins and prostaglandin analogs[990].

No further information is yet available about the relevance of this enzyme in physiological or clinical situations.

6.32. P450 4F22

No information is available except the existence of the *CYP4F22* gene in the human genome[705].

6.33. P450 4V2

No further information is available except for the existence of the human *CYP4V2* gene[705].

6.34. P450 4X1

Relatively little is known beyond the existence of the human *CYP4X1* gene except for one recent paper on rat P450 4X1 (ref. [993]). mRNA expression was highly selective in brain (cortex, hippocampus, cerebellum, brainstem). The rat protein was expressed in yeast but has not been examined for catalytic activity.

6.35. P450 4Z1

The only information available is the existence of the *CYP4Z1* gene in the human genome[705].

6.36. P450 5A1

P450 5A1 is the classification of thromboxane synthase, which converts prostaglandin H_2 to thromboxane (Figure 10.12). Thromboxane causes vasoconstriction and platelet aggregation, which are of considerable interest.

6.36.1. Sites of Expression and Abundance

P450 5A1 is expressed in platelets and also erythroleukemia cells[995]. The enzyme is also found in human monocytes[996], leukocytes[997], and kidney interstitial dendritic reticulum cells

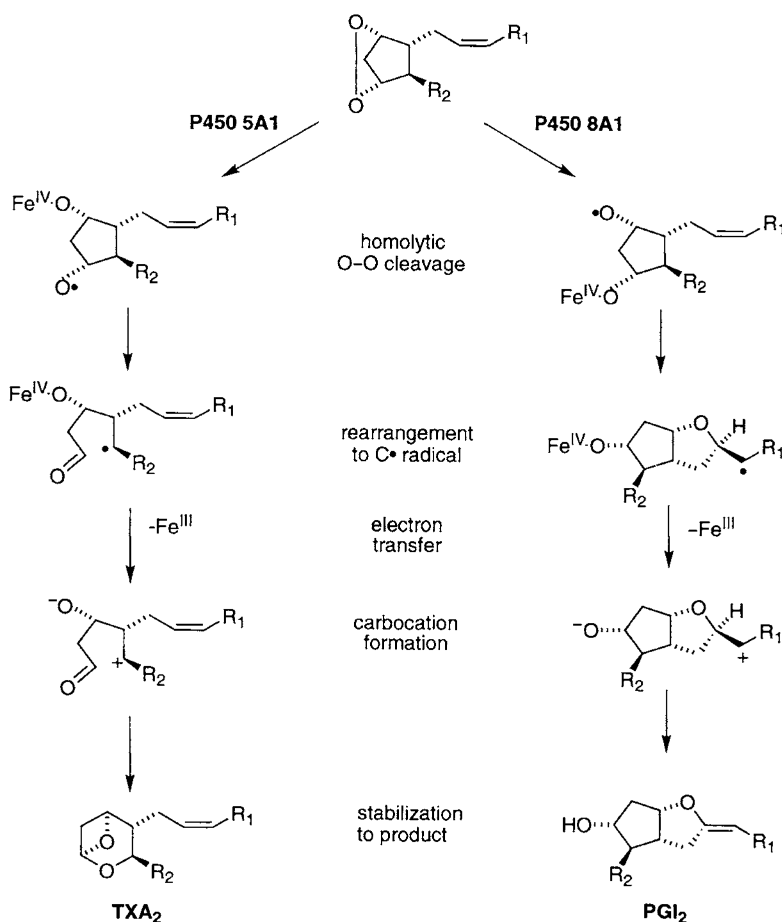

Figure 10.12. Rearrangement of prostaglandin H_2 to prostacyclin (PGI_2) by P450 8A1 and thromboxane (TXA_2) by P450 5A1 (ref. [994]).

surrounding the tubules[998]. Some expression is also seen in lung and liver[996].

6.36.2. Regulation and Polymorphism

As one might expect from its function, P450 5A1 is a highly regulated system. Dexamethasone induces P450 5A1 in human monocytes[996]. Phorbol esters also induce P450 5A1 (e.g., 12-*O*-tetradecanoylphorbol-13-acetate) in human erythroleukemia cells[999]. Patients with systemic sclerosis showed 6-fold enhanced levels of leukocyte P450 5A1 (ref. [997]).

Promoter analysis indicates a 39-bp core promoter, containing TATA and initiator elements that control transcription. Binding of the transcription factor NF-E2 is critical for both alteration of the nucleosomal structure and activation of the P450 5A1 promoter[1000].

Chevalier *et al.*[1001] identified 11 polymorphic variants in the *CYP5A1* gene, including 8 missense changes in the coding region. The effects of these changes have not been reported yet.

6.36.3. Substrates and Reactions

The thromboxane synthase reaction has been known for many years but was associated with a P450 by Ullrich and his associates, first in spectral studies[1002] and then by purification[1003]. With the purified enzyme or one expressed in a baculovirus system[1004], prostaglandin H_2 was

converted to thromboxane A_2 and 12-hydroxyheptatrienoic acid (HHT) plus malondialdehyde, in equimolar amounts[1005]. Prostaglandin G_2 was transformed to malondialdehyde and the corresponding 15- and 12-hydroperoxy products. Prostaglandin H_1 was enzymatically transformed into 12(*L*)-hydroxy-8, 10-heptadecadienoic acid, and prostaglandin H_3 yielded thromboxane B_3 and 12(*L*)-hydroxy-5,8,10,14-heptadecatetraenoic acid[1005] (Figure 10.12).

These are all rearrangement reactions, not involving input of O_2 or electrons from pyridine nucleotides. The reaction of the "oxygen-surrogate" iodosylbenzene with a P450 5A1-containing preparation and the stable prostaglandin H_2 analog 15(*S*)-hydroxy-11α,9α-epoxymethano-5(*Z*), 13(*E*)-prostadienoic acid (U46619) yielded three oxidation products (that could also be formed in a similar system using rat liver microsomes)[1006]. These and other studies led Hecker and Ullrich[1007] to propose a mechanism involving homolytic cleavage of the prostaglandin endoperoxide (with the Fe^{IV} bonded to one oxygen and the other oxygen bearing a radical), transfer of the radical to a carbon, further electron transfer to generate Fe^{III} plus a carbocation, and collapse of the *bis*-ionic structure to yield thromboxane A_2 (ref. [994]). Fragmentation competes with the electron transfer step to also yield malondialdehyde and heptatrienoic acid[994].

6.36.4. Knowledge about Active Site

Relatively little is known about the active site of P450 5A1 beyond the information about the reactions presented above. As indicated, the protein does not bind NADPH-P450 reductase. Presumably, the active site is rather specific, although iodosylbenzene could be utilized as an oxygen surrogate.

6.36.5. Inhibitors

Thromboxane synthase inhibitors have been a matter of interest for many years because of their potential use in preventing plugs of platelets, and efforts at development preceded the characterization of the enzyme as a P450 (refs [1008–1010]). Many of these inhibitors have a basic nitrogen atom that binds to the P450 5A1 heme[1011]. The search for inhibitors as drug candidates has continued[1012].

6.36.6. Clinical Issues

As indicated earlier, platelet aggregation due to the produced thromboxanes is important but overproduction can yield plugs, so control of homeostasis is desirable.

6.37. P450 7A1

P450 7A1 catalyzes cholesterol 7α-hydroxylation, the rate-limiting step in bile acid synthesis. Much has been done in several animal models, including purification of the enzyme from rabbit and rat liver[1013, 1014]. The enzyme was partially purified from human liver[1015] and the cDNA was cloned by several groups in 1990[1016–1018].

6.37.1. Sites of Expression

Apparently the only site of P450 7A1 expression is the liver. The *CYP7A1* gene is on chromosome 8q11–q12 and contains recognition sequences for a number of liver-specific transcription factors[1019–1021].

The level of the enzyme in liver appears to be similar to some of the low-to-moderately abundant xenobiotic-metabolizing enzymes in liver.

6.37.2. Regulation and Polymorphism

The regulation of the *CYP7A1* gene is very complex, as might be expected from the important physiological role this enzyme plays.

P450 7A1 activity has long been known to be upregulated by dietary cholesterol in most animal models[1017], although there are some exceptions[1022]. Feeding rats the competitive inhibitor 7-oxocholesterol led to reduced bile acid synthesis (due to inhibition) and a compensatory increase in P450 7A1 synthesis[1023]. Chiang[1024] identified a bile acid-responsive element in the *CYP7A1* promoter.

Studies with P450 7A1-knockout mice show that this reaction (cholesterol 7α-hydroxylation) is essential for proper absorption of dietary lipids and fat-soluble vitamins in newborn mice, but not for maintenance of cholesterol and lipid levels[1025].

The mice exhibit a complex phenotype with abnormal lipid excretion, skin pathologies, and behavioral irregularities. The cholesterol levels were not altered. Interestingly, vitamin D_3 and E levels were low to undetectable.

A new era in the regulation of P450 7A1 began with reports of the involvement of some of the orphan steroid receptors. The proximal promoter region interacts with LXRα. The oxysterols 24(*S*)-hydroxycholesterol and 24(*S*)-epoxycholesterol activate LXRα (and LXRβ)[1026]. Further, mice devoid of LXRα fail to induce *CYP7A1* transcription[1027]. Two other proteins, FXR and CPF, are also involved[1028–1030]. Chenodeoxycholate, a bile acid derived from cholesterol, interacts with FXR to suppress *CYP7A1* transcription[1031]. However, the action of FXR has been reported to be indirect[1031]. PXR binds lithocholic acid and downregulates *CYP7A1* (ref. [1032]). Thus, cholesterol metabolites control their synthesis in the liver through feedback suppression of *CYP7A1* (ref. [1028]). Hylemon[1033] has concluded that the dominant factor is LXRα. CPF binds to the promoter (as a monomer) and leads to *CYP7A1* transcription[1030].

Other studies have addressed the role of PPARα in P450 7A1 downregulation[1034]. However, differences exist between humans and mice gene responses have been observed, with the mouse gene showing an enhanced response to ligands because of an additional binding site[1035] (further, humans have much less PPARα than rodents[1036]). Chiang[1037] analyzed the PPARα response and provided evidence that the downregulation by PPARα-agonist complex is due to competition with HNF-4 for the DR-1 sequence.

The regulation of P450 7A1 by other factors has been considered. Downregulation by TNFα has been interpreted in the context of MEKK1, an upstream nitrogen-activated protein kinase, affecting HNF-4 (ref. [1038]). The same mechanism may be involved in the repression by endotoxin and interleukin-1 (ref. [1039]). A novel *CYP7A1* site appears to be involved in the repression of *CYP7A1* by thyroid hormone (T_3)[1040]. Studies with rats indicate differences in the regulation of P450 7A1 and P450 27A1, a sterol 27-hydroxylase[1041]. Human *CYP7A1* expression is also repressed by insulin and phorbol esters[1042]. Estrogen (100 μg/kg/week) increased hepatic cholesterol 7α-hydroxylation 2.7-fold in ovariectomized baboons[1043]. Retinoic acid increased (rat) *CYP7A1* expression in a reporter assay[1044].

In addition to the mouse *CYP7A1* knockouts, work has been done with overexpression in mice[1045, 1046]. The mice did not exhibit altered cholesterol levels[1046]. The lack of an LXR element in a region (−56 to −49) of the human promoter may dictate some of the differences seen in mouse and human models. With regard to humans, one study of biopsy samples from gallstone patients led to the conclusion that there was no correlation between levels of total bile acids and P450 7A1 activity[1047]. A correlation was seen with levels of chenodeoxycholic acid.

A long-standing observation from rodent studies is the apparent circadian rhythm of P450 7A1 (ref. [1048]). This phenomenon has been suggested to be indicative of a short halflife of the enzyme[1049, 1050]. The phenomenon has also been reported in nonhuman primates[1051]. The circadian rhythm can be demonstrated at the level of actual P450 7A1 in rats[1052]. The molecular mechanism of the rhythm is still not clear. One aspect is the instability of P450 7A1 in microsomes (*in vitro*), with a $t_{1/2}$ of ~1–2 hr in humans and rats[1053]. Alternatively, the mRNA has a short $t_{1/2}$ and the circadian rhythm can be seen at the mRNA level[1054]. Another unresolved aspect of P450 7A1 research is the issue of phosphorylation, postulated early in the field[1055]. *In vitro* experiments with microsomes show some effects of various treatments[1056, 1057]. More recent work with microsomes and recombinant proteins also shows effects[1058], although the *in vivo* significance is yet unclear.

Polymorphisms in the coding and noncoding regions of the *CYP7A1* gene are known[1059]. Some have been associated with clinical changes[1060], but others have not[1061].

6.37.3. Substrates and Reactions

The classic reaction of P450 7A1 is cholesterol 7α-hydroxylation[18], and esterified cholesterol is not a substrate[1062]. However, recent experiments have established that the enzyme also catalyzes the 7α-hydroxylation of 24-hydroxycholesterol, with preference for the (*S*)-isomer[1063]. 7α-Hydroxylation (with recombinant human P450 7A1) was observed with 20(*S*)-hydroxycholesterol, 25-hydroxycholesterol, and

27-hydroxycholesterol[1064]. The relevance of the activity toward 25(*S*)-hydroxycholesterol is unknown compared to P450 39 (ref. [1065]).

6.37.4. Knowledge about Active Site

Relatively little has been done with site-directed mutagenesis or modeling. As indicated (*vide supra*), the enzyme only catalyzes 7α-hydroxylation but is not very sensitive to side-chain hydroxyls.

The region 214–227 has been postulated to interact with the membrane and to serve as a substrate-access channel[1066]. Mutations in this region yielded some changes in kinetic parameters toward cholesterol.

6.37.5. Inhibitors

Limited information about inhibitors is available. As indicated earlier, 7-oxocholesterol is a (n) (competitive) inhibitor[1023].

6.37.6. Clinical Issues

P450 7A1 has been a topic of considerable interest in the areas of hepatology and gastroenterology.

The hypersecretion of cholesterol in obesity does not appear to be due to reduced 7α-hydroxylation[1067]. Coffee terpenes (e.g., cafestol) inhibit P450 7A1 and also raise cholesterol levels[1068], although it is not clear that the two phenomena are linked. The complex regulation of P450 7A1 makes interpretation of some experiments difficult. Overexpression of P450 7A1 in HepG2 cells increased bile acid synthesis but led to decreased hydroxymethylglutarate (HMG) CoA reductase activity (rate-limiting step in cholesterol biosynthesis)[1069].

Alterations in P450 7A1 were not seen in hypo- or hyperthyroidism[1070].

A 10-week old child with a stop-codon mutation and lacking P450 7A1 presented with severe cholestasis, cirrhosis, and liver synthetic failure[1060]. A frameshift leading to (homozygous) lack of P450 7A1 was associated with high low-density lipoprotein (LDL) cholesterol, but not total cholesterol[1071]. Heterozygotes were also hyperlipidemic. However, Beigneux *et al.*[1072] have discussed some of the caveats associated with interpretation of results of family and experimental studies with P450 7A1.

6.38. P450 7B1

Almost all of the work with P450 7B1 is from rodent models and application to the human *CYP7B1* gene system is by inference. A P450 7B1 transcript was first characterized in a rat (brain) hippocampus cDNA library[1073]. A heterologously expressed protein was shown to catalyze the 7α-hydroxylation of the steroids DHEA and pregnenolone[1074]. Expression has also been reported in liver and kidney[1073, 1075]. Disruption of the mouse *CYP7B1* gene yielded animals that were viable and apparently normal, but *ex vivo* 7α-hydroxylation of DHEA and 25-hydroxycholesterol was blocked in brain, spleen, thymus, heart, lung, prostate, uterus, and mammary gland[1075].

Although extrapolation to humans has not been reported, P450 7B1 is considered to be a neurosteroid hydroxylase and have a potentially important role[1065, 1075]. Functional polymorphisms in the human *CYP7B1* gene have not been reported, but have been postulated to lead to severe hypercholesterolemia and neonatal liver disease[29].

6.39. P450 8A1

Prostacyclin (prostaglandin I_2) has strong vasodilation and anti-aggregation effects on platelets, and the imbalance of prostacyclin and thromboxane A_2 (product of P450 5A1) is a factor in several diseases, for example, myocardial infarction, stroke, atherosclerosis[1076, 1077]. The reaction yielding prostacyclin from prostaglandin H_2 is another "internal" oxygen transfer, without the input of O_2 and electrons from NADPH (Figure 10.12), and the involvement of a P450 was not immediately obvious. Ullrich hypothesized P450 involvement on the basis of spectral interaction studies[1078]. DeWitt and Smith[1079] used a monoclonal antibody to purify catalytically active prostacyclin synthase from bovine aorta and demonstrated a P450 Fe^{2+}·CO spectrum. Subsequently, P450 8A1 was cloned from bovine endothelial cells[1080].

6.39.1. Sites of Expression and Abundance

Human P450 8A1 was cloned from aorta endothelial cells by Tanabe's group[1077]. The mRNA is widely expressed in human tissues, including ovary, heart, skeletal muscle, lung, prostate[1077], and umbilical vein[1081]. More recent work has shown some localization in the brain, including neurons[1082, 1083]. Another site of expression is fallopian tubes, with expression in luminal epithelia, tubal smooth muscle, vascular endothelial cells, and vascular smooth muscle cells[1084].

6.39.2. Regulation and Polymorphism

P450 8A1 is constitutively expressed in human endothelial cells[1081]. The human *CYP8A1* gene (chromosome 20) has 10 exons[1085–1087] and has consensus sequences for Sp1, AP-2, an interferon-γ response element, GATA $NF_{\kappa}B$, a CACCC box, glucocorticoid receptor, and a shear stress responsive element (GAGACC)[1085]. Whether or not all of these are functional and how they interact to maintain constitutive expression is not well understood yet.

Polymorphisms have been of interest because of disease relevance. The coding sequence contains at least five alleles[1088]. In the 5′-region, these are polymorphisms involving a variable number of tandem repeats (VNTR) that affect transcription, as demonstrated in reporter systems *in vitro*[1089]. At least nine of these allelic variants are known[1089]. An association between this VNTR polymorphism and cerebral infarction has been reported[1090].

A SNP in exon 8 has been reported to be linked to myocardial infarction, although no amino acid change occurs[1091]. However, the VNTR polymorphism does not appear to be related to essential hypertension[1092], nor does the 5′-flanking region SNP T192G (ref. [1093]). However, a novel splicing variation leading to skipping of exon 9 has been linked to hypertension[1094].

6.39.3. Substrates and Reactions

P450 8A1 has a very limited catalytic specificity, functioning only as the prostacyclin synthase (Figure 10.12). Prostaglandins G_2, H_2, 13(*S*)-hydroxy H_2, 15-keto H_2, and H_3 are isomerized to the corresponding prostacyclins[1007]. Spectral binding studies with 9,11-epoxymethano prostaglandins F_2 and $F_{2\alpha}$ lead to the view that the binding juxtaposition is the key determinant in distinguishing the courses of catalysis by P450s 5A1 and 8A1 (ref. [1007]). A mechanism consistent with available data has been proposed (Figure 10.12)[994, 1007].

6.39.4. Knowledge about Active Site

Mutagenesis of Cys441 (heme binding Cys) or Glu347 or Arg350 (EXXR motif) abolished catalytic activity, suggesting that the placement of these residues is correct[1095]. Other site-directed mutagenesis studies suggest roles of Ile67, Val76, Leu384, Pro355, Glu360, and Asp364, which have been suggested in models[1096]. However, the level of residual activity was 5–10% and only a single substrate concentration was used; another caveat is that the expression work was done in COS cells and the level of expression of holoprotein was not measured.

Other work has been on membrane topology, and antibody studies indicate that P450 8A1 is mainly exposed on the cytoplasmic site of the endoplasmic reticulum with a single transmembrane anchor[1097, 1098]. The (unstable) substrate, prostaglandin H_2, is produced in the lumen and apparently passes through the membrane to reach P450 8A1. Antibodies raised to the peptides of the putative substrate channel (66–75 and 95–116) interact only after membrane solubilization, implying that the substrate-access channel is very near the membrane[1099].

6.39.5. Inhibitors

Relatively little interest has been shown in the development of drugs that inhibit P450 8A1 because inhibition is generally considered to be deleterious. Phenylbutazone has been reported to inhibit[1100].

P450 8A1 is slowly inactivated during the normal reaction itself, apparently by one of the reactive intermediates in the catalytic cycle (Figure 10.12)[1101]. A $k_{\mathrm{inactivation}}$ of $0.06\ s^{-1}$ was reported[1101].

Peroxynitrite is a powerful inhibitor of P450 8A1, with a reported K_i of 50 nM[1102]. Peroxynitrite is formed by the chemical reaction

of NO$^\bullet$ and $O_2^{-\bullet}$ (ref. [1103]). The mechanism is believed to involve tyrosine nitration[1104], and recently Tyr430 has been implicated as the site of nitration[1105].

6.39.6. Clinical Issues

As mentioned earlier, prostacyclin is a powerful vasodilator and inhibits platelet adhesion and undesired cell growth. Although this view may be overly simplistic, prostacyclins are a counterbalance to thromboxanes in a "yin–yang" relationship. Thus, the action of P450 8A1 balances that of P450 5A1.

Decreased expression of P450 8A1 has been reported in severe pulmonary hypertension[1106]. With regard to general cardiovascular disease, a study of Japanese subjects associated the VNTR polymorphism with hypertension (odds ratio 1.9)[1107]. Individuals with 3–4 repeats had less promoter activity and higher risk. In experimental studies, the overexpression of P450 8A1 in transgenic mice protected against the development of hypoxic pulmonary hypertension[1108]. In another study, the expression of human P450 8A1 in the carotid arteries of rats after arterial balloon injury (using a virus) led to increased synthesis of prostacyclin and to reduced neointimal formation[1109].

P450 8A1 also has relevance in cancer treatment. Transfection of colon adenocarcinoma cells with P450 8A1 led to slower growth and reduced vascular development following inoculation into syngeneic mice[1110].

Finally, antibodies in the sera of some patients with hypersensitivity reactions to phenytoin and carbamazepine recognize rat P450 3A1 but not human P450 3A (ref. [1111]). The antisera also recognize peptides derived from P450s 8A1 and 5A1, although relationships of etiology and causality are unclear.

6.40. P450 8B1

P450 8B1 is a sterol 12α-hydroxylase expressed in the liver. The human *CYP8B1* gene was characterized on the basis of the rabbit and mouse orthologs[1112]. Of interest is the finding that this gene is devoid of introns, unique for this gene among the P450 family[1112].

Regulation of the gene is of interest, in that P450 8B1 catalyzes the synthesis of cholic acid and controls the ratio of cholic acid to chenodeoxycholic acid in the bile[1113]. HNF-4α activates human *CYP8B1* expression in HepG2 cells[1113]. Bile acids and farnesoid X receptor (FXR) downregulate HNFα expression. Inflammation in liver cells causes increased synthesis of α_1-antitrypsin, a serum protease inhibitor, and in a derived peptide (C-36). C-36 appears to interact with the α_1-fetoprotein transcription factor (FTF) site in the human *CYP8B1* promoter, inducing a conformational change to lower DNA binding ability, and suppressing the transcription of the *CYP8B1* (and *CYP7A1*) genes[1114, 1115]. HNFα could overcome the inhibitory effects of FTF and bile acids[1115]. Thus, regulation of P450 8B1 is involved in bile acid feedback inhibition.

6.41. P450 11A1

P450 11A1 is the enzyme involved in the initiation of steroid synthesis (Figures 10.13 and 10.14). It catalyzes the conversion of cholesterol to pregnenolone by side-chain cleavage and has been referred to in the older literature as $P450_{scc}$ or cholesterol desmolase. The enzyme was purified from bovine adrenal cortex mitochondria[1116]. The human gene was cloned by Omura and Fujii-Kuriyama in 1987[1117] and includes nine exons. Of historical significance is the fact that this P450 only contains a single cysteine and further establishes the position of the heme thiolate peptide in P450s, extending the work on the location from the crystal structure of bacterial P450 101 (ref. [1118]).

6.41.1. Sites of Expression

P450 11A1 is found primarily in steroidogenic tissues, that is, adrenal cortex and gonads, including ovary (corpus luteum[1119, 1120] and theca interna cells[1121] and others[1122]). Of interest are recent reports of P450 11A1 in brain[1123–1126] and pancreas[1127].

P450 11A1 is one of the few P450s localized in the mitochondria (Table 10.1 and Figure 10.14). Studies with the bovine enzyme demonstrated that

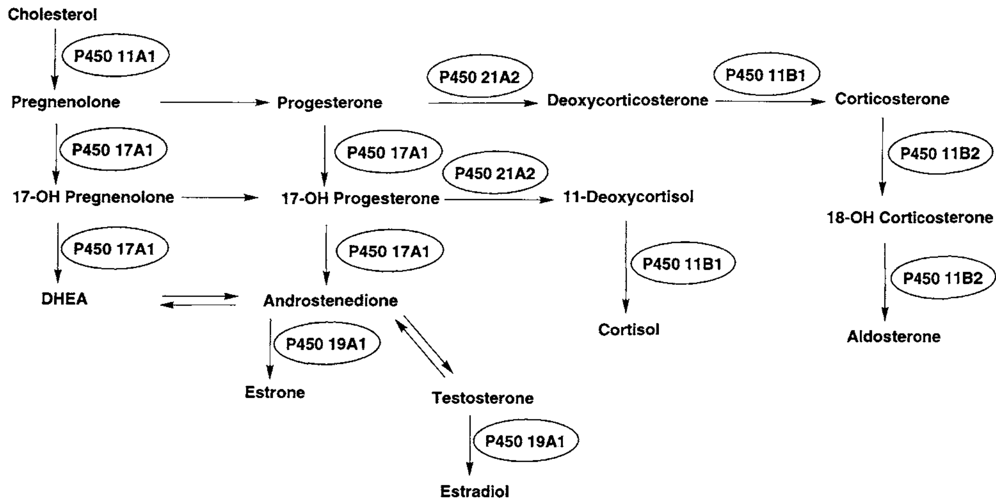

Figure 10.13. A view of the metabolic pathway of steroidogenesis and the major P450s involved[29].

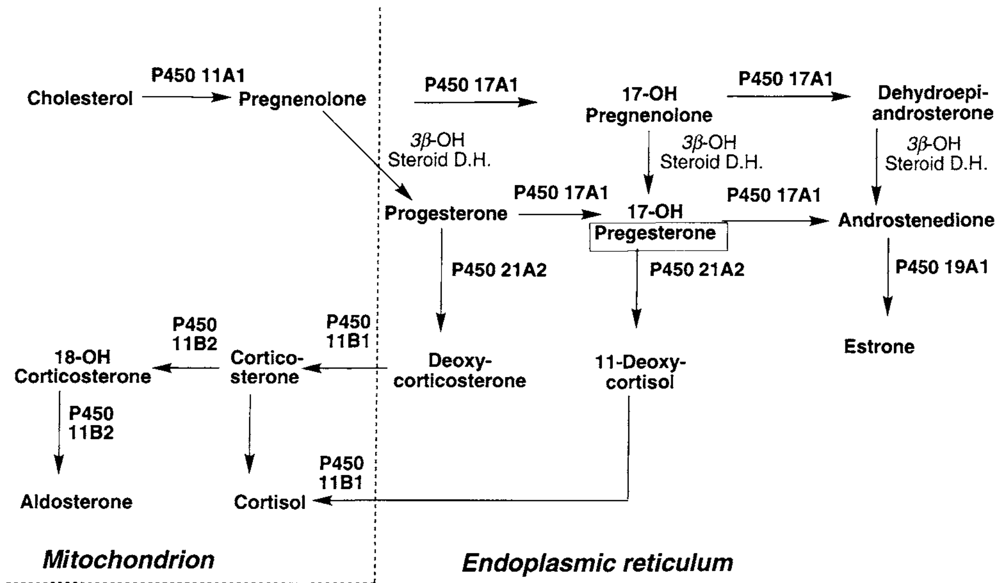

Figure 10.14. Overview of steroidogenic pathway and cellular compartmentalization[23].

P450 11A1, synthesized on ribosomes in the cytosol, is imported into mitochondria without processing of the amino terminal extension peptide[1128]. The protein moves to the mitochondrial inner membrane and is then cleaved to yield the mature form[1128]. Alteration of the basic amino acid residues of the *N*-terminus resulted in less efficient mitochondrial import[1129]. Miller's group constructed vectors that could be used to direct P450 11A1 to the endoplasmic reticulum and found that the enzyme was inactive[1130]. The membrane environment was concluded to be more important in modulating catalytic function than the electron transfer partners.

6.41.2. Regulation and Polymorphism

The regulation of P450 11A1 is relatively complex, as might be expected for the initial step in steroid formation[1122]. Moreover, the system must be able to respond to signals in many different tissues. Much of our understanding of the regulation of P450 11A1 expression is based on studies with *CYP11A1* genes of experimental animals and reinvestigated with human *CYP11A1*.

P450 11A1 has long been known to be regulated by ACTH and cyclic AMP. In the bovine *CYP11A1* gene two Sp1-binding sites mediate cyclic AMP transcription through the protein kinase A signaling pathway, utilizing the rather ubiquitous transcription factor Sp1 (ref. [1131]). The steroidogenic factor 1 (SF1) activates *CYP11A1* transcription through interaction with protein factors upstream[1122]. An upstream CREB-binding region and an AP-1 site are also involved in the cyclic AMP response. Sp3 can also be involved[1132]. The TATA box drives cell type-specific cyclic AMP-dependent transcription[1133]. SF-1 also interacts with Sp1 (refs [1134–1136]). Thus, the regulation of the human *CYP11A1* gene involves all the above factors plus an AdE element[1122]. More recently, the expression of the human gene has been shown to involve the zinc finger protein TreP-132, interacting with both CBP/p300 (ref. [1137]) and SF-1 (ref. [1138]). Also, salt-inducible kinase (SIK) represses cyclic AMP-dependent protein kinase-mediated activation through the CREB basic leucine zipper domain[1139].

Other recent work with human placenta show that activating protein-2 (AP-2) assumes the role of SF-1 by binding to an overlapping promoter element[1140].

Mutations are also found in *CYP11A1* and can cause congenital adrenal insufficiency. Arg353 was found to be critical in a study with an afflicted patient[1141].

6.41.3. Substrates and Reaction

P450 11A1 appears to be quite specific in using cholesterol as a substrate. The reaction proceeds in a three-step sequence, with generation of (22*R*)-20α, 22-dihydroxycholesterol as an intermediate[1142]. Oxidative cleavage of the diol to pregnenolone and 4-methylpentanal (isocaproic aldehyde) completes the overall reaction. The mechanism of the last step is not completely clear, but some proposals have been presented[1143–1145].

The rate of electron transfer from adrenodoxin is important and appears to be the rate-limiting step for the enzyme in human placenta[1146]. The redox potential of adrenodoxin can be varied by site-directed mutagenesis, but had little effect on rates of electron transfer, consistent with the view that other factors such as protein–protein interactions are more important than the intrinsic thermodynamics[1147]. When P450s 11A1 and 11B1 are expressed together in cells, they can compete for reducing equivalents from adrenodoxin[1148]; exactly how important the competition is in tissues is unclear. Another report indicates interaction of P450 11A1 with and enhancement by b_5 (ref. [1149]) although the relevance is unclear because of the compartmental separation of P450 11A1 (mitochondria) and b_5 (endoplasmic reticulum) (Figure 10.14).

6.41.4. Knowledge about Active Site

Knowledge about this enzyme is still relatively limited. Studies with bovine P450 11A1 indicated the significance of Lys377 and Lys381 in adrenodoxin binding[1150]. As indicated earlier, a mutation at Arg353 was found to attenuate the function of P450 11A1 in a patient[1141]. Site-directed mutagenesis of human P450 11A1 (in *E. coli*) indicated that Ile462 had some effect on kinetic parameters[1151].

6.41.5. Inhibitors

A number of inhibitors of P450 11A1 have been reported, although some were studied only with the bovine enzyme[1152, 1153], including some acetylenic mechanism-based inactivators[1154]. With regard to the human enzyme, there is some potential for use of inhibitors in treatment of prostatic cancer, and prodrug forms of aminoglutethimide have been examined[1155].

Anti-convulsants have been reported to inhibit P450 11A1, but the interaction is not strong[1156].

6.41.6. Clinical Issues

Two major issues are of interest. Because of the nature of P450 11A1 in initiating steroidogenesis, deficient P450 11A1 can lead to (congenital) adrenal hyperplasia[29]. Rabbit and mouse models show the effects[1157, 1158]. *CYP11A1*-null mice die shortly after birth, but can be rescued by steroid injection[1158]. ACTH levels become very high due to lack of feedback regulation by glucocorticoids. Male null mice are feminized with female external genitalia and underdeveloped male accessory sex organs. These manifestations resemble various human steroid deficiency syndromes.

Another issue is autoantibodies to P450 11A1 (and also P450 17A1) in patients with autoimmune polyglandular syndrome types I and II, and Addison's disease[1159, 1160]. As with other P450s recognized by autoantibodies, causal relationships between immunity and disease are unclear.

6.42. P450 11B1

P450s 11B1 and 11B2 differ in only 32 residues. P450 11B1 catalyzes the 11β-hydroxylation of deoxycortisol to yield cortisol, which is the main glucocorticoid in the body. Deficiencies in the enzyme are known, causing congenital adrenal hyperplasia[29, 1161].

6.42.1. Sites of Expression

P450 11B1 is expressed in the adrenal cortex, specifically the *zona fasciculate/reticularis*[1161]. In rats, some expression has been detected in brain but the relevance is not clear.

P450 11B1 is synthesized in the cytosol and directed to the mitochondria with a 24-residue *N*-terminal targeting sequence (where this is lost after entry). As with the other four (exclusively) mitochondrial P450s (Table 10.1 and Figure 10.14), P450 11B1 receives electrons from adrenodoxin instead of NADPH-P450 reductase.

The characterization of the *CYP11B* gene has developed considerably in recent years. Much of the early research in this field was done with bovine adrenal glands because of the need for large amounts of material, and the bovine P450 11B protein has the function that P450 11B1 (11-hydroxylation) and the P450 11B2 (11-hydroxylation, 18-hydroxylation, and oxidation of the 18-alcohol to an aldehyde) have in most other species, including humans[1162]. The two human genes (*CYP11B1*, *CYP11B2*) were characterized and clearly shown in both to be essential[1163–1166].

P450 11B1 expression has also been deleted in human fetal adrenal gland, particularly in the "fetal zone" (as opposed to neocortex)[1167].

6.42.2. Regulation and Polymorphism

Much of the background on regulation of P450 11B1 came from studies with the bovine gene, which responds to ACTH and has six *cis*-acting regulatory elements[1168]. The protein (Ad4BP) that binds to one of these (Ad4) is a member of the steroid hormone receptor superfamily[1169]. Other studies by Omura[1170] indicated the cooperative nature of these elements in transcription. Work with rat *CYP11B1* showed that ACTH stimulates transcription by changing composition in AP-1 factors (Fos, Jun)[1171].

The human gene also has a cyclic AMP response element (CRE)[1172]. The Ad1 element bound CRE-binding protein, activating transcription factor-1 (ATF-1), and ATF-2. Steroidogenic factor-1 (SF-1) interacted at the Ad4 site (−242/−234) and was required for transcription[1172, 1173], which contrasts with the lack of response of *CYP11B2*.

Many mutations are known because of the relationship of the gene with congenital adrenal hyperplasia[1161]. These include a 5-base duplication[1174] and clusters of mutations in exons 6–8 (ref. [1175]). The high similarity and proximity of the *CYP11B1* and *CYP11B2* genes appear to lead to mutant generated by unequal crossover and inactive chimeric product[1176–1179]. Splice donor site mutations are also known[1180].

6.42.3. Substrates and Reactions

As indicated previously, the only substrate for P450 11B1 is deoxycortisol, which undergoes

11β-hydroxylation to yield cortisol (Figures 10.13 and 10.14).

6.42.4. Knowledge about Active Site

One of the concerns about studies on the function of particular residues in site-directed mutagenesis is that expressions in some cellular systems lead to competition between P450s 11A1 and 11B1 for (adrenodoxin) reducing equivalents in cellular systems[1148]. Another issue is that human P450s 11B1 and 11B2 have been difficult to express in bacteria, so that most experiments have relied on mammalian cells (*Schizosaccharomyces pombe* has provided some success[1161]). Nevertheless, much information about function has been obtained from patients' samples[1161].

The close similarity of P450s 11B1 and 11B2 (and their reactions) has also facilitated studies. Making the changes S288G and V320A yielded an enzyme with both P450 11B1 and 11B2 activities[1181]. Changes at positions 147 (refs [1182], [1183]), and 301/355 (ref. [1184]) have also had the same effect.

Homology models of P450 11B1 have also been published[1161, 1185, 1186], although the effects of all of the mutants known to alter function have not been systematically rationalized.

6.42.5. Inhibitors

Compared with some of the other steroidogenic P450s, there is some reason to develop P450 11B1 inhibitors. High levels of cortisol are associated with Cushing's syndrome[1161]. Cellular expression systems have been set up to assay for inhibitors, using measurements of concentrations of steroids[1187, 1188].

18-Vinylprogesterone and 18-ethynylprogesterone have been reported to be mechanism-based inactivators of bovine P450 11B, but apparently have not been tested with human P450 11B1 (ref. [1189]).

6.42.6. Clinical Issues

As indicated previously, the main issue with P450 11B1 is the impaired synthesis of cortisol and congenital adrenal hyperplasia, characterized by hypertension and signs of androgen excess[1190, 1191]. Overproduction of glucocorticoids, which could have any of several causes including overactive P450 11B1, is associated with Cushing's syndrome[1161].

6.43. P450 11B2

P450 11B2 is highly related to P450 11B1 (*vide supra*) and has a somewhat similar function. P450 11B2 catalyzes the 11β-hydroxylation of 11-deoxycorticosterone followed by 18-hydroxylation and 2-electron oxidation of the 18-alcohol to an aldehyde (Figures 10.13 and 10.14). Changes in the gene can lead to corticosterone methyloxidase deficiency and hyperaldosteronism[29, 1165, 1192, 1193]. In the older literature, this P450 is sometimes termed as "$P450_{aldo}$."

6.43.1. Sites of Expression

P450 11B2 is expressed in the adrenal cortex (zona glomerulosa) and is involved in the synthesis of aldosterone (the 11β-hydroxy, 19-aldehyde product). It is a mitochondrial P450, as are the other 11 family P450s. The cDNA was first cloned from an adrenal tumor of a patient suffering from primary aldosteronism[1194]. Another early study showed higher levels of P450 11B2 in aldosterone-secreting tumors[1195].

There is some evidence for the synthesis of aldosterone outside of the adrenals, and Li *et al.*[1196] reported expression of P450 11B2 in hepatic stellate cells of liver; the activation of these cells is a key event in liver fibrogenesis.

6.43.2. Regulation and Polymorphism

Some of the research on regulation overlaps that presented for *CYP11B1* (*vide supra*). A CRE/Ad1 element and ATF-1 (and ATF-2?) play a role with both *CYP11B1* and *CYP11B2* (ref. [1197]). However, SF-1 does not appear to regulate *CYP11B2*, in contrast with *CYP11B1* (ref. [1173]). Many aspects of regulation remain to be further investigated, including the mechanisms of the observed Ca^{2+} and cyclic AMP signaling[1198] and the effects of kinase inhibitors[1199, 1200].

As in the case of *CYP11B1*, many mutations have now been defined from clinical studies. The "crossovers" between P450s 11B1 and 11B2 yield inactive P450 11B2, as well as P450 11B1 (refs [1178], [1179], [1201], [1202]). Other mutations in *CYP11B2* were associated with corticosterone methyloxidase I and II deficiency[1192, 1193, 1203]. Polymorphisms in *CYP11B2* have also been linked to idiopathic hyperaldosteronism, a condition characterized by autonomous production of aldosterone and arterial hypertension[1204]. A polymorphism in the promoter region of *CYP11B2* (−344 TK) has been associated with predisposition to essential hypertension[1205].

6.43.3. Substrates and Reactions

P450 11B2 catalyzes the three-step conversion of 11-deoxycorticosterone to aldosterone, with 11β-hydroxylation, 18-hydroxylation, and 2-electron oxidation of the 18-carbinol (Figures 10.13 and 10.14). No other substrates are known. Information about the processivity of the human enzyme (i.e., extent of release of intermediate products) is not available at this time.

6.43.4. Knowledge about Active Site

Some studies with the closely related P450 11B1 have already been mentioned. Bernhardt's laboratory found that changes only at positions 320 and 335 conferred some 18-hydroxylation activity to P450 11B1 (ref. [1184]). Homology modeling has also been done[1161, 1186]. In other site-directed mutagenesis work, residues that differed among species were changed and residues 112, 147, and 152 were found to have effects[1206]. Modeling suggested an indirect effect of residue 147 and that residue 112 might be in the substrate-access channel.

6.43.5. Inhibitors

Elevated aldosterone levels can be detrimental and some interest exists in targeting P450 11B2. A yeast system has been developed that can be used for screening for inhibitors[1207].

The literature contains one older report of the use of an acetylenic P450 19 inhibitor to inhibit the activity of what may have been P450 11B1 (ref. [1208]), although that paper reported that the enzyme used catalyzed 11β-, 18-, and 19-hydroxylation.

6.43.6. Clinical Issues

The issues of congenital adrenal hyperplasia and Types I and II corticosterone methyloxidase deficiency in individuals with attenuated P450 11B2 activity have already been mentioned. The other issue also mentioned is elevated aldosterone. Several studies have reported an association between polymorphisms and essential hypertension, although the measurements of aldosterone excretion are still lacking in some studies[1209]. Other studies show association of the –344C allele with increased left ventricular size[1210–1212]. The hypertension association has been seen in several studies[1209, 1210, 1213, 1214], but not in a Japanese study[1215].

6.44. P450 17A1

17-Hydroxylation and the 17, 20-lyase reaction ("desmolase") are two important reactions in steroid biosynthesis (Figures 10.13 and 10.14). Cloning of a cDNA which, when expressed, yielded both activities that established the role of what is now known as human P450 17A1 (previously termed $P450_{17\alpha}$, etc.)[1216]. The gene[1217] showed similarity to *CYP21A1*. The demonstration of both catalytic activities in a single protein established work previously done with purified hog protein[1218]. The two activities have long been known to be regulated by b_5 (refs. [1219], [1220]), and aspects of this duality of function still remain unclear.

6.44.1. Sites of Expression

Human P450 17A is expressed in steroidogenic tissues, including adrenals and gonads. The enzyme has also been reported in fetal kidney, thymus, and spleen[1221]. The enzyme has also been found in human (adult) heart[1222] and adipose tissue[1223].

P450 17A1 is a microsomal enzyme. A proline rich region in the *N*-terminus has been found to be important for efficient folding, but not for subsequent maintenance of the folded structure[1224].

6.44.2. Regulation and Polymorphism

As with the other steroidogenic P450s, the regulation of the *CYP17A1* gene is relatively complex. Induction of P450 17A1 has long been known to be cyclic AMP-mediated and the induction is suppressed by testosterone (mouse model)[1225], and a cyclic AMP response region was mapped in porcine Leydig cells[1226].

With the human *CYP17A1* gene, the homeodomain protein Pbx1 was shown to interact with protein kinase A in the cyclic AMP-dependent regulation (at −250/−241)[1227]. Further analysis showed interaction at a cyclic AMP-related site (−80/−40) by SF-1 (ref. [1228]). Further, interactions were shown for Sp1 and Sp3 (−227/−184) and NF-1C (−107/−85 and −178/−152)[1229]. SF-1 (*vide supra*) also interacts with $p54^{nrb}$, NonO, and protein-associated splicing factor[1230]. The ACTH/cyclic AMP response is dependent upon phosphatase activity, as well as kinase activity[1231, 1232]. The cyclic AMP-dependent protein kinase enhances transcription via MKP-1 activation, involving phosphorylation of SF-1 (ref. [1233]).

Polymorphisms of *CYP17A1* are known, but most of the attention has been given to mutations that result in serious defects in patients[1234]. Most of the mutations have been SNPs in the coding region[1234–1236], but others include a 2-bp deletion yielding a frameshift and premature stop codon[1237], a 4-bp duplication changing the *C*-terminal 28 amino acids[1238], and a 5′-splice site mutation[1239]. Some of the patients presenting with symptoms yielded P450 17A1 that, upon heterologous expression, retained 17-hydroxylation but not 17,20-lyase activity[1240, 1241]. Mutations of the latter type led Auchus[1242] to propose a model in which neutralization of positive charges in the redox partner binding surface of P450 17A may block the lyase activity but not 17-hydroxylation.

6.44.3. Substrates and Reactions

The generally accepted reactions of P450 17A are the 17α-hydroxylation of pregnenolone to 17-hydroxypregnenolone and of progesterone to 17-hydroxyprogesterone. 17-Hydroxypregnenolone is also oxidized to DHEA in the lyase reaction (Figures 10.13 and 10.14)[1241, 1243]. The mechanism of the lyase reaction is not completely established, but mechanisms have been proposed using analogs[1244]. Lieberman[1245] has proposed alternative reactions, although the favored pathway involves what would be a very unstable diradical. No other substrates are known presently, other than pregnenolone and progesterone and possibly closely related analogs. Very recently, Soucy *et al.*[1246] have provided evidence that human P450 17A1 also converts pregnenolone into 5,16-androstadien-3β-ol, a "16-ene synthase" reaction (without intermediate formation of an alcohol).

A key to research on the protein was the development of a robust *E. coli* expression system by Waterman's group in 1991[19]. Further work on the differential effects of b_5 on individual catalytic activities has been reported[1247]. The ratio of b_5 to P450 is high in testis and this phenomenon might regulate the two activities of P450 17A1. Miller's group has proposed that phosphorylation of Ser and Thr residues in P450 17A1 may alternatively influence the two activities[1248], although any experimental evidence in support of this hypothesis is still very limited[1248, 1249].

A second b_5 gene has been identified recently, and this protein also has the same stimulatory effect on lyase activity[1250]. Auchus *et al.*[809] also demonstrated that the same stimulatory effect of b_5 could be obtained with apo-b_5, arguing against the requirement for electron transfer. P450 17A enzymes from other species vary in their ability to catalyze the 17,20-lyase reaction, and comparisons of the rat and human enzymes also led to the conclusion that selective enhancement of the lyase reaction was not due to changes in electron transfer[1251].

The concertedness of the P450 17A1 lyase reaction has been examined, and both the studies both reached the conclusion that much of the 17α-hydroxypregnenolone dissociates[1252, 1253]. In one of the studies[1252], the authors concluded that the off-rate was an important factor in determining the balance between 17-hydroxypregnenolone and DHEA. Exactly how b_5 would control this rate, which was modeled to be rather slow (2.6–29 min^{-1}), is unclear, unless the effect is on the protein conformation. However, a classic burst kinetic experiment was not done in the cited work and the hypothesis remains to be addressed in more detail.

6.44.4. Knowledge about Active Site

Much of the information about the significance of active site residues comes from the analysis of mutations in patients presenting with diseases (see Section 6.44.2.). The changes H373L (ref. [1254]) and P409R (ref. [1255]) led to a loss of heme incorporation. Mutation at Thr306, possibly involved in protonation of $Fe–OO^-$ or O–O cleavage, impaired 17α-hydroxylation more than the lyase reaction[1256]. However, the change R346A selectively abolished lyase activity[1257], as did F417C (ref. [1258]). Mutations at Lys83, Arg347, Arg358, and Arg449 produced proteins that were refractory to b_5 stimulation and attenuated in lyase activity[1259–1261]. Of these, only R347H and R358Q have been found in patients[1262]. Some mutants found in patients do cause the loss of both 17-hydroxylation and the lyase reaction, however[1263, 1264].

Some animal P450 17A1 enzymes have different ratios of 17-hydroxylation/lyase activity and efforts have been made to use these properties to define more elements controlling the latter steps, although the results have been limited to date[1265, 1266].

Several homology models of human P450 17A1 have been published and some of the mutagenesis results can be interpreted[1185, 1267–1271].

6.44.5. Inhibitors

Inhibitors of P450 17A1 have been studied for some time. Interestingly, ketoconazole inhibits lyase activity but not 17-hydroxylation activity[1272]. 7α-Thiospirolactone is a mechanism-based inhibitor of (guinea pig) P450 17A1 (ref. [1273]).

A number of steroidal inhibitors have been studied, primarily with the goal of treating cancers[1274–1278]. The enantiomer of progesterone (*ent*-progesterone) is reported to be a competitive inhibitor of P450 17A (K_i = 0.2 μM)[1279].

Nonsteroidal inhibitors have also been studied[1280, 1281].

Molecular modeling (Section 6.44.4) has also been applied to searches for inhibitors[1271, 1282]. Other approaches utilize P450 17A expressed in *E. coli* to screen for P450 17A inhibition in medium-to-high throughput systems[1283, 1284].

6.44.6. Clinical Issues

P450 17A1 has a central role in human steroid metabolism because of its role in regulating steroid flux (Figures 10.13 and 10.14). Perturbations lead to problems in adrenarche, aging, and polycistric ovary syndrome[1241, 1285]. Some of the more serious mutations have been mentioned already; another is a case of pseudohermaphroditism due to lack of lyase activity[1286].

Some of the other possible disease conditions or risks are being studied in relationship to less serious polymorphisms. In most of these cases, the relationships are more difficult to establish in the serious diseases. A possible link of *CYP17A1* polymorphism has been made with rheumatoid arthritis[1287]. Little influence of polymorphism was seen on age of menarche[1288]. However, a link was made between a particular polymorphism and the prediction to use hormone replacement therapy (i.e., postmenopausal estrogen therapy)[1289]. No association was found with polycistronic ovarian syndrome in a study with an SNP at the regulatory Sp1 site[1290].

Much attention has been given to the possibility of a link between *CYP17A1* allelic SNPs and breast cancer risk[1291]. The epidemiology results are mixed at best[1292–1295] and a conclusion in favor of a relationship cannot be made at this time[1296–1298].

Some positive epidemiology has been presented for a relationship with prostate cancer[1299], although probably not a strong one[1300]. Some positive results have also been reported for endometrial cancer and *CYP17A1* alleles[1301].

As with some other P450s, circulating antibodies to P450 17A are seen in some autoimmune diseases, for example, autoimmune polyglandular syndrome and Addison's disease[1159, 1302], but no causal relationship has been demonstrated.

6.45. P450 19A1

P450 19A1 is the classic "aromatase," often known by that name in endocrinology. This enzyme oxidizes the androgens androstandione and testosterone to the estrogens estrone and 17β-estradiol, respectively (Figure 10.15). This process is very important in normal physiology and also a target for inhibition in some tumors.

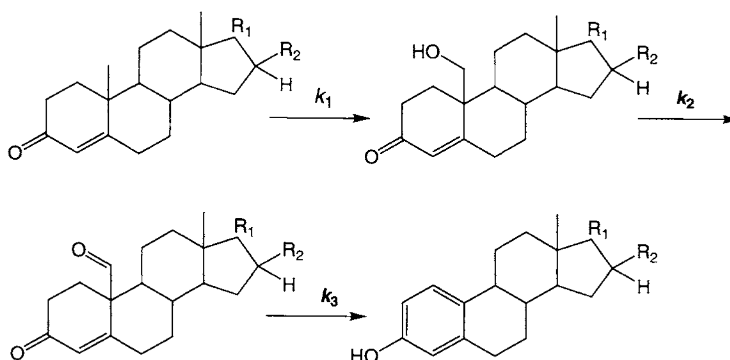

Figure 10.15. Aromatization reactions catalyzed by P450 19. The three distinct steps are shown, with the possible substrates: Testosterone, R_1: –OH, R_2: H; androstenedione, R_1: =O, R_2: –OH; 16-hydroxytestosterone, R_1=R_2=–OH.

6.45.1. Sites of Expression

Estrogens have a number of functions and not only feminization. Sites of (human) expression include the ovaries, testes, placenta, fetal (but not adult) liver, adipose tissue, chondrocytes and osteoblasts of bone, vasculature smooth muscle, and several sites in brain, including parts of the hypothalamus, limbic system, and cerebral cortex[1303]. As discussed later, regulatory mechanisms differ considerably in these tissues. P450 19A1 is also expressed in some tumors, particularly those derived from these tissues.

The actions of androgens and estrogens in the gonadal tissues are fairly well understood, but less is known in the brain. Androgens and androgen-derived estrogens regulate complementary and interacting genes in many neural networks[1304].

6.45.2. Regulation and Polymorphism

The regulation of the *CYP19A1* gene is quite complex, primarily because of the use of four tissue-selective promoters[1303, 1305]. Either the I.1, I.4, I.f, or I.6 sequence is read as exon I and spliced into the mRNA, depending upon the tissue. However, exon I does not code for the protein, so the P450 19A1 enzyme is always the same.

In preovulatory follicles and corpora lutea of human ovary, the 5′-untranslated region of P450 19A1 transcripts is encoded by exon IIa (ref. [1306]). The major operatives here are CRE and SF-1 elements[1303].

In adipose tissue, the promoter from exon I.4 is utilized[1303]. The same exon is utilized in bone and skin[1303], and in leiomyoma tissue derived from myometrium[1307]. This system is regulated with Sp1, a glucocorticoid regulatory element, STAT3, and possibly PPARγ[1303, 1308]. Pre-adipocytes also involve regulation with liver receptor homolog-1 (LRH-1)[1309].

In placenta exon I.1, an 89 kb upstream element is utilized[1303]. This is a strong promoter and involves C/EBP-β[1303]. A strong positive enhancer element between −42 and −501 is present[1310]. The possibility exists that vitamin D receptor/RXRα heterodimers and PPARγ may have effects[1303].

Regulation in bone uses exon I.6 (ref. [1303]). The study of regulation in bone is less extensive than in other sites, and 1,25-dihydroxycholecalciferol, interleukins, TNFα, and TGF-β_1 have had stimulatory activity.

Regulation in brain uses exon I.f and has also not been as extensively studied[1303]. P450 19A1 does seem to be upregulated by androgens.

Regulation in fetal liver involves exon I.4, as with adipose tissue[1303]. The same pattern appears to apply in skin fibroblasts and intestine.

In cancer cells, alternate regulatory pathways are utilized[1303].

A number of polymorphisms have been reported in the *CYP19A1* gene. These have been studied in relationship to breast but without convincing relationships (*vide infra*); also, there was no relationship with breast density[1311].

The number of cases of serious P450 19A1 deficiency is apparently only about ten, including two adult males[1303].

6.45.3. Substrates and Reactions

The reaction involves three steps and has been the subject of considerable mechanistic interest (Figure 10.15). Androstanedione is converted to estrone, testosterone to 17β-estradiol, and 16-hydroxy DHEA to estriol. The first two steps are relatively straightforward, for example, $RCH_3 \rightarrow RCH_2OH \rightarrow CHO$ (at C19). The third step was difficult to rationalize with "classic" FeO^{3+} chemistry, and there has been general acceptance of a $FeOO^-$-based mechanism originally developed by Robinson[1312] and Akhtar[1313], and further developed in models by Coon and Vaz[1314].

The possibility of utilization of DHEA as a substrate for estrone synthesis has been proposed but not addressed directly[1315].

6.45.4. Knowledge about Active Site

One of the historic problems in studying structure–function relationships in P450 19A1 has been the availability of expression systems. Recently two *E. coli* systems have been developed[1316, 1317].

Site-directed mutagenesis has been done using mammalian and insect cell-based systems, and models have been in existence for some time[1318]. More recent modeling work[1319] has been done, with an emphasis on docking of inhibitors in SRS-1.

6.45.5. Inhibitors

P450 19A1 inhibitors have long been of interest for treatment of estrogen-dependent cancers in a variety of tissues[30, 1320]. Today, the process has reached the stage of "3rd-generation" inhibitors[1321], moving beyond early drugs such as aminoglutethimide[1322]. The newer inhibitors are more effective in lowering the body limit of estrogens[1323]. One example of a newer drug is exemestane, a mechanism-based inactivator currently in Phase III clinical trials and being compared with the estrogen receptor antagonist tamoxifen[1322–1325]. The inactivation appears to involve generation of a Michael acceptor in the active site[1322].

Another report suggests generation of inhibitory Michael agents from prostaglandin J_2, but detailed characterization has not been done[1326].

Other nonsteroidal inhibitors of P450 19A1 are also under consideration[1327].

Breast cancer is the major target area for P450 19A1 inhibition, but other cancers are also under investigation[1328].

The point has been made by Simpson *et al.*[1303] that a future goal of P450 19A1 inhibition should be tissue selectivity. The diverse role of P450 19A1 in different tissues might indicate that generalized inhibition of estrogen synthesis may be less than desirable. Targeted inhibition of P450 19A1 could, in principle, be achieved by (a) selective targeting of inhibitors of P450 19A1 catalysis to tumors/individual organs or (b) targeted down-regulation of P450 19A1 synthesis in selected areas.

6.45.6. Clinical Issues

Several clinical issues have already been alluded to. The first issue is congenital aromatase deficiency. Serious cases in adults appear to be relatively rare[1303, 1329] and have been treated with estrogen replacement therapy[1330]. However, some children are considered to have attenuated P450 19A1 activity[1331].

Studies with P450 19A1 knockout mice show expected reproductive and sexual phenotypes and also adipose and bone phenotypes[1329, 1332], as well as a sociosexual behavior phenotype[1333].

The issue of using P450 19A1 inhibitors in the treatment of a variety of estrogen-dependent tumors has already been presented. In addition, there is consideration of the use of inhibitors for breast cancer prevention in high-risk individuals[1334].

A number of studies have been made on the relationship of *CYP19A1* polymorphisms with breast cancer, but the evidence has not shown a change in risk[1311, 1335]. No strong association was seen for endometriosis either[1336].

6.46. P450 20A1

At the time of writing this chapter, the only available information was the existence of the *CYP20* gene in the human genome[705]. The gene

appeared to be vertebrate specific and had been speculated to be involved in development.

6.47. P450 21A2

P450 21A2 is the enzyme involved in the 21-hydroxylation of progesterone and 17-hydroxy-progesterone, yielding deoxycorticosterone and 11-deoxycortisol from the two substrates, respectively (Figures 10.13 and 10.14). The 21-hydroxylation reaction is an important step in the synthesis of glucocorticoids and mineralcorticoids, and deficiencies lead to "salt-wasting syndrome," if not treated, and to congenital adrenal hyperplasia in the worst cases.

6.47.1. Sites of Expression

The major site of expression is the adrenal cortex. This reaction has been known for some time, and many of the early biochemical studies were done with bovine adrenals because of the need for large amounts of tissue[1337].

Low amounts of P450 21A2 have been reported in human lymphocytes[1338] and brain[1339]. Any specific function in these tissues is unknown at this time.

6.47.2. Regulation and Polymorphism

The regulation of P450 21A2 has some similarity to that of P450 17A1, in that both are regulated by ACTH. The cyclic AMP responsive sequence in the 5′-flanking region[1340] uses adrenal-specific protein factor and an Ad4-like sequence[1341]. One issue in the regulation of the *CYP21A2* gene is the neighboring homologous but nonfunctional *CYP21A1* pseudogene, which can compete for transcription factors and other regulatory proteins[1342]. In other work, protein kinases A and C and Ca^{2+} were found to regulate *CYP21A2* gene expression in a human cortical cell line[1343].

Another interesting aspect of the regulation of the *CYP21A2* gene is that it is located very close to the major histocompatibility locus, 2.3 kb downstream from the *C4* gene. Transcriptional regulatory elements for the *CYP21A2* gene lie within intron 35 kb of the *C4* gene[1344].

Steroid 21-hydroxylase deficiency is the most common cause of congenital adrenal hyperplasia, and many mutations are now known to be associated with the disease. Many are the result of recombination with the related pseudogene[1345, 1346]. Some are in the coding region[1346–1348] and the 5′-flanking region[1349]. The incidence of carriers of congenital adrenal hyperplasia is 1–2% in the population, and many deleterious mutations have now been identified[1350–1357].

6.47.3. Substrates and Reactions

The only known substrates are progesterone and 17-hydroxyprogesterone, which are hydroxylated only at the 21-position (Figures 10.13 and 10.14).

6.47.4. Knowledge about Active Site

Homology models have been reported[1185, 1358]. The amount of site-directed mutagenesis has been limited, but the disease has yielded many locations for loss of function because the severity of the disease is (inversely) correlated to the residual 21-hydroxylation activity. Many of the mutants could be rationalized in the context of a homology model[1358], although some associated with disease are more subtle (e.g., E380D).

6.47.5. Inhibitors

Relatively little has been published about inhibitors. Detrimental effects of spironolactone have been attributed to inhibition of 21-hydroxylation[1359], although further details with this P450 are lacking. Recently, Auchus[1279] reported that the enantiomeric form of progesterone (*ent*-progesterone) is a competitive inhibitor of P450 21A2 (although not as effective as with P450 17).

6.47.6. Clinical Issues

As mentioned earlier, the incidence of defects is relatively frequent and the ability to form cortisol is a problem. At least 56 different mutations have been identified[1351]. Patients who cannot synthesize sufficient aldosterone may lose sodium balance and can develop a fatal "salt-wasting" syndrome. Treatment involves administration of mineralocorticoids and glucocorticoids. Females with severe, classic P450 21A2 deficiency are

exposed to excess androgens prenatally and born with virilized external genitalia, but prenatal diagnosis permits prenatal treatment of affected females[1350]. Experimental research is being done on gene therapy to transfer active *CYP21A2* genes; work done on mice suggests feasibility[1360].

6.48. P450 24A1

The next three P450s (24A1, 27A1, 27B1) are involved in vitamin D metabolism (Figure 10.16). All three are mitochrondrial and receive electrons from the iron sulfur protein adrenodoxin (via the flavoprotein adrenodoxin reductase) (Table 10.1).

6.48.1. Sites of Expression and Abundance

The 24-hydroxylation of 25-hydroxyvitamin D_3 has long been known to occur in the kidney mitochondrial membrane[1361]. Following the purification of a rat P450 with this activity[1362], cDNA clones for chicken[1363] and human[1364] homologs were obtained.

The enzyme is expressed in both proximal and distal kidney tubules[1365], but has also been found in human nonsmall cell lung carcinomas[1366]. This would appear to be a relatively low abundance P450. Expression has also been reported in human keratinocytes[1367, 1368], colon carcinoma cells[1369], and prostatic cancer cells[1370].

6.48.2. Regulation and Polymorphism

The regulation of the *CYP24A1* gene appears to be complex, although some phenomena observed in animal models have not been examined in as much detail in humans. The activity has long been known to be inducible by vitamin D, perhaps to relieve the cells of an overload, and a vitamin D receptor element has been found in the 5′-region of the *CYP24A1* gene[1371, 1372]. Parathyroid hormone and cyclic AMP both enhance induction by the vitamin D receptor[1365].

In human keratinocytes, P450 24A1 mRNA was also elevated by 1α,25-dihydroxyvitamin D_3 (ref. [1367]). Studies with rat systems indicate that this response is also mediated by vitamin D response elements and that two of these (VDRE-1, VDRE-2) operate synergistically[1373]. A functional Ras-dependent Ets-binding site is located downstream from the proximal VDRE site and was critical; the model indicates transcriptional cooperation between Ras-activated Ets proteins and the vitamin D receptor–RXR complex in mediating 1α,25-dihydroxyvitamin D action on the P450 24A1 promoter[1374]. The YY1 transcription factor has been reported to repress 1α,25-dihydroxyvitamin D_3-induced transcription in cell culture[1375]. The isoflavone genistein was reported to block the transcription of the *CYP24A1* gene in human prostatic cancer cells and this block could be relieved with the histone deacetylase inhibitor trichostatin A[1370]. Finally, the earlier results with Ets proteins (*vide supra*) have been expanded to show distinct roles of the MAP kinases

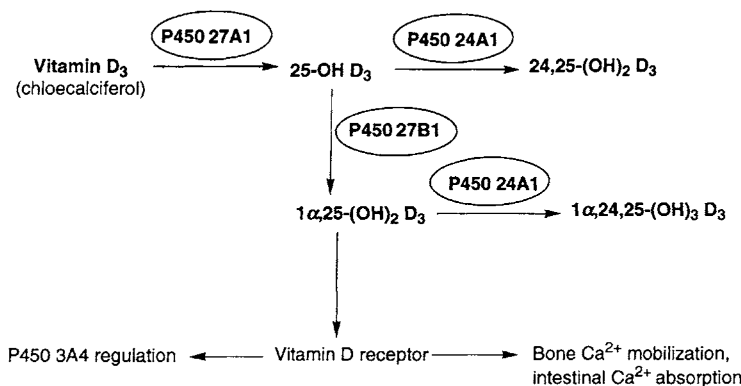

Figure 10.16. Overview of P450s involved in key steps of vitamin D activation[29].

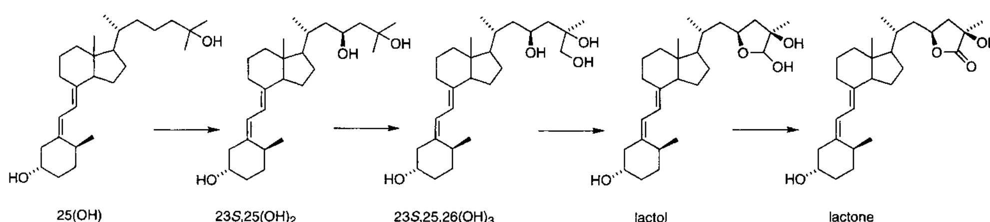

Figure 10.17. C-23 hydroxylation pathway for 25-hydroxyvitamin D_3 (25(OH)) oxidation catalyzed by P450 24A1 (ref. [1378]).

ERK1/ERK2 and ERK5 (ref. [1376]). Induction of P450 24A1 by 1α,25-dihydroxyvitamin D_3 involves Ets-1 phosphorylation at Thr38, but 1α,25-dihydroxyvitamin D_3 stimulation of ERK1/ ERK2 required RXRα phosphorylation on Ser260 (ref. [1376]).

Polymorphisms in P450 24A1 have apparently not been reported.

6.48.3. Substrates and Reactions

Both 25-hydroxyvitamin D_3 and 1α,25-dihydroxyvitamin D_3 are substrates for 24-hydroxylation (Figure 10.16), with the latter being the preferred substrate[1377]. However, human P450 24A1 can also catalyze other side-chain reactions (Figure 10.17). Studies with side-chain-fluorinated vitamin D analogs also provide evidence for some flexibility of this side chain in allowing P450 24A1 to oxidize[1380, 1381]. Rat P450 24A1 differs from the human ortholog in taking 1α,25-dihydroxyvitamin D_3 on to calcitroic acid instead of the products shown in Figure 10.17[1382–1384].

6.48.4. Knowledge about Active Site

Limited site-directed mutagenesis has been done with P450 24A1. Expression studies using *E. coli* indicate species conservation in the residues 245–253 (F-helix), and the mutants M246F and F249T showed changes in the ratio of 24-hydroxylation to other pathways[1384].

Studies by Jones' group[1385] with analogs indicate that the site of hydroxylation by P450 24A1 depends on the distance from the ring to the site (23 or 24), and not by the distance beyond this. Schuster's group has developed pharmacophore models[1386].

6.48.5. Inhibitors

Inhibition of P450 24A1 is of some interest in the context of raising levels of active vitamin D metabolites. Schuster[1368, 1386, 1387] has identified some inhibitors that differ in their selectivity between P450 24A1 and P450 27B1 and have sub-μM IC_{50} values.

6.48.6. Clinical Issues

The scheme presented in Figure 10.16 depicts P450 24A1 as an enzyme involved in deactivating the activated form of vitamin D. The possibility has been considered that defects in P450 24A1 might lead to hypervitaminosis D[29]. An overactive P450 24A1 could lead to vitamin D deficiency. Henry[1388] has reviewed the role of P450 24A1 and made comparisons to other "multistep" P450 enzymes. The possibility is raised that P450 24 could serve to generate products with their own biological activities, with P450 24 thus being involved in an anabolic pathway. Recently, transgenic rats overexpressing (rat) P450 24A1 were found to have *low* plasma levels of 24,25-dihydroxy vitamin D_3[1389], which was unexpected. Further, the transgenic rats developed albuminuria and hyperlipidemia shortly after weaning, and later developed atherosclerotic lesions in the aorta. These results raise the possibility that P450 24A1 is involved in functions other than vitamin D metabolism[1389].

6.49. P450 26A1

Retinoic acid is a vitamin A derivative that plays an important role in gene regulation and development. The metabolism of retinoic acid has been a matter of interest for some time, and

the oxidation of retinoic acid by some hepatic P450s has been demonstrated[386, 942, 1390]. However, the relevance of these transformations to retinoic acid homeostasis in target tissues is not clear. White *et al.*[1391] probed a panel of mRNAs from mammalian cell lines with a cDNA from a zebrafish P450 shown to be involved in retinoic acid-inducible retinoic acid oxidation and characterized P450 26A1 (ref. [1391]). The heterologously expressed enzyme converted all-*trans*-retinoic acid to the 4-hydroxy-, and 4-oxo-, and 18-hydroxy products. The turnover numbers are unknown because the amount of P450 was not quantified, but the enzyme is clearly able to catalyze the oxidation of sub-μM additions of all-*trans*-retinoic acid[1391]. Apparently other retinoic acid isomers are not substrates.

The enzyme is expressed in cell lines derived from several different tissues (kidney, lung, liver, breast)[1391]. P450 26A1 has also been shown to be expressed in human fetal liver and brain tissues[1392, 1393], with a pattern differing from P450 26B1.

P450 26A1 (and 26B1, *vide infra*) may function to protect fetal brain and possibly other tissues from excess retinoic acid, which is known to be teratogenic, by metabolism. The interplay between P450s 26A1 and 26B1, and the significance of changes in their levels to normal tissue function is not yet known.

6.50. P450 26B1

The *CYP26B1* gene has 44% sequence identity with *CYP26A1*; it was found by searches of genomic databases[1394]. The gene expression pattern for P450 26B1 is different from that of P450 26A1 in mice[1395] and also in human fetal brain[1396]. The localization of P450 26B1 in adult human brain differs from that of P450 26A1, with a higher level of P450 26B1 mRNA in the cerebellum[1396]. Exactly how critical this enzyme is in brain function is unknown[1392]. The catalytic specificity is very similar to that of P450 26A1, acting only on all-*trans* retinoic acid and catalyzing the formation of 4-hydroxy-, 4-oxo-, and 18-hydroxy (*trans*) retinoic acid[1392].

As with P450 26A1, further studies are needed to define the presence of any defects in the gene and what the consequences might be.

6.51. P450 26C1

The only information presently available about the *CYP26C1* gene is its existence in the human genome[705]. Any suggestion of a role in retinoid metabolism is only speculatory at this time.

6.52. P450 27A1

This is a mitochondrial enzyme that was characterized on the basis of two rather divergent catalytic activities, the 25-hydroxylation of vitamin D_3 (Figure 10.16) and the oxidation of cholesterol at the C27 position (Figure 10.18). Thus, the enzyme bridges between hormone (vitamin D) and oxysterol pathways, and the clinical relevance of P450 27A1 is considerable.

6.52.1. Sites of Expression and Abundance

The enzyme is localized in liver mitochondria. Confusion existed in the early literature because some animal species have liver microsomal vitamin D_3 25-hydroxylases (e.g., hog liver and kidney P450 2D25, (refs [1398], [1399])), but not humans[1400]. The rat and human liver mitochrondrial P450 27A1 recombinant enzymes were clearly shown to catalyze both vitamin D_3 25-hydroxylation and the 27-hydroxylation of the side chains of cholesterol and several derivatives[1401, 1402].

Expression, at least at the mRNA level, has also been reported in leukocytes[1403], skin fibroblasts[1404], kidney[1405] (and fetal liver and kidney[1405]), and the arterial wall[1406].

6.52.2. Regulation and Induction

In a "normal" human population, the variation in the steady-state P450 27A1 mRNA level was reported to be ~25-fold, compared with 60-fold for P450 7A1 in the same study[1041]. However, at least two polymorphisms ($\geq$1% incidence, no dramatic effect) and 42 mutations (rare alleles, usually debilitating) are known[1404, 1407]. Truncation mutations are known[1403], as well as splice variants[1408]. Defects in the *CYP27A1* gene are associated with a condition known as cerebrotendinous xanthomatosis (CTX), a rare, autosomal recessive disorder characterized by accumulation of

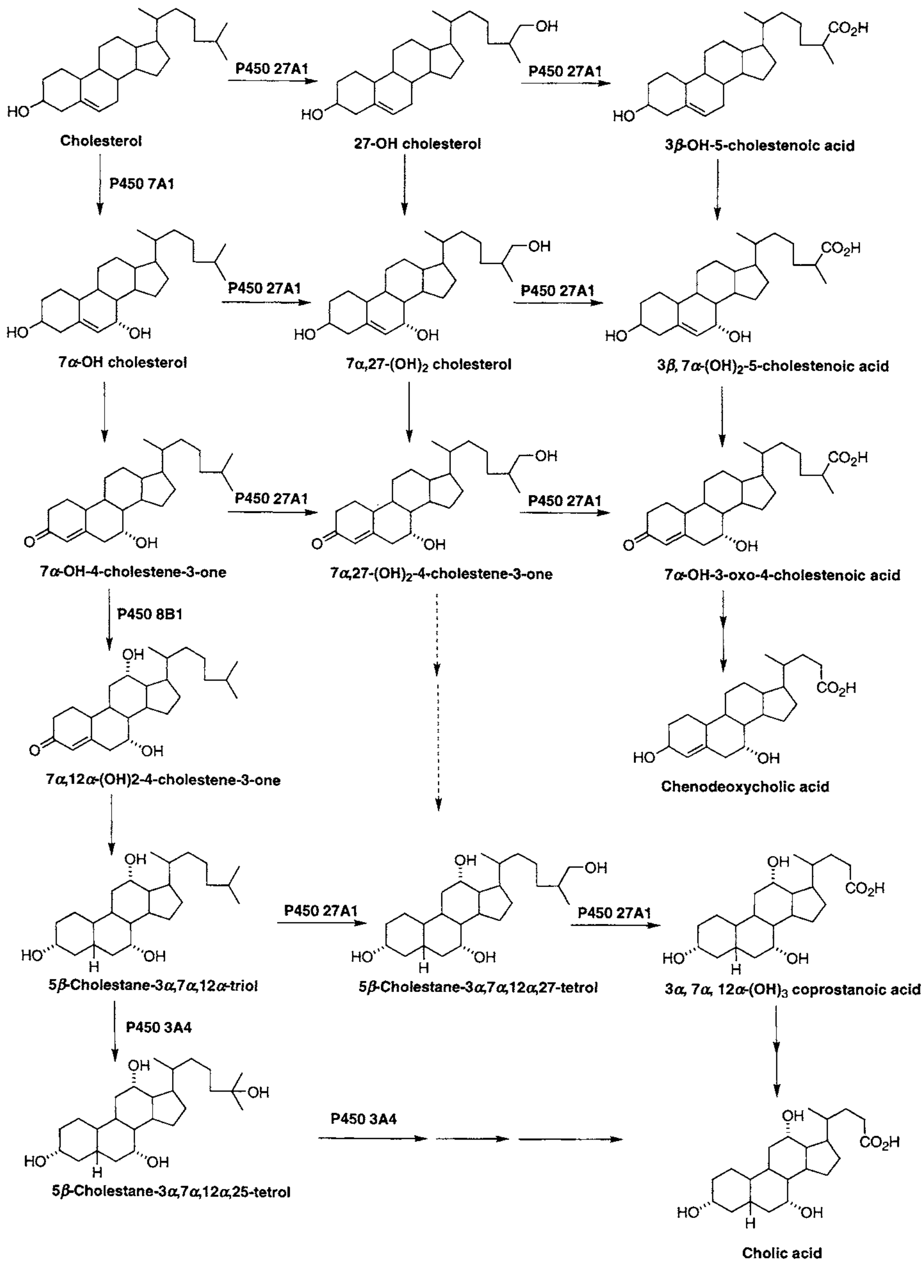

Figure 10.18. Bile acid synthesis from cholesterol[1397]. The steps shown with dashed arrows are tentative.

cholestanol and cholesterol in many tissues. The clinical manifestations include tendon xanthoma, premature cataracts, juvenile atherosclerosis, and a progressive neurological syndrome involving mental retardation, cerebellar atoxia, pyramidal tract signs, myelopathy, and peripheral neuropathy[29, 1407].

Several aspects of regulation of the *CYP27A1* gene have been studied. In rats, the enzyme can be induced by gonadotropin[1409]. A hamster model

showed downregulation of the gene in cholestatic liver[1397], although human P450 27A1 (used in HepG2 cells) was not subject to negative feedback regulation[1041].

6.52.3. Substrates and Reactions

Expanding on previous discussion, P450 27A1 catalyzes the 25-hydroxylation of vitamin D_3 (Figure 10.16), 1α-hydroxyvitamin D_3, vitamin D_2, and 1α-hydroxyvitamin D_2, and also the 27-hydroxylation of cholesterol and several derivatives (Figure 10.18)[1410, 1411]. The cholesterol alcohols are further oxidized by the enzyme to aldehydes and then carboxylic acids[1412]. The available information suggests release of the intermediates in the pathway[1412]. The regioselectivity of the enzyme is considered to be a function of the distance of the hydroxylation site to the end of the side chain[1385].

More detailed analysis of the vitamin D_3 reaction has been with *E. coli* recombinant P450 27A1, with evidence for the following products (from vitamin D_3): 25-hydroxy, 26-hydroxy, 27-hydroxy, 24*R*-25-dihydroxy, 1α,25-dihydroxy, 25, 26-dihydroxy, 25-,27-dihydroxy, 27-oxo, and an unidentified dehydrogenated product[1402, 1413].

6.52.4. Knowledge about Active Site

Some information about the roles of amino acids can be inferred from the knowledge of alleles involved in CTX; many of these proteins were unstable when attempts were made at heterologous expression[1414]. Other work by Pikuleva *et al.*[1415] with the putative F and G helices has shown differences due to substitution of Phe207, Ile211, Phe215, Trp235, and Tyr238. Interestingly, the I211K and F215K mutations affected the regioselectivity and enabled the enzyme to catalyze C–C bond cleavage. Further work with mutants in this region led to weaker association of P450 27A1 with the membrane, and some of the nonconservative changes yielded impaired catalytic activity[1416].

Human P450 27A1 can be contrasted with porcine P450 2D25, which also catalyzes vitamin D_3 25-hydroxylation. The only human P450 2D subfamily enzyme which does not have activity toward vitamin D is P450 2D6. Further, changing a set of residues of porcine 2D25 to their counterparts in (human) P450 2D6 abolished the activity toward vitamin D_3[1417].

6.52.5. Inhibitors

Apparently little specific work has been done on inhibition of this enzyme. Inhibition of this enzyme by a drug would probably be undesirable.

6.52.6. Clinical Issues

Low serum 25-hydroxyvitamin D_3 concentrations have been reported in a variety of other medical conditions and are considered to be potential problems[1418]. Although CTX is linked with defective P450 27A1 (ref. [29]), there are a number of enigmas about the etiology. A heterozygote showed frontal lobe dementia and abnormal cholesterol metabolism[1419]. Compound heterozygous mutations have also been reported to cause a variation of CTX[1407].

Björkhem has recently reviewed the issue of whether oxysterols (e.g., hydroxycholesterol) control cholesterol homeostasis[1420]. Studies with rodents and cultured cells have not been very clear to date. For instance, disruption of the mouse *CYP27A1* gene yielded reduced bile acid synthesis but apparently caused no change in levels of cholesterol or 1α,25-dihydroxyvitamin D_3[1421]. P450 27A1 is constitutively expressed in the normal artery wall and is substantially upregulated in atherosclerosis, and the possibility has been raised that P450 27A1 may be a protective mechanism for removing cholesterol[1406]. Further, immune complexes and IFN-γ decreased P450 27A1 expression in human aortic endothelial cells, peripheral blood mononuclear cells, monocytes-derived macrophages, and a human monocytoid cell line, suggesting downregulation of P450 27A1 to maintain cholesterol homeostasis in the arterial wall[1422].

In *Cyp27A1*$^{-/-}$ mice, a dramatic increase in the level of P450 3A enzymes is seen; some sterols accumulate and induce via the mouse PXR system[1423]. P450 3A4 has some side-chain hydroxylation activities toward cholesterol-derived sterols[771]. However, elevated P450 3A4 activities were not increased in CTX[771], indicating a difference in the murine and human systems. Recently, Escher *et al.*[1424] have reported that cholesterol

efflux in CHOP cells is enhanced by (heterologous) expression of human P450 27A1, and the authors suggest this as part of a protective system against atherosclerosis. The basis is probably the ability of 27-hydroxycholesterol to act as an endogenous ligand for the liver X receptor in cholesterol-loaded cells[1425].

In considering the general question of whether oxysterols (e.g., 27-hydroxycholesterol) control cholesterol homeostasis, the hypothesis is still open and the rodent data are not totally clear here. Björkhem[1041] has made the point that humans lacking P450 27A1 have normal circulating levels of cholesterol.

6.53. P450 27B1

As discussed earlier, vitamin D is an important hormone. A critical step in activation is 1α-hydroxylation[1426] (Figure 10.16). Early work established the P450 nature of the enzyme, localized in kidney mitochondria[1427]. Subsequent work demonstrated that the 1α- and 24-hydroxylation activities could be attributed to different enzymes[1428, 1429]. Some early work had suggested that the 1α- and 25-hydroxylation activities were associated with the same enzyme[1430], but later work showed that these activities were due to P450 27B1 and 27A1, respectively.

6.53.1. Sites of Expression and Abundance

The cloning of the human cDNA for what is now known as P450 27B1 established the kidney mitochondrial P450 (27B1) as the vitamin D_3 1α-hydroxylase[1431]. The gene has nine exons and spans only 5 kb[1432].

P450 27B1 is expressed in many parts of the human kidney, including the distal convoluted tubule, the cortical and medullary part of the collecting ducts, and the papillary epithelia[1433]. Lower expression was observed along the thick ascending limb of the loop of Henle and Bowman's capsule. Some weaker expression was observed in glomeruli or vascular structures. In normal humans, the distal nephron is the predominant site of expression[1433].

P450 27B1 is also expressed in many extrarenal sites (human) where it is involved in vitamin D-related activities, including skin (basal keratinocytes, hair follicles), lymph nodes (granulomata), colon (epithelial cells and parasympathetic ganglia), pancreas (islets), adrenal medulla, brain (cerebellum and cerebral cortex)[1434], placenta (decidual and trophoblastic cells)[1434–1436], cervix[1437], and parathyroid glands[1438]. Thus, P450 27B1 may be an intracrine modulator of vitamin D function in peripheral tissues[1434]. The expression of P450 27B1 was elevated in parathyroid adenomas, but attenuated in carcinomas, relative to normal parathyroid tissue[1438].

6.53.2. Regulation and Polymorphism

Although the *CYP27B1* gene is only 5 kb in size, the regulation is quite complex. The promoter is in the −85/+22 region and requires a functional CCATT element. Three consensus AP-1 sites are upstream[1439]. Enzyme activity has long been known to be stimulated by low phosphorus diets (in animal models)[1440], and more recently, this phenomenon has been linked to a growth hormone mechanism[1441, 1442]; its relevance in humans is not known.

Expression is also regulated by calcium, parathyroid hormone, and by the product 1α,25-dihydroxyvitamin D_3[1443, 1444]. Regulatory regions involving the responses to parathyroid hormone, calcitonin, and 1α,25-hydroxyvitamin D_3 are located in the region −0.4 to −0.5 kb[1445]. Forskolin also regulates gene expression, and an Sp1 site is involved in aspects of regulation[1446, 1447].

Complexity is seen in different models. Parathyroid hormone-related protein and Ca^{2+} have conflicting actions in a nude rat model of humoral hypercalcemia of malignancy[1448]. In differentiated Caco cells, there is upregulation of P450 27B1 expression by 1α,25-dihydroxyvitamin D_3 and epidermal growth factor, but downregulation in less differentiated Caco cell lines[1369].

Another aspect of regulation of P450 27B1 is genetic. P450 27B1 results in Type I vitamin D-dependent rickets[1449]. The genetics have been established in more than 30 patients involving at least 20 mutations[1450, 1451]. At least 13 missense mutations have been observed, none of which encode an active protein. Some of the mutants are splicing defects[1452]. Some mutations in *CYP27B1* are also involved in what is termed pseudovitamin D-deficiency rickets[1453, 1454]. Beyond these

debilitating mutations, little information is available about actual polymorphisms.

6.53.3. Substrates and Reactions

P450 27B1 can catalyze the 1α-hydroxylation of both 25-hydroxy and 24(*R*),25-dihydroxyvitamin D_3[1383, 1455] (Figure 10.16). The intrinsic activity (k_{cat}/K_m) for the recombinant human enzyme is better for 24(*R*),25-hydroxy vitamin D_3, but this does not mean that this is the favored substrate in the cell, because of the balance of vitamin D metabolites regulated by P450s 24A1 and 27A1 (ref. [29]). Apparently the 25-hydroxy group is an obligatory requirement[1378, 1455].

6.53.4. Knowledge about Active Site

Some information is available from the natural mutants of P450 27B1, even if the basis for loss of activity is not obvious. Inouye's group[1414] has provided evidence that Arg107, Gly125, and Pro497 are not simply involved in binding substrate but required for proper folding. It was also suggested that Arg389 and Arg453 are involved in heme binding and that Asp164 stabilizes the bundle of the four helices D, E, I, and J. Thr321 is suggested to be involved in O_2 activation[1414]. The natural mutants L343F and E189G show partial activity and the individuals bearing these have only marginal impairment[1456].

6.53.5. Inhibitors

Little has been done because impairment of this enzyme is a clinical problem. Some thiavitamin D analogs have been evaluated in animal models[1457].

6.53.6. Clinical Issues

The significance of the enzyme is due to the pleiotropic actions of the active form of vitamin D, 1α,25-dihydroxyvitamin D_3, which include regulation of calcium homeostasis, control of bone cell differentiation, and modification of immune responses[1458]. The 1α-hydroxylation reaction is rate limiting and hormonally controlled. The expression of the gene is usually tightly regulated (*vide supra*), but gene defects are responsible for vitamin D-dependent rickets Type I[1459]. At least 30 different mutations are known in patients[1449, 1460]. Even the "mild" phenotype of Type I rickets is due to deficiency in P450 27B1 (ref. [1461]).

CYP27B1 knockout mice have been characterized and show a typical rickets phenotype[1462]. Another mouse model in which the gene has been ablated showed skeletal, reproductive, and immune dysfunction[1463]. Rickets was also observed in a conditional knockout model[1464].

Patients with severe renal insufficiency show attenuated 1α-hydroxylation activity[1465].

Another aspect of P450 27B1 research involves cancer. Increased activity was reported in parathyroid tumors[1466]. Some splice variants of the *CYP27B1* gene (coding for truncated proteins) were amplified in human (brain) gliomas[1467]. Reports have also appeared on the relationship of P450 27B1 expression to various biological processes in human nonsmall cell lung carcinomas[1366], colon tumors[1468–1470], and prostate cancers[1471, 1472], generally with decreased expression in tumors.

Finally, 1α,25-dihydroxyvitamin D_3 is used to treat psoriasis, and patients can develop resistance. An experimental model for therapy involves enhancement of the endogenous production of 1α,25-dihydroxyvitamin D_3 by gene therapy[1473].

6.54. P450 27C1

As with some of the other P450s, the only knowledge currently available is the existence of the *CYP27C1* gene in the human genome[705]. Suggestions that this enzyme is involved in vitamin D metabolism are still only speculatory.

6.55. P450 39A1

An expression-cloning approach was utilized to isolate a cDNA from (*Cyp7b1*$^{-/-}$) mice that could, when expressed, catalyze the 7α-hydroxylation of 24-hydroxycholesterol[1474]. P450 39 has a microsomal location with a preference for the substrate 24-hydroxycholesterol and is expressed in liver. Presumably these characteristics of mouse P450 39 apply to the human ortholog but no further information is yet available. Potential relevance of this enzyme is in the inactivation of

24-hydroxycholesterol, a ligand for the LXR nuclear hormone receptor (see Section 6.56 on P450 46, *vide infra*).

6.56. P450 46A1

An expression cloning approach was utilized to clone cDNAs encoding both murine and human cholesterol 24-hydroxylase, P450 46A1 (ref. [1475]). The mouse and human sequences are 95% identical. Expression is predominantly in brain (neurons in several regions). The enzyme is in the endoplasmic reticulum; alternate substrates have not been explored but may be unlikely.

The significance of P450 46A1 rests in the fact that the brain is the most cholesterol-rich tissue in the body[1476]. LXRs, members of the nuclear hormone receptor family, are activated by 24-hydroxycholesterol. LXRβ and P450 46 have overlapping expression patterns in brain[1476]. The system may be counter-balanced by P450 39, which catalyzes the 7α-hydroxylation of 25-hydroxycholesterol (Section 6.55).

Recently, there has been considerable interest in the relationship between P450 46A1 and Alzheimer's Disease. P450 46A1 had a marked difference in distribution in the brains of normal and Alzheimer's patients, with less staining of neuronal cells but more of glial cells in diseased patients[1477]. Also, elevated levels of 25(*S*)-hydroxycholesterol were found in cerebral spinal fluid in the early stages of dementia[1478]. Associations[1479] and lacks of associations[1480] between *CYP46* SNPs and Alzheimer's Disease have been reported. One of the issues in the research with P450 46A1 is the apparently very low rate of cholesterol 24-hydroxylation (although an exact rate can be deduced from the published information). Recently, Pikuleva's laboratory has found that 24-hydroxycholesterol is a much better substrate for recombinant P450 46A1 than is cholesterol, being oxidized to as yet uncharacterized product[1480a].

6.57. P450 51A1

Lanosterol is an important intermediate in cholesterol synthesis, and 14α-demethylation has been established as a step in the pathway. Yoshida's laboratory had studied the yeast enzyme for many years and then demonstrated the reaction in rat liver microsomes in 1994[1481]. Subsequently the reaction was also demonstrated in rat brain microsomes[1482].

6.57.1. Sites of Expression and Abundance

Waterman's group identified the human *CYP51A1* gene and two pseudogenes[1483]. mRNA blot analysis showed the highest levels in testis, ovary, adrenal, prostate, liver, kidney, and lung. In mouse testis, P450 51A1 was localized in both round and elongated spermatids[1484]. The enzyme is also found in (rodent) oocytes[1485].

6.57.2. Regulation and Polymorphism

Polymorphisms in the human *CYP51A1* gene have not been reported nor have debilitating mutants been defined. However, Kelley *et al.*[1486] reported a patient with Antley–Bixler syndrome and ambiguous genitalia with lanosterol accumulation and an apparent defect in P450 51A1.

With regard to regulation of the human gene, primer extension studies indicated predominant transcription initiation sites in liver, lung, and kidney, and placenta 250 and 249 bp upstream from the translation start site and a second major site at −100 bp, with the absence of TATA and CAAT patterns and a GC-rich sequence in the promoter region[1483]. Multiple (rat) testis-specific transcripts arise from differential polyadenylation site usage[1487].

In human adrenocortical H295R cells (in culture), cholesterol deprivation led to a 2.6–3.8-fold induction of P450 51A1 mRNA, which was suppressed by the addition of 25-hydroxycholesterol[1488]. In the liver and other somatic tissues, *CYP51* is regulated by a sterol/sterol-regulatory element binding protein (SREBP)-dependent pathway[1489]. In testis, cAMP/cAMP-responsive element modulator $(CREM)_1$-dependent regulation predominates. Sp1 functions to maximize the sterol regulatory pathway of P450 51 (ref. [1490]).

Insulin is an essential factor in "basal" expression of P450 51 in rat liver, with possible involvement of SREBP-1c involvement[1491]. In a porcine vascular endothelial cell model (and in arterial wall), LDLs downregulated P450 51 through an SREBP-2 mechanism[1492].

6.57.3. Substrates and Reactions

Stimulation of human P450 51 activity by b_5 in a reconstituted system has been reported by Kelly's laboratory[1493].

The normal mammalian substrate for P450 51 is 24,25-dihydrolanosterol[1494], with the 14α-demethylation process proceeding in what are assumed to be three consecutive steps, as with some other P450s, for example, 11A1, 17A1, 19A1. Interestingly, both human and yeast (*Candida albicans*) P450 51 showed relatively little selectivity among a closely related group of analogs[1494]. It is also interesting to note that even though this P450 has a relatively defined role in a physiological process, the kinetic parameters are relatively poor among P450s (k_{cat}/K_m = 300 $M^{-1}s^{-1}$)[1494].

6.57.4. Knowledge about Active Site

A crystal structure of human P450 51 is not yet available but high resolution structure of the soluble *Mycobacterium tuberculosis* P450 51 is (ref. [1495]). Two notable features are a bent I helix and an open conformation of the BC loop. The bacterial structure has been utilized in consideration of mammalian models, and the SRS predictions do not seem to apply well[1495]. Further, the mutation hotspots for known azole-resistant *C. albicans* P450 51 mutants tend to be outside the predicted active site and suggest the contribution of long-range effects on ligand binding[1496, 1497].

Studies on Arg448 indicate that, despite sequence conservation, aspects of the role of this and other residues are different for human and yeast P450 51 (ref. [1498]).

6.57.5. Inhibitors

Most of the interest in inhibition has been with fungal P450 51, as a target for antimycotic drugs. The goal is to select candidate drugs inhibitory to fungal P450 51, but not human P450 51.

Some work on the interaction of azoles with human P450 51 has been published[1499]. Although human P450 51 has been suggested as a target for cholesterol-lowering drugs, apparently little has been done and potential toxicity due to the steroidogenic and potential germ cell side effects (*vide infra*) could be an issue.

6.57.6. Clinical Issues

Most of the work discussed here is from experimental studies on the possible role of P450 51 in reproduction, and the translation of phenomena from animal models to humans is still somewhat speculatory. However, the very high level of P450 51 expression in postmeiotic haploid spermatids is striking. The action of P450 51 is proposed to lead to the production of signaling steroids in haploid germ cells[1500]. Meiosis-activating substances (MAS) are produced by 14-reduction of products of the action of P450 51 on lanosterol[1500]. Follicular fluid MAS (FF-MAS) is formed from lanosterol in rat spermatids[1501]. Yoshida's group has reported gonadotropin-dependent expression of P450 51 in rat ovaries and the production of MAS[1502].

The reaction and possible physiological significance of the system in reproduction have been reviewed recently by Rozman[1503]. Leydig cells and acrosomes of spermatids have the highest P450 51 levels, and primary mouse oocytes and granulose cells also contain P450 51. The MAS may have a role in fertilization[1503].

7. Concluding Remarks

Some information has been presented about the known P450s. Because the genome is nearly completed, no more human P450s are likely to be added unless our views of marker sequences change.

Although much of the interest in human P450 is directed toward drug metabolism, the majority of P450s appear to be involved in the metabolism of endobiotics (Table 10.2). In one sense, the fact that about 5 or 6 of the P450s are so dominant in xenobiotic metabolism (Figure 10.3) is not surprising when we recognize that only about 15 of the 57 use xenobiotics as substrates (Table 10.1) and consider the dominant levels of expression of a few P450s that have rather broad selectivity (Figure 10.4).

Aside from the current problems already mentioned, including practical issues, what are some of the future challenges and in what areas should work be done? Some of the problems are very basic, such as the goals of deriving more experimental three-dimensional structures, answering questions about catalytic mechanisms, and understanding the complexities of regulation of these genes.

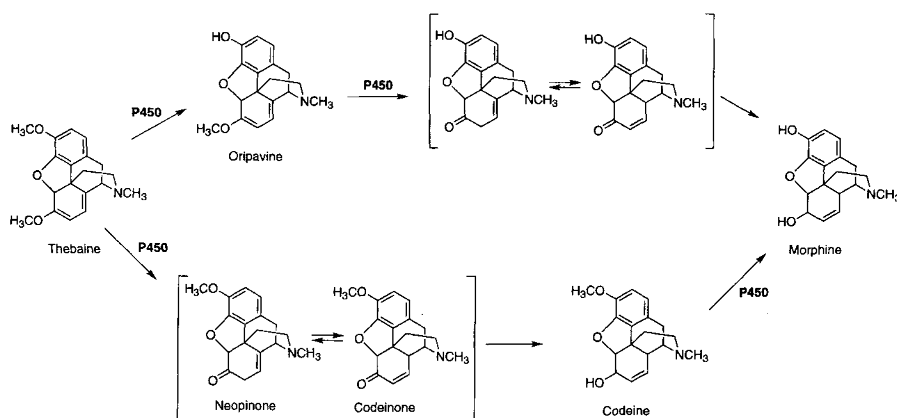

Figure 10.19. Steps in mammalian morphine synthesis[1504, 1505].

There is considerable opportunity to improve screening and predictability for use with new chemical entities in the pharmaceutical industry. Another open issue is whether large-scale SNP analysis can be practical in drug development.

Another area is understanding the relevance of variations in P450s (both SNPs and expression levels) to diseases, not only drug therapies. For instance, one can ask if some of the "minor" sterol products produced by P450 3A4 have any impact on cardiovascular disease. How important are some of the P450s that oxidize arachidonic acid to various products (in humans)? Does a P450 2D6 deficit have any relevance to long-term health?

Finally, there are two more major problems in understanding what human P450s do. One problem is the oxidation reactions, largely involving endobiotics, for which no P450s have been defined. One example is the synthesis of endogenous morphine in the body, which appears to require multiple oxidations and is subject to short-term regulation[1504, 1505] (Figure 10.19). Although P450 2D6 can oxidize codeine to morphine[1506], this enzyme does not contribute to the endogenous formation of morphine[1507]. What other oxidative reactions remain to be discovered and characterized? In the past year, the oxidative dealkylation in DNA repair was demonstrated to involve an α-ketoglutarate-dependent dioxygenase[1508, 1509]. Perhaps similar roles for P450s may be found. The other issue is identifying functions for the remaining human P450s, of which there are at least 15 (Table 10.2). Doing this on a step-by-step basis as in the past will not be very efficient, and there is an opportunity for the introduction of novel approaches.

Note added in proof. Recently Pai *et al.*[1510] have demonstrated an unusual phenomenon with the pseudogene, *CYP2D7*. A frame shifted allelic variant yields expression of an enzyme specifically in the brain, not liver, and this enzyme is more efficient than P450 2D6 in converting codeine to morphine (Figure 10.19).

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