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# $^1$H Fourier Transform Nuclear Magnetic Resonance Relaxation Rate Studies on the Interaction of Acetanilide with Purified Isozymes of Rabbit Liver Microsomal Cytochrome P-450 and with Cytochrome $b_5$

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SUMMARY

$^1$H Fourier transform NMR longitudinal ($T_1$) relaxation rate measurements were used to study the interaction of the phenyl and methyl protons of acetanilide with the paramagnetic heme iron of highly purified cytochromes P-450$_{LM_2}$, P-450$_{LM_4}$, and $b_5$. The relaxation rates of the phenyl and methyl protons of acetanilide were found to increase from 0.017 and 0.028 sec$^{-1}$ in the absence of hemeprotein to 1.35 and 1.50 sec$^{-1}$, respectively, in the presence of P-450$_{LM_2}$. The paramagnetic contribution to the enhanced relaxation rate values was determined by converting P-450$_{LM_2}$ to the ferrous carbonyl derivative *in situ*. The difference between the relaxation rates obtained with the paramagnetic ferric form and the diamagnetic ferrous carbonyl form of P-450$_{LM_2}$ yields the paramagnetic relaxation rate value, $T_{1_p}^{-1}$. In the presence of P-450$_{LM_2}$, the $T_{1_p}^{-1}$ values of the phenyl and methyl protons are 0.53 and 1.08 sec$^{-1}$, respectively, demonstrating that methyl protons relax almost 2-fold more rapidly than the phenyl protons as a result of the paramagnetic interaction with the catalytic center. Addition of P-450$_{LM_4}$ also leads to a substantial enhancement in the $T_1$ relaxation rate of the phenyl and methyl protons of acetanilide, but the phenyl protons relax approximately 1.7 times faster than the methyl protons as a result of the paramagnetic interaction with the heme iron atom of this P-450$_{LM}$ isozyme. In comparison, cytochrome $b_5$ produces substantially smaller enhancements in the $T_1$ relaxation rates. Using reduced cytochrome $b_5$ as the diamagnetic derivative, virtually identical $T_{1_p}^{-1}$ values are obtained for the phenyl and methyl protons at 28 $\mu$M cytochrome $b_5$, and a small differential effect in these values is seen at higher concentrations of the cytochrome (84 $\mu$M). Variable temperature studies suggest that acetanilide is in rapid exchange between free and hemeprotein-complexed environments. Distance estimates for the approach of the phenyl and methyl protons of acetanilide to the heme iron atom of P-450$_{LM_4}$ are provided, and range from 4.9 to 7.2 Å for the phenyl protons and 5.3 to 8.0 Å for the methyl protons. These data implicate acetanilide in specific complex formation with P-450$_{LM_2}$ and P-450$_{LM_4}$, and are in agreement with the specificity of the aromatic hydroxylase activity exhibited by the respective cytochrome in metabolic studies.

## INTRODUCTION

Cytochrome P-450, the major membranous enzyme of mammalian hepatocytes, displays an extraordinary metabolic versatility, for in liver microsomes in the presence of NADPH and oxygen, it catalyzes the hydroxylation or other chemical modification of naturally occurring steroids, fatty acids, and prostaglandins (3–5) as well as of a host of xenobiotics such as drugs, insecticides, anesthetics, petroleum products, and carcinogens (3, 4). Many foreign compounds are detoxified by P-450$_{LM}$,[1] while in other cases metabolism of these agents results in their activation to cytotoxic, mutagenic, or carcinogenic products (6). As reviewed elsewhere (7, 8), numerous studies have provided compelling evidence that the remarkably broad substrate specificity of the hepatic microsomal monooxygenase system(s) can be largely accounted for by the existence of P-450$_{LM}$ isozymes, which have recently been purified to varying extents by several investigators (cf. refs. 9 and 10). The two major isozymes of

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[1] The abbreviations used are: P-450$_{LM}$, liver microsomal cytochrome P-450 (the various isozymes of rabbit P-450$_{LM}$ are numbered according to their relative mobilities when submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis); FT, Fourier transform.

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the cytochrome in rabbit liver microsomes, phenobarbital-inducible P-450$_{LM_2}$ and carcinogen-inducible P-450$_{LM_4}$, have been purified to homogeneity and shown to be distinct proteins with different subunit molecular weights, spin states, spectral characteristics, and immunochemical and kinetic properties (7, 10–12). Furthermore, these enzymes possess different but often overlapping substrate specificities (7, 8, 10), and exhibit pronounced regio- and stereoselectivities both toward the substrate and with respect to product formation (10, 13).

Among the several hepatic monooxygenase activities presently known to be associated with the genetic locus (or loci) controlling the formation of cytochrome P-448 in mice and rats, only acetanilide 4-hydroxylase and 2-acetylaminofluorene *N*-hydroxylase activities were found to be inducible in rabbit liver by compounds of the polycyclic aromatic hydrocarbon class (14). Both microsomal activities were stimulated to the same level by pretreatment of rabbits with 5,6-benzoflavone, 3-methylcholanthrene, or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, and the increase in hydroxylation rates was accompanied by a 3-fold enhancement in the specific content of cytochrome P-448 (P-450$_{LM_4}$) (14). In accordance with these findings, NADPH-dependent aromatic ring hydroxylation of acetanilide in the reconstituted rabbit liver microsomal enzyme system, consisting of P-450$_{LM}$, NADPH-cytochrome P-450 reductase, and phospholipid, is catalyzed almost exclusively by P-450$_{LM_4}$ (8),[2] while, contrary to expectations (14), P-450$_{LM_2}$ is devoid of acetanilide *p*-hydroxylase activity (8).[3] Similar results have been reported by Johnson and Muller-Eberhard with partially purified cytochrome P-450 isozyme 2 (15) and homogeneous preparations of isozyme 4 (P-450$_c$) (15, 16) isolated from liver microsomes of phenobarbital- and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-treated adult rabbits, respectively. The latter cytochrome and P-450$_{LM_4}$ purified from phenobarbital-, 5,6-benzoflavone-, or 3-methylcholanthrene-treated rabbits are believed to be the same protein, as judged by a number of independent criteria (7, 8, 10, 15).

Although the mechanism of cytochrome P-450-catalyzed reactions has been intensively studied in several laboratories in the last decade (for a review see ref. 17), the basis for the observed selectivity of individual isozymes of P-450$_{LM}$ toward different substrates, different sites on a given molecule, or different optical isomers of the same substrate, remains poorly understood. In view of its ability to detect relatively low concentrations of material ($10^{-3}$–$10^{-5}$ M) and distinguish individual chemical sites, $^1$H FT NMR spectroscopy represents a powerful technique for investigations of molecular interactions in solution. Specifically, $^1$H $T_1$ relaxation rate measurements are particularly sensitive and highly useful for monitoring the interaction of electronically distinct sites of a molecule with a paramagnetic center. In this regard, relaxation rate changes resulting from the dipolar interaction of an individual molecular site with a paramagnetic metal ion are inversely proportional to the sixth power of the distance of separation from the paramagnetic center, thereby providing valuable information on the distance of approach and relative orientation of the substrate molecule in proximity to the paramagnetic center. The present paper describes the interaction of acetanilide with homogeneous P-450$_{LM_2}$, P-450$_{LM_4}$, and cytochrome $b_5$, as studied by proton $T_1$ relaxation rate measurements. The differential changes in the paramagnetic relaxation rates obtained for the phenyl and methyl protons of acetanilide suggest that this substrate is oriented in a manner such that the phenyl protons are closer to the catalytic site of P-450$_{LM_4}$, whereas the methyl protons are closer to the iron atom in the heme prosthetic group of P-450$_{LM_2}$.

## EXPERIMENTAL PROCEDURES

*Microsomal enzymes.* Homogeneous P-450$_{LM_2}$ and P-450$_{LM_4}$, purified from liver microsomes of phenobarbital-treated rabbits according to previously published procedures (18), were generous gifts of Professor M. J. Coon, University of Michigan (Ann Arbor, Mich.). Both Ouchterlony double-diffusion analysis (7) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7, 18) have shown that P-450$_{LM_2}$ is present in only trace amounts in microsomes from untreated and 5,6-benzoflavone-treated rabbits, whereas P-450$_{LM_4}$ is present at significant levels in microsomes from untreated and phenobarbital-treated animals and increased to about twice as high a level by induction with 5,6-benzoflavone. The purification of detergent-solubilized (intact) cytochrome $b_5$ from liver microsomes of phenobarbital-treated rabbits was carried out as described by Spatz and Strittmatter (19), and the resulting preparations were homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence of $\beta$-mercaptoethanol. The proteins were dialyzed extensively against $^2H_2O$ in potassium phosphate buffer, pH$_{obs}$ 7.5 (0.1 M with P-450$_{LM_2}$ and cytochrome $b_5$ or 0.3 M with P-450$_{LM_4}$), to remove glycerol and achieve the exchange of water and labile protons for $^2H_2O$.

The concentration of P-450$_{LM}$, before and after dialysis in $^2H_2O$, was determined from the absolute spectra of the ferrous carbonyl complexes using absorption coefficients of 110 mM$^{-1}$ cm$^{-1}$ at 450 nm and 119 mM$^{-1}$ cm$^{-1}$ at 448 nm for P-450$_{LM_2}$ and P-450$_{LM_4}$, respectively (18). The concentration of cytochrome $b_5$ was determined from the absolute spectrum of the oxidized cytochrome using an absorption coefficient of 117 mM$^{-1}$ cm$^{-1}$ at 413 nm (20). Optical spectra were recorded with a Cary Model 219 double-beam recording spectrophotometer, while the temperature was maintained at 30° by means of a Lauda Model K-2/R constant-temperature water circulator.

*NMR spectra.* $^1$H FT NMR spectra were recorded with a Varian Model CFT-20 spectrometer operating at 80 MHz and internally locked on the $^2$H signal of the $^2H_2O$ solvent. Irradiation of the residual HDO signal was employed in order to reduce the residual HDO signal

[2] K. P. Vatsis and M. J. Coon, unpublished results.

[3] Recent experiments utilizing a high-pressure liquid chromatography assay for acetanilide *p*-hydroxylase activity have shown that P-450$_{LM_2}$ catalyzes a small amount of acetaminophen formation from acetanilide (E. T. Morgan and M. J. Coon, personal communication). The colorimetric assay for acetaminophen, however, has repeatedly failed to detect product even under supraoptimal conditions (8). Thus, P-450$_{LM_2}$ may catalyze a small amount of acetaminophen formation which is detectable only by high-pressure liquid chromatography.

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intensity and enhance the signal-to-noise ratio. Spectra were recorded at ambient magnet temperature (29°), except for the variable-temperature experiments in which the sample temperature was varied from 5° to 35°. Temperatures were measured directly with a thermometer inserted into the probe. The $^1$H longitudinal relaxation rates, $T_1^{-1}$, were determined using the standard (180-$\tau$-90)$_n$ inversion-recovery sequence. The 90° pulse width was 21 $\mu$sec, and a pulse delay of 5 times the largest $T_1$ value was employed throughout the $T_1$ relaxation rate experiments.

*Treatment of data.* $T_1^{-1}$, the experimentally determined longitudinal relaxation rate of the phenyl and methyl protons of acetanilide in a solution containing P-450$_{LM}$, represents the sum of several separate contributions. These include the paramagnetic effect, which results from the interaction of acetanilide with or in proximity to the heme iron atom; the diamagnetic apoprotein contribution, which results from the interaction of the substrate with the apoprotein moiety; and the contributions of buffer and dissolved oxygen present in the sample. Therefore, it becomes necessary to separate the contributions to the change in $T_1^{-1}$ which may occur as the result of interactions involving the apoprotein, buffer, or dissolved oxygen, from those which reflect an interaction occurring at or in proximity to the paramagnetic heme iron atom. This is accomplished by converting the hemeprotein *in situ* to a diamagnetic form, such as the ferrous carbonyl complex of P-450$_{LM}$[4] or the reduced (ferrous) form of cytochrome $b_5$. In this manner, the paramagnetic contribution to the observed relaxation rate for P-450$_{LM_2}$, P-450$_{LM_4}$, and cytochrome $b_5$ may be expressed by the following equations:

$$T_{1_{pP\text{-}450_{LM}}}^{-1} = T_{1_{P\text{-}450^{3+}_{LM}}}^{-1} - T_{1_{P\text{-}450^{2+}_{LM}\text{-}CO}}^{-1} \quad (1)$$

$$T_{1_{pb_5}}^{-1} = T_{1_{b_5}3+}^{-1} - T_{1_{b_5}2+}^{-1} \quad (2)$$

The paramagnetic and diamagnetic contributions should vary linearly with protein concentration, while the contribution of buffer and dissolved oxygen should be independent of this parameter in the absence of selectively enhanced oxygen solubility by the protein. Hence, use of the diamagnetic form of the cytochrome also permits the selective removal of additional contributions, such as those of the buffer and dissolved oxygen, to the enhanced relaxation rate.

*Estimation of distances.* The effect of electron spin ($S$) and nuclear spin ($I$) interaction on the longitudinal relaxation time ($T_1$) is described by the Solomon-Bloembergen equation (22–24):

$$\frac{1}{T_{1_M}} = \frac{2\hbar^2\gamma_I^2\gamma_S^2(S)(S+1)}{15\langle r^6\rangle} \cdot\left[\frac{3\tau_c}{1+\omega_I^2\tau_c^2}+\frac{7\tau_c}{1+\omega_S^2\tau_c^2}\right] + \frac{2}{3}\frac{S(S+1)A^2}{\hbar^2}\left[\frac{\tau_e}{1+\omega_S^2\tau_e^2}\right] = \frac{\text{const.}}{\langle r^6\rangle}[f(\tau_c)] \quad (3)$$

$T_{1_M}$ is the relaxation time of a nucleus bound in the proximity of a paramagnetic metal ion. Here $\gamma_I$ is the nuclear gyromagnetic ratio, $\gamma_S$ is the electron gyromagnetic ratio, $\hbar$ is Planck's constant divided by $2\pi$, $S$ is the total electron spin, $r^6$ is the length of the vector between the nuclear-spin dipole and the electron-spin dipole, $\tau_c$ is the correlation time which describes molecular motions that modulate the electron-nuclear dipolar coupling, and $\omega_I$ and $\omega_S$ are the nuclear and electron precession frequencies, respectively. The second part of Eq. 3 is the contact term, or isotropic hyperfine term. $A/\hbar$ is the hyperfine coupling constant, and $\tau_e$ is the correlation time for the hyperfine interaction. Since the typical value of the hyperfine coupling constant $A/\hbar$ is $< 1$ MHz (25), this hyperfine term of Eq. 3 is considered negligible relative to the dipolar term for values of $r < 20$ Å. Hence, for the high-spin form ($S = 5/2$) of the ferrihemeprotein, P-450$_{LM_4}$, Eq. 3 reduces to

$$r(\text{Å}) = 812(T_{1_M}f(\tau_c))^{1/6} \quad (4)$$

where $f(\tau_c)$ refers to the expression in brackets in the first term of Eq. 3.

The correlation time, $\tau_c$, for the dipolar term of Eq. 3 is given by

$$\frac{1}{\tau_c} = \frac{1}{\tau_r} + \frac{1}{\tau_s} + \frac{1}{\tau_M} \quad (5)$$

where $\tau_r$ is the rotational correlation time, $\tau_s$ is the electron-spin relaxation time, and $\tau_M$ is the mean residence time of the species complexed to the metal ion (or the reciprocal of the pseudo-first order dissociation rate constant of the metal-ion complex). The components of $\tau_c$ are given in Eq. (5), and it follows that the fastest process (shortest correlation time) will contribute most significantly to $\tau_c$. In general, $\tau_s$ is on the order of $10^{-11}$–$10^{-10}$ sec for high- and low-spin ferric hemeproteins while $\tau_r \sim 10^{-9}$–$10^{-8}$ sec and $\tau_M \sim 10^{-4}$–$10^{-6}$ sec (26–28). Since $\tau_r$ and $\tau_M$ are one to seven orders of magnitude greater than $\tau_s$, they would be expected to contribute negligibly to the value of $1/\tau_c$. Equation 4 then reduces to $1/\tau_c \simeq 1/\tau_s$. Correlation times have been reported for ligand-hemeprotein complexes under various conditions. The formate-Mb complex gave $\tau_c = 3 \times 10^{-11}$ sec while for the formate-catalase complex it was $5 \times 10^{-11}$ sec (29). Horseradish peroxidase and fluorohorseradish peroxidase gave $\tau_c$ values of $9.0 \times 10^{-11}$ to $2.7 \times 10^{-10}$ sec, respectively, as determined from EPR linewidth measurements (30). Val-

[4] The ferrous carbonyl complex of P-450$_{LM}$ was used as the diamagnetic control to evaluate the paramagnetic contribution to the relaxation rate change. Conversion of oxidized P-450$_{LM}$ to the ferrous carbonyl complex may be accompanied by protein conformational changes which may alter the acetanilide binding site and thus contribute to the estimated paramagnetic relaxation rate values. This possibility has been investigated with imidazole, a nitrogenous base which is known to coordinate directly to the heme iron atom of various hemeproteins. The relaxation rate difference for the phenyl and methyl protons of acetanilide seen in the presence of imidazole is identical with that obtained for the respective group when oxidized P-450$_{LM}$ is converted to the diamagnetic ferrous carbonyl complex (in the absence of imidazole). These data suggest that changes in protein conformation upon reduction of ferric P-450$_{LM}$ do not contribute significantly to the paramagnetic relaxation rate values (21) (also see Discussion).

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ues of $\tau_c$ determined from extrapolated EPR linewidth data for cytochrome P-450$_{cam}$ ranged from $1.5 \times 10^{-11}$ sec to $1.4 \times 10^{-10}$ sec for the camphor and metyrapone complexes, respectively (31). However, the values of $\tau_c$ determined from EPR linewidth data plotted against reciprocal temperature were reported to be $3.0 \times 10^{-10}$ and $3.8 \times 10^{-10}$ sec, respectively, for the same complexes. Also, $\tau_s$ values reported for other $Fe^{3+}$-containing proteins have been approximately $10^{-10}$ sec (32, 33). Hence, a reasonable estimate of $\tau_c$ for substrate-P-450$_{LM}$ complexes would be $10^{-11} < \tau_c < 10^{-10}$ sec.

For a given substrate moiety, the contribution to the observed longitudinal relaxation rate caused by the paramagnetic ion, $T_{1_p}$, is given by Eqs. 1 and 2. $T_{1_M}$ may be calculated from the following equation (34):

$$\frac{1}{T_{1_p}} = \frac{\alpha_M}{T_{1_M} + \tau_M} \qquad (6)$$

where $\alpha_M$ is the mole fraction of a given species complexed to the paramagnetic ion. When $\tau_M \ll T_{1_M}$, "fast exchange" prevails, then $T_{1_M} \simeq \alpha_M T_{1_p}$.

Hence the calculation of the distance from various parts of the substrate to the paramagnetic heme iron atom using Eq. 3 requires (a) knowledge of the spin state of the heme paramagnetic iron atom of the hemeprotein under ambient conditions, (b) a dissociation constant for the substrate-hemeprotein complex ($K_S$), (c) a value of the correlation time $\tau_c$, and (d) a measure of the $T_{1_p}^{-1}$ values for the individual sites of the substrate.

*Spectral dissociation constants.* Difference spectra were determined at 30° in 3.0-ml solutions containing 1.1 μM P-450$_{LM_4}$ and 0.1 M potassium phosphate buffer (pH 7.4). Aliquots (2–40 μl) of a 1.0 M solution of acetanilide (in methanol) were added to the sample cell, and equal volumes of methanol were added to the reference cell. The difference spectra were recorded (from 360 to 480 nm) with a Cary 219 spectrophotometer after the attainment of equilibrium, which occurred within a few minutes or less. The results were corrected for the effect of dilution on the absorbance changes, and double-reciprocal plots of $\Delta A$ versus acetanilide concentration yielded a spectral dissociation constant ($K_s$)[5] of 8.4 ± 0.7 mM.

## RESULTS

The $^1$H FT NMR spectrum of acetanilide in buffer is shown in the last scan of the sequence presented in Fig. 1. The proton spectrum of this compound consists of two signals: a singlet at $\delta = 2.0$ ppm indicative of the methyl protons, and an apparent singlet at $\delta = 7.5$ ppm ascribable to the phenyl protons. These signals are well-defined, relatively sharp singlets ideally suited for monitoring intermolecular interactions by the $T_1$ relaxation rate technique.

A typical 180-$\tau$-90 inversion-recovery sequence for acetanilide, consisting of partially relaxed FT NMR spectra employed in the calculation of $T_1$ relaxation rates, is also depicted in Fig. 1, where $M_\tau$ represents the peak amplitude (either positive or negative) obtained at a particular value of delay time ($\tau$) in the two-pulse sequence. $M_\infty$ refers to the maximal signal amplitude obtained when

[5] R. F. Novak and K. P. Vatsis, unpublished results.

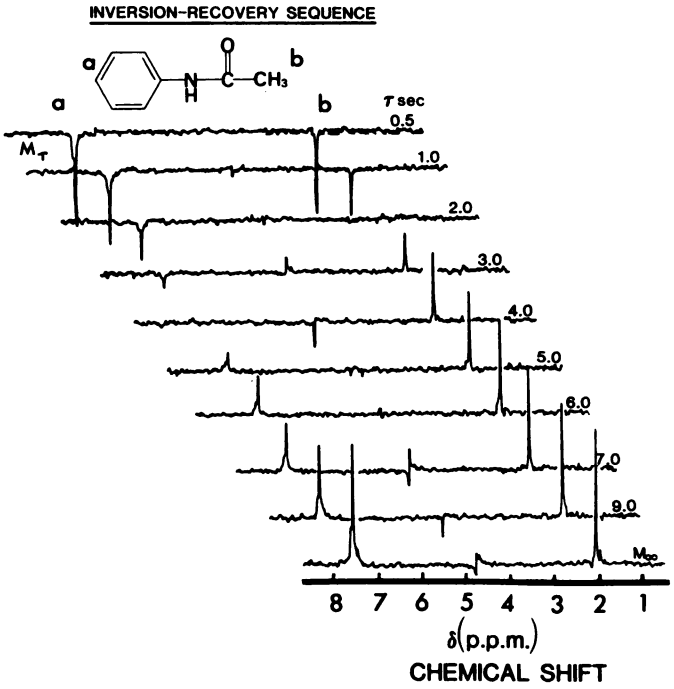


FIG. 1. *$^1$H FT NMR spectrum and inversion-recovery sequence of acetanilide*

A solution of acetanilide (4 mM) in 0.1 M potassium phosphate buffer (pH 7.5) was prepared (total volume, 0.4 ml), and the spectrum was recorded at 28° as described under Experimental Procedures. The chemical shifts of the phenyl and methyl protons of acetanilide were 7.5 and 2.0 ppm, respectively, from the internal reference DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate).

The parameter $\tau$ is the delay time (in seconds) between the 180° and 90° pulse. A pulse delay of 5 times the longest $T_1$ was employed, and 25 acquisitions per spectrum were required. The data shown were used for the calculation of the $T_1$ relaxation rates ($T_1^{-1}$).

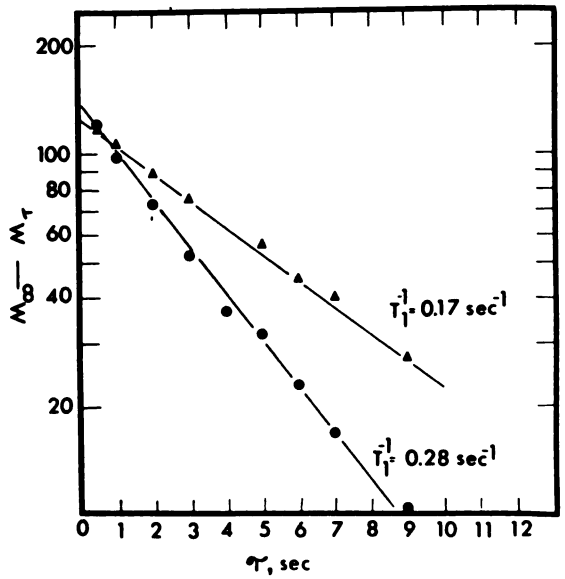


FIG. 2. *Semilogarithmic plot of $M_\infty - M_\tau$ for the phenyl and methyl protons of acetanilide*

$M_\tau$ refers to the signal amplitude (either positive or negative) at time $\tau$. $M_\infty$ is the signal amplitude obtained from the fully relaxed spectrum. ▲, Phenyl protons; ●, methyl protons.

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the system is allowed to relax fully between the two-pulse sequence, i.e., when $\tau$ is sufficiently long (5 times the value of the longest $T_1$, in this case, that of the phenyl protons) to allow for complete relaxation to occur. A semilogarithmic plot of $M_\infty - M_\tau$ versus $\tau$, used for calculating $T_1^{-1}$, is presented in Fig. 2.

The addition of a hemeprotein such as P-450$_{LM}$ to a buffered solution of acetanilide should result in an increase in the relaxation rates of the phenyl and methyl protons, since under these conditions interactions are expected to occur between the substrate and the apoprotein moiety and/or the paramagnetic center of P-450$_{LM}$. Figure 3 (*inset*) illustrates the linear dependence of $T_1$ relaxation rates on P-450$_{LM_2}$ concentration. The observed increase in $T_1$ values with increasing cytochrome concentration consists of a diamagnetic contribution that results from the interaction of acetanilide with the apoprotein moiety, as well as a paramagnetic contribution attributable to the interaction of the substrate with the catalytic heme iron atom. In order to evaluate the paramagnetic contribution to the observed relaxation rate increase, P-450$_{LM_2}$ was converted *in situ* to the diamagnetic ferrous carbonyl complex. Values for $T_{1_p}^{-1}$, the paramagnetic contribution to the relaxation rate, were determined from Eq. 1 and are shown as a function of P-450$_{LM_2}$ concentration in Fig. 3. The results demonstrate that in the presence of P-450$_{LM_2}$, which possesses essentially no acetanilide *p*-hydroxylase activity, the paramagnetic relaxation rate of the methyl protons is substantially greater than that of the phenyl protons. The $T_1$ relaxation rates of the phenyl and methyl protons of acetanilide also display a linear dependence on P-450$_{LM_4}$ concentration, as shown in the *inset* to Fig. 4. In this case, however, the $T_1^{-1}$ of the phenyl protons increases more rapidly than does that of the methyl protons. As with P-450$_{LM_2}$, conversion of P-450$_{LM_4}$ to the diamagnetic ferrous carbonyl complex allows for an evaluation of paramagnetic contributions to the increase in $T_1^{-1}$. The resulting $T_{1_p}^{-1}$ values indicate a considerably faster paramagnetic relaxation rate for the phenyl relative to the methyl protons of acetanilide in the presence of P-450$_{LM_4}$ (Fig. 4).

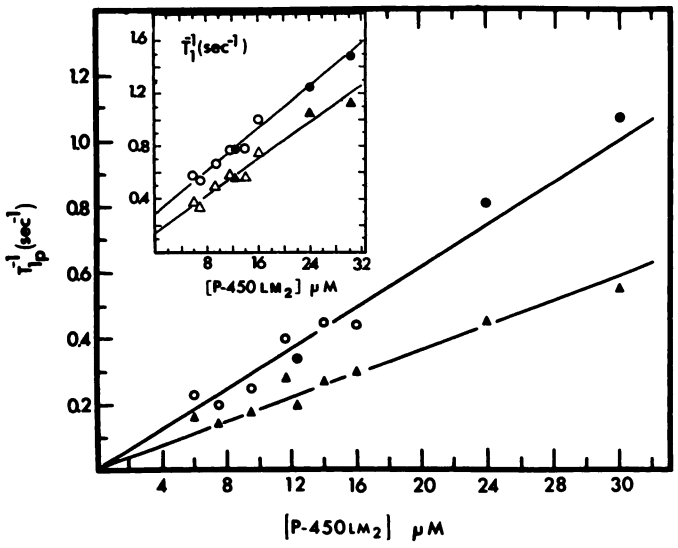


FIG. 3. *Paramagnetic contribution of P-450$_{LM_2}$ to the relaxation rates of the phenyl and methyl protons of acetanilide*

*Open* and *closed symbols* represent data obtained with different P-450$_{LM_2}$ preparations. △, ▲, phenyl protons; ○, ●, methyl protons.

Inset. Effect of P-450$_{LM}$ concentration on the relaxation rates of the phenyl and methyl protons of acetanilide. The conditions were as described in Fig. 1, except that P-450$_{LM_2}$ was also added as indicated. *Open* and *closed symbols* represent data obtained with two different P-450$_{LM_2}$ preparations (isolated at different times). △, ▲, Phenyl protons; ○, ●, methyl protons.

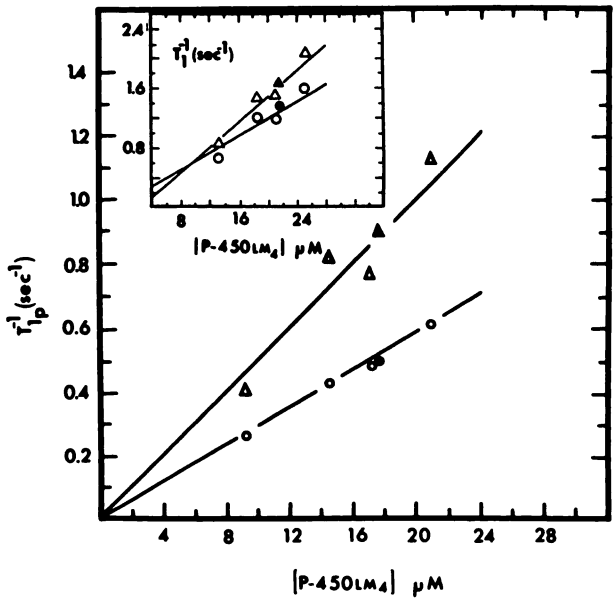


FIG. 4. *Paramagnetic contribution of P-450$_{LM_4}$ to the relaxation rate change of the phenyl and methyl protons of acetanilide*

*Open* and *closed symbols* represent data obtained with different P-450$_{LM_4}$ preparations; both preparations were isolated from phenobarbital-treated rabbits. △, ▲, Phenyl protons; ○, ●, methyl protons.

Inset. Relaxation rates of the phenyl and methyl protons of acetanilide as a function of P-450$_{LM_4}$ concentration. Solutions contained acetanilide (4 mM) and P-450$_{LM_4}$ at the indicated final concentration in 0.3 M potassium phosphate buffer (pH 7.5). *Open* and *closed symbols* represent data gathered with two different P-450$_{LM_4}$ preparations (isolated at different times). The relative contents of high-spin and low-spin ferric heme were similar in the two preparations, which were purified from liver microsomes of phenobarbital-treated rabbits. △, ▲, Phenyl protons; ○, ●, methyl protons.

The relaxation rate change of the methyl and phenyl protons of acetanilide in the presence of varying concentrations of homogeneous, detergent-solubilized (intact) cytochrome $b_5$ is presented in the *inset* to Fig. 5. Since cytochrome $b_5$ does not form a ferrous carbonyl complex, the reduced (ferrous) form of this cytochrome was used as the diamagnetic derivative in assessing the apoprotein contributions to the increase in the $T_1$ relaxation rates (Eq. 2). The paramagnetic contribution of cytochrome $b_5$ to the $T_1$ relaxation rates of the phenyl and methyl protons of acetanilide is shown in Fig. 5. It may be seen that both the phenyl and methyl protons exhibit virtually identical paramagnetic relaxation rates at cytochrome $b_5$ concentrations of 20–30 μM. Furthermore, the $T_{1_p}^{-1}$ values in the presence of 60–100 μM cytochrome $b_5$ are significantly smaller than those obtained with either P-450$_{LM_2}$ or P-450$_{LM_4}$ at substantially lower concentrations (5–30 μM). The apparent differential change in paramagnetic relaxation rates at the higher cytochrome $b_5$ concentra-

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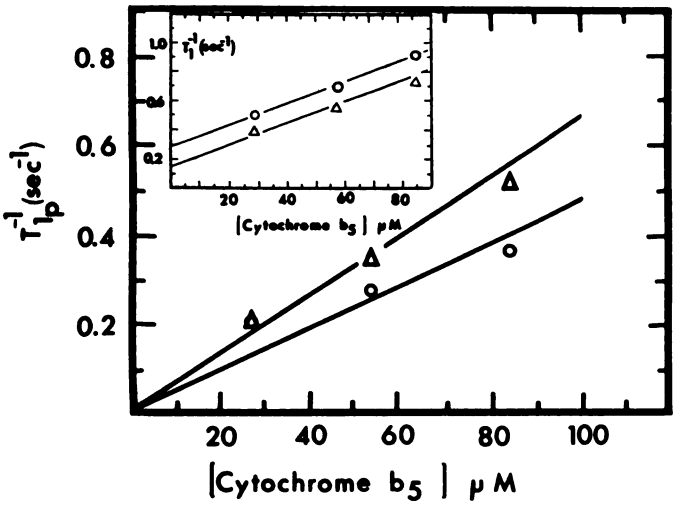


FIG. 5. *Effect of cytochrome $b_5$ concentration on the paramagnetic relaxation rates of the phenyl and methyl protons of acetanilide*

△, Phenyl protons; ○, methyl protons.

Inset. Relaxation rates of the phenyl and methyl protons of acetanilide as a function of cytochrome $b_5$ concentration. The conditions were as described in Fig. 1, except that cytochrome $b_5$ was also added as indicated. △, phenyl protons; ○, methyl protons.

tions (>50 μM) may be attributed to incomplete reduction of cytochrome $b_5$, an alteration in exchange rates between free acetanilide and that in complex with the cytochrome, or decreased accessibility of acetanilide to cytochrome $b_5$

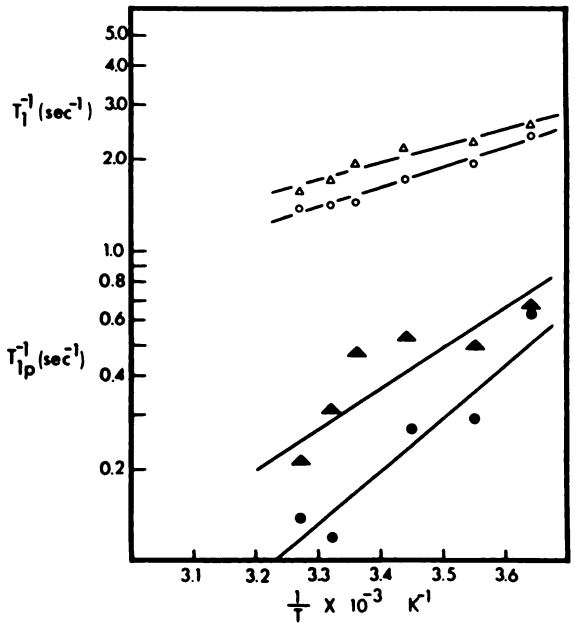


FIG. 6. *Temperature dependence of the relaxation rates of the phenyl and methyl protons of acetanilide in the presence of 17.3 μM P-450$_{LM_4}$*

△, ▲, Phenyl protons; ○, ●, methyl protons; *open symbols*, temperature dependence of the $T_1$ relaxation rate; *closed symbols*, temperature dependence of the paramagnetic relaxation rate. The activation energies obtained for the phenyl and methyl protons of acetanilide from $T_1^{-1}$ data (*open symbols*) were 2.97 and 3.38 kcal/mole, respectively. Activation energies obtained from $T_{1_p}^{-1}$ data (*closed symbols*) were 6.80 and 8.64 kcal/mole for the phenyl and methyl protons of acetanilide, respectively.

owing to aggregation of the purified protein into larger units.

Figure 6 is an Arrhenius plot depicting the temperature dependence of the paramagnetic relaxation rates for acetanilide in the presence of 17.3 μM P-450$_{LM_4}$. Similar results were obtained for acetanilide in the presence of P-450$_{LM_2}$. These data suggest that acetanilide is in rapid exchange (34, 35) between free and hemeprotein-complexed ($\tau_M \ll T_{1_M}$) species in solution and yield activation energies of 6.8 and 8.6 kcal/mole for the phenyl and methyl protons of acetanilide, respectively, in the presence of P-450$_{LM_4}$. For 20 μM P-450$_{LM_2}$, activation energies of 6.8 and 2.8 kcal/mole were obtained for the phenyl and methyl protons of acetanilide, respectively.

The spectral dissociation constant, in conjunction with the paramagnetic relaxation rate values, $T_{1_p}^{-1}$, for the acetanilide-P-450$_{LM_4}$ complex permits an estimation of the distance of approach of the phenyl and methyl protons of acetanilide to the paramagnetic heme iron atom in P-450$_{LM_4}$. Since no spectral dissociation constant could be obtained for the P-450$_{LM_2}$-acetanilide complex, a comparable estimation of the distances of approach of the phenyl and methyl protons to the heme iron atom of this isozyme was not possible. The distance estimates obtained for the acetanilide-P-450$_{LM_4}$ complex using Eqs. 1, 3, 4, and 6 are provided in Table 1 and show that the phenyl group approaches approximately 0.4–0.8 Å closer to the heme iron atom relative to the methyl protons. Since the exact value of the correlation time, $\tau_c$, for the acetanilide-P-450$_{LM_4}$ complex is not known, a range of $\tau_c$ values was used for estimation of the distance of approach. The values of $\tau_c$ used in the distance calculation are $10^{-10}$ and $10^{-11}$ sec, which represent the range of $\tau_c$ values determined for various ferrihemeproteins as estimated from $\tau_s$ or variable frequency paramagnetic relaxation rate studies (26–33). These values were used in estimating the distances of approach (Table 1), and it can be seen that one order of magnitude change in $\tau_c$ results in a difference in distances of approach to the heme iron atom of 2.29 Å and 2.68 Å for the phenyl and methyl protons of acetanilide, respectively.

TABLE 1

*Acetanilide P-450$_{LM_4}$ distances*

The distances of approach of the phenyl and methyl protons of acetanilide to the paramagnetic heme iron atom of cytochrome P-450$_{LM_4}$ for two values of the correlation time, $\tau_c$.

| Acetanilide | $\tau_c$ | $\bar{r}$(Fe-H)Å; S = 5/2[a] |
|---|---|---|
| | *sec* | |
| Phenyl | $10^{-11}$ | 4.91 ± 0.13 |
| Methyl | $10^{-11}$ | 5.32 ± 0.10 |
| Phenyl | $10^{-10}$ | 7.20 ± 0.19 |
| Methyl | $10^{-10}$ | 8.00 ± 0.19 |

[a] Distances were calculated using Eqs. 1, 3, 4, and 6. The spectral dissociation constant was employed in calculation of the mole fraction of complex, $\alpha_M$, and $T_{1_M}$ was calculated under the assumption that rapid exchange prevailed (i.e., $T_{1_M} \simeq \alpha_M T_{1_p}$). Distances are the average values ± standard deviation and were calculated from paramagnetic relaxation rate values obtained at four different concentrations of P-450$_{LM_4}$ (10, 14, 18, and 21 μM).

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## DISCUSSION

It was demonstrated in previous studies that the interaction of xylidine (2,6-dimethylaniline) with hemoglobin or partially purified cytochrome P-450, isolated from liver microsomes of phenobarbital-treated rats, gave rise to differential paramagnetic relaxation rate changes for the methyl and phenyl protons of this compound. Thus, with either of these hemeproteins, the phenyl protons of 2,6-dimethylaniline gave a paramagnetic relaxation rate which was about 1.5-fold greater than that seen for the methyl protons. In contrast, virtually identical $T_{1_p}^{-1}$ values were obtained for the phenyl and methyl protons of xylidine in the presence of myoglobin (36). These differential $T_{1_p}^{-1}$ values for the phenyl and methyl protons of xylidine in the presence of cytochrome P-450 or hemoglobin suggested that xylidine was oriented in complex with these hemeproteins such that the phenyl protons most closely approached the paramagnetic heme iron atom (36). We have presently expanded these investigations to include relaxation rate studies on the interaction of acetanilide with P-450$_{LM_2}$, P-450$_{LM_4}$, and cytochrome $b_5$. In the reconstituted enzyme system, phenobarbital-inducible P-450$_{LM_2}$ displays no aromatic hydroxylase activity toward acetanilide (8),[3] whereas P-450$_{LM_4}$ is the most active of several rabbit P-450$_{LM}$ isozymes examined[2] in converting acetanilide to acetaminophen. $^1$H FT NMR $T_1$ relaxation rate studies on the interaction of acetanilide with P-450$_{LM}$ have shown that the phenyl and methyl protons of acetanilide are differentially affected by P-450$_{LM}$, with the phenyl protons experiencing the greatest paramagnetic effect in the presence of P-450$_{LM_4}$ and the methyl protons having the greatest $T_{1_p}^{-1}$ in the presence of P-450$_{LM_2}$. Attempts to obtain a spectral dissociation constant and thus utilize the Solomon-Bloembergen (22–24) equation to obtain distance estimates of the phenyl and methyl protons of acetanilide to the heme iron atom were successful only with P-450$_{LM_4}$. A sufficiently high concentration of acetanilide required to produce a saturable change in the Soret band of P-450$_{LM_2}$ could not be achieved. Nevertheless, since the paramagnetic relaxation rate, $T_{1_p}^{-1}$, is inversely proportional to the sixth power of the distance of separation between a given molecular site and the paramagnetic center, it may be inferred from these $T_{1_p}^{-1}$ data for P-450$_{LM_2}$ that acetanilide is oriented with the methyl protons most closely approaching the paramagnetic center, whereas the phenyl protons are in closest proximity to the heme iron atom of P-450$_{LM_4}$ (Fig. 7). These results are in agreement with the catalytic specificity of P-450$_{LM_2}$ and P-450$_{LM_4}$ toward acetanilide (8).[3] The virtually equivalent relaxation rate changes obtained in the presence of 28 $\mu$M cytochrome $b_5$ suggests that both the methyl and phenyl groups may be equidistant from the heme iron atom. Furthermore, the smaller magnitude of $T_{1_p}^{-1}$ values for the methyl and phenyl protons of acetanilide (as compared with those obtained for P-450$_{LM}$) achieved in the presence of substantially higher concentrations of cytochrome $b_5$ suggests that the acetanilide molecule has less affinity and/or accessibility to the heme environment of cytochrome $b_5$ or that the acetanilide molecule is located at a greater distance from the paramagnetic center.

In order to investigate whether such paramagnetic relaxation rate data may indeed be associated with a specific preparation and hence be of limited generalized value, we also elected to investigate the effect of two different samples of P-450$_{LM_2}$ and P-450$_{LM_4}$ (purified on separate dates) on the methyl and phenyl relaxation rates of acetanilide. The results of these studies were consistent with those obtained using the initial samples of P-450$_{LM_2}$ and P-450$_{LM_4}$ utilized in the investigation.

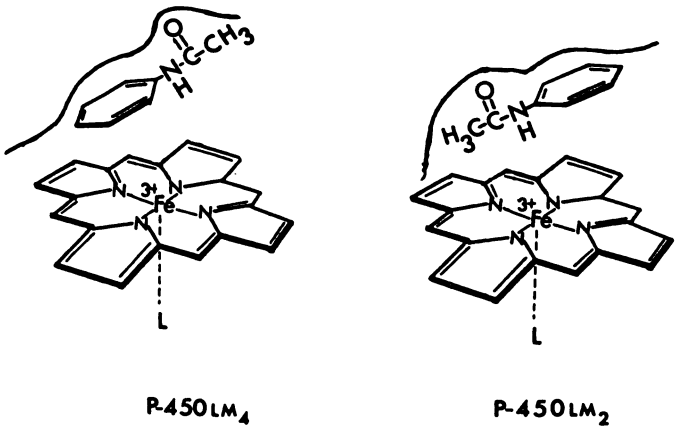


FIG. 7. *Proposed model of acetanilide-P-450$_{LM}$ interactions showing the orientation of the molecule with respect to the paramagnetic heme iron atom*

The values of $T_{1_p}^{-1}$ for the phenyl and methyl protons of acetanilide are less in the presence of P-450$_{LM_2}$ than in the presence of an identical concentration of P-450$_{LM_4}$, possibly because the latter is high-spin as compared with P-450$_{LM_2}$, which is low-spin.[6] In order to preclude the effects of heme iron anisotropy associated with the high- and low-spin forms of P-450$_{LM}$ as being completely responsible for the differential paramagnetic relaxation rate values of the phenyl and methyl protons, a preparation of P-450$_{LM_4}$ which contained a substantially greater proportion (approximately 50%) of the low-spin form was also used.[5] Again, the differential paramagnetic relaxation rate values were observed with $T_{1_p}^{-1}$ phenyl > $T_{1_p}^{-1}$ methyl, but the absolute magnitudes of the $T_{1_p}^{-1}$ values were smaller owing to the greater percentage of P-450$_{LM_4}$ in the low-spin form.[5] Variable temperature studies conducted over the range 4–35° suggest that acetanilide was in rapid exchange between the pool of uncomplexed substrate and that which was in complex with either P-450$_{LM_2}$ or P-450$_{LM_4}$. Hence, it appears that acetanilide maintains a reasonable degree of dynamic accessibility to the binding site in proximity to the heme iron atom.

Recent experiments have also shown that the H(2), H(4,5) protons of imidazole yield virtually identical values of $T_{1_p}^{-1}$ in the presence of equivalent concentrations of P-450$_{LM_2}$ and P-450$_{LM_4}$ (37), and that addition of imidazole to a solution of acetanilide and P-450$_{LM_2}$ or P-450$_{LM_4}$ results in a decrease in the relaxation rate of solvent and of the phenyl and methyl protons of acetanilide (21, 37, 38). These data imply that imidazole

[6] A major difference between P-450$_{LM_2}$ and P-450$_{LM_4}$ relates to their spin state. As isolated, P-450$_{LM_2}$ is low-spin and P-450$_{LM_4}$ is high-spin. The relative proportion of high- and low-spin states may vary in different P-450$_{LM_4}$ preparations and with experimental conditions (18).

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experiences virtually identical heme environments in P-450$_{LM_2}$ or P-450$_{LM_4}$, and that imidazole either prevents the interaction of acetanilide with P-450$_{LM}$ or, in a dynamic sense, renders the paramagnetic heme iron atom inaccessible to the substrate (21, 37, 38).

The paramagnetic contribution to the P-450$_{LM}$-enhanced relaxation rate of the phenyl and methyl protons of acetanilide was evaluated using the diamagnetic ferrous carbonyl derivative of the respective P-450$_{LM}$ isozyme. The change in hemeprotein oxidation state from ferric to ferrous form may well result in conformational changes of the apoprotein, and such alterations in quaternary or tertiary structure of the apoprotein may be reflected in the paramagnetic relaxation rate value. We have recently demonstrated that addition of imidazole (8 mM) to solutions of acetanilide and P-450$_{LM_2}$ or P-450$_{LM_4}$ results in $T_{1_p}^{-1}$ values virtually identical with those obtained with the ferrous carbonyl derivatives. These results are in agreement with distance estimates for the acetanilide-P-450$_{LM_4}$ complex (Table 1) and further demonstrate that alterations in apoprotein structure contribute negligibly to the paramagnetic relaxation rate values determined using the ferrous carbonyl derivative of the hemeprotein. In addition, the results of experiments using imidazole with acetanilide and P-450$_{LM_2}$ suggest that the acetanilide binding site for this hemeprotein may also lie within the distance estimates for acetanilide-P-450$_{LM_4}$ (i.e., 5–8 Å from the heme iron atom).

The results of the above experiments further suggest that imidazole, in decreasing the interaction of acetanilide with P-450$_{LM_2}$ or P-450$_{LM_4}$, should serve as a noncompetitive inhibitor of cytochrome P-450-catalyzed oxidation reactions. We have recently confirmed that imidazole is an effective noncompetitive inhibitor of cytochrome P-450-catalyzed *N*-demethylation, *O*-dealkylation, and *p*-hydroxylation reactions in uninduced, phenobarbital-induced, and 5,6-benzoflavone-induced rabbit liver microsomes (39).

The NMR relaxation rate approach to the study of substrate-P-450$_{LM}$ interactions as illustrated herein is capable of providing specific information regarding hemeprotein structure via dynamic accessibility as evidenced by substrate orientation in proximity to the catalytic center. This approach may also be expanded to include investigations of the effects of the individual components (lipid, NADPH-cytochrome P-450 reductase) of the reconstituted enzyme system upon substrate orientation and accessibility. Preliminary experiments performed in this laboratory indicate that dilauroylphosphatidylcholine causes a comparable enhancement in the paramagnetic relaxation rates of the phenyl and methyl protons of acetanilide when added to P-450$_{LM}$ (40).

Further efforts may be directed to studies of interactions occurring between the isolated components in order to understand more fully the molecular basis of substrate specificity exhibited by the different isozymes of cytochrome P-450 from the same species as well as a comparison of data with similar isozymes isolated and purified from different animal species. The combination of such molecular level results with those obtained from metabolic studies using the reconstituted enzyme system or microsomal suspensions may well provide a composite view of the basis for the substrate specificity of cytochrome P-450-catalyzed oxidation reactions and the relative interactions among the various individual components of the system. Such molecular level studies may also provide a basis for predicting the effects of other drugs (i.e., inhibitors) on cytochrome P-450-catalyzed oxidation reactions.

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## REFERENCES

1. Novak, R. F., and K. P. Vatsis. $^1$H FT NMR studies on the interaction of acetanilide with purified forms of liver microsomal cytochrome P-450. *Fed. Proc.* **38**:526 (1979).
2. Novak, R. F., and K. P. Vatsis. Characterization of acetanilide-P-450$_{LM}$ interactions: implications regarding substrate specificity, in *Microsomes, Drug Oxidations, and Chemical Carcinogenesis* (M. J. Coon, A. H. Conney, R. W. Estabrook, H. V. Gelboin, J. R. Gillette, and P. J. O'Brien, eds.), Vol. 1. Academic Press, New York, 159–162 (1980).
3. Gillette, J. R. Biochemistry of drug oxidation and reduction by enzymes in hepatic endoplasmic reticulum. *Adv. Pharmacol.* **4**:219–261 (1966).
4. Conney, A. H. Pharmacological implications of microsomal enzyme induction. *Pharmacol. Rev.* **19**:317–366 (1967).
5. Vatsis, K. P., A. D. Theoharides, D. Kupfer, and M. J. Coon. Marked stimulatory effect of cytochrome $b_5$ on NADPH-dependent hydroxylation of $PGE_1$ by rabbit phenobarbital-inducible P-450$_{LM_2}$ in a reconstituted enzyme system, in *Biochemistry, Biophysics, and Regulation of Cytochrome P-450* (J.-Å. Gustafsson, J. Carlsted-Duke, A. Mode, and J. Rafter, eds.). Elsevier/North-Holland Biomedical Press, Amsterdam, 73–76 (1980).
6. Gelboin, H. V., N. Kinoshita, and F. J. Wiebel. Microsomal hydroxylases: induction and role in polycyclic hydrocarbon carcinogenesis and toxicity. *Fed. Proc.* **31**:1298–1309 (1972).
7. Coon, M. J., J. L. Vermilion, K. P. Vatsis, J. S. French, W. L. Dean, and D. A. Haugen. Biochemical studies on drug metabolism: isolation of multiple forms of liver microsomal cytochrome P-450, in *Drug Metabolism Concepts* (D. M. Jerina, ed.). American Chemical Society Symposium Series 44, American Chemical Society, Washington, D. C., 46–71 (1977).
8. Coon, M. J., and K. P. Vatsis. Biochemical studies on chemical carcinogenesis: role of multiple forms of liver microsomal cytochrome P-450 in the metabolism of benzo[*a*]pyrene and other foreign compounds, in *Polycyclic Hydrocarbons and Cancer: Environment, Chemistry, and Metabolism* (H. V. Gelboin and P. O. P. Ts'O, eds.), Vol. 1. Academic Press, New York, 335–360 (1978).
9. Guengerich, F. P. Isolation and purification of cytochrome P-450, and the existence of multiple forms. *Pharmacol. Ther.* **6**:99–121 (1979).
10. Lu, A. Y. H., and S. B. West. Multiplicity of mammalian microsomal cytochromes P-450. *Pharmacol. Rev.* **31**:277–295 (1980).
11. Coon, M. J., and R. E. White. Cytochrome P-450, a versatile catalyst in monooxygenation reactions, in *Metal Ion Activation of Dioxygen* (T. G. Spiro, ed.). John Wiley & Sons, New York, 73–123 (1980).
12. Vatsis, K. P., D. D. Oprian, and M. J. Coon. Kinetics of reduction of purified liver microsomal cytochrome P-450 in the reconstituted enzyme system studied by stopped flow spectrophotometry. *Acta Biol. Med. Ger.* **38**:459–473 (1979).
13. Vatsis, K. P., J. Deutsch, H. V. Gelboin, and M. J. Coon. Catalytic activity and stereoselectivity of phenobarbital-inducible and 5,6-benzoflavone-inducible isozymes of liver microsomal cytochrome P-450 in the oxygenation of benzo[*a*]pyrene and *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene, in *Microsomes, Drug Oxidations, and Chemical Carcinogenesis* (M. J. Coon, A. H. Conney, R. W. Estabrook, H. V. Gelboin, J. R. Gillette, and P. J. O'Brien, eds.), Vol. 2. Academic Press, New York, 1065–1077 (1980).
14. Atlas, S. A., S. S. Thorgeirsson, A. R. Boobis, K. Kumaki, and D. W. Nebert. Differential induction of murine *Ah* locus-associated monooxygenase activities in rabbit liver and kidney. *Biochem. Pharmacol.* **24**:2111–2116 (1975).
15. Johnson, E. F., and U. Muller-Eberhard. Resolution of multiple forms of rabbit liver cytochrome P-450, in *Drug Metabolism Concepts* (D. M. Jerina, ed.). American Chemical Society Symposium Series 44, American Chemical Society, Washington, D. C., 72–80 (1977).
16. Johnson, E. F., and U. Muller-Eberhard. Resolution of two forms of cytochrome P-450 from liver microsomes of rabbits treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *J. Biol. Chem.* **252**:2839–2845 (1977).
17. White, R. E., and M. J. Coon. Oxygen activation by cytochrome P-450. *Annu. Rev. Biochem.* **49**:315–356 (1980).
18. Haugen, D. A., and M. J. Coon. Properties of electrophoretically homogeneous

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phenobarbital-inducible and β-naphthoflavone-inducible forms of liver microsomal cytochrome P-450. *J. Biol. Chem.* **251**:7929–7939 (1976).
19. Spatz, L., and P. Strittmatter. A form of cytochrome $b_5$ that contains an additional hydrophobic sequence of 40 amino acid residues. *Proc. Natl. Acad. Sci. U. S. A.* **68**:1042–1046 (1971).
20. Strittmatter, P., and S. F. Velick. The isolation and properties of microsomal cytochrome. *J. Biol. Chem.* **221**:253–264 (1956).
21. Kaul, K. L., R. F. Novak, and K. P. Vatsis. The interaction of imidazole with purified forms of liver microsomal cytochrome P-450 as studied by $^1$H FT-NMR spectroscopy. *Fed. Proc.* **39**:393 (1980).
22. Solomon, I. Relaxation processes in a system of two spins. *Phys. Rev.* **99**:559–565 (1955).
23. Solomon, I., and N. Bloembergen. Nuclear magnetic interactions in the HF molecule. *J. Chem. Phys.* **25**:261–266 (1956).
24. Bloembergen, N. Proton relaxation times in paramagnetic solutions. *J. Chem. Phys.* **27**:572–573 (1957).
25. Swift, T. J., and R. E. Connick. NMR relaxation mechanisms of $O^{17}$ in aqueous solutions of paramagnetic cations and the lifetime of water molecules in the first coordination sphere. *J. Chem. Phys.* **37**:307–320 (1962).
26. Asakura, T. Heme spin-label studies on human hemoglobin. *Ann. N. Y. Acad. Sci.* **222**:68–85 (1973).
27. Gupta, R. K., and S. H. Koenig. Some aspects of pH and temperature dependence of the NMR spectra of cytochrome *c*. *Biochem. Biophys. Res. Commun.* **45**:1134–1143 (1975).
28. Gupta, R. K., and A. S. Mildvan. Nuclear relaxation studies on human methemoglobin. *J. Biol. Chem.* **250**:246–253 (1975).
29. Hershberg, R. D., and B. Chance. Optical and magnetic resonance studies of formate binding to horse liver catalase and sperm whale myoglobin. *Biochemistry* **14**:3885–3891 (1975).
30. Vuk-Pavlovic, S., and B. Benko. The heme-environment in horseradish peroxidase as seen by $^1$H magnetic resonance. *Biochem. Biophys. Res. Commun.* **66**:1154–1159 (1975).
31. Griffin, B. W., and J. A. Peterson. *Pseudomonas putida* cytochrome P-450. *J. Biol. Chem.* **250**:6445–6451 (1975).
32. Asakura, T., J. S. Leigh, Jr., H. R. Drott, T. Yonetani, and B. Chance. Structural measurements in hemeproteins: use of spin-labelled protoheme as a probe of heme environment. *Proc. Natl. Acad. Sci. U. S. A.* **68**:861–865 (1971).
33. Koenig, S. H., and W. E. Schillinger. Nuclear magnetic relaxation dispersion in protein solutions. II. Transferrin. *J. Biol. Chem.* **244**:6520–6526 (1969).
34. Luz, Z., and S. Meiboom. Proton relaxation in dilute solutions of cobalt (II) and nickel (II) ions in methanol and the rate of methanol exchange of the solvation sphere. *J. Chem. Phys.* **40**:2686–2692 (1964).
35. Swift, T. J. The paramagnetic linewidth, in *NMR of Paramagnetic Molecules* (G. N. La Mar, R. Holm, and W. D. Horrocks, eds.). Academic Press, New York, 53–86 (1974).
36. Novak, R. F., I. M. Kapetanovic, and J. J. Mieyal. Nuclear magnetic resonance studies of substrate-hemoprotein complexes in solution. *Mol. Pharmacol.* **13**:15–30 (1977).
37. Vatsis, K. P., K. L. Kaul, and R. F. Novak. $^1$H FT NMR studies of the effects of acetanilide and imidazole on the solvation sphere of the heme iron atom in cytochromes P-450$_{I,M_2}$, P-450$_{I,M_4}$, and $b_5$, in *Microsomes, Drug Oxidations, and Chemical Carcinogenesis* (M. J. Coon, A. H. Conney, R. W. Estabrook, H. V. Gelboin, J. R. Gillette, and P. J. O'Brien, eds.), Vol. 1. Academic Press, New York, 183–186 (1980).
38. Novak, R. F., K. P. Vatsis, and K. L. Kaul. $^1$H FT NMR $T_1$ relaxation rate studies on the competitive interaction of imidazole and acetanilide with purified isozymes of rabbit liver microsomal cytochrome P-450, in *Biochemistry, Biophysics, and Regulation of Cytochrome P-450* (J-Å. Gustafsson, J. Carlsted-Duke, A. Mode, and J. Rafter, eds.). Elsevier/North-Holland Biomedical Press, Amsterdam, 583–586 (1980).
39. Hajek, K. K., N. I. Cook, and R. F. Novak. Noncompetitive inhibition by imidazole of cytochrome P-450-catalyzed oxidation reactions in rabbit liver microsomes. *Pharmacologist* **23**:5 (1981).
40. Novak, R. F., and K. P. Vatsis. The effect of phospholipid on the interaction of acetanilide with cytochromes P-450$_{I,M_2}$ and P-450$_{I,M_4}$ as studied by $^1$H FT NMR spectroscopy. *Pharmacologist* **21**:231 (1979).

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