Ligand Interactions with Cytochrome P-450. I. Binding of Primary Amines*

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ABSTRACT: Primary aliphatic amines bind to oxidized P-450 of rabbit liver microsomes to produce two superimposed difference spectra: (a) λ_{max} 427 mμ, λ_{min} 392 mμ; (b) λ_{max} 432 mμ, λ_{min} 410 mμ. Binding to the separate forms of P-450 has provided an easy method to quantitatively estimate the relative amounts of the different forms of P-450. A simple equation relating absorbancy and relative amounts of types *a* and *b* is given.

Using this method we have shown that pretreatment of rabbits with methylcholanthrene increases the specific content of type *a* P-450 in liver microsomes, while pretreatment with phenobarbital yields increases in both forms of P-450, although favoring type *b*. The binding energy for *n*-alkylamines with P-450 is related linearly with alkyl chain length, providing evidence for a large hydrophobic cavity in the heme region of P-450 which must be sufficient to accommodate large, hydrophobic substrates. Furthermore, type *b* P-450 showed two distinct binding constants for alkylamines with an approximately equal contribution to the total binding. The binding of cyanide ions, 1-butanol, and phenobarbital to P-450 has also been investigated, together with the effect of amines upon this binding. Accordingly, we have been able to show selectivity by these different ligands for the two different forms of P-450.

Cytochrome P-450 is the terminal oxidase for many mixed-function oxidations associated with the endoplasmic reticular membranes of liver and other tissues (Mason *et al.*, 1965). P-450 has been characterized as a hemoprotein (Sato *et al.*, 1965). In liver microsomes, P-450-dependent processes account for the metabolism of many drugs, carcinogenic hydrocarbons, and steroids. Oxygen is consumed along with either NADPH or NADH to hydroxylate or demethylate the various substrates (Klingenberg, 1958; Garfinkel, 1958; Estabrook *et al.*, 1963). The only striking similarity between many of these substrates is that each molecule contains a relatively hydrophobic region.

Investigations of binding of certain ligands to P-450 have been reported. For example, changes in both electron paramagnetic resonance and light absorption spectra of P-450 are produced by the addition of substrates of P-450-dependent oxidations (Table I). The binding constants of aniline and hexobarbital determined spectrally were found to be similar to Michaelis constants for hydroxylation of these substrates by liver microsomes (Schenkman *et al.*, 1967). Thus, a relationship between ligand binding and hydroxylation is suggested.

In Table I, ligands have been divided into two classes, as suggested by others, according to the spectral changes produced in P-450. We have studied the binding sites on P-450 corresponding to these spectral changes by variations of the ligand structure and hydrophobicity. In addition, ligands producing one type of spectral change were added to P-450-containing preparations in the presence of a second ligand to determine the relationship between their respective sites.

Essentially all of the previous investigations of ligand binding to P-450 have been carried out with microsomal suspensions by measurements only of difference spectra. A preparation of a submicrosomal particle, suitable for measurements of absolute spectra of P-450, has recently been obtained by treatment of rabbit liver microsomes with the nonionic detergent Lubrol-WX (Miyake *et al.*, 1968). In this preparation, cytochrome b_5 is undetectable and P-420 is limited to les than 5% of the hemochromagens. In this paper we have compared ligand binding with P-450 in microsomes by difference spectral studies, and both difference and absolute spectra of the P-450 in the submicrosomal particle were obtained afters Lubrol treatment.

Pretreatment of animals with phenobarbital or methylcholanthrene (MC)[1] (Ernster and Orrenius, 1965) increases the specific activities of many liver microsomal enzymes. The effect of these pretreatments differs both with respect to enzyme distribution of P-450 between rough and smooth microsomes (Fouts, 1968) and in the properties of P-450. For example, MC induces changes relative to either phenobarbital treatment or untreated animals in the light absorption of the reduced P-450–CO complex (Alvares *et al.*, 1967), and in hydroxylation of testosterone at carbon atoms 6, 7, and 17 (Conney *et al.*, 1968). These observations may be explained, in part, if microsomal P-450 exists in two forms and if the proportion of the two forms may be altered by pretreatment. The effect of pretreatment on the binding of different ligands was explored for ligand selectivity for each form of P-450.

Experimental Procedures

Methods and Materials. Male rabbits, weighing approxi-

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[1] MC = methylcholanthrene.

TABLE I: Ligand Binding to Liver Microsomes.

Type	Ligand	Reference	Difference Spectrum (mμ) (500–350)	Electron Paramagnetic Resonance Shifts	Reference
I	Hexobarbital	*e*	λ_{min} 420	g = 2.41 (2.42)[f]	*a*
	Phenobarbital	*e*	λ_{max} 390		
	Aminopyrine	*e*			
	Phenacetin	*b*			
	Testosterone	*c*			
II	Aniline	*b*	λ_{max} 430		
	Pyridine	*b*	λ_{min} 390	g = 2.45 (2.42)	*a*
	Ethylisocyanide	*b*			
	Alcohols	*b*	λ_{max} 420		
			λ_{min} 390		
	CN^-			g = 1.83 (1.91)	*d*
				g = 2.30 (2.25)	
				g = 2.52 (2.41)	

[a] Cammer *et al.* (1966). [b] Imai and Sato (1966). [c] Estabrook *et al.* (1966). [d] Ichikawa *et al.* (1967). [e] Remmer *et al.* (1966) [f] Values in parentheses refer to uncomplexed P-450.

mately 2 kg and of the New Zealand strain, were used for all experiments. The rabbits were fed Purina rabbit chow (Medicated). For phenobarbital treatments, rabbits were injected with a dose of 80 mg/kg three times in 5 days. For MC treatment, a single dose of 3-methylcholanthrene (20 mg/kg) in corn oil was injected intraperitoneally 4 days before sacrifice.

The preparation of liver microsomes has been described in detail (Mason *et al.*, 1965). The preparation of the submicrosomal particle was carried out exactly as described by Miyake *et al.* (1968). The resulting preparation contains Lubrol. Because P-450 decomposes rapidly when microsomes are suspended in solutions containing high concentrations of salt (Imai and Sato, 1967a), the earlier procedure of removal of Lubrol from the protein by salt fractionation was abandoned (Miyake *et al.*, 1968). Submicrosomal particles dissolved in glycerol–phosphate buffer (1:1, pH 7.5, containing 10 mg of protein/ml) were chilled to −20° and centrifuged. The dense floating layer of Lubrol was discarded. The solution containing the P-450 was warmed slowly to room temperature. The solution was concentrated approximately fivefold by ultrafiltration (Diaflo, Amicon Corp., Boston, Mass.). The remaining detergent was removed by chromatography on Sephadex LH-20 as described by Gaylor and Delwiche (1969). Glycerol–phosphate buffer eluted the protein; most of the Lubrol was retained on the column. Based on recovery of [^{3}H]Lubrol (Gaylor and Delwiche, 1969), the concentration of Lubrol in the collected protein was less than 0.04 mg of Lubrol/mg of protein. Thus, the Lubrol content of the preparation was reduced to less than 0.5% of the original.

All spectra were measured in 1-cm cells at room temperature in a Cary recording spectrophotometer equipped with a high-sensitivity slide-wire. The concentrations of cytochromes b_5 and P-450 were determined by the difference spectral techniques described by Omura and Sato (1964) and Hildebrandt *et al.* (1968). Sodium dithionite was used as the reducing agent.

Difference spectra were obtained by addition of aqueous glycerol solutions of the ligand to P-450-containing preparations, also in aqueous glycerol. A microliter syringe was used to inject the ligand solution into the sample cuvet while an equal volume of solvent was added to the reference cuvet. The pH of amine solutions was adjusted to neutrality. Protein was determined by the method of Lowry *et al.* (1951).

Lubrol-WX was the generous gift of Imperial Chemical Industries, Providence, R. I.

Results

Oxidized P-450 interacted with many nitrogen bases to produce spectral changes grossly characteristic of type II binding (Table I and Figure 1), although only a few of the bases are substrates of P-450-dependent processes. Furthermore, the interaction of primary aliphatic alkylamines (*e.g.*, *n*-octylamine) with P-450 varied, according to the pretreatment of the rabbits, in a way which clearly suggested that these difference spectra were comprised of two component spectra (Figure 1). Thus, for simplicity of reporting, we have further classified the type II spectra as: type *a*, having λ_{max} 427 mμ and λ_{min} 392 mμ; and type *b*, having λ_{max} 432 mμ and λ_{min} 410 mμ.

Binding of Amines to P-450 in Submicrosomal Particles. Difference spectra observed by addition of *n*-octylamine to submicrosomal particles obtained from phenobarbital, MC, or control-treated rabbits are represented in Figure 1. The shape of the difference spectrum changed only slightly as the concentration of *n*-octylamine was increased, and the large differences between the three types of preparation were retained. Type *a* dominated in the MC-induced preparation, type *b* dominated in the phenobarbital-induced preparation, and the control was obviously intermediate between the induced preparations.

Apparently, type *a* and type *b* amine difference spectra corresponded directly to the formation of amine complexes with distinct forms of P-450, whose proportions depended upon the pretreatment. Accordingly, the relative contributions of types *a* and *b* were measured. The contribution of the type *a*

TABLE II: Binding of Primary Amines to Oxidized P-450.[a]

Amine	Phenobarbital Prepn (−410 mμ) (mM)		MC Prepn (−392 mμ) (mM)	
	K_1	K_2	K_1	K_2
n-Propyl-	17–19			
n-Butyl-	4	20	1.3	11
n-Pentyl-	1.8	5		
n-Hexyl-	0.45	2		
Cyclohexyl-	90			
n-Heptyl-	0.13	0.6		
n-Octyl-	0.015	0.2	0.012	0.24
	0.11[b]	1.0[b]		
n-Decyl-	0.010	0.07		
Dodecyl-	0.013	0.06		
Benzyl-	1.2			
Cysteamine	1.0			
t-Butyl-	No spectrum			
t-Pentyl-	No spectrum			
Octadecyl-	No spectrum			

[a] Submicrosomal particles; pH 7.4, 0.1 M phosphate buffer, 50% glycerol. [b] pH 6.5.

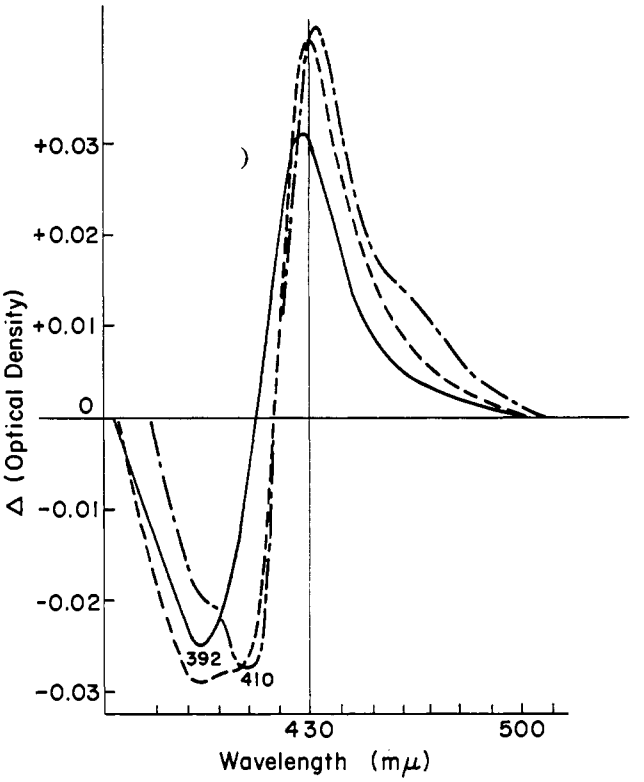

FIGURE 1: Effect of pretreatment on the difference spectra of *n*-octylamine with the P-450 submicrosomal particle from rabbit liver. (———) Methylcholanthrene preparation, CO difference spectrum at 448 mμ = 0.35, protein = 2.0 mg/ml, [*n*-octylamine] = 0.06 mM. (- - - -) Control preparation, CO difference spectrum at 450 mμ = 0.40, protein = 3.0 mg/ml, and [*n*-octylamine] = 0.18 mM. (— — — —) Phenobarbital preparation, CO difference spectrum at 450 mμ = 0.19, protein = 0.7 mg/ml, and [*n*-octylamine] = 0.32 mM. Spectra were taken in 0.1 M potassium phosphate buffer (pH 7.4) containing 50% glycerol.

spectrum at 410 mμ was small, thus absorbancy change at this wavelength measured essentially type *b* binding. Type *b* binding contributed two-fifths as much absorbancy change at 392 mμ as at 410 mμ, thus type *a* binding was measured by $[\Delta OD_{392} - {}^2/_5\Delta OD_{410}]$.[2]

All aliphatic alkylamines, which caused difference spectra, required two binding constants (K_1 and K_2) for *both* type *a* binding on the MC-induced preparation and type *b* binding on phenobarbital-induced preparation (Figure 2a,b). In each case, the plots showed two distinct slopes ($-1/K$). Extrapolation of each line to the *x* axis indicated the change of absorbancy at saturation of the corresponding binding site with amine. The change of absorbancy after saturation of the second (weak) binding site was consistently double the change produced by saturation of the first (strong) binding site, indicating equal contributions to the *total* spectral change from each site. Submicrosomal preparations of P-450, whatever the pretreatment, scarcely showed any change in the ratio of absorbancy changes at 392 and 410 mμ (*i.e.*, type *a*/type *b* binding) when the concentration was varied. Thus, a change from type *a* to type *b* binding, or *vice versa*, cannot be the source of the biphasic binding phenomena.

Values of K_1 and K_2 for different amines which were evaluated for both type *a* and type *b* binding are reported in Table II. The values of K_1 and K_2 obtained from type *a* binding were similar to the values obtained from type *b* binding, and also suggested that binding to both forms of P-450 was primarily determined by the hydrophobicity of the amine.

For hydrophobic binding without steric restriction, a linear increase in binding energy with chain length has been predicted by theory (Nemethy and Scheraga, 1963a,b) and found in practice for inhibitors of alcohol dehydrogenase (Anderson *et al.*, 1965). Accordingly, the effect of variation of alkyl chain length upon the type *b* binding energies (proportional to log K) is shown in Figure 3. Excellent, linear correlations were obtained when both log K_1 and log K_2 were plotted against the number of alkyl carbons in the amine (straight chain) through C_8. Thus, from C_3 to C_8, addition of each methylene group to the alkyl chain of the amine increased the binding energies by 0.75 (K_1) and 0.65 (K_2) kcal, respectively. These magnitudes are typical for hydrophobic binding of an alkyl side chain in a nonpolar cavity (Nemethy, 1967) which could either be provided by the P-450 protein or the microsomal membrane.

The binding of *n*-octylamine was weakened by a factor of 7 (type *b*, K_1; Table II) when the pH of the medium was lowered from 7.4 to 6.5; aniline binding remains constant throughout this pH range (Imai and Sato, 1966). Thus, a proton must be released in binding *n*-octylamine but not aniline. Since *n*-octylamine was protonated at pH 7.4, two explanations of this effect can be considered: (1) P-450 equilibrated only with *neutral* amine bound within the membrane; and (2) P-450 equilibrated with protonated amine in the aqueous phase, but the displaced protein ligand did not take up a

[2] In microsomes, *n*-octylamine binds sequentially to form type *b* P-450 then type *a* P-450 while the isobestic point changes from 419 to 408 mμ. Since type *a* binding shows an isobestic point at 408 mμ, the contribution at 410 mμ is small (Figure 7, factor A).

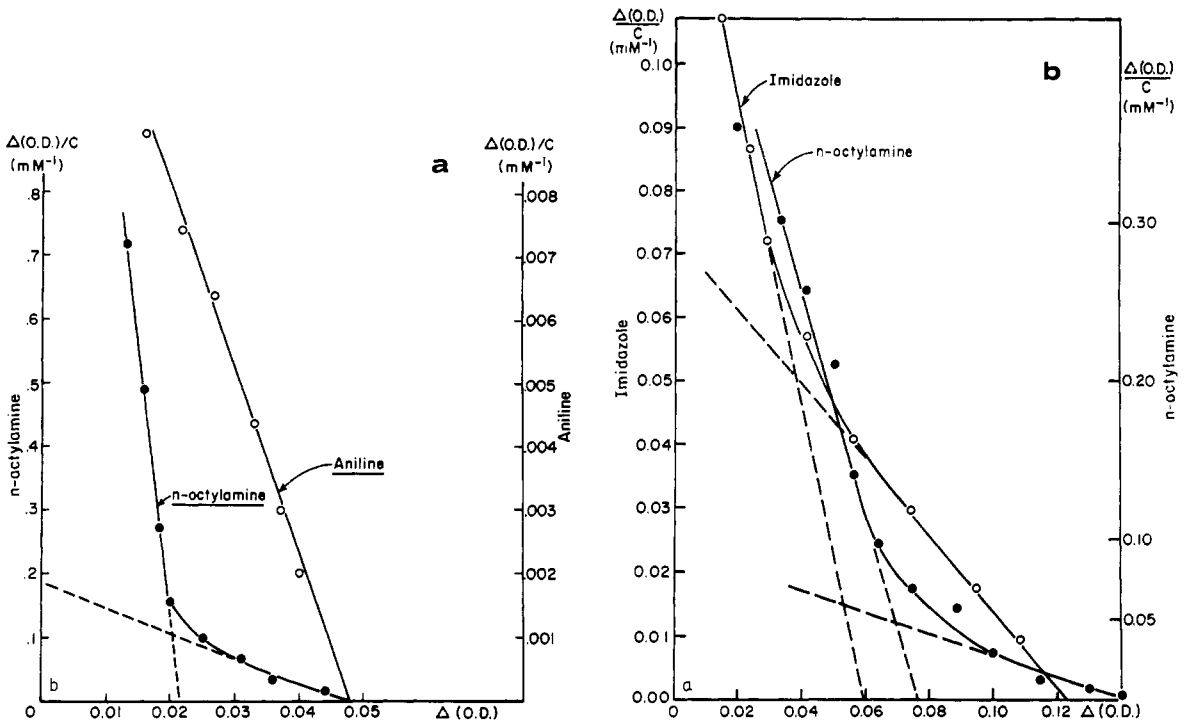

FIGURE 2: Binding of *n*-octylamine. (a) With aniline to P-450 submicrosomal particles from rabbit livers, after pretreatment with methylcholanthrene. CO difference spectrum at 448 mμ, 0.14; protein, 1.2 mg/ml. c = [ligand]; slope = $-1/K$; x-axis intercept = $\Delta(OD)_{sat.}$. In both cases, ΔOD values refer to difference maxima at 428 mμ for complete binding and an isobestic point at 500 mμ. (b) With imidazole to P-450 subparticles from rabbit livers after pretreatment with phenobarbital. CO difference spectra at 450 mμ = 0.42 (*n*-octylamine) and 0.35 (imidazole). Protein = 1.45 mg/ml (*n*-octylamine) and 1.3 mg/ml (imidazole). In both cases ΔOD values refer to difference maxima at 432 mμ and an isobestic point at 500 mμ. Conditions were as described in Figure 1.

proton at pH 7.4, *i.e.*, pK (protein ligand) = <7.4 (George *et al.*, 1967).

$$\begin{array}{c} | \\ \text{YFeX} \\ | \quad | \end{array} + \overset{+}{\text{R}}\text{NH}_3 \underset{}{\overset{\text{pH 7.4}}{\rightleftharpoons}} \begin{array}{c} | \\ \text{YFeNH}_2\text{R} \\ | \quad | \\ \quad \text{X} \end{array} + \text{H}^+$$

Since the values of pK_b for aliphatic alkylamines are approximately 10.4, binding of neutral amine according to either mechanism requires a further reduction in binding constants by a factor of 10^3, in comparison with aniline, to allow for protonation of the amine at pH 7.4. Accordingly, a second "corrected" scale has been included in Figure 3 (right-hand legend) allowing for this factor.

An estimate of the interaction energy of the amine nitrogen in the binding site was made by extrapolation of the lines in Figure 3 to zero chain length. Allowing for the effect of protonation, K_1 and K_2 binding sites on the type *b* P-450 showed nitrogen interactions of 4.3 and 3.6 kcal, respectively. Thus, the major energetic difference between K_1 and K_2 is derived from the nitrogen interaction.

For chain lengths greater than eight carbon atoms, a limiting value to binding appears to be reached. We failed to observe a difference spectrum using *n*-octadecylamine at the limit of solubility ($\sim 10^{-4}$ M), but there remains uncertainty of the concentration retained in solution. Branching of the alkyl chain severely weakened binding as was shown by cyclohexylamine, *t*-butylamine, and *t*-pentylamine (Table II).

In liver microsomes, cysteamine exerts a specific effect on cholesterol 7α-hydroxylase which is considered to be a P-450-dependent process (Scholan and Boyd, 1968, and unpublished results). We observed that cysteamine added to the Lubrol preparation of P-450 produced typical amine difference spectra indicating interaction with nitrogen rather than sulfur (*cf.* metmyoglobin). The binding was low (1.5 mM) compared with the corresponding *n*-propylamine (17–19 mM), but this was probably due to the considerable hydrophobicity of the sulfhydryl group.

Aniline binding to P-450 contrasted with amine binding in showing only a single binding constant for either phenobarbital- or MC-induced preparations of P-450 (Figure 2), in agreement with reported experiments on liver microsomes (Imai and Sato, 1966). The change produced by pyridine resembled that observed for aniline, since only a single binding constant with P-450 preparations was found. On the other hand, binding constants and difference spectra for several aromatic nitrogen bases (Table III) showed considerable variation with structure (compare aniline and *N*-methylaniline, pyridine, and nicotinamide).

Imidazole (Imai and Sato, 1967b) was the only nitrogen-containing ligand, apart from alkylamines, to show biphasic binding (Figure 2). However, the two binding constants (Table III) were closer than generally found with alkylamines, thus representing an intermediate case between alkylamines and weak aromatic bases.

A size or steric factor was also found to be important in the binding of aromatic bases to P-450. 1-Naphthylamine, whose

TABLE III: Binding of Aromatic Bases to P-450 Phenobarbital Preparation.[a]

Base	Spectrum (mμ) λ_{max}	Spectrum (mμ) λ_{min}	K_s (mM)
Aniline	430	400	2.1
	429[b]	395[b]	3.1[b]
N-Methylaniline	425	395	3.2
N,N'-Dimethylaniline	Very small spectral change		
1-Naphthylamine	No spectrum to 1 mM		
Pyridine	428	395	0.5
Nicotinamide	425	~390	90
Phenylhydrazine	428	400	3.5
Imidazole	432	410	0.44, 1.7

[a] pH 7.4, 0.1 M phosphate buffer, 50% glycerol. [b] Methylcholanthrene preparation.

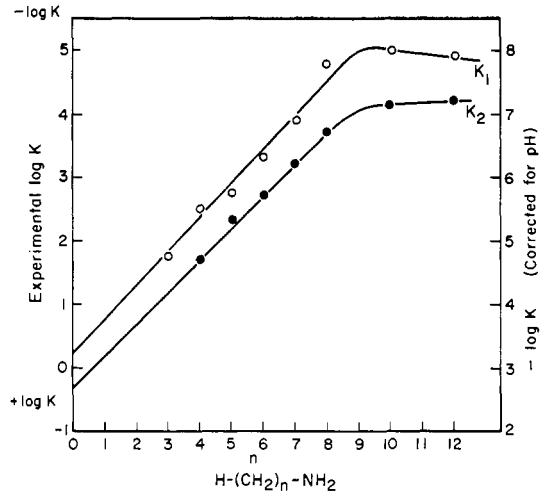

FIGURE 3: Type *b* binding constants of *n*-alkylamines with P-450, as a function of the chain length of the amine. Determinations were carried out on P-450 submicrosomal particles from rabbit livers after pretreatment with phenobarbital.

potential for hydrophobic interaction exceeds that of aniline, showed no difference spectrum. *N-N'*-Dimethylaniline also showed scarcely any difference spectrum to the limit of its solubility (10 mM).

Binding of Amines to P-450 in Microsomes. Phenobarbital-induced microsomes, either suspended directly in a solution of 50% aqueous glycerol or clarified in the same medium by the addition of 1% of solid sodium deoxycholate, gave very similar titrations with *n*-octylamine. On the other hand, difference spectra of the microsomal preparations differed somewhat from the corresponding submicrosomal preparation of P-450 reported above. The major distinction from the submicrosomal particles was that, in microsomes, binding of *n*-octylamine to type *a* P-450 was much weaker than to type *b* P-450, resulting in a shift of the difference minimum from 410 to 392 mμ at high concentrations of *n*-octylamine (Figure 4). However, type *b* binding of *n*-octylamine to microsomes, calculated from the spectral change at 410 mμ, indicated two binding constants which were similar to those obtained from the submicrosomal preparation (*cf.* Tables II and IV). Seemingly, the type *b* P-450 or the immediate membrane environment has been specifically affected during the Lubrol preparation. This effect was less pronounced with *n*-butylamine. A lower limit to the type *a* binding constant for *n*-octylamine in phenobarbital-induced microsomes was calculated from the spectral changes at 392 mμ. This was consistent with the type *a* binding constant evaluated for MC-induced microsomes. This encouraged the view that we were observing binding to the same form of P-450 in each case. Type *a* binding constants were also determined on MC-induced microsomes for *n*-butylamine, *n*-hexylamine, and *n*-decylamine (Table IV). The type *a* binding constant of *n*-butylamine (17 mM) was only 50 times greater than that of *n*-octylamine (0.3 mM), compared with a factor of 400 for the analogous type *b* binding constants on microsomes. This effect apparently results from low selectivity by *n*-butylamine between P-450's of types *a* and *b* compared to high selectivity by *n*-octylamine.

Effect of Other Ligands on Amine Binding. CYANIDE. Cyanide ions produced a difference spectrum with phenobarbital-induced P-450 which is shown in Figure 5 (λ_{max} 445, λ_{min}

TABLE IV: Amine Binding to Rabbit Liver Microsomes.[a]

Amine	Phenobarbital-Induced Microsomes: Type *b* (−410 mμ) K_1 (mM)	Phenobarbital-Induced Microsomes: Type *b* (−410 mμ) K_2 (mM)	Phenobarbital-Induced Microsomes: Type *a* (−392 mμ) K_a (mM)	MC-Induced Microsomes: Type *a* (−392 mμ) K_a (mM)
n-Butyl-	1.5	7		17
n-Hexyl-				5.3
n-Octyl-	0.005	0.034	>0.075	0.3
n-Decyl-				0.3

[a] pH 7.4, 0.1 M potassium phosphate buffer, 50% glycerol.

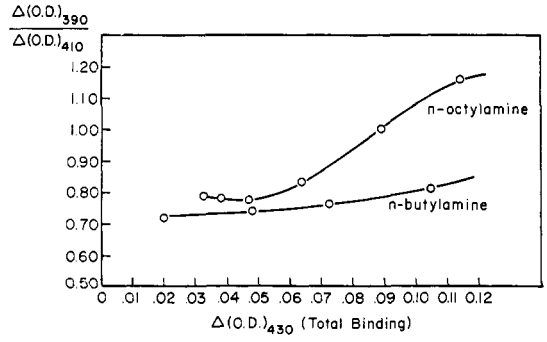

FIGURE 4: Binding of *n*-alkylamines to P-450 in liver microsomes. Variation in the shape of the difference spectrum with increased binding of amines. Liver microsomes from phenobarbital-treated rabbits (1 mg/ml) were suspended as described in Figure 1.

TABLE V: Binding of Other Ligands to P-450.

Ligand	Preparation	λ_{max}	λ_{min}	K (mM)	ΔOD_{max}[a]/ ΔOD_{CO}[b]
CN^-	Phenobarbital microsomes	445	410	7–8	0.47
	Lubrol–phenobarbital microsomes	445	412	15	
1-Butanol	MC microsomes	420	390	90	0.30
Phenobarbital	Lubrol–phenobarbital microsomes	420	390	10	
	Phenobarbital microsomes	420	390	17	0.12
	MC microsomes	420	390	25	0.31

[a] Absorbancy change produced by the saturating concentrations of ligand at the difference maximum. [b] CO-reduced minus reduced absorbancy change at 450 mμ.

412). A binding constant of 6–8 mM was determined for cyanide binding to these microsomes (Table V); a similar value for the corresponding submicrosomal preparation was observed (k = 15 mM). We also examined the binding of cyanide to a suspension of MC-induced liver microsomes, maintaining the same magnitude of CO difference spectrum as the above phenobarbital-induced microsomes [ΔOD_{448} compared with ΔOD_{450}]. At cyanide concentrations below 10 mM, the difference spectrum was similar to that for the phenobarbital-induced microsomes, but the spectral change at 445 mμ was reduced by a factor of 4–4.5. When a cyanide concentration of 15 mM was reached, an additional difference minimum at 395 mμ appeared and the ratio between spectral changes in the two preparations started to diminish. Thus, cyanide binding distinguishes type *a* and type *b* P-450 proteins in a way similar to amines, while showing higher affinity for type *b* P-450.

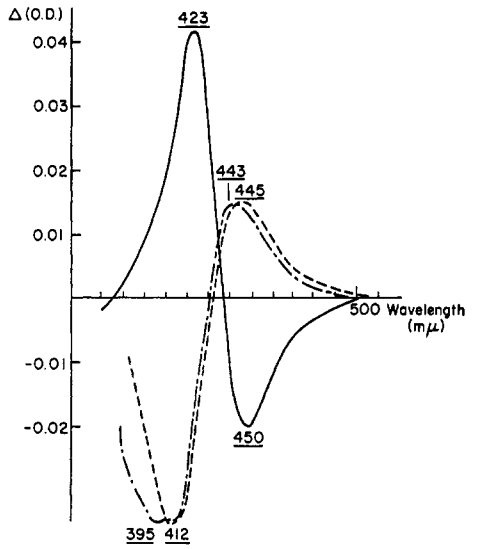

FIGURE 5: Difference spectra produced by addition of cyanide ions to P-450 and by displacement of cyanide ions from P-450 by *n*-octylamine. (a) P-450 submicrosomal particles from rabbit livers after pretreatment with phenobarbital. (———) [NaCN] = 18 mM (in both cuvets); [*n*-octylamine] = 0.07 mM. (– – – –) [NaCN] = 2.6 mM; without *n*-octylamine. CO difference spectrum at 450 mμ = 0.19, protein 0.7 mg/ml. (b) Liver microsomes from rabbits pretreated with methylcholanthrene. (— · — · —) [NaCN] = 15 mM. CO difference spectrum at 448 mμ = 0.3, protein = 1.65 mg/ml. Conditions were as described in Figure 1.

We examined the effect of cyanide ions upon amine binding by adding 18 mM cyanide and *n*-octylamine to submicrosomal particles obtained from phenobarbital-induced rabbits. The resulting difference spectrum (Figure 5; λ_{min} 450, λ_{max} 423), and increased K for *n*-octalamine (twofold) corresponded to direct 1:1 displacement of cyanide by *n*-octylamine.

ALCOHOLS. 1-Butanol produced a difference spectrum (λ_{max} 420, λ_{min} 390), both with liver microsomes (Imai and Sato, 1967b) and the submicrosomal preparation of P-450 (Table V), which was much more pronounced after MC induction than after phenobarbital induction (k = 90 mM).

Binding of 1-butanol to P-450 was in competition with both cyanide and *n*-octylamine; 70 mM 1-butanol was added to MC-induced microsomes in the presence of 35 mM cyanide. The difference spectrum (λ_{min} 445 mμ, λ_{max} 417 mμ) was similar to that obtained from the *n*-octylamine cyanide dependent experiment described above and indicated that binding of 1-butanol to P-450 had effected a displacement of cyanide from P-450. However, the weak inhibition by cyanide suggested that 1-butanol competed only with cyanide ions which bound to type *a* P-450, which had a weaker affinity for cyanide ions. This conclusion was consistent with the earlier observation that 1-butanol produced much larger difference spectra on MC-induced microsomes compared with phenobarbital-induced microsomes and, thus, probably was bound selectively to type *a* P-450.

An analogous displacement experiment was carried out with *n*-octylamine (2 mM) [difference spectrum λ_{min} 434, λ_{max} 413], which was much more effective in inhibiting binding of 1-butanol than the high concentration of cyanide used previously; this is consistent with the stronger binding constant of *n*-octylamine to type *a* P-450 microsomes (0.3 mM, Table IV).

Phenobarbital Binding. Phenobarbital binding to liver microsomes has been reported with two distinct difference spectra: (i) λ_{min} 420 mμ, λ_{max} 385 mμ (rats, Schenkman *et al.*, 1967); and (ii) λ_{max} 420 mμ, λ_{min} 390 mμ (rabbits, Imai and Sato, 1966). Addition of phenobarbital to liver microsomes from

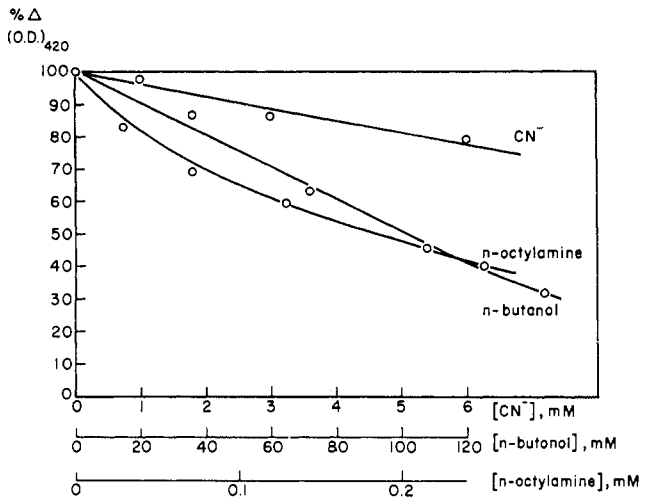

FIGURE 6: Displacement of phenobarbital from P-450. Rabbit liver microsomes obtained after MC pretreatments were suspended as described in Figure 1. Phenobarbital was added to the sample cuvet; other ligands were added to both sample and reference cuvet. CO difference spectrum at 448 mμ = 0.30, protein = 1.65 mg/ml. [Phenobarbital] = 25 mM with *n*-octylamine and cyanide; 45 mM with 1-butanol.

untreated immature rabbits (weighing about 2 kg) produced first spectrum followed by a change to spectrum ii at higher concentrations of phenobarbital (Table V). After pretreatment of the immature rabbits with phenobarbital or MC, only spectrum ii was detected, while even in untreated rabbits spectrum ii was of much greater magnitude than the spectrum i, obtained at lower phenobarbital concentrations. Difference spectrum ii was indistinguishable from the difference spectrum produced by 1-butanol. Further, for microsomes with the same reduced-CO difference spectrum, the complete difference spectrum at saturation produced by phenobarbital was 2.5 times greater on MC-induced microsomes than phenobarbital-induced microsomes (Table V). The magnitude of the saturated difference spectrum produced by phenobarbital on MC microsomes was also equal to that produced by 1-butanol. Thus, phenobarbital, like 1-butanol, produced a difference spectrum by binding to the P-450, preferentially induced by MC, *i.e.*, type *a* P-450. Exactly the same spectra could be repeated in absence of glycerol.

Phenobarbital was displaced from P-450 by addition of either 1-butanol, *n*-octylamine, or cyanide ions, or conversely, phenobarbital displaced these ligands from P-450 when they were present initially. Figure 6 shows the effect of other ligands on the phenobarbital difference spectrum. Displacement by *n*-octylamine clearly fell in the range of the type *a* *n*-octylamine binding constant rather than the lower concentration range observed for type *b* binding to *n*-octylamine. Displacement by cyanide ions also required very high concentrations consistent with involvement of only the type *a* P-450. The reversibility of binding of amines, 1-butanol, cyanide, and phenobarbital was completely confirmed by complete formation of the reduced CO complex found in the absence of the ligands, with retention of the maximum at 448 mμ. At very high concentrations of 1-butanol (>100 mM) conversion into P-420 occurred, probably accounting for the sharp decline of the phenobarbital difference spectrum at this level of 1-butanol.

$$\frac{[\text{type } a]}{[\text{type } b]} = \frac{\Delta OD_{392} - B\Delta OD_{410}}{C[\Delta OD_{410} - A\Delta OD_{392}]}$$

$A = -0.15$, $B = +0.4$, and $C = 2.5$

FIGURE 7: The ratio of type *a* P-450 to type *b* P-450 which is given by *n*-octylamine difference spectra. Spectral changes refer to saturation of P-450 by *n*-octylamine (1 mM). Differences were taken relative to an isobestic point at 500 mμ, with *decrease* of absorption assigned as positive. Factors A and B represent the respective overlapping contributions of the type *a* spectrum at the minimum of the type *b* spectrum and *vice versa*. *C* equals the ratio of Δε for the difference minima from type *a* P-450 (392 mμ) and type *b* P-450 (410 mμ).

Discussion

The spectral changes that we have observed with P-450 from rabbit liver microsomes in the presence of different ligands (amines, alcohols, barbiturates, and cyanide) have further demonstrated the existence of two forms of P-450 after phenobarbital and control, as well as MC treatments of the rabbits. The type *a* and type *b* difference spectra produced by the interaction of amines with P-450 derived from a red shift of the Soret maxima on formation of the corresponding amine complex. The types *a* and *b* P-450 correspond exactly to the two forms termed, respectively, high- and low-spin P-450 by Hildebrandt *et al.* (1968). These authors reported a major difference in the extinction coefficients of the Soret absorptions of reduced P-450–CO complexes of these two forms of P-450 (Δε, reduced CO-reduced 220 mM^{-1} type *a* or high spin; 50 mM^{-1} type *b* or low spin). A distinction of this magnitude was quite obviously required by our data and these figures were consistent when used to estimate the P-450 content of our microsomal preparations.

The difference spectra obtained with primary aliphatic amines affords a sensitive method to estimate the proportions of type *a* (high spin) and type *b* (low spin) P-450's in microsomes after different treatments and also after purification procedures such as the Lubrol method. For the determination of absolute amounts of P-450, the molar ratio of the two forms of P-450 must be known for one preparation. We have used MC-induced liver microsomes as the standard since we have determined an approximate ratio of 1:1 between the two forms from electron paramagnetic resonance and visible absorption spectra, relating only to P-450 (Jefcoate and Gaylor, 1969). An empirical relationship is shown in Figure 7.

Although there is a slight uncertainty about the absolute magnitudes of each type of P-450, amine difference spectra show two features very clearly. The proportion of type *a* to type *b* P-450 in liver microsomes increases according to treatment, as phenobarbital < control < MC, and also decreases during the Lubrol purification procedure (Table VI). The change upon MC induction would seem to result from MC selectively stimulating type *a* P-450, while scarcely affecting type *b* P-450. Phenobarbital induction, however, stimulated both forms, although favoring the type *b* P-450. The increase in type *b* P-450 during the Lubrol preparation seems to indicate selectivity in the process. However, control and phenobarbital microsomes were affected differently, suggesting that phenobarbital induction may have produced changes in P-450 unrealized by the spectra.

The type *a* and type *b* difference minima reflected *directly* the absorption spectrum of *oxidized* P-450 and in this sense

TABLE VI: Proportions of Type *a* (High Spin) and Type *b* (Low Spin) P-450 Obtained from *n*-Octylamine Difference Spectra.

Prepn and Method of Induction	$\Delta OD_{410}/\Delta OD_{392}$[a]	Type *a*/ Type *b*	Total P-450[b] (mμmoles/mg of protein)	
			Type *a*	Type *b*
Microsomes				
MC	0.2	1:1	0.8	0.8
Control	0.7	1:2.9	0.35	1.0
Phenobarbital	0.8	1:3.5	0.65	2.35
Lubrol Purification[c]				
MC	0.35	1:1.5	0.55	0.8
Control	0.8	1:3.5	0.35	1.2
Phenobarbital	1.2	1:6.5	0.5	3.3

[a] Obtained at saturation with neutralized *n*-octylamine (1.0 mM) in 50% glycerol–0.1 M phosphate (pH 7.4). [b] From CO difference spectra using Δε values of Hildebrandt *et al.* [c] After removal of Lubrol on Sephadex LH-20.

provided complementary data to that from ethyl isocyanide complexes of *reduced* P-450 (Sladek and Mannering, 1966). Further, amine complexes of oxidized P-450 were found to be much more stable than reduced ethyl isocyanide complexes; their shape was pH independent in the range of stability, and P-420 did not combine with *n*-octylamine (1 mM), unlike ethyl isocyanide. The exact relationship between the ethyl isocyanide complexes (430 and 455 mμ) and the different forms of P-450 has not been established. However, amine difference spectra showed distinct minima for each type of P-450, which should be a useful additional method of estimation [for 1 mM *n*-octylamine, and $\Delta\epsilon_{392} = 65$ mM^{-1} (type *a*), and $\Delta\epsilon_{410} = 25$ mM^{-1} (type *b*)].

The outstanding features of binding by *n*-alkylamines to the type *b* P-450 were (a) the existence of two binding constants (K_1 and K_2), and (b) the increase of *both* K_1 and K_2 with chain length to a limiting value at chain lengths of 8–12 carbons. Most of the difference in energy between K_1 and K_2 came from a 0.7-kcal/mole difference in the nitrogen interaction. When allowance was made for the effect of protonation, *n*-butylamine (K_1) was bound 1000 times more strongly than aniline, representing a difference of 4 kcal/mole in the binding energy. Since the hydrophobic interaction of a phenyl ring approximately equals that of an *n*-butyl chain (Nemethy, 1967), the amino group of aniline probably contributed less than 0.5 kcal to the binding of aniline to P-450. Thus, since the nitrogen interaction contributed so little, aniline could not discriminate between type *a* and *b* P-450 binding sites by the same mechanisms as primary amines. Providing the same Δε is associated with K_1 and K_2, both sites were distributed about equally among type *b* P-450's. The most attractive explanation is that P-450 hemes associate in pairs and that binding to one slightly weakens binding to the other. Imai and Sato (1968) have elegantly demonstrated that ethyl isocyanide forms a reduced hemin complex which absorbs at 450 mμ, only at high hemin concentrations where aggregation occurs. However, type *b* P-450's could equally well be distributed heterogeneously in the microsomes or exist in two forms.

The small changes in the difference spectra found with aromatic nitrogen bases probably arise from a direct interaction of the aromatic ring with the heme through a direct coordination of the nitrogen to the heme iron. The capacity of P-450 to bind long-chain amines indicates a large cavity adjacent to the porphyrin which could also accommodate the frequently bulky lipophilic substrates of P-450. The substituents on the α-carbon to the nitrogen probably restrict binding by their interaction with the displaced protein ligand, as has been reported for metmyoglobin (Keilin, 1966). This would be of less importance for substrates occupying this region since coordination to iron is not obligatory and may even be a disadvantage for a substrate such as aniline.

The spectral changes produced by high concentrations of phenobarbital and 1-butanol were identical within experimental error (Table V) and directly competitive (Figure 6). The relative magnitude of the difference spectra obtained by high concentrations of phenobarbital and 1-butanol on MC- and phenobarbital-induced microsomes corresponded to an exclusive binding to the type *a* (high spin) P-450 ($\Delta\epsilon_{420} = 80$ mM^{-1}). Although the binding of phenobarbital and 1-butanol to type *a* P-450 was reduced competitively by *n*-octylamine and cyanide (Figure 6), the associated spectral change indicates a change from high-spin to low-spin iron, which could not be produced by direct coordination. Rather, their binding at a second site must effect an increase in the interaction by one of the protein ligands of P-450 which could provide the ligand field necessary for the observed spectral change and also inhibit coordination by external ligands. The inverted difference spectrum (420 mμ min) obtained by the addition of low concentrations of phenobarbital to the microsomes probably derived from an interaction with type *b* P-450.

The best criterion that ligand binding has occurred exclusively to one form of P-450 is that the difference spectrum does not change in shape from MC- to phenobarbital-induced microsomes. In addition, the magnitude of the difference spectrum must be consistent with the change in the proportion of type *a* to type *b* P-450's in the two preparations. Thus, in liver microsomes we have determined that aliphatic primary amines and cyanide will bind selectively to type *b* (low spin) P-450, with the selectivity of the amines increasing greatly with alkyl chain length. Phenobarbital, and alcohols, which are ligands with a weak affinity for iron, produce spectral changes in type *a* P-450, which were observed only in the oxidized state of P-450, while amines and cyanide showed similar affinity for P-450 in the reduced state. This further suggests a separate binding site for phenobarbital and alcohols on type *a* P-450, which affects the heme allosterically.

A study of selective binding ligands, although only started in this paper, provides a useful approach to the problem of clarifying P-450-dependent hydroxylations according to the type of P-450 involved and also provides useful information about the heme regions of these P-450's. For example, Staudinger *et al.* (1965) have shown that hydroxylation of acetanilide and oxidation of NADPH by liver microsomes can both utilize two oxidases; one of low affinity toward oxygen (K_m–$O_2 = 1.5 \times 10^{-4}$ M) which is partially inhibited by cyanide, and one of high affinity toward oxygen (K_m–$O_2 = 2 \times 10^{-6}$ M) which is insensitive to cyanide. The existence of type *a* and

type *b* (higher affinity toward cyanide) P-450's simply account for these results.

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