Ligand Interactions with Hemoprotein P-450. II. Influence of Phenobarbital and Methylcholanthrene Induction Processes on P-450 Spectra*

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ABSTRACT: The absolute spectrum of submicrosomal particles (free of cytochrome b_5) which have been prepared from liver microsomes shows the induction of a second type of P-450 (oxidized: λ_{max} 395, 505, and 650 mμ) after pretreatment of rabbits with 3-methylcholanthrene. Correspondingly, electron paramagnetic resonance spectra of fresh 3-methylcholanthrene microsomes show a new signal at $g = 6.6$, suggesting the presence of a high-spin hemoprotein in addition to the usual triplet of low-spin P-450. Double integration of these signals has shown that the two hemoproteins are present in a ratio of about 1:1. Similar proportions are calculated from difference spectra of reduced P-450 with CO. By use of labeled 3-methylcholanthrene it has been shown that this high-spin P-450 cannot arise from a direct 1:1 binding of 3-methylcholanthrene and P-450. The difference in spin state probably arises from different interactions of protein ligands. Thus, investigation of ligand interaction with the heme center of metmyoglobin revealed a close similarity between the electron paramagnetic resonance spectrum of metmyoglobin–*n*-propylmercaptide and the low-spin form of P-450. Further, the relative affinities of organic ligands (alkylamines, imidazole, and propylmercaptan) for P-450 appeared in a reverse order to those for metmyoglobin. Based on light absorption and electron paramagnetic resonance spectroscopy and ligand binding a scheme is proposed for protein ligand binding to P-450 in the two oxidation states and the two spin states of iron.

Evidence for a new form of P-450[1] has recently been obtained from a difference spectrum between liver microsomes prepared from MC- and control-treated rats (Hildebrandt *et al.*, 1968). When the concentrations of cytochrome b_5 in these suspensions were balanced, the difference spectrum of oxidized hemoprotein showed maxima at 395 and 650 mμ, while the reduced-CO spectrum showed a maximum at 446 mμ. These spectra were interpreted as the absorptions of a new hemoprotein of the P-450 class. In the previous paper (Jefcoate *et al.*, 1969), we showed that certain ligands, when bound to oxidized P-450 from rabbit liver microsomes, produced separate difference minima at close to either 390 or 410 mμ concomitant with a shift of the Soret bands of distinct P-450 species to a longer wavelength. Since the difference minimum observed near 390 mμ predominated after MC induction, it was attributed to the new form of P-450 indicated by the experiment of Hildebrandt *et al.* (1968).

The electron paramagnetic resonance examination of liver microsomes has invariably exhibited an anisotropic three-line spectrum ($g = 2.41$, 2.25, and 1.91) that is typical of ferric iron with one unpaired electron ("low-spin" iron) (Mason *et al.*, 1965). For either control animals or those pretreated with phenobarbital there was a close correlation between the concentration of low-spin iron detected by electron paramagnetic resonance spectroscopy and the concentration of P-450 estimated from the absorbance of the reduced-CO complex. Furthermore, this electron paramagnetic resonance signal was not detected following NADPH reduction of these microsomes. Thus, it was concluded that the electron paramagnetic resonance spectrum was due to P-450, whose iron was consequently in a low-spin state. This was further established by absolute light absorption and electron paramagnetic resonance spectroscopy carried out on a submicrosomal particle isolated from microsomes (Miyake *et al.*, 1968a).

Hildebrandt *et al.* (1968) pointed out that the spectral changes produced in microsomes by MC induction indicated a high-spin form of P-450 (five unpaired electrons). We have investigated the possibility of a high-spin form of P-450 in microsomes by measurements of absolute visible spectra of the submicrosomal particle and by electron paramagnetic resonance spectrometry of microsomes and the particle.

Changes in the spin states of oxidized P-450 presumably result from changes in the interaction of the protein ligands with the heme center of P-450. The environment of iron in low-spin-oxidized P-450 resembles metmyoglobin to the extent of coordination by protoporphyrin IX and probably one histidine, all within a hydrophobic cleft. Metmyoglobin, however, has one coordination position occupied by a labile water molecule which can be interchanged by other ligands (Schoenborn *et al.*, 1965). We have investigated the effect of ligand substitution upon the visible absorption and electron paramagnetic resonance spectra of metmyoglobin as a model for the P-450 heme environment.

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[1] Abbreviations are as follows: MC, 3-methylcholanthrene; P-450, microsomal hemoprotein P-450.

Experimental Procedures

Male rabbits (weighing approximately 2.2 kg) of the New Zealand strain were used for all preparations of liver microsomes. The procedures for pretreatment, preparation of microsomes, and Lubrol purification were exactly as described previously (Jefcoate *et al.*, 1969). Microsomes were also prepared free of cytochrome b_5 by digestion for 12 hr with Nagarse proteinase (Teikaku Chemical Co., Osaka, Japan; 1 mg for 10 mg of microsomal protein) in 0.01 M sodium phosphate buffer (pH 7.4) containing 25% glycerol. After digestion, the suspension was centrifuged at 105,000*g* for 2 hr, which concentrated P-450 in the pellet, while leaving cytochrome b_5 in the supernatant fraction (Nishibayashi and Sato, 1968).

Horse heart myoglobin (Calbiochem) was completely converted into metmyoglobin with oxidation by potassium ferricyanide (10^{-3} M). The potassium ferricyanide was subsequently removed by dialysis against distilled water for 12 hr.

The treatment of rabbits with [6-^{14}C]3-methylcholanthrene (New England Nuclear Corp.) was as previously described for unlabeled MC, with each rabbit receiving 50 μCi of ^{14}C in 40 mg of compound (specific activity = 737 dpm/mμmole). The microsomes and P-450 particle were prepared as described above. ^{14}C was assayed by counting in Bray's solution (Bray, 1960) or, for isolated MC, toluene-counting solution. The induction of the high-spin P-450 was assayed from the CO-reduced P-450 difference spectrum and by the *n*-octylamine difference spectrum of the oxidized P-450 (Jefcoate *et al.*, 1969).

Electron paramagnetic resonance spectra were recorded on a Varian E3 spectrometer equipped with a 100-Kc field modulation and a variable-temperature accessory. Determination of *g* values and signal integration was carried out by comparison with a pitch standard. Light absorption spectra were measured at room temperature in 1-cm cells on a Cary spectrophotometer.

NADPH–cytochrome *c* reductase was isolated and partially purified as described by Williams and Kamin (1962). NADPH–P-450 reductase was measured spectrophotometrically by reduction of the hemoprotein in an atmosphere of CO as described earlier (Miyake *et al.*, 1968a). Protein was determined spectrophotometrically by the method of Lowry *et al.* (1951). Heme content was determined by the pyridine hemochromogen method (Omura and Sato, 1964).

All common chemicals were of the highest grade of purity available commercially. NADPH, cytochrome *c* (type VI), glucose oxidase (type II), and isocitric dehydrogenase (type IV) were purchased from Sigma Chemical Co., St. Louis, Mo. Lubrol-WX was the generous gift of Imperial Chemical Industries, Providence, R. I.

Results

Effect of Pretreatment on P-450 Spectra. The submicrosomal particle was prepared from liver microsomes by the Lubrol procedure following pretreatment of the rabbits with either MC or phenobarbital. The absolute spectra of P-450 after treatment of the rabbits with phenobarbital and MC, respectively, are shown in Figure 1a,b. Similarly, in Figure 1c, the absorption spectrum of MC-induced microsomes is shown, after removal of cytochrome b_5 by Nagarse digestion. A comparison of the oxidized spectra clearly indicated the presence

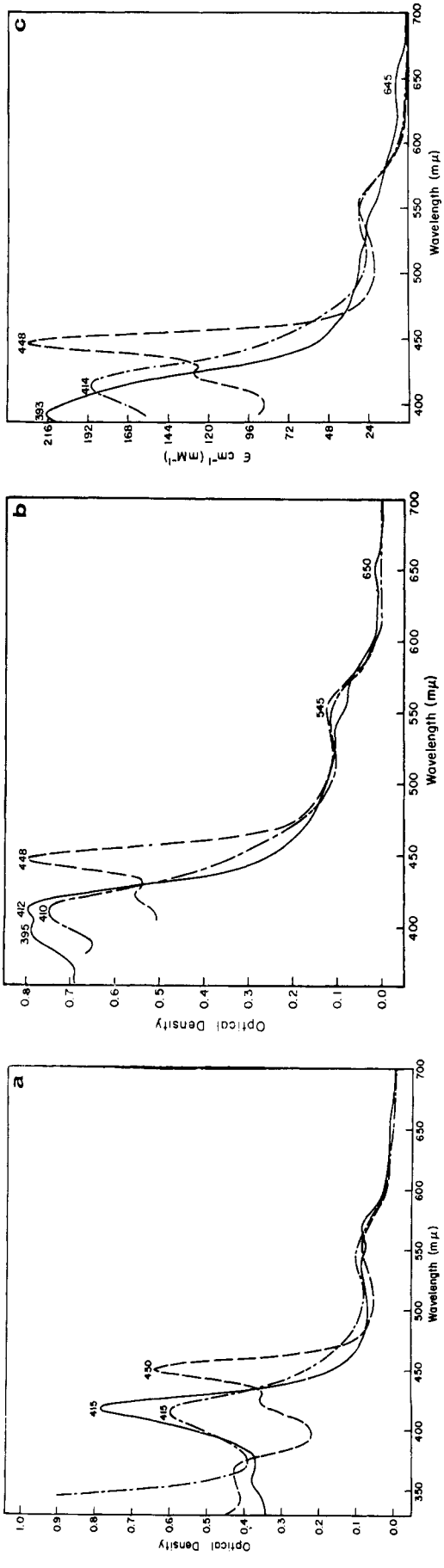


FIGURE 1: Absolute spectra of P-450 subparticles suspended in 0.1 M potassium phosphate buffer (pH 7.4), containing 50% glycerol. (———) Oxidized, (— · —) reduced ($S_2O_4^-$), and (– – –) reduced CO. (a) P-450 subparticle after Lubrol treatment of liver microsomes from rabbits treated with phenobarbital. Protein = 1.7 mg/ml. (b) P-450 subparticle after Lubrol treatment of liver microsomes from rabbits treated with MC. Protein, 3.3 mg/ml. (c) P-450 subparticle after Nagarse digestion of liver microsomes from rabbits treated with MC. Protein = 2.2 mg/ml. The solution was partially clarified with 1% of solid sodium deoxycholate and absorption was balanced at 700 mμ against a turbid blank obtained by treatment of microsomes with H_2O_2 (turbidity optical density at 700 mμ = 0.02). Extinction values were based on total heme content estimated by the pyridine hemochromogen method (Omura and Sato, 1964).

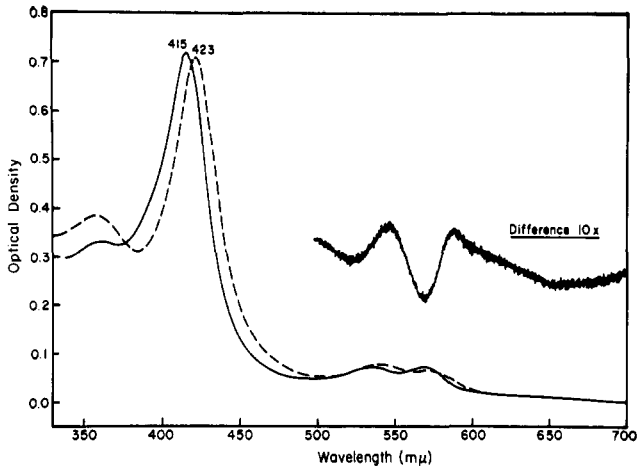


FIGURE 2: The absolute spectrum of oxidized P-450 complexes by *n*-octylamine. (———) Oxidized P-450 and (- - - -) + *n*-octyl amine (3 mM). The Lubrol subparticle, obtained from rabbit livers after phenobarbital treatment, was dissolved in 0.1 M phosphate buffer (pH 7.4) containing 50% glycerol. Protein = 1.6 mg/ml.

of a new hemoprotein, following treatment with MC. For the submicrosomal particle the absorbancy of oxidized P-450 at 395 mμ was equal to the absorbancy at 415 mμ, which was the only Soret peak observed with the phenobarbital preparation. In the Nagarse-treated preparation, the absorbancy of oxidized P-450 at 395 mμ was clearly greater than at 415 mμ. Difference spectra obtained with phenobarbital and *n*-octylamine upon the microsomes were not affected by Nagarse treatment; thus, Lubrol treatment must cause a small relative diminution of the type of P-450 which absorbs at 395 mμ. The distinguishing peaks at 395 and 650 mμ (and perhaps 505 mμ) are characteristic of a high-spin hemoprotein (Braterman *et al.*, 1964), which, however, retains the properties of P-450 in the reduced state.

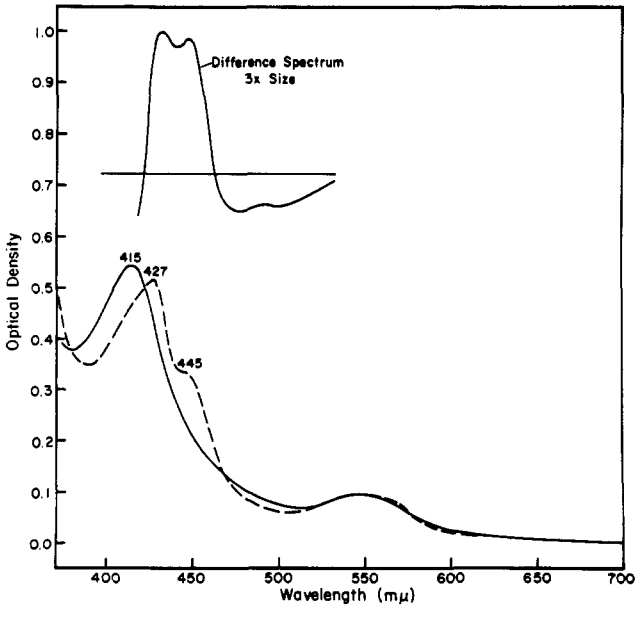


FIGURE 3: The absolute spectrum of reduced P-450 complexed by *n*-octylamine. (———) Reduced P-450 and (- - - -) + 10 mM *n*-octylamine. Conditions are exactly as for Figure 2.

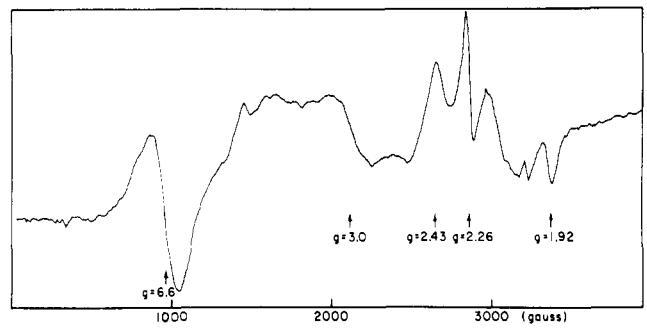


FIGURE 4: The electron paramagnetic resonance spectrum of liver microsomes freshly prepared from rabbits treated with MC. Microsomes (protein = 75 mg/ml) were suspended in 0.1 M potassium phosphate buffer (pH 7.4), containing 50% glycerol. Temperature, −160°; microwave power = 25 mW. Microwave frequency = 9.14 GHz; modulation amplitude = 20 gauss; receiver gain = 1.5×10^5.

The absorption maxima of reduced P-450-CO complexes in the Soret region for these two preparations were separated, however (448 mμ for MC pretreatment *vs.* 450 mμ for phenobarbital pretreatment) (Alvares *et al.*, 1967). An increase of absorbancy in the Soret region for the CO complex of reduced P-450 was also apparent after the MC treatment (Hildebrandt *et al.*, 1968).

Spectra of Amine Complexes of P-450. Difference spectra showing interactions of aniline and pyridine with both oxidized and reduced P-450 in microsomes have been reported (Imai and Sato, 1966b, 1967a,b). The spectra produced by *n*-octylamine saturation of oxidized and reduced P-450 from the livers of phenobarbital-treated rabbits are shown in Figures 2 and 3, respectively. The final absolute spectra after addition of *n*-octylamine were similar for both phenobarbital and MC pretreatments of P-450. Thus, both types of P-450 appear to have been converted into *n*-octylamine complexes which were spectrally identical.

The binding of *n*-octylamine to reduced P-450 produced two distinguished Soret peaks showing maxima at 427 and 445 mμ (difference maxima 428 and 450 mμ) (Figure 3). The maxima were similar to those exhibited by reduced P-450 and ethyl isocyanide, though the extinction coefficient at 427 mμ for the amine complex was much lower (Imai and Sato, 1966a). On the other hand, the combined α, β-band was scarcely changed by *n*-octylamine by contrast with ethyl isocyanide which increased the α band at 558 mμ (Miyake *et al.*, 1968a). The *n*-octylamine complex shows a high 450/428 difference ratio, but this may be ascribed to a low extinction coefficient for the 427-mμ absorbancy compared with the ethyl isocyanide complexes.

Electron Paramagnetic Resonance Spectra. The electron paramagnetic resonance spectrum of oxidized P-450 (Mason *et al.*, 1965) indicated iron in a low-spin state, showing three lines which corresponded to different energies of *dxy*, *dxz*, and *dyz* orbitals of iron (Griffith, 1957). Microsomes from MC-treated rabbits showed a strong signal with $g = 6.6$ (Figure 4), which was indicative of a high-spin hemoprotein (Ehrenberg, 1962). No such signal could be detected for microsomes from phenobarbital-treated rabbits. A second, smaller signal could be observed with $g = 3$, which showed almost

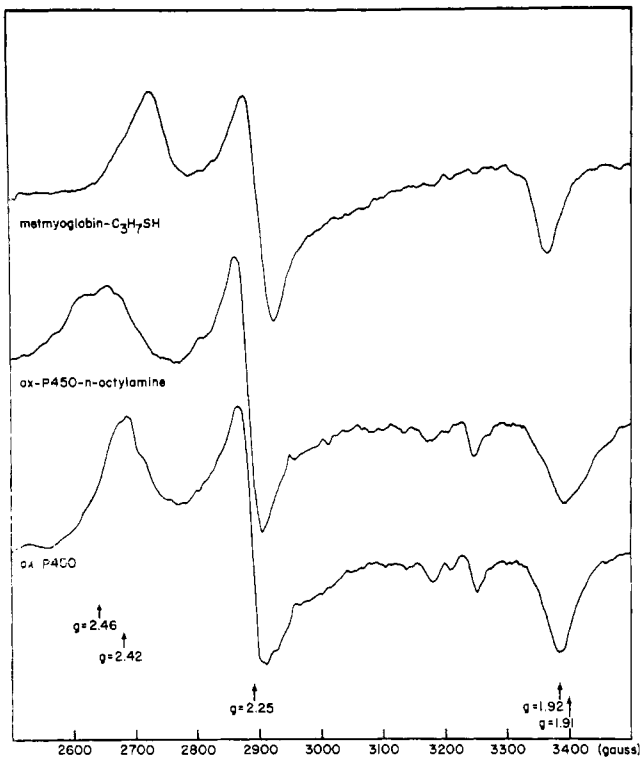


FIGURE 5: Low-spin electron paramagnetic resonance signals. (1) Metmyoglobin-*n*-propylmercaptide (1.2×10^{-4} M) in 0.1 M potassium phosphate buffer (pH 7.4). (2, 3) Liver microsomes from phenobarbital-treated rabbits. The microsomes were suspended in potassium phosphate buffer containing 50% glycerol (protein = 35 mg/ml). The concentration of *n*-octylamine was 3 mM. Temperature = −160°, microwave power = 25 mW, microwave frequency = 9.14 GHz, modulation amplitude = 20 gauss; receiver gain (1) = 1.5×10^5, (2, 3) = 2.5×10^5.

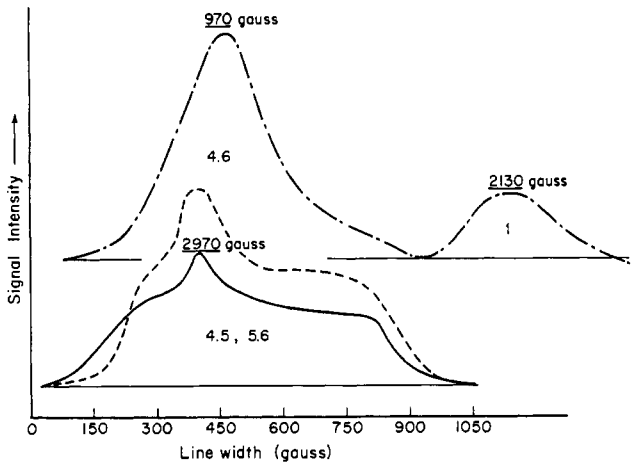


FIGURE 6: Integration of first derivative electron paramagnetic resonance signals (from Figures 4 and 5). (— · —) High-spin signals from methylcholanthrene microsomes (g = 6.6, 3.0). (———) Low-spin signal from methylcholanthrene microsomes. (– – – –) Signal from metmyoglobin-C_3H_7SH (2×10^{-4} M) size reduced by two-thirds relative to P-450 signals.

identical temperature and microwave power dependencies as the $g = 6.6$ signal. Both signals may be associated with the same anisotropic signal since high-spin hemoproteins typically show strong low-field ($g = 6$) and weak high-field ($g = 2$) components (Ehrenberg, 1962). Although a soluble heme fragment of cytochrome b_5 exhibited an anisotropic electron paramagnetic resonance signal ($g = 3.03$, 2.23, and 1.43; Bois-Poltoratsky and Ehrenberg, 1967), the line observed in microsomes was greatly enhanced by the MC treatment and showed a different line shape from that exhibited by cytochrome b_5.

After aging for 1 day at −15°, the $g = 6.6$ signal could not be detected, although broad absorption at intermediate fields (1500–3000 gauss) was apparent. However, the CO difference spectrum after this period retained the 448-mμ maximum which was characteristic of the high-spin hemoprotein and no formation of P-420 was observed. Thus, the loss of the electron paramagnetic resonance signal must be associated with a small modification of the high-spin form of P-450 which may only become apparent at low temperature.

The spectral changes observed at room temperature indicated that addition of *n*-octylamine caused the high-spin P-450 induced by MC to change its spin state. However, although 5 mM *n*-octylamine caused an electron paramagnetic resonance spectral change in the low-spin P-450 similar to that produced by aniline (Cammer *et al.*, 1966) (Figure 5), no transfer of the $g = 6.6$ or 3 high-spin signals to the low-spin region ($g = 2$) occurred at −160°.

Integration of the $g = 6.6$, 3, and 2 signals gave intensity ratios of 4.6:1:4.5, respectively (Figure 6). Thus, the electron paramagnetic resonance spectrum inferred a ratio of high-spin P-450 to low-spin P-450 of 1 or 1.2:1, depending upon whether or not the $g = 3$ signal is included in the high-spin signal. Visible absorption spectra of microsomes indicate that only the high-spin form of P-450 could be present at this concentration. We also found that the low-spin electron paramagnetic resonance signal increased by a factor of 1.8 in the change from MC-induced microsomes to phenobarbital-induced microsomes, which had the same *total* P-450 concentration (estimated by CO difference spectra). A factor of 1.6 was predicted from the proportions estimated by amine difference spectra (Jefcoate *et al.*, 1969).

Induction by Labeled Methylcholanthrene. A possible explanation of the change in the proportions of the two forms of P-450 in liver microsomes after treatment of rabbits with MC is that MC remains bound to P-450. This hypothesis was tested by treating rabbits with [6-^{14}C]3-methylcholanthrene. Induction of the high-spin form of P-450 was indicated by the shift of the reduced-CO difference spectrum to a 448-mμ maximum and by a 395-mμ Soret absorbance in the Lubrol subparticle. The amounts of MC retained in the preparations and the estimated levels of high-spin P-450. are shown in Table I. The concentration of MC was only a half of that of high-spin P-450. The proportion of [^{14}C]MC *increased* greatly in the Lubrol particle, even though the proportion of high-spin P-450 *decreased*. Furthermore, approximately 75% of the [^{14}C]MC in the microsomes was sufficiently loosely bound to be extracted by warm acetone. When the Lubrol was separated from P-450 by chromatography on Sephadex LH-20, MC was retained with the Lubrol while 85% of P-450 protein was recovered in a separate fraction. Thus, MC content does not relate to the proportion of high-spin P-450, but associates with the most nonpolar component (Lubrol) of the medium. No attempt was made to determine whether the

TABLE I: Induction of P-450 by [6-^{14}C]3-Methylcholanthrene.

Preparation	Sp Content of MC (mμmoles/mg of protein[a])	Sp Content of P-450 (mμmoles/mg protein[b])	
		High Spin	Low Spin
Microsomes	0.39	0.8	0.8
Acetone-extractable ^{14}C from microsomes	0.29		
Supernatant fraction from Lubrol treatment	0.31		
P-450 particle containing Lubrol[c]	1.22	0.7	1.1
P-450 particle with Lubrol removed[d]	0.28		

[a] Samples containing 8–25 mg of protein were assayed. Each value is the average of results of duplicate samples from three separate rabbits. [b] Proportions estimated from *n*-octylamine difference spectra, total amounts estimated from reduced-CO difference spectra as described by Hildebrandt *et al.* (1968). [c] Particle contains about six times as much Lubrol as protein (see Miyake *et al.*, 1968a). [d] Lubrol particle was chromatographed on a column (1 × 29 cm) of Sephadex LH-20 as described previously (Jefcoate *et al.*, 1969); 83% of protein was recovered free of Lubrol while most of the ^{14}C radioactivity was associated with chromatographic fractions containing Lubrol.

^{14}C radioactivity was associated with MC or a metabolite of MC.

Metmyoglobin Model. It has been suggested by Mason *et al.* (1965) that a sulfhydryl group could be involved in the heme coordination of P-450. At pH 7, metmyoglobin contains iron bound strongly by protoporphyrin IX and histidine, the coordination sphere of the iron being completed by a labile water molecule (Schoenborn *et al.*, 1965). The iron in this environment exists in a high-spin state. From pH 7 to 9, the water ligand ionizes to form a hydroxymetmyoglobin complex which is indicated by visible absorption (Lemberg and Legge, 1949) and magnetic methods (Ehrenberg, 1962) to be a mixture of high- and low-spin forms.

Electron paramagnetic resonance spectra at −160° showed only a high-spin (g = 5.9) signal in metmyoglobin at pH 7.4 and 10. However, upon addition of imidazole or *n*-butylamine (at pH 10.6), the high-spin signal at g = 5.9 (at −160°) was completely replaced by a broad anisotropic triplet centered around g = 2 (Table II) which was similar to that reported for cytochrome b_5.

Further, addition of imidazole or *n*-butylamine (pH 10.6) to metmyoglobin at room temperature produced visible absorption spectra characteristic of a low-spin heme (Table III).

Addition of *n*-propylmercaptan to metmyoglobin at pH 7.4 produced remarkable changes in both light absorbancy (Table III) and electron paramagnetic resonance (Table II) spectra. The absorption at 630 mμ (Figure 7) which characterized the high-spin state, was largely removed, while the Soret band was shifted to 427 mμ and was greatly reduced in intensity (Table III). The α,β absorbancies at 570 and 540 mμ were, however, very similar in their position and relative magnitudes to the imidazole and *n*-butylamine complexes of metmyoglobin. The electron paramagnetic resonance spectrum of the metmyoglobin–*n*-propylmercaptan complex showed a complete conversion of the g = 5.9 signal into an anisotropic triplet characteristic of low-spin iron (Figure 5 and Table II). This spectrum (g = 2.39, 2.24, and 1.94) was almost superimposable with that obtained from the low-spin form of P-450 or the *n*-octylamine complex of P-450. The signal amplitudes for electron paramagnetic resonance spectra of P-450 and metmyoglobin–*n*-propylmercaptide both increased as dependents of the square root of the microwave power up to 200 mW. However, this was found to be common to both high- and low-spin iron hemoproteins.

Integration of the electron paramagnetic resonance signals by graphical means produced a greater integral (5.5 units) from the low-spin mercaptide complex than high-spin metmyoglobin (3.3 units) at the same concentration. However, the asymmetry of the g = 5.9 line of metmyoglobin leads to an underestimation of the correct integral.

The binding constants for the interactions of *n*-butylamine, imidazole, and *n*-propylmercaptan were determined and were found to decrease sharply in that order (Table III). Consequently, the relative affinities of these ligands for metmyoglobin were exactly the reverse of their affinities for P-450.

Enzymic Activity. Convincing evidence has been reported that the microsomal flavoprotein, NADPH–cytochrome *c* reductase, is necessary for reduction of P-450 by NADPH (Sato *et al.*, 1965). It was recently shown that P-450 in the Lubrol subparticle could not be reduced by NADPH, even upon addition of purified NADPH–cytochrome *c* reductase (Miyake *et al.*, 1968a). However, Lubrol is a relatively strong inhibitor of P-450 reduction by NADPH even in intact microsomes (Miyake *et al.*, 1968a). When Lubrol was removed from P-450 subparticle obtained from rat liver microsomes (Gaylor and Mason, 1968), P-450 was reduced by NADPH (maintained at 0.1 mM with a NADPH-generating system[2]) at an initial rate of 0.04 mμmole/min per mg of protein (25°, in 50% aqueous glycerol, under 10% CO in 90% nitrogen). When NADPH–cytochrome *c* reductase was added so that the relative levels of reductase activity and P-450 specific content were similar to those found in liver microsomes, the rate of reduction of the P-450 in the subparticle by NADPH was approximately one-fifth of the initial rate observed for rat liver microsomal P-450 reduction (0.24 mμmole/min per mg of protein). After an extended period of time about 40% of P-450 in the subparticle was reduced by NADPH relative to full reduction by dithionite.

Discussion

MC induced the formation of an alternate form of P-450 compared with that normally predominating in microsomes. The new form exhibited light absorbancies and an electron paramagnetic resonance spectrum characteristic of iron in a

[2] The cuvet contained 2.6 mg of isocitric acid, 0.1 μmole of NADPH, 6 μmoles of Mg^{2+}, and 0.25 mg of isocitric dehydrogenase in a 1-ml final volume.

TABLE II: Complexes of Metmyoglobin and Other Hemoproteins Measured by Electron Paramagnetic Resonance Spectroscopy.

		Iron Porphyrins	Electron Paramagnetic Resonance Spectra			Ref
Class I	High spin					
		Metmyoglobin F^- Metmyoglobin (H_2O)	$g_{\|\|} = 2$	$g_\perp = 6$		*b*
Class II	Low spin		g_z	g_y	g_x	
		Metmyoglobin N_3^-	2.80	2.25	1.75	*b*
		[a] Metmyoglobin OH^-	2.61	2.19	1.82	*b*
		Horseradish peroxidase OH^-	2.86	2.12	1.67	*c*
		[a] Cytochrome *c* peroxidase	2.70	2.22	1.83	*d*
		Cytochrome b_5	3.03	2.23	1.43	*e*
		Metmyoglobin (RNH_2)	2.86	2.25	1.63	*f*
		Metmyoglobin (imidazole)	2.86	2.27	1.53	*f*
		P-450	2.42	2.25	1.92	*f*
		Metmyoglobin (RSH)	2.39	2.24	1.94	*f*

[a] Equilibria between high- and low-spin forms. [b] Ehrenberg (1962). [c] Morita and Mason (1965). [d] Yonetani *et al.* (1967). [e] Bois-Poltorataky and Ehrenberg (1967). [f] This report.

TABLE III: Complexes of Metmyoglobin and Oxidized P-450 Measured by Visible Absorption Spectroscopy.

		Ligands			
	Hemoprotein	Native	*n*-Butylamine	Imidazole	Propyl-SH
	Metmyoglobin		~0.4[a]	2×10^{-2}	1.7×10^{-4}
Measurement of *K* (M)	P-450[b]		1.3×10^{-6} [c]	4×10^{-4}	$>10^{-2}$
	Metmyoglobin	407 (144)	415 (100)	417 (102)	427 (80)
		502 (8.0)	540 (8.8)	535 (9.15)	542 (8.5)
		633 (2.6)			510 (6.9)
Absorbance maxima	P-450 (low spin)	415			
(ϵ mM^{-1})		535			
		565			
	P-450 (high spin)	395			
		650			

[a] pH 10.6. [b] First binding to low-spin P-450 in microsomes (50% glycerol). [c] Factor 10^{-3} included for protonation of *n*-butylamine at pH 7.4.

high-spin state. Peroxidase, catalase, metmyoglobin, and methemoglobin, either in absence of an added ligand or with fluoride coordinated to the heme, have been shown by magnetic susceptibility measurements to contain iron with five unpaired electrons (Lemberg and Legge, 1949). In all cases, the Soret absorption band lies between 403 and 408 mμ and a charge-transfer band appears between 600 and 650 mμ (ϵ 3–10). When ligands which interact more strongly (π acceptors, *e.g.*, CN^- and N_3^-) were added, the Soret bands invariably shifted to longer wavelength (415–425 mμ) and the charge-transfer band virtually disappeared. Analogy with the other hemoproteins discussed above would suggest that the MC-induced P-450 differs in the oxidized state from the normal P-450 by one iron–ligand interaction. This could be either a completely different substituent group or a weakening of an interaction. Significantly, the Soret peak of high-spin P-450 at 395 mμ is 10 mμ to shorter wavelength than other heme complexes containing protoporphyrin IX, while the charge-transfer band is at the longest wavelength observed thus far for this class of hemes.

In the reduced state the two types of P-450 were very similar, but different from other *ferroporphyrins*. A 450-mμ Soret band was observed for reduced P-450 complexes with CO, ethyl isocyanide, aromatic nitrogen bases, and aliphatic amines. The 450-mμ complexes formed by aliphatic amines with reduced P-450 are particularly significant since these ligands have none of the *π-bonding* potential of the other ligands forming these abnormal complexes. Thus, the ligands which bind to reduced P-450 can generally produce an absorbancy maximum at 450 mμ while cyanide (Miyake *et al.*,

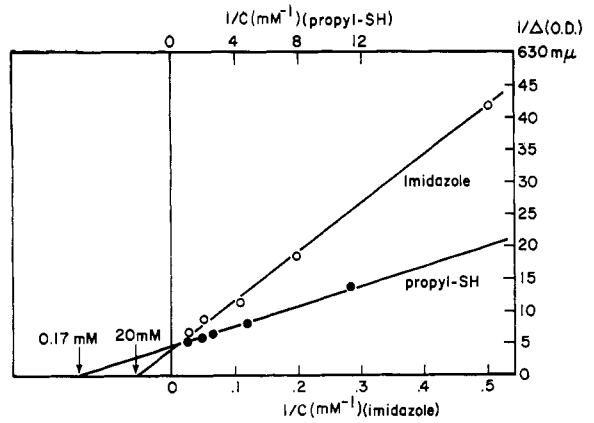


FIGURE 7: Binding of imidazole and *n*-propylmercaptan to metmyoglobin in 0.1 M potassium phosphate buffer. Lineweaver–Burk plots.

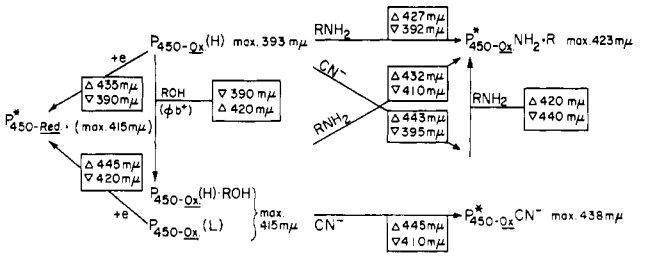


FIGURE 8: Difference and absolute spectra from two forms of P-450 in the Soret region. (*) both forms of P-450 have spectrally similar heme regions, (Δ) difference maximum, (▽) difference minimum; (H) high-spin P-450, (L) low-spin P-450, (+) phenobarbital, and depending upon the animal and treatment, barbiturates behave either like alcohols or bind to low-spin P-450 with the inverted difference spectrum (420 mμ/min).

1968b) and imidazole (Imai and Sato, 1967b) remain exceptional since they only form complexes with absorbancy maxima at 428 mμ. Although the difference spectrum produced by *n*-octylamine showed equal contributions at 450 and 428 mμ (Figure 3), the absolute spectrum showed that this change in favor of the 450-mμ complex, when compared with ethyl isocyanide complexes, resulted from a decrease in the extinction coefficient of the form exhibiting λ_{max} at 428 mμ. Similarly, the increased absorbancy of the peak at 455 mμ in the difference spectrum of ethyl isocyanide with MC-induced reduced P-450 (Sladek and Mannering, 1966) probably arises from a higher extinction coefficient for this ethyl isocyanide complex when it is formed by the high-spin form of P-450. The change would be analogous to the change of extinction coefficient in the CO complex.

Extinction coefficients from the absorption spectrum of rabbit liver MC-induced P-450 were determined on microsomes after removal of cytochrome b_5 by Nagarse treatment. The $\Delta\epsilon$ for the CO complex of 143 cm^{-1} mM^{-1} agrees very well with a value of 135 cm^{-1} mM^{-1} which was predicted for rat liver MC-induced P-450 by Hildebrandt *et al.* (1968) (50 cm^{-1} mM^{-1} (low spin), 220 cm^{-1} mM^{-1} (high spin)) and our electron paramagnetic resonance determination of an approximately 1:1 mixture of the two forms. The absolute spectrum probably contains an appreciable contribution from turbidity at 395 mμ, but nevertheless, a very high extinction coefficient is required for the Soret absorbancy (395 mμ) in the oxidized form of the high-spin P-450 (~160 cm^{-1} mM^{-1}). Comparison of the absolute spectrum of the Lubrol–P-450 subparticle and the P-450 in microsomes which were treated with Nagarse confirmed the partial loss of the high-spin form of P-450 during the Lubrol preparation, which was first observed in the amine difference spectrum (Jefcoate *et al.*, 1969).

The concentration of MC retained by the microsomes has been shown to be about half the concentration of high-spin P-450. Furthermore, 75% of the MC was removed by extraction with acetone, and thus MC cannot be covalently bound. Therefore, equimolar amounts of MC cannot be bound to yield the high-spin form of P-450, but a cooperative change effected through the membrane structure by less than 1-equiv amount of MC cannot be excluded.

The electron paramagnetic resonance signal which we have assigned to the high-spin form of P-450 is unusual in several respects. The signal lies to the low-field (g = 6.6) side of the usual position for high-spin heme signals (g = 5.9–6.0; *e.g.*, P-420 at pH 3.5) and it appears as a symmetrical line compared with the usual *asymmetric* lines detected around g = 6 from high-spin hemes. This asymmetry generally arises because high-spin hemoproteins show an additional small contribution at higher fields (g = 2) and consequently "broaden" across the intervening fields. The minor line at g = 3, which also appears symmetrical, may be the low-field component of the high-spin spectrum, but similar power and temperature effects for g = 3 and g = 6 lines do not provide a very conclusive criterion since all electron paramagnetic resonance signals from iron-oxidized hemoproteins which were subjected to this test showed closely matched effects. The most remarkable feature of the electron paramagnetic resonance signal of high-spin P-450 is that it is quite labile. There was no associated lability of the reduced-CO difference spectrum. Thus, the signal was probably lost through a specific magnetic effect related to the organization of spin centers which results in line broadening effects.

The low-spin electron paramagnetic resonance signal of P-450 was almost exactly replicated by the signal from metmyoglobin *n*-propylmercaptide. Apparently the weakly coordinated water molecule of metmyoglobin was readily replaced by *n*-propylmercaptan. The anisotropic triplet signal was normal for low-spin iron (Table II) and essentially identical with P-450 that exhibits, by far, the narrowest separation of the component lines that have been reported. Thus, the similarity of the electron paramagnetic resonance spectrum of P-450 and metmyoglobin mercaptides provides strong evidence for the coordination of histidine and the sulfhydryl from a cysteine in P-450 proposed earlier by Mason *et al.* (1965). Substitution of either imidazole or *n*-butylamine in metmyoglobin produced low-spin triplet signals which were extremely broad compared with the sulfhydryl complex; thus, the electron paramagnetic resonance spectrum of P-450 excludes two histidines or a histidine and ε-amino group from lysine as the extra protein ligands in P-450. Complexes of methionine with iron in heme have also been reported to give low-spin complexes and thus remain a possibility for coordination in P-450 (Harbury *et al.*, 1965).

The visible absorption spectrum of oxidized P-450, however, was different in the Soret region from metmyoglobin–*n*-propylmercaptide (Table III). Thus, the coordination

TABLE IV: Summary of Properties of High- and Low-Spin Forms of P-450 in Liver Microsomes.

Properties	High-Spin P-450	Low-Spin P-450
Oxidized		
Spin of Fe^{3+}	5/2	1/2
Electron paramagnetic resonance	g = 6.6 (3.0?), unstable	g = 2.42, 2.25, and 1.92; stable
Light absorption	λ_{max} = 393, ~505, and 650 mμ	λ_{max} 415, 535, and 565 mμ
Ligand binding		
Heme binding[a]		
n-Octylamine	Weak[c]	Strong[c]
CN^-	Very weak[c]	Weak[c]
Second site binding[b]		
1-Butanol	Very weak[c]	
Phenobarbital	Very weak[c]	
Effect of Lubrol procedure compared with microsomes	Proportion lower, amine binding stronger	Effect small
Reduced		
Light absorption free	λ_{max} 415 and 555	Same
CO complex	λ_{max} 448 (ϵ 220)[a]	λ_{max} 450 (ϵ 50)[a]
EtNC complex	λ_{max} 428 and 453 (high ϵ)[b]	λ_{max} 428 and 455 (low ϵ)[b]
Proportions after induction		
MC	50–55%; specific concentration increased twofold over control	45–50%; specific concentration unchanged
Phenobarbital	20–25%; specific concentration increased threefold	75–80%; specific concentration increased threefold
Control	20–25%	75–80%

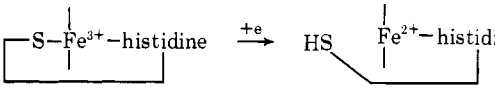
[a] Hildebrandt *et al.* (1968). [b] Imai and Sato (1966a). [c] Jefcoate *et al.* (1969).

spheres of iron in low-spin P-450 and metmyoglobin–*n*-propylmercaptide cannot be identical.

The binding constants of *n*-butylamine, imidazole, and *n*-propylmercaptan decreased in that order for metmyoglobin while they increased for P-450. This suggested that the ligand *trans* to the displacement process was different in the two cases. Therefore, the ligand which remained bound to P-450 during these ligand substitutions was *not* a histidine. This was also implied by the considerable red shifts of the oxidized P-450 spectra when imidazole or primary amines completed the coordination sphere. The great ease with which lipophilic ligands substituted in P-450 compared even with metmyoglobin indicates that the displaced protein ligand is rather weakly bound to iron. The strong binding of the sulfhydryl group to metmyoglobin, despite an adverse interaction with the distal histidine, would further suggest the probable existence of natural sulfhydryl–heme complexes.

Anaerobic reduction of the metmyoglobin–mercaptide produced the absorption spectrum of myoglobin. Thus, the high affinity for a sulfhydryl ligand was lost upon reduction producing a change in spin state from low to high spin. This change of spin state seems also to occur upon reduction of low-spin-oxidized P-450 and requires explanation since generally low-spin complexes are more prevalent in the *lower* oxidation state. On the basis of these analogies, the scheme for low-spin P-450 shown in eq 1 seems plausible. The high-spin character of the reduced P-450 has been assigned primarily on the basis of the rapid autoxidation of P-450 (Omura *et al.*, 1965), compared with the generally slow autoxidation of diamagnetic iron complexes (Williams, 1959), and by its spectral characteristics. Hoard *et al.* (1965) has shown that high-spin iron porphyrins invariably have iron about 0.45 Å out of the plane of the porphyrin, and thus, reduced P-450 should be considered in this way.

Imai and Sato (1968) have provided evidence that the association of two hemes can cause the anomalous 450-mμ absorptions of reduced P-450 complexes. The biphasic binding of amines to oxidized P-450 may also be an indication of the close association of hemes (Jefcoate *et al.*, 1969). Thus, the weakness of the interaction of a sulfhydryl group with reduced heme could provide the open cleft structure necessary for the direct interaction of iron with oxygen and also with an adjacent heme. Significantly, a major change in the protein–heme interaction upon reduction has been indicated by optical rotatory dispersion measurements in the visible region (H. S. Mason, unpublished results). This change is represented by



(1)

The similarity of the absorption spectra of the two forms of reduced P-450 suggest that the same two protein ligands bind the heme in each case. Furthermore, addition of either amine or cyanide to the oxidized forms of both types of P-450 produced heme complexes which showed similar spectra and, therefore, have similar heme regions. Thus, both forms of oxidized P-450 have at least *one* common type of protein ligand.

The distinction between the two forms of P-450 in the oxidized state may arise from a weakening of the interaction of one protein ligand due to small changes in the conformation of the protein between the two species.

The spectral changes that occurred in the heme regions of the two forms of P-450 have been summarized in Figure 8. It was exceptional that in the microsomal state of P-450, ligands which almost certainly coordinated directly to the heme (CN^- and RNH_2) bound more weakly to the high-spin form, where a weaker interaction of one protein–ligand was expected. This difference might arise if high-spin P-450 is less accessible to ligands than low-spin P-450. In this respect it may be significant that the differences in amine binding do not appear in the Lubrol–P-450 subparticle.

The weak interactions with metal ions derived from 1-butanol or phenobarbital could not produce the observed change from a high-spin to a low-spin form of P-450 by direct coordination but only by providing an alternative, or stronger protein ligand by means of an allosteric effect. The properties of P-450 which have been discussed in this and the preceding paper (Jefcoate *et al.*, 1969) have been summarized in Table IV. Methods by which the two forms of P-450 can be distinguished and estimated quantitatively are suggested. Further, gradual variation of the proportion of the forms of P-450 by carefully controlled pretreatments of the animals, in conjunction with the use of ligands which bind selectively on one form of P-450 during enzymic processes should provide an important approach to the assignment of P-450 reactions within liver microsomes.

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