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COMMENTARY

MULTIPLE FORMS OF CYTOCHROME P-450 AND THE IMPORTANCE OF MOLECULAR BIOLOGY AND EVOLUTION

DANIEL W. NEBERT and MASAHIKO NEGISHI

Developmental Pharmacology Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20205, U.S.A.

Throughout evolution, development of a functional protein is an unlikely event. Once a successful solution to a chemical problem (e.g. monooxygenation) is achieved, most likely the solution is repeated and modified to suit any new metabolic task. This phenomenon is now known to have occurred for numerous multigene families [1] by means of duplication of the ancestral gene and subsequent modification of extra genes for evolving functions. Gene duplication is believed to occur by gene conversion and/or unequal crossing-over [1, 2].

As the result of this recent discovery in molecular biology, there are two immediate conclusions.

(a) In a given species, there exist families of proteins with strikingly similar structure. Insulin, insulin-like growth factor, relaxin, and nerve growth factor, for example, are four insulin-related growth factors within the same multigene family whose genes have evolved from a common ancestral gene [3–5]. Albumin and α-fetoprotein evoke different antibodies yet have evolved from a common ancestral gene [6, 7]. The genes for prolactin, growth hormone and chorionic somatomammotropin probably have arisen from a single ancestral gene [8]. The genes that encode a single protein may be arranged in tandem fashion with spacer regions between each gene on the same chromosome (as in the case of globin [9, 10], interferon [11] or albumin and α-fetoprotein [12]) or on two or more chromosomes, as in the case of the immunoglobulins [13]. 'Silent' genes (or pseudogenes') for α-globin have been described on a chromosome distinct from that containing the regular α-globin multigene family [14].

(b) Among many different species, homologous enzymes and other proteins are structurally similar. A corollary to this conclusion is that the genes encoding homologous proteins will have considerable nucleotide sequence homology. By DNA hybridization techniques, therefore, the growth hormone mRNA in cow, rat and human were shown to be related [15]. The yeast cytochrome *c* gene was used for isolating the rat cytochrome *c* gene [16]. The mouse P_1-450 gene was used to isolate the rat P_1-450 gene [17].

How improbable during evolution is the search for a functional protein?

If we assume 20 amino acids can be aranged randomly in a polypeptide of 500 residues (M_r ~55,000), there are 20^{500}, or 3.3×10^{650}, different proteins possible. In higher vertebrates, there is enough DNA to encode less than 10^7 proteins [18]. Hence, the problem is not whether the potential for diversity exists, but rather how functional proteins during evolution are 'discovered.' And once a successful enzyme or other protein is found, it is far more efficient for the organism to modify than to seek an entirely new solution. Determination of the amino acid sequence for the first 20 NH_2-terminal residues and the first five COOH-terminal residues, for example, of a polypeptide of 500 amino acids, therefore, gives us no indication of how identical two forms of P-450 are for their remaining 475 residues. In fact, any report in the literature suggesting that two forms of P-450 are 'identical', based on the determined sequence of 25 residues or less, must be viewed as totally naive.

If one considers secondary and tertiary structure, proteins must be able to fold on a reasonable time scale. For a 500-residue protein, if every amino acid can assume three different rotation orientations, 3^{500}, or 3.6×10^{238}, possible states exist. Molecular rotations have rate constants of ~10^{13} sec^{-1}. If this protein were to carry out a totally random search for the correct conformation, the time required would be 3.6×10^{225} sec, or 1.14×10^{218} years! During evolution, therefore, efficient folding pathways for protein structures obviously were not discarded; it has been proposed [19] that proteins use α-helical regions and hydrophobic contacts as nucleation sites in folding. These domains appear to coincide with exons in split genes [1, 18, 19]. Virtually all proteins thus should be expected to have 'constant' and 'variable' regions.

How many of the same forms of P-450 exist in various species?

In 1964 there was biochemical evidence for at least one form of P-450 [20]. Two years later there was spectral evidence for at least two forms [21]. Evidence was recently presented [22] for eleven forms in liver microsomes from untreated rabbits. Most of these eleven forms were characterized only by electrophoretic bands and catalytic activities; NH_2-terminal and COOH-terminal sequencing, immunologic studies, and peptide mapping have not yet been done on all of the forms, nor have the authors ruled out proteolysis as a possible source of so many forms. In any case, the number of well characterized forms of P-450 has continued to increase in recent years, with more forms having been described in rabbit than in rat or mouse.

As stated above, P-450 structural genes expressing

Table 1. Comparisons of different forms of liver microsomal P-450 among four species*

Rabbit	Rat	Mouse	Fish
'Form 1' <50 kd	'P-450$_a$' 48 kd		
'LM$_2$' 'form 2' 'P-450$_1$' 49 or 50 kd	'P-450$_b$' 52 kd 'P-450$_e$' 52.5 kd	'P-450-PB' 52 kd	
'LM$_{3a}$' 'LM$_{3b}$' 'P-450$_5$'(?) 51 or 52 kd 'Form 3b' 'P-450$_5$'(?) 51 or 52 kd 'LM$_{3c}$' 'P-450$_9$'(?) 53 kd 'P-450$_{B1}$' 'P-450$_{10b}$' (?) 50 or 52 kd			
	'P-450-PCN' 51 kd 'P-446' 53.4 kd 'P-448' 55 kd	'P-450-PCN' 53 kd	
'LM$_4$' 'form 4' 'P-448$_1$' 54 kd 'Form 5' 55 kd	'P-450$_c$'(?) 56 kd	'P-448' 55 kd	'P-448'
'Form 6' 'P-448$_2$'(?) 56 kd 'LM$_6$' 'P-448$_2$'(?) 56 kd	'P-450$_c$'(?) 56 kd 'P-450$_d$' 53 kd	'P$_1$-450' 55 kd 'P$_2$-450' 55 kd	'P$_1$-450'

* The isozymes are arranged in their approximate order of increasing molecular weight. The approximate molecular weights (in kilodaltons, kd) are sometimes included, although it should be appreciated that these can vary among laboratories by several thousand daltons, depending upon experimental conditions employed. Sato's rabbit 'P-450$_2$', 'P-450$_3$', 'P-450$_4$', 'P-450$_6$', 'P-450$_7$', 'P-450$_{8a}$', 'P-450$_{8b}$', 'P-450$_{10a}$', and 'P-448$_9$' are not included in this table. Mitochondrial, bacterial, and nonvertebrate forms of P-450 are beyond the scope of this review.

constitutive or inducible monooxygenase activity should, like any other protein undergoing divergent evolution, be similar among various species. What follows is an attempt to make such a comparison amongst four species (Table 1). In spite of the viewpoint that no clear similarities have been shown among different species [23], we contend that some interesting and striking parallelisms are now evident.

Rabbit 'form 1' [24], originally named 'P-450$_a$' [25], migrates slightly faster on electrophoretic gels than 'form 2,' is a major form among nontreated rabbits, and catalyzes very well the 21-hydroxylation of progesterone [26]. Differences in progesterone 21-hydroxylase activity and form 1 P-450 were recently found among individual New Zealand White rabbits [26]; this intrastrain genetic variability illustrates the potential difficulties of using randombred or outbred laboratory animals. Any laboratory animal that is not strictly an inbred strain (i.e. brother–sister mating only for at least 20 generations) can be expected to display some degree of heterogeneity [27, 28]. Use of heterogeneous species of laboratory animals [29], as well as human clinical data (see Ref. 30 for recent review), on the other hand, can be very advantageous in demonstrating P-450 multiplicity and may be useful in uncovering new P-450 polymorphisms. We propose that the gene for rat 'P-450$_a$,' the major form seen in nontreated rats [31], corresponds to the gene for rabbit form 1; this hypothesis can be proven or disproven by recombinant DNA techniques. No equivalent form of P-450 in mouse or fish has been adequately characterized yet.

Rabbit 'LM$_2$' [32], form 2 [24], and 'P-450$_1$' [22, 33, 34] appear to be the same protein and represent the major phenobarbital-inducible form. Rat 'P-450$_b$' [31] and mouse (J. S. Felton and D. W. Nebert, unpublished data; M. A. Lang and D. W. Nerbert, manuscript in preparation) 'P-450-PB' represent the major phenobarbital-inducible form in their respective species and have estimated molecular weights also in the 50–52 kd range. Trout (and also frog and snake) exhibit no P-450 induction by phenobarbital [35]. Recent protein chemistry studies [36] showed a small difference in the typtic peptide fingerprinting of P-450$_b$ between Holtzman and Long-Evans rats. Although this interstrain genetic difference should come as no surprise, it is interesting that P-450$_b$ is immunologically identical between these two rat strains [36]. These data support the statement made earlier in this Commentary that genetic differences will be found where immunologic differences cannot. We propose that the structural gene for rabbit LM$_2$ corresponds to the structural gene for rat P-450$_b$ and mouse P-450-PB.

Multiple forms of phenobarbital-induced P-450

Rat 'P-450$_e$' can be separated from P-450$_b$ by use of a long chromatographic column [36]; no equivalent for rat P-450$_e$ has been identified in the other species. P-450$_e$, like P-450$_b$, is induced* at least 50-fold by phenobarbital [36]. P-450$_a$ can be induced several-fold by phenobarbital [31]. These data indicate the induction of at least three forms of rat liver P-450 by phenobarbital.

An antibody that is not monospecific was developed against rat liver microsomal phenobarbital-induced P-450 [37], and polysome-bound mRNA was used in preparing cloned cDNA [38] believed to represent a single gene. When the partial nucleotide sequence of two separate clones was determined, however, 14 nucleotide differences between the two clones were found [39]. These dissimilarities rep-

* The term 'fold-induction' is relative, since trace amounts of protein or mRNA from untreated animals can be variable. It is not yet known, for certain, whether any of these inducible forms of P-450 exist in the naive untreated control animal. In numerous immunologic studies, a form of P-450 immunoprecipitated in untreated animals appears to be identical to increased amounts of a form precipitated by the same antibody in inducer-treated animals, but absolute proof must await total amino acid and/or nucleotide sequencing and perhaps a better understanding of the genetic mechanisms regulating P-450 induction.

resent six amino-acid differences out of total of 491 residues estimated [39]. Both clones, when hybridized to mRNA during phenobarbital induction, exhibited maximal increases in phenobarbital-induced P-450 mRNA of no more than 3-fold [39]; however, P-450$_b$ in rat liver is induced more than 50-fold by phenobarbital [36]. Adesnik *et al.* [40] also reported cloning of a portion of the gene which encodes the major phenobarbital-induced P-450 in rat liver; they have demonstrated that phenobarbital induces 30- to 100-fold increases in the P-450 mRNA. Both of the clones in Fujii-Kuriyama's laboratory, thus, may reflect structural genes encoding either endogenous P-450 forms and/or minor phenobarbital-inducible forms* rather than the major phenobarbital-inducible (rat P-450$_b$) structural gene. This conclusion is possible in spite of the fact that the NH_2-terminal residues 7 through 19 and several COOH-terminal residues of the P-450$_b$ protein are in perfect agreement with the nucleotide sequence of one or the other clone [39]. What remains to be determined, therefore, is how closely the remaining ~475 amino acids of the rat P-450$_b$ protein correspond to the remaining ~1,425 nucleotides of these clones—if the P-450$_b$ protein can ever be totally sequenced.

Rabbit 'LM$_{3a}$' catalyzes best aniline and ethanol [42]. 'LM$_{3b}$' [43], 'form 3b' [24, 41] and 'P-450$_5$' [22] were believed to represent the same protein, because all three laboratories have found similar molecular weights (~51 kd), spectral properties, and capacities to metabolize aminopyrine well. However, the NH_2-terminal residue number 10 of rabbit LM$_{3b}$ is Thr [44] and that of 3b is Tyr in New Zealand White rabbits [41] and Leu in 3BO inbred rabbits from The Jackson Laboratory (E. F. Johnson, personal communication) 'LM$_{3c}$' [42, 44], 'P-450$_9$' [22] and 'P-450$_{B1}$' [45] had appeared to be the same; however, among the first 11 NH_2-terminal residues of LM$_{3c}$ [44] and P-450$_{B1}$ [46, 47] two residues (numbers 8 and 9) differ. P-450$_{B1}$ has a high affinity for cytochrome b_5 and reflects NADH-, as well as NADPH-, dependent *p*-nitroanisole *O*-demethylase activity [48]. No equivalents to the rabbit LM$_{3a}$, LM$_{3b}$, LM$_{3c}$ or P-450$_{B1}$ proteins have been described in other species. The induction of these proteins by phenobarbital, pregnenolone 16α-carbonitrile, 3-methylcholanthrene (MC), or isosafrole has not been reported; it is therefore suspected that these proteins exist principally for the metabolism of endogenous substrates, although ethanol induces LM$_{3a}$ [42].

Pregnenolone 16α-carbonitrile (PCN)-induced P-450

A form of rat P-450 induced by pregnenolone 16α-carbonitrile has been purified to apparent electrophoretic homogeneity and shown [49] to differ from phenobarbital- or MC-inducible forms. A major electrophoretic band of approximately 53 kd was demonstrated for mouse liver microsomes [50]. Although this mouse pregnenolone 16α-carbonitrile-induced form [50] was not further purified or characterized, we propose that the structural gene for rat 'P-450-PCN' corresponds to that for mouse P-450-PCN. No equivalent has been reported yet for other species.

Polycyclic-aromatic-induced P-450

'Rabbit LM$_4$' was originally described as a single form of β-naphthoflavone-inducible P-450 in adult rabbit [32, 51], but now appears to represent at least two major forms and one minor form with indistinguishable molecular weights [52, 53]. 'P-448$_1$' [22, 33, 34] has as the NH_2-terminal residue only alanine, which is one of the three NH_2-terminal residues detected in LM$_4$ [52].

LM$_4$ has at least a 2-nm blue spectral shift in the Soret maximum of the reduced hemoprotein·CO complex, appears to be a glycoprotein, and has its ferric iron predominantly in the high-spin state [51]. Mouse 'P-448' [54] also is inducible by β-naphthoflavone [and MC or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin] (TCDD), has at least a 2-nm blue shift in the Soret peak of the reduced hemoprotein·CO complex, is associated genetically with high-spin P-450 [55], and is apparently a glycoprotein [56]. Acetanilide 4-hydroxylase activity induced by polycyclic aromatic compounds (β-naphthoflavone, MC, TCDD) is quite closely associated with rabbit P-448$_1$ [22], mouse P-448 [54] and rat 'P-448' [57] or 'P-446' [58]. The developmental study by Guenthner and Nebert [57] included only analysis of electrophoretic bands and catalytic activities of rat liver microsomes, rather than a study of a highly purified protein. The antibody to mouse P-448, however, cross-reacts with rat P-448, blocks quite specifically rat liver acetanilide 4-hydroxylase activity induced by MC, and immunoprecipitates a 55-kd electrophoretic band [17].

Although it had been reported that MC treatment of fish produces no detectable blue shift in the Soret maximum of the reduced hemoprotein·CO complex [35, 59], other laboratories now have found a detectable blue shift if the induction time is extended to more chronic treatment with MC [60, 61]. Recently fish liver 'P-448' of high purity has been isolated and characterized [62]. We, therefore, propose that a major form of polycyclic aromatic-inducible P-450 having at least a 2-nm blue shift in the Soret peak of the reduced hemoprotein·CO complex represents a structural gene common to rabbit, rat, mouse and fish; further, the protein product of this gene is not as closely associated with induced aryl hydrocarbon hydroxylase activity as another form of polycyclic-aromatic-inducible P-450 having a higher molecular weight.

* Ozols *et al.* [41] recently reported that rabbit form 2 and form 3b have almost analogous amino acid sequences of an internal tridecapeptide fragment:
form 2: Met–Pro–Tyr–Thr–Asp–Ala–Val–*Ile*–His–Glu–Ile–Gln–Arg,
form 3b: Met–Pro–Tyr–Thr–Asp–Ala–Val–*Met*–His–Glu–Ile–Gln–Arg.
Of interest, the sequence of 39 nucleotides in one of the clones from Fujii-Kuriyama's laboratory [39] corresponds exactly with this form 2 tridecapeptide fragment (at residues 346–358). This internal fragment therefore appears to be a relatively constant region, since it is so similar in an endogenous and a phenobarbital-inducible form of rabbit P-450 and identical in phenobarbital-inducible forms of rabbit and rat P-450. These data are therefore consistent with the argument about evolution presented earlier in this Commentary.

Rabbit lung P-450$_{II}$ [63, 64] migrates between form 4 and form 6, and immunologic evidence and NH_2-terminal sequence data (E. F. Johnson, personal communication) indicate its presence in rabbit liver. This protein thus might be designated as rabbit 'form 5.' No equivalent proteins have been described for other species.

Rabbit 'form 6' [24] is highly induced by TCDD in neonates but poorly induced in adults; 'P-448$_2$' [22] might be the same as form 6. Polycyclic-aromatic-inducible aryl hydrocarbon hydroxylase activity is more closely associated with form 6 than with form 4 of LM_4 in the adult rabbit, because this inducible activity with intact microsomes is very high in the neonate but is not detectable after 2 weeks of postpartum age in the rabbit [65]. The blue spectral shift in the Soret maximum of the reduced hemoprotein·CO complex is at least 1 nm less in 'form 6' than in 'form 4' [24, 25]; these data are the same for mouse MC-induced 'P_1-450' and 'P-448,' respectively [54]. Aryl hydrocarbon hydroxylase activity is markedly induced by polycyclic aromatic compounds in fish [59–61] and, therefore, a similar 'P_1-450' protein presumably exists.

Rat 'P-450$_c$' [31] is induced by MC, is closely associated with MC-induced aryl hydrocarbon hydroxylase activity, but P-450$_c$ has at least a 2-nm blue shift in the Soret peak of the reduced hemoprotein·CO complex and has predominantly low-spin ferric iron. P-450$_c$ was compared with rabbit LM_4 [66]; these two proteins differed in estimated molecular weights, antigenic determinants, paramagnetic properties, and the number of sulfhydryl groups.* Most likely rat P-450$_c$ corresponds to rabbit 'form 6' and mouse P_1-450. The antibody to mouse P_1-450 cross-reacts with a rat form (P-450$_c$?), blocks quite specifically rat liver aryl hydrocarbon hydroxylase activity induced by MC, and immunoprecipitates a 55-kd electrophoretic band [17]. MC-induced rat P-446 [58] and rat P-450$_c$ [31] are believed to be the same, since both forms are claimed to be most specific for MC-induced aryl hydrocarbon hydroxylase activity. There is a large discrepancy in molecular weights (M_r of P-446 ~53,400; M_r of P-450$_c$ ~56,000), however, and a slight discrepancy in the Soret maximum of the absolute CO-binding spectrum. Moreover, β-naphthoflavone-induced P-446 has a higher turnover number for aryl hydrocarbon hydroxylase activity [58] than MC-induced P-450$_c$ [31].

We propose that the rabbit 'form 6' structural gene most likely corresponds to the P_1-450 structural gene in both mouse and fish. Whether these genes are related to the rat P-450$_c$ structural gene or the rat P-446 structural gene will require further study.

Multiple forms of polycyclic aromatic-inducible P-450

β-Naphthoflavone or TCDD induces in the rabbit at least two P-450s, 'form 4' and 'form 6' [24]. MC induces in the mouse at least two forms, P-448 and P_1-450 [54]. MC in the rat appears to induce at least three forms: P-450$_c$ [31] is the major form, inducible more than 50-fold; 'P-448' is more closely correlated to induced acetanilide 4-hydroxylase activity and mouse P-448 than to induced aryl hydrocarbon hydroxylase activity [17, 57]; and P-450$_a$ is induced several-fold [36]. The recent purification of five forms of P-450 from β-naphthoflavone-treated rats [74] showed two β-naphthoflavone-induced P-450s, with only one having highly induced aryl hydrocarbon hydroxylase activity.

The mouse P_1-450 mRNA was estimated [75, 76] to be 3500 bp long; this mRNA was reverse-transcribed to cDNA, from which clone 46, an 1100-bp piece of double-stranded cDNA, was isolated and characterized [75, 77]. Clone 46 was shown genetically and immunologically to be associated with the mouse P_1-450 structural gene [77, 78]. Clone 46 has internal *PstI* and *XbaI* sites and no *AvaI, BamHI* or *SalI* sites [77]. Clone pEB339 recently was demonstrated immunologically to be correlated to the rat 'P-450$_c$' gene and is about 1000 bp long; clone pEB339 has *PstI* and *AvaI* internal sites (among others) and no *XabI, BamHI or SalI* sites [79]. The mouse and rat clones, thus, exhibit very little similarity. The mouse clone 46 has been used to characterize the rat P_1-450 structural gene and P_1-450 mRNA: the *EcoRI*- and *BamHI*-digested DNA fragments and the size of mRNA [17] are strikingly similar to those in mouse. It therefore appears quite likely that clone pEB339 does not represent rat P_1-450; alternatively, the 1100 bp of the rat P_1-450 structural gene portion, of which mouse clone 46 has a large degree of homology, might have very little or no overlap with the 1000 bp of rat pE339.

Mouse P_1-450 induction by MC or TCDD [78] and more recently rat P-450$_b$ induction by phenobarbital [40] and rat P_1-450 induction by MC [17] have been shown to be under transcriptional control. A large precursor mRNA in the nucleus was demonstrated to appear during the P_1-450 induction process [78]. Putative pre-mRNA processing intermediates containing linked intron and exon transcripts so far have been identified for the genes of chicken ovalbumin, ovomucoid, conalbumin, various globins, *Xenopus* vitellogenin, chicken α-2 collagen, heavy- and light-chain immunoglobulins, many adeno-

* Still, these protein data [66] cannot prove that the rabbit LM_4 and rat P-450$_c$ structural genes are not associated in an evolutionary sense. The argument is the same as that described earlier for albumin and α-fetoprotein. Insertion of one of the termination codons (UAA, UAG, or UGA) in the rabbit LM_4 structural gene about 54 nucleotides further upstream (5'-ward) than in the rat P-450$_c$ structural gene, for example, would lead to a 54-kd protein for rabbit LM_4 and a 56-kd protein for rat P-450$_c$. Replacement of as little as a single nucleotide could lead to a different amino acid and therefore a different protein conformation; this nucleotide change could cause a species difference in electrophoretic mobility, catalytic activity, paramagnetic properties of the ferric iron, spectral maximum of the reduced hemoprotein·CO complex, and/or antigenic specificity. In fact, there are numerous examples of such single nucleotide changes among the many hemoglobinopathies [67, 68]. Hemoglobin Constant Spring, for example, has a late termination codon, resulting in 31 extra residues and an affinity for oxygen different from that for hemoglobin A [68]. Hemoglobin Tak [70] and Hemoglobin Icaria [71] are other examples of termination mutants [68, 72, 73]. This same sort of thinking must be considered for any minor differences in molecular weight, catalytic activity, etc. for all the P-450 forms among various species listed in Table 1.

viruses and yeast mitochondria [1]. The P-450 multigene family therefore can be placed in this same category of genes.

From clone 46, a portion of the mouse genomic structural gene recently has been located from a mouse library [80, 81]. From extensions of such studies with the chromosomal P-450 genes, a far better understanding of the absolute number of genes, the evolution of these genes, and the mechanism of P-450 induction will soon be accomplished.

Sizing liver mRNA from MC-treated rats with a relatively nonspecific antibody resulted in isolation of three control and three MC-inducible forms of P-450 mRNA [82]. In all molecular weight studies to date with rabbit, rat, mouse and fish P-450s induced by polycyclic aromatic compounds, it is of interest that the highest molecular weight form in each species is associated most closely with the polycyclic aromatic-induced aryl hydrocarbon hydroxylase activity—the activity believed [83] to be most important in the 'initiation' step of polycyclic hydrocarbon carcinogenesis. Cloning of genes encoding MC-inducible forms of lower molecular weight P-450 [84] thus should aid in characterizing structural genes other than that encoding polycyclic-aromatic-induced aryl hydrocarbon hydroxylase activity.

Isosafrole-induced P-450

Isosafrole induces 'P-450$_d$' in the rat [85, 86], 'LM$_6$' in the rabbit [42] and 'P$_2$-450' in the mouse [87]. Rat P-450$_d$ is distinctly different from P-450$_c$ [85, 86]. Mouse 'P$_2$-450' is also distinctly different from P$_1$-450, when we compare antibodies, mRNA, or DNA (M. Negishi, R. H. Tukey, T. Ohyama and D. W. Nebert (unpublished data). Mouse 'P$_2$-450' is not associated with polycyclic-aromatic-induced aryl hydrocarbon hydroxylase activity [87]. Rabbit P-448$_2$ [22] might be the same as LM$_6$. Rabbit LM$_6$ is believed [42] to be the same as form 6. Indeed, the two proteins appear identical by means of sodium dodecylsulfate-polyacrylamide gel electrophoresis, one-dimensional peptide mapping following limited proteolytic digestion, antigenic specificity, spectral properties and aryl hydrocarbon hydroxylase activity that is sensitive to α-naphthoflavone inhibition *in vitro* (E. F. Johnson, D. R. Koop and M. J. Coon, personal communication). LM$_6$ has the same rate of aryl hydrocarbon hydroxylase activity as rabbit form 6 (E. Eisenstadt, D. Hamilton, D. R. Koop and M. J. Coon, manuscript in preparation). Yet, by all the evolutionary and molecular genetic arguments presented earlier in this Commentary, we contend that rabbit LM$_6$ and 'form 6' must correspond to two distinctly different structural genes, just as they do in the rat and mouse.

How many possible forms of P-450 might exist?

To understand the multiplicity of P-450s and the mechanism of P-450 induction by literally hundreds of foreign chemicals [88] is among the most exciting problems today in pharmacology and molecular genetics research. At the present time, only six or eight forms of P-450 have been adequately isolated and characterized biochemically to the extent that these forms are proven to be distinctly different, beyond any shadow of a doubt [23]; this work has been carried out predominantly with rabbit and rat liver. At the other extreme is the provocative hypothesis [89] that any individual mammal has the genetic capacity to produce hundreds or thousands of unique P-450 proteins. Having the capacity to respond to various P-450 inducers does not mean that the P-450 proteins are present at all times; the same argument can be stated for the immunoglobulins.

It now has become obvious that recombinant DNA and ancillary technologies will provide us with the best answers as to the absolute number of forms of P-450. This is not to say that protein chemistry, studies of catalytic activities, the development of reasonably specific antibodies*, and the sequencing of NH_2-terminal, COOH-terminal, and internal fragments of P-450 peptides are no longer important. Quite to the contrary, the protein data base has been (and will continue to be) absolutely essential in attempting to correlate specific P-450 genes with their corresponding final protein products.

To date there has been a great deal of reluctance among many laboratories in agreeing to any standardized nomenclature of the multiple forms of P-450. Any further revisions in P-450 nomenclature probably should await the outcome of recombinant DNA studies, during which time the tandem arrangement of the various P-450 structural genes on one or more chromosomes will be determined.

Speculation

How large might the P-450 multigene family be? (a) Some believe [23, 42] that the ultimate maximal number will be only between ten and perhaps twenty-five; this quantity would make the P-450 gene family similar in size to the globin [9, 10] or interferon [11] multigene families. (b) Others believe [89] that hundreds or thousands of P-450 structural gene products may be involved. This size would be similar to the *H-2* system. Little is known about the mechanism of gene regulation and expression for the histocompatibility multigene family [90–93], but at least 3500 tissue-specific membrane-bound antigens have been described. (c) The mechanism of P-450 multiplicity may involve DNA intragenic rearrangements, combined with RNA processing, similar to those seen for the immunoglobulin system in dealing with approximately one million antigens [13].

This speculative comparison of different forms of P-450 across four species (Table 1) is intended as a useful guide, not a final answer. Studies of partially purified liver P-450 from control and drug-treated pigs [94] could have caused us to include the pig as a fifth species in Table 1. Perhaps guinea pig, ham-

* With regard to P-450 cloning studies, we see no special advantage in the expensive and tedious task of developing monospecific antibodies. One might isolate a single monospecific antibody, for example, that recognizes an antigenic determinant site common to numerous forms of P-450, and the conclusion would be that these multiple forms represent a single form. Conversely, two or more monospecific antibodies may 'see' different antigenic determinant sites on a single P-450 protein, and the conclusion would be that this single purified protein represents two or more forms of P-450.

ster, *Drosophila* and yeast should be included eventually. This Commentary is designed to stimulate new ideas, to encourage new avenues of approach. We believe that there are advantages to speculation and to postulating theories which then can be corroborated or rejected by further biochemical and genetic experiments. Presently there are many more questions than answers, but the molecular biology and evolution of P-450 multiplicity have emerged as one of the most interesting biomedical research fields today.

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Note added in proof—Since this Commentary was submitted, rabbit liver P-450 form 1 has been further characterized [95], and the primary nucleotide structure of two phenobarbital-inducible rat P-450 cDNA clones have been almost totally sequenced [96]. The mouse P_1-450 chromosomal gene spans about 5 kbp and contains at least five exons and four intervening sequences [97]. A cDNA clone (1710 bp in length) representing a portion of the mouse P_2-450 gene has been isolated and characterized by both immunologic and genetic criteria [98].