MOLECULAR PHARMACOLOGY, 13, 15-30

Nuclear Magnetic Resonance Studies of Substrate-Hemoprotein Complexes in Solution

I. The Interaction of Xylidine with Myoglobin, Hemoglobin, and Cytochrome P450

R. F. NOVAK,[1] I. M. KAPETANOVIC,[2] AND J. J. MIEYAL

Department of Pharmacology, Northwestern University Medical and Dental Schools, Chicago, Illinois 60611

(Received March 26, 1976)

(Accepted August 19, 1976)

SUMMARY

NOVAK, R. F., KAPETANOVIC, I. M. & MIEYAL, J. J. (1977) Nuclear magnetic resonance studies of substrate-hemoprotein complexes in solution. I. The interaction of xylidine with myoglobin, hemoglobin, and cytochrome P450. *Mol. Pharmacol.*, **13**, 15-30.

^{1}H nuclear magnetic resonance longitudinal relaxation time (T_1) measurements were used to study the interaction of xylidine (2,6-dimethylaniline) with the hemoproteins myoglobin, hemoglobin, and solubilized rat liver microsomal cytochrome P450. Upon addition of various amounts of ferrimyoglobin, ferrihemoglobin, or ferricytochrome P450 to solutions of xylidine, the T_1 values for the methyl and phenyl protons of the xylidine molecule decreased markedly. The observed changes showed that xylidine was much more sensitive to addition of cytochrome P450 than to myoglobin or hemoglobin. The relative effects upon the specific moieties of the substrate also differed; whereas myoglobin produced essentially the same effect upon the relaxation rates ($T_{1_p}^{-1}$) of the phenyl and methyl protons of xylidine, hemoglobin and cytochrome P450 produced differential changes in the ($T_{1_p}^{-1}$) values, phenyl > methyl. These results were shown to reflect specific xylidine-ferrihemoprotein complexes. A nonsubstrate, noninteracting internal reference (tetramethylammonium phosphate) was added to each sample as a control both for experimental variation and for effects due to changes in solution viscosity; in no case was a substantial change in the T_1 value of the reference observed. Variable-temperature studies confirmed that the residence times for the xylidine-ferrimyoglobin and xylidine-cytochrome P450 interactions were in the region of fast exchange with respect to the NMR time scale (i.e., $\tau_M \ll T_{1_M}$). Formation of the cyano derivative of ferrimyoglobin or ferrihemoglobin changes the paramagnetic spin state from $S = 5/2$ to $S = 1/2$, and conversion to the carbonmonoxyferrous derivative results in a diamagnetic species, $S = 0$. When the ferrihemoproteins were so converted *in situ* in the presence of xylidine, the T_1 values for the xylidine moieties increased. Since the carbonmonoxyferrous derivatives of all three hemoproteins are diamagnetic, the latter type of experiment was performed in all cases to give the control values ($1/T_1^0$),

This work was supported in part by Grants (to J. J. M.) GM 20050 from the National Institute of General Medical Sciences and C75-21 from the Chicago and Illinois Heart Associations. (Present address: Department of Pharmacology, Case Western Reserve University, Cleveland, Ohio.) The results of this study were presented in part at the Fourth Annual Pharmacology-Toxicology Program Symposium, Washington, D. C., November 1975.

[1] National Institutes of Health Postdoctoral Fellow (GM 05225-01), 1975-1976.

[2] National Institutes of Health Predoctoral Trainee (Training Grant GM 11062 to the Department of Pharmacology).

which allowed calculation of the paramagnetic relaxation rate values, $1/T_{1_p}$. The ^1H T_1 results, in conjunction with the value obtained for the dissociation constant of the xylidine–cytochrome P450 complex ($K_D = 4.1 \times 10^{-4}$ M) and the estimated correlation time for the complex, allowed calculation of distances between the heme iron atom and specific portions of the substrate molecule.

INTRODUCTION

The hemoprotein cytochrome P450 from liver microsomes is the terminal oxidase enzyme localized to the liver endoplasmic reticulum, which catalyzes the oxidative metabolism of a wide variety of chemical agents, including fatty acids, steroids, drugs, insecticides, and carcinogens (1, 2). Although this enzyme system has been widely studied, the detailed mechanism by which it carries out the selective transfer of activated oxygen to specific moieties on substrate molecules remains unknown. Ultraviolet difference spectroscopy has been used extensively to investigate the binding of drug molecules to cytochrome P450 (3–5). Most drugs when combined with ferricytochrome P450 cause a shift in the Soret absorbance band of the hemoprotein. The resulting difference spectra have been classified as type I or type II, depending upon the direction of the observed shift, i.e., hypsochromic (type I) or bathochromic (type II). These shifts have been interpreted to be a reflection of direct coordination (type II) or indirect (type I) interaction of the substrate with the iron atom of the heme group (4, 6). The purpose of the present work was to provide further information on the nature of the interaction of substrates with cytochrome P450 by directly monitoring changes in specific substrate moieties via nuclear magnetic resonance spectroscopy.

NMR spectroscopy is a powerful technique for investigation of complex formation in solution, ultimately allowing one to elucidate the molecular structure of the complex. The technique is ideally suited for investigation of the interaction of substrates with proteins that contain paramagnetic centers (such as the hemoproteins), because marked changes may be observed in the NMR properties of those substrate moieties which most closely approach the paramagnetic site in the complex. In particular, NMR longitudinal relaxation time (T_1) measurements can be used to observe differential relaxation effects on different parts of a substrate molecule and thereby allow evaluation of the substrate orientation and distance from the paramagnetic center. Such data should allow evaluation of the type of substrate binding and, potentially, deduction of the structural details of the substrate-binding cavity. For example, substrates may interact with the paramagnetic heme metal ion by direct coordination, by indirect or "outer sphere" coordination, or by association with a binding cavity in the protein which is located proximal to the heme moiety. NMR techniques analogous to those described in this article have been employed recently to study certain ligand interactions with the hemoproteins myoglobin, horseradish peroxidase, catalase, and cytochrome P450 (7–10).

In previous publications (11, 12) we reported that the oxygen-dependent hydroxylation of aniline, which is a reaction typically catalyzed by cytochrome P450, can be catalyzed about as effectively by hemoglobin and myoglobin under similar conditions. The 2,6-dimethyl analogue of aniline, xylidine (whose NMR spectrum is better suited for differential T_1 studies; *see below*) is also hydroxylated by these three hemoproteins. Therefore xylidine seemed well suited for the study of how the nature of the substrate-hemoprotein interaction might correlate with the subsequent hemoprotein-catalyzed hydroxylation reaction. We report here a comparative study of the interaction of xylidine with Mb,[3] Hb, and solubilized P450 as delineated by longitudinal relaxation time changes for the proton moieties of the substrate. The observed changes reflected that xylidine was much more sen-

[3] The abbreviations used are: Mb, myoglobin; Hb, hemoglobin; KPi, potassium phosphate buffer; DSS, Sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

sitive (approximately 300 times) to the addition of P450 than to Hb or Mb. Mb produced essentially the same changes in T_1 for the methyl and phenyl protons of xylidine, but Hb and P450 produced differential changes; i.e., phenyl > methyl. These changes may reflect different affinities and molecular orientations for the different xylidine-hemoprotein complexes.

METHODS

Ferrimyoglobin from sperm whale skeletal muscle (type II, twice recrystallized, Sigma Chemical Company) was treated with Chelex resin (Bio-Rad Chelex 100, 50–100 mesh, sodium form) to remove contaminating paramagnetic metal ions and passed over Sephadex G-25 equilibrated with 0.02 M KPi, pH_{obs} 7.5 in D_2O. The latter step accomplished both removal of small molecules and exchange of protons for deuterium.

Hemoglobin (human, type IV, twice recrystallized, Sigma) was treated with $K_3Fe(CN)_6$ to convert it fully to the oxidized form, Hb^{3+} and was then passed over a Sephadex G-25 column previously equilibrated with 0.02 M KPi, pH_{obs} 7.5 in D_2O. The concentration of hemoglobin in solution was determined according to the assay of Van Kampen and Zijlstra (13), whereby the hemoglobin is converted to the cyanoferric form by the action of $K_3Fe(CN)_6$ and KCN and the absorbance is measured at 541 nm; ϵ per heme = 11 mM^{-1} cm^{-1} (internationally accepted value; i.e., 44/hemoglobin tetramer). The concentrations of myoglobin solutions were estimated analogously, ignoring the small difference in extinction coefficient. The spin states of Mb and Hb in solution were checked by recording the absolute uv-visible spectra of the solutions. The positions of the Soret bands were compared with the fluoro (S = 5/2) and cyano (S = 1/2) derivatives of the respective hemoproteins (14). At 35° aquoferrimyoglobin and aquoferrihemoglobin exist 88% in the high-spin (S = 5/2) state.

Cytochrome P450 was solubilized from the liver microsomes of phenobarbital-treated rats. Thirty-five male Holtzman rats, 70–80 g, were treated with phenobarbital (75 mg/kg of body weight) for 3 days and fasted for 24 hr prior to sacrifice. Liver microsomes were isolated as previously described (15). The microsomes were diluted to a protein concentration of 13–15 mg/ml with 10 mM EDTA, 10 mM dithiothreitol, and 20% glycerol and sonicated for 5 × 1 min at ≤ 8°. Cholic acid sodium salt (Sigma) was added at 1 mg/mg of protein, and the solution was stirred in the cold (4°) for 1 hr. The ammonium sulfate extraction procedure of Autor *et al.* (16) was then used for partial purification of the solubilized cytochrome P450. The concentrations of P450 and P420 were determined by the dithionite-reduced carbon monoxide minus dithionite-reduced difference spectrum method of Omura and Sato (17). P450 content was quantitated from $\Delta\epsilon_{450-490\ nm}$ = 91 mM^{-1} cm^{-1}. P420 content was obtained from $\Delta\epsilon_{420-490\ nm}$ = 111 mM^{-1} cm^{-1} after correction for the contribution of $P450^{2+}$ at 420 nm; i.e., $\Delta\epsilon_{420-490\ nm} = -41\ mM^{-1}\ cm^{-1}$ (17). Alternatively, the sum of P450 and P420 was determined by converting the P450 completely to P420 with KSCN. The values obtained from all these procedures agreed within 5%. Total heme content was determined from the dithionite reduced minus pyridine-oxidized difference spectra, using $\Delta\epsilon_{555-575\ nm}$ = 32.4 mM^{-1} cm^{-1} (18). The concentration of the solubilized cytochrome P450 in our preparations after the two-step ammonium sulfate precipitation ranged from 15.4 to 18.8 nmoles/ml, with a specific content of 2.3–2.8 nmoles/mg of protein. Protein was determined according to Lowry *et al.* (19). Cytochrome P420 content ranged from 2%, for freshly prepared cytochrome P450, to ≦15%, for samples which were stored frozen. It was confirmed that no significant change in the relative concentrations of P450 and P420 would occur at 37° during the time course of a typical NMR experiment. The sum of the P450 and P420 content accounted for at least 95% of the total heme content. Therefore no more than 5% non-P450-related hemoprotein contaminants (i.e., cytochrome b_5, Hb) were present in these preparations. The actual concentration of P450 (not total heme) was employed in the NMR ex-

periments (see legend to Table 3). Xylidine (2,6-dimethylaniline, Eastman Organic Chemicals), D_2O (99.8%, Aldrich), and KCN (Fisher) were used as purchased. Tetramethylammonium phosphate was prepared by neutralization of tetramethylammonium hydroxide (Eastman) with H_3PO_4 (Fisher). All solutions were treated with Chelex resin (Bio-Rad Chelex 100, 50–100 mesh, sodium form) prior to use in order to remove contaminating paramagnetic ions. The cyanometmyoglobin and cyanomethemoglobin derivatives were prepared directly in the NMR tubes by addition of microliter volumes of 0.1 M KCN in D_2O. The corresponding carbonmonoxyferrous derivatives were prepared by reduction with dithionite (Fisher) under a stream of carbon monoxide (Matheson).

UV-Visible spectra were recorded using an Aminco DW-2 dual-beam spectrophotometer operating in the split beam mode. Spectra were recorded under ambient conditions unless otherwise noted.

^1H NMR spectra were recorded using a Perkin-Elmer R-32 spectrometer operating at 90 MHz and interfaced for Fourier transform technique with a Nicolet TT-7 computer package. The ^1H longitudinal relaxation times, T_1, of the signals were measured using the standard inversion-recovery sequence, 180°–τ–90° (see Fig. 1B).

Treatment of data. The effect of electron spin–nuclear spin interaction upon the longitudinal relaxation time (T_1) is described by the Solomon-Bloembergen equation[4] (21, 22):

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

T_{1_M} is the relaxation time of a nucleus bound in the proximity of a paramagnetic metal ion. Here γ_I is the nuclear gyromagnetic ratio, γ_S is the electron gyromagnetic ratio, $\hbar$ is Planck's constant divided by 2π, S is the total electron spin, r is the length of the vector between the nuclear spin dipole and the electron spin dipole, τ_c is the correlation time which describes molecular motions that modulate the electron-nuclear dipolar coupling, and ω_I and ω_S are the nuclear and electron precession frequencies, respectively. The second part of Eq. 1 is the contact term, or isotropic hyperfine term. A/h is the hyperfine coupling constant, and τ_e is the correlation time for the hyperfine interaction. Since the typical value of the hyperfine coupling constant A/h is less than 1 MHz (23), this hyperfine term of Eq. 1 is considered negligible relative to the dipolar term for values of $r < 20$ Å. Hence for high-spin ($S = 5/2$) and low-spin ($S = 1/2$) forms of the ferrihemoprotein, Eq. 1 reduces to

$$r\ (\text{A}) = 812\ (T_{1_M}f(\tau_c))^{1/6} \quad (2a)$$

$$r\ (\text{A}) = 540\ (T_{1_M}f(\tau_c))^{1/6} \quad (2b)$$

where $f(\tau_c)$ refers to the expression in brackets in the first term of Eq. 1.

The correlation time τ_c for the dipolar term of Eq. 1 is given by

$$\frac{1}{\tau_c} = \frac{1}{\tau_r} + \frac{1}{\tau_s} + \frac{1}{\tau_M} \quad (3)$$

where τ_r is the rotational correlation time, τ_s is the electron spin relaxation time, and τ_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 τ_c are given in Eq. 3, and it follows that the fastest

[4] Inherent in the use of Eq. 1 as given is the assumption that the substrate approaches along the axis normal to the plane of the heme. This assumption appears justified at least for the well-characterized hemoproteins, because our estimate of the proton-to-Fe^{3+} distances for water in aquometmyoglobin, using Eq. 1, agreed well (2.50 Å) with previously reported values obtained from NMR data (7), and the distance estimates are also consistent with the oxygen-to-Fe^{3+} distance for this aquometmyoglobin as determined from X-ray crystallographic data [i.e., 2.1 Å (20)].

process (shortest correlation time) will contribute most significantly to τ_c. In general, τ_s is on the order of 10^{-11}–10^{-10} sec for high- and low-spin ferric hemoproteins while $\tau_r \sim 10^{-9}$–10^{-8} sec and $\tau_M \sim 10^{-4}$–10^{-6} sec (24–26). Since τ_r and τ_M are one to seven orders of magnitude greater than τ_s, they would be expected to contribute negligibly to the value of $1/\tau_c$. Equation 2 then reduces to $1/\tau_c \cong 1/\tau_s$. Correlation times have been reported for ligand-hemoprotein complexes under various conditions. The formate-Mb complex gave $\tau_c = 3 \times 10^{-11}$ sec, while for the formate-catalase complex it was 5×10^{-11} sec (7). Horseradish peroxidase and fluorohorseradish peroxidase gave τ_c values of 9.0×10^{-11} and 2.7×10^{-10} sec, respectively, as determined from EPR linewidth measurements (8). Values of τ_c determined from extrapolated EPR linewidth data for cytochrome P450 ranged from 1.5×10^{-11} to 1.4×10^{-10} sec for the camphor and metyrapone complexes, respectively (9). However, the values of τ_c determined from EPR linewidth data plotted against reciprocal temperature were reported to be 3.0×10^{-10} and $3.8\ 10^{-10}$ sec, respectively, for the same complexes. Also, τ_s values reported for other Fe^{3+}-containing proteins have been approximately 10^{-10} sec (27, 28). Hence, for the xylidine-ferrihemoprotein complexes, τ_c can be expected to lie in the range $10^{-11} < \tau_c < 10^{-10}$.

For a given substrate moiety, the contribution to the observed longitudinal relaxation rate due to the paramagnetic ion, $T_{1_p}^{-1}$, is given by

$$\frac{1}{T_{1_p}} = \frac{1}{T_{1_{obs}}} - \frac{1}{T_1^0} \qquad (4)$$

where $1/T_1^0$ is the relaxation rate in the absence of the paramagnetism. T_{1_M} can then be calculated from the following equation (29):

$$\frac{1}{T_{1_p}} = \frac{\alpha M}{T_{1_M} + \tau_M} \qquad (5)$$

where α_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 $T_{1_M} \cong \alpha_M T_{1_p}$.

Hence the calculation of the distance from various parts of the substrate to the paramagnetic heme iron atom using Eq. 2a or b requires (a) a knowledge of the spin state of the heme paramagnetic iron atom of the hemoprotein under ambient conditions, (b) a dissociation constant for the substrate-hemoprotein complex (K_D), (c) a value of the correlation time τ_c, and (d) a measure of the $T_{1_p}^{-1}$ values for the proton moieties of the substrate.

RESULTS

The ^1H NMR spectrum of xylidine, with internal reference $(CH_3)_4N^+$, is given in Fig. 1A. The assignment of signals is straightforward, with the phenyl protons giving an AB_2 spectrum (30) at $\delta = 6.6$–7.4 ppm and the methyl groups giving a singlet at $\delta = 2.2$ ppm; this allows observation of individual changes in T_1 for the phenyl and methyl moieties of xylidine (Fig. 1A). A typical series of partially relaxed Fourier transform spectra, from which T_1 values can be calculated for the signals of the internal reference and the methyl group of xylidine, is presented in Fig. 1B. Additions of aliquots of solutions of Mb^{3+} to a solution of 14 mM xylidine produced marked decreases in the ^1H T_1 relaxation times of the phenyl and methyl protons, with virtually no change in the T_1 of the internal reference (Fig. 2 and Table 1). Similar qualitative results were obtained for Hb^{3+} and $P450^{3+}$ (Tables 2 and 3). The outstanding quantitative difference was that much lower concentrations of P450 were required to produce effects comparable to those of Hb and Mb. Approximately the same increases in relaxation rates $[\Delta(1/T_1)]$ for the methyl and phenyl protons of xylidine were effected by Mb^{3+}, and the plot of $\Delta(1/T_1)$ vs. Mb^{3+} concentration was linear through 300 μM Mb^{3+} (Fig. 3A). On the other hand, significantly different increases in the relaxation rates for the methyl and phenyl ring protons were observed when Hb^{3+} or $P450^{3+}$ was added (Fig. 3B and C). For example, at 300 μM heme, myoglobin gave 0.84 sec^{-1} and 0.83 sec^{-1} for the methyl and phenyl relaxation rates $\Delta(1/T_1)$, respectively, while Hb^{3+} at the same heme concentration gave 0.46 sec^{-1} and 0.67 sec^{-1}, respectively. Cyto-

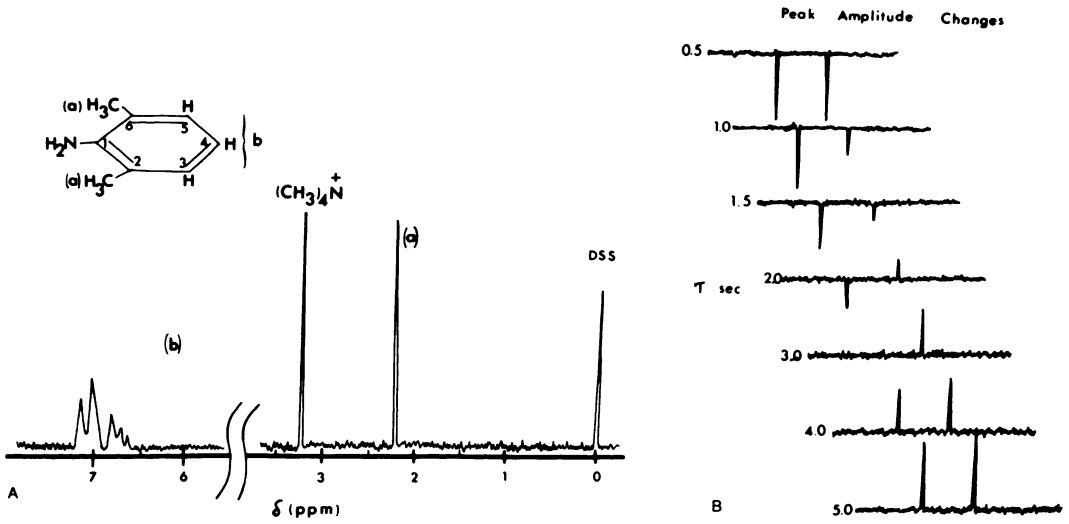


FIG. 1. *NMR Spectra.*

A. ^{1}H spectrum of 14.2 mM xylidine (2,6-dimethylaniline) in 0.1 M KPi, pH 7.5 (99.8% D_2O), at 34.5°. The tetramethylammonium phosphate reference occurs at 3.2 ppm. The phenyl protons give rise to the multiplet at $\delta \cong 7$ (signals *b*), and the methyl groups give rise to a singlet (signal *a*). The chemical shift values are given relative to DSS.

B. Typical T_1 spectra obtained from use of the inversion-recovery sequence (180°–τ–90°). T_1 values were calculated from the slope of a line obtained by plotting $\ln(M_\infty - M_\tau)$ vs. τ, where M_∞ is the signal amplitude obtained after a 90° pulse and M_τ is the signal amplitude obtained using the appropriate delay time τ in the 180°–τ–90° sequence.

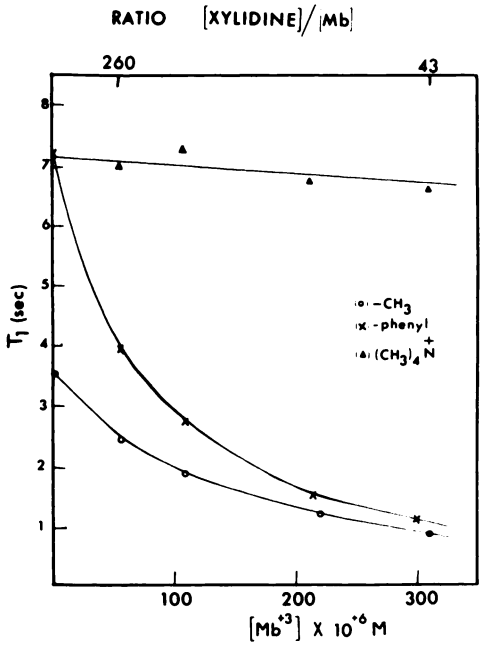


FIG. 2. *Variation in T_1 for phenyl and methyl protons with increasing myoglobin concentration*

Little change is observed in the T_1 of the internal reference, $(CH_3)_4N^+$ (7.4–6.9 sec).

chrome P450 at 1.3 μM gave 0.52 sec^{-1} and 0.86 sec^{-1} for the methyl and phenyl relaxation rates, respectively.

In order to verify that the observed T_1 changes were associated with specific substrate-hemoprotein interactions (i.e., the result of interactions involving sites at or near the paramagnetic iron atom), we modified the state of the heme iron atom *in situ* in such a fashion as to cause predictable changes. Formation of the cyano derivative of ferrimyoglobin or ferrihemoglobin changes the paramagnetic spin state from high-spin ($S = 5/2$) to low-spin ($S = 1/2$). Therefore, upon formation of the cyanoferrihemoprotein derivative *in situ*, the $T_{1_{obs}}$ value of the substrate should increase as a result of either the diminished paramagnetism or displacement of the substrate by the directly coordinated cyanide anion. Therefore the addition of cyanide to the solution of xylidine containing Mb^{3+} or Hb^{3+} should cause $T_{1_{obs}}$ to increase in value. This was the observed result. $T_{1_{obs}}$ for the methyl and phenyl protons changed from 0.89 sec and 1.03 sec

TABLE 1

Effects of myoglobin on T_1 values for xylidine

A. Ferrimyoglobin

Group	Heme	Xylidine	Xylidine/Mb	KCN	$Na_2S_2O_4$-CO	$T_{1_{obs}}$	$\Delta(1/T_1)$[a]	$T_{1_p}^{-1}$[b]
	μM	*mM*		*mM*		*sec*	sec^{-1}	sec^{-1}
$-CH_3$		14.2				3.50		
Phenyl		14.2				7.20		
$-CH_3$	300	14.2	47		–[c]	3.52	~0.00	
Phenyl	300	14.2	47		–[c]	7.14	~0.00	
$-CH_3$	300	14.2	47			0.89	0.84	0.84
Phenyl	300	14.2	47			1.03	0.83	0.83
$-CH_3$	300	14.2	47	3.0		2.32	0.15	0.15
Phenyl	300	14.2	47	3.0		2.92	0.20	0.20

B. Ferromyoglobin

Group	Heme	Xylidine	Xylidine/heme	$Na_2S_2O_4$	$Na_2S_2O_4$-CO	$T_{1_{obs}}$	$\Delta(1/T_1)_{obs}$[a]	$T_{1_p}^{-1}$[b]
	μM	*mM*				*sec*	sec^{-1}	sec^{-1}
$-CH_3$		14.2		–[d]		3.97		
Phenyl		14.2		–[d]		11.71		
$-CH_3$	300	14.2	47		–[c]	3.52	0.03	
Phenyl	300	14.2	47		–[c]	7.14	0.05	
$-CH_3$	300	14.2	47			0.89	0.87	0.84
Phenyl	300	14.2	47			1.03	0.88	0.83
$-CH_3$	300	14.2	47	–[d]		2.87	0.10	0.06
Phenyl	300	14.2	47	–[d]		5.08	0.11	0.06

[a] $\Delta(1/T_1) = 1/T_{1_{obs}} - 1/T_{1_{blank}}$, where $\Delta(1/T_1)$ refers to the difference between $1/T_1$ of the xylidine proton moieties in the presence of a certain hemoprotein (in any form) and the $1/T_1$ value of the xylidine moieties in the total absence of hemoprotein.

[b] $T_{1_p}^{-1} = 1/T_{1_{obs}} - 1/T_{1_{Mb^{3+}\text{-}CO}}$, where $T_{1_p}^{-1}$ is defined as the difference between $1/T_1$ in the presence of a certain hemoprotein (in any form) and the $1/T_1$ value of the xylidine moieties in the presence of the reduced carbonyl form (diamagnetic) of the hemoprotein.

[c] Excess-saturated.

[d] Excess.

at 300 μM Mb^{3+} without KCN to 2.92 sec and 2.3 sec, respectively, at 3 mM KCN (Fig. 4 and Table 1). Similar results were observed for Hb^{3+} (Table 2). This $T_{1_{obs}}$ value, however, does not approach the $T_{1_{obs}}$ value of the substrate either in the absence of hemoprotein (i.e., 2.3 sec vs. 3.5 sec) or in the presence of the reduced carbonyl form, which is diamagnetic, suggesting that xylidine continues to interact with the cyano derivative (see DISCUSSION).

An additional experiment to modify the equilibrium spin state was carried out by the addition of F^- ion. Mb^{3+} and Hb^{3+} exist as 88% high-spin under ambient conditions as determined from ultraviolet spectroscopy (see METHODS). The addition of fluoride ion will convert this to the 100% high-spin form (14). By analogy to the case for CN^-, F^- should cause a small decrease in $T_{1_{obs}}$ if the xylidine continues to interact with the heme iron atom in the presence of fluoride (see DISCUSSION). The addition of 15 mM F^- to a solution of xylidine containing 300 μM Mb^{3+} did cause the $T_{1_{obs}}$ value of the methyl protons to decrease further, going from approximately 1.0 sec to 0.65 sec (Fig. 4).

Formation of the reduced carbonyl forms of Mb, Hb, and cytochrome P450, which are most likely diamagnetic (14, 31, 32), provided a means for evaluation of protein contributions to the measured relaxation time changes (see DISCUSSION). The $1/T_1^0$ so obtained allowed determi-

TABLE 2

Effects of hemoglobin on T_1 values for xylidine

For definition of symbols, see footnotes to Table 1.

A. Ferrihemoglobin

Group	Heme	Xylidine	Xylidine/heme	KCN	$Na_2S_2O_4$-CO	$T_{1_{obs}}$	$\Delta(1/T_1)^a$	$T_{1_p}^{-1\,b}$
	μM	*mM*		*mM*		*sec*	sec^{-1}	sec^{-1}
—CH_3		14.2				3.50		
Phenyl		14.2				7.20		
—CH_3	300	14.2	47		—[c]	3.36	0.01	
Phenyl	300	14.2	47		—[c]	6.49	0.02	
—CH_3	300	14.2	47			1.34	0.46	0.45
Phenyl	300	14.2	47			1.24	0.67	0.65
—CH_3	300	14.2	47	3.5		2.51	0.11	0.10
Phenyl	300	14.2	47	3.5		3.86	0.12	0.10

B. Ferrohemoglobin

	Heme	Xylidine	Xylidine/heme	$Na_2S_2O_4$	$Na_2S_2O_4$-CO	$T_{1_{obs}}$	$\Delta(1/T_1)^a$	$T_{1_p}^{-1\,b}$
	μM	*mM*				*sec*	sec^{-1}	sec^{-1}
—CH_3		14.2		—[d]		3.97		
Phenyl		14.2		—[d]		11.71		
—CH_3	300	14.2	47		—[c]	3.36	0.05	
Phenyl	300	14.2	47		—[c]	6.49	0.07	
—CH_3	300	14.2	47			1.34	0.49	0.45
Phenyl	300	14.2	47			1.24	0.72	0.65
—CH_3	300	14.2	47	—[d]		2.31	0.18	0.14
Phenyl	300	14.2	47	—[d]		3.85	0.17	0.11

[a] $\Delta(1/T_1) = 1/T_{1_{obs}} - 1/T_{1_{blank}}$.

[b] $1/T_{1_p} = 1/T_{1_{obs}} - 1/T_{1_{Hb^{2+}\text{-}CO}}$.

[c] Excess-saturated.

[d] Excess.

TABLE 3

Effects of ferricytochrome P450 on T_1 values for xylidine.

For definition of symbols, see footnotes to Table 1. In a separate experiment, utilizing samples of P450 which contained different amounts of P420, it was confirmed that changes in T_1 were dependent upon P450 concentration rather than on total heme concentration.

Group	Cytochrome P450	Xylidine	Xylidine/P450	$Na_2S_2O_4$-CO	T_1	$\Delta(1/T_1)^a$	$T_{1_p}^{-1\,b}$
	μM	*mM*			*sec*	sec^{-1}	sec^{-1}
—CH_3		14.2			3.50		
Phenyl		14.2			7.20		
—CH_3	1.28	14.2	11,094		1.24	0.52	
Phenyl	1.28	14.2	11,094		1.00	0.86	
—CH_3	1.28	14.2	11,094	—[c]	2.14	0.18	0.34
Phenyl	1.28	14.2	11,094	—[c]	2.06	0.35	0.51
—CH_3	2.56	14.2	5,500		0.85	0.89	
Phenyl	2.56	14.2	5,500		0.52	1.78	

[a] $\Delta T_1^{-1} = 1/T_{1_{obs}} - 1/T_{1_{blank}}$.

[b] $T_{1_p}^{-1} = 1/T_{1_{obs}} - 1/T_{1_{P_{450}^{2+}\text{-}CO}}^{0}$.

[c] Excess-saturated.

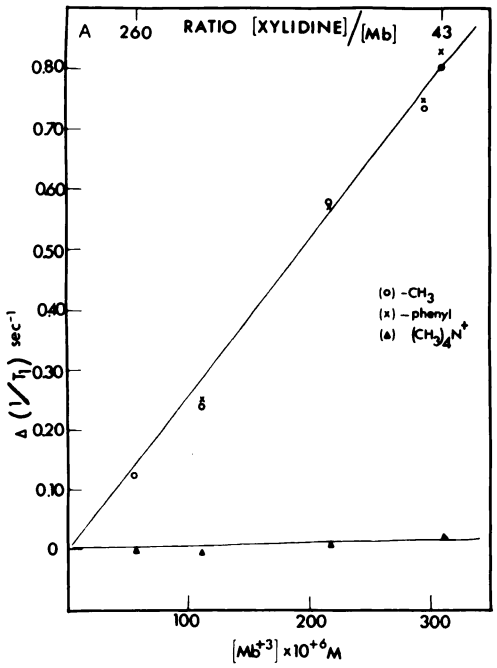
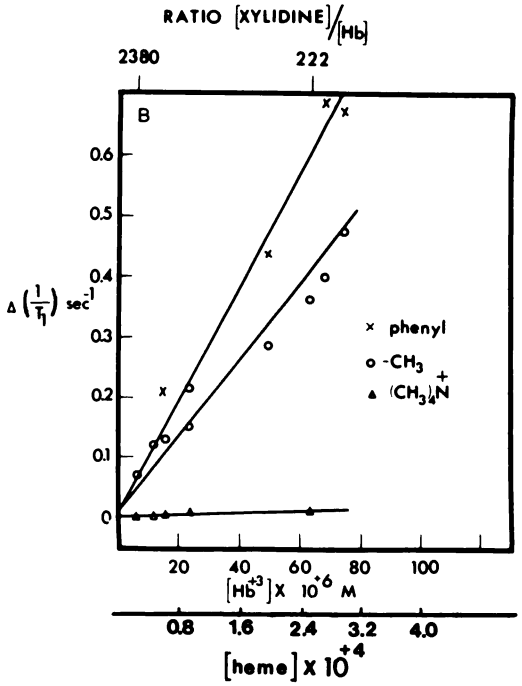
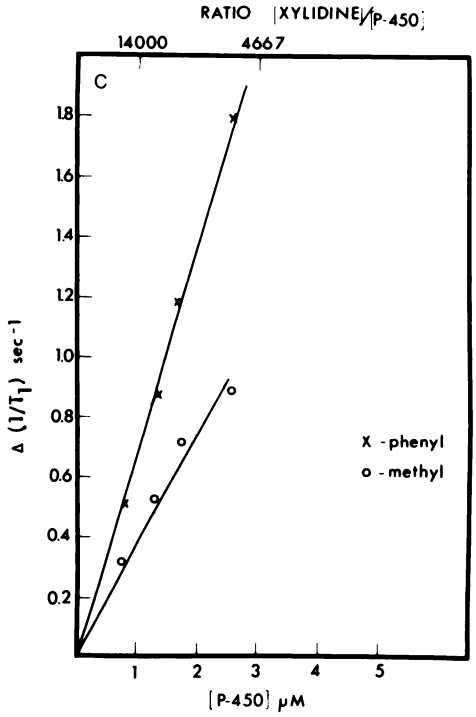


FIG. 3. *H Relaxation Rate Changes*

A. A plot of $\Delta(1/T_1)$ for the phenyl and methyl protons of xylidine as a function of increasing myoglobin concentration. Both phenyl and methyl protons give the same $\Delta(1/T_1)$ values, $\Delta(1/T_1) = 1/T_{1_{obs}} - 1/T_{1_{blank}}$. See footnotes to Table 1 for definition of terms.



B. $\Delta(1/T_1)$ values for the phenyl and methyl protons of xylidine with increasing concentration of hemoglobin. $\Delta(1/T_1) = 1/T_{1_{obs}} - 1/T_{1_{blank}}$. See footnotes to Table 1 for definition of terms.



C. $\Delta(1/T_1)$ values for phenyl and methyl protons of xylidine with increasing concentration of cytochrome P450. $\Delta(1/T_1) = 1/T_{1_{obs}} - 1/T_{1_{blank}}$. See footnotes to Table 1 for definition of terms.

nation of $1/T_{1_p}$ values, where $1/T_{1_p}$ represents relaxation due only to the paramagnetic ion. Formation of the ferrous carbonyl form of each of the hemoproteins in the presence of xylidine resulted in an increase in $T_{1_{obs}}$ values for xylidine. For example, at 300 μM Mb, the $T_{1_{obs}}$ values for the methyl and phenyl protons were 0.89 sec and 1.03 sec, respectively. Upon formation of the carbonmonoxyferromyoglobin derivative, the methyl and phenyl protons gave $T_{1_{obs}}$ times of 3.5 sec and 7.2 sec, respectively, which more closely approach the values obtained in the absence of hemoprotein. The complete results for Mb, Hb, and cytochrome P450 are given in Tables 1, 2, and 3. In contrast, cytochrome P450 appears to have a greater effect upon the substrate even in the reduced CO form (Table 3).

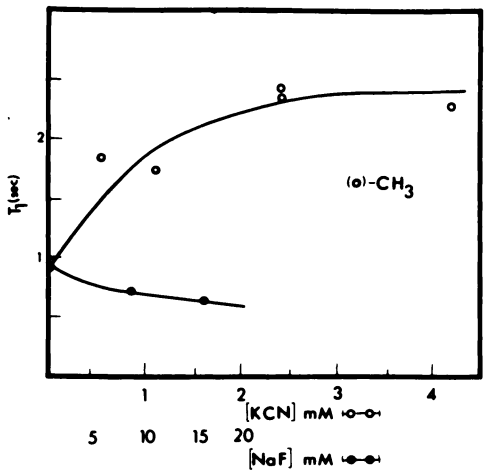


FIG. 4. *Changes in T_1 for xylidine methyl protons at 300 μM Mb^{3+} upon addition of NaF and KCN*

Higher concentrations of NaF were used, since fluoride anion is a much weaker ligand than cyanide anion. The hemoprotein was added to the xylidine solution, which contained the NaF.

Temperature profile. Arrhenius plots of the temperature dependence of the longitudinal relaxation times are presented in Fig. 5A and B. The increase in the proton magnetic relaxation rate of the methyl group with decreasing temperature in the presence of Mb^{3+} and cytochrome $P450^{3+}$, along with the value of the activation energy derived from these data E_{act} = 2.66 kcal/mole, demonstrates that the interaction is in the fast exchange region with respect to the NMR time scale (29, 33) (see DISCUSSION).

Ferrous forms of hemoproteins. It was also of interest to investigate the possibility of interaction between substrate and the reduced form of the hemoproteins. Sodium dithionite ($Na_2S_2O_4$) is both an effective reducing agent for Mb^{3+}, Hb^{3+}, and cytochrome $P450^{3+}$ and a scavenger of dissolved oxygen. Reduction of the hemoprotein changes the spin state of the iron atom from S = 5/2 (Fe^{3+}) to S = 2 (Fe^{2+}) (14). The results presented in Tables 1B and 2B for ferromyoglobin and ferrohemoglobin suggest that very little interaction occurs between xylidine and the reduced form of these hemoproteins at the concentration tested, provided that the residence time of xylidine in the complex remains in the fast exchange region of the NMR time scale (see DISCUSSION).

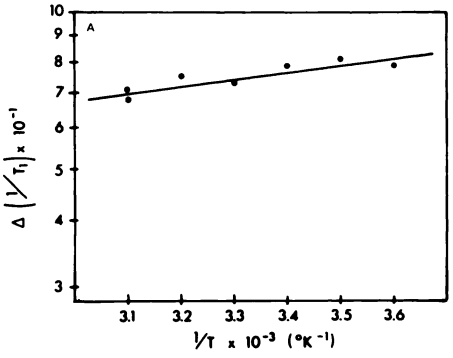
Optical studies. Addition of xylidine to cytochrome P450 produces slight changes in the extinction coefficient and a small shift in the maximum of the Soret band. The difference spectrum obtained from cytochrome P450 plus xylidine minus free cytochrome P450 was used to determine the dissociation constant of the xylidine–cytochrome P450 complex. The data obtained from the difference spectra were plotted in a Hofstee plot (34) as shown in Fig. 6, and the average K_D for xylidine–cytochrome P450 was found to be 4.1×10^{-4} M.[5]

DISCUSSION

The above results may be summarized as follows: (a) changes in the T_1 values of particular xylidine moieties indicated specific interactions with the added hemoproteins; (b) differential T_1 changes for methyl and phenyl groups occurred, depending upon the particular hemoprotein; (c) P450 was about 300 times more effective than Mb or Hb in changing the xylidine T_1 values.

Experiments in which the oxidation state and/or spin state of the iron atom in the heme moiety was modified demonstrated that the observed changes in T_1 resulted from substrate interaction with the paramagnetic center of the heme (Tables 1–3). The differential effects upon the methyl and phenyl T_1 values (phenyl > methyl) observed with Hb and cytochrome P450 may reflect a molecular orientation of the substrate on each of these hemoproteins, where the average position of the phenyl protons is in closer proximity to the paramagnetic center than that of the methyl protons. For example, as calculated for xylidine-P450 (see legend to Fig. 7), the phenyl protons appear to approach about 0.3 A closer to the iron atom than

[5] K_D values were not obtained for xylidine-Hb^{3+} or xylidine-Mb^{3+}, because in attempts to do so the solubility limit (approximately 30 mM) of xylidine was exceeded before the dependence of ΔAbsorbance on xylidine concentration exceeded the linear region; i.e., saturation of these hemoproteins by xylidine could not be achieved.



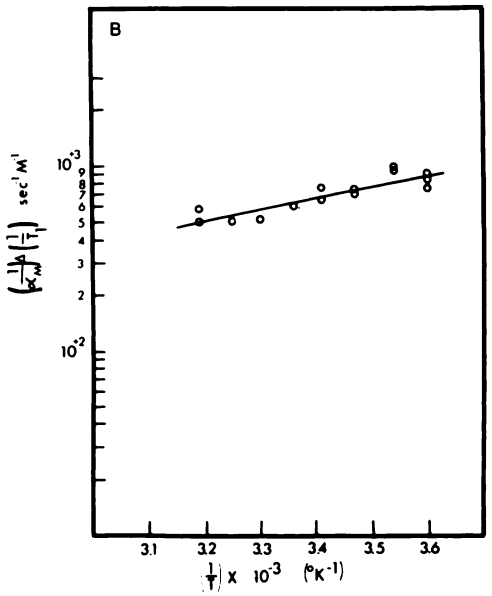
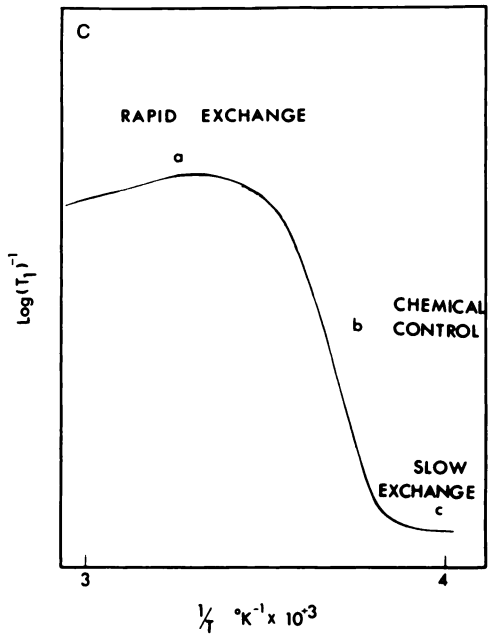


FIG. 5. *Variable Temperature Studies*

A. Arrhenius temperature plot of $\Delta(1/T_1)$ for xylidine-myoglobin, where $\Delta(1/T_1) = 1/T_{1_{obs}} - 1/T_{1_{blank}}$.

B. Arrhenius temperature plot of $(1/\alpha_M)\ \Delta(1/T_1)$ for xylidine–cytochrome P450, where α_M is the mole fraction of the xylidine-P450 complex.



C. Theoretical T_1 temperature profile (29, 33).

the methyl protons. Xylidine is hydroxylated predominantly in the *para* position[6] [as is aniline (11)]; hence the enhanced relaxation rate of the phenyl protons relative to the methyl protons correlates with the site of hydroxylation. Whether or not such differential T_1 changes may prove generally useful as a means of predicting the site of metabolic alteration on substrates by the P450 system requires a much more extensive survey of substrate-P450 interactions via this technique.

The greater sensitivity of the substrate to additions of cytochrome P450 may indicate a greater affinity of P450 for xylidine. The association constant, K_{assoc}, for xylidine–cytochrome P450 is approximately $2.5 \times 10^3\ M^{-1}$. By comparison with the aniline-Hb complex, for which $K_{assoc} \sim 10\ M^{-1}$ (11), it may be inferred that the K_{assoc} for xylidine with Hb or Mb would be of a similar magnitude.[5] Hence one would expect that cytochrome P450, with a K_{assoc} approximately 100 times greater than that of xylidine-Hb, would have a pronounced effect upon the substrate at much lower concentration, provided that the nature of the complexes (i.e., iron-substrate distances) were essentially the same (see below and Fig. 7).

The observed changes in the relaxation rates of xylidine are composed of a contri-

[6] J. M. Strong and J. J. Mieyal, unpublished observations.

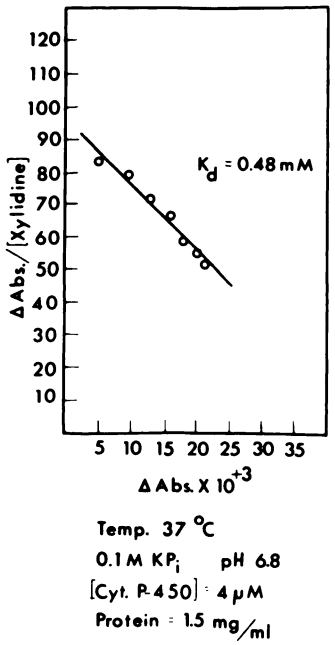


FIG. 6. *Hofstee plot (34) giving value of K_D for xylidine-cytochrome P450*

The average value of K_D in the text is given as 0.41 mM. (Duplicate determinations gave an average K_D value of 0.41 mM; standard error, 0.07 mM; linear regression, 0.98.)

bution from the paramagnetic metal ion, as well as possible contributions from the protein moieties of Mb, Hb, and cytochrome P450. The reduced carbonyl forms of myoglobin and hemoglobin are diamagnetic (14), and the reduced CO form of the camphor-induced cytochrome P450 from *Pseudomonas putida* has also been judged to be diamagnetic (31, 32); therefore it seems reasonable to assume that the liver microsomal $P450^{2+}$-CO is also diamagnetic. Hence the evaluation of diamagnetic protein interactions upon the T_1 of the substrate can be taken into account by changing the hemoproteins to the carbonmonoxyferrous form *in situ* and comparing the T_1 values in the presence of these diamagnetic derivatives with the T_1 values in the absence of hemoproteins, provided that substrate does not displace carbon monoxide.[7] The addition of dithionite alone to solutions of xylidine increased the T_1 values of the methyl and phenyl protons in the blank, probably as a result of elimination of dissolved oxygen, which is a paramagnetic species. When this increase was taken into consideration (Tables 1A and B and 2A and B), a protein contribution to $\Delta(1/T_1)$ of approximately 0.05 sec^{-1} for Mb and Hb (Tables 1B and 2B) was determined. For cytochrome P450 the protein contribution appeared to be much larger, about 0.17 sec^{-1} (Table 3); this might reflect a stronger interaction with the protein moiety of P450. Since the P450 was only partially purified, it must also be considered that other factors, such as contamination (by other proteins possibly containing paramagnetic centers), might contribute. Nevertheless, the ability to assess the protein contribution suggests that it may be possible to characterize diamagnetic substrate-ferrohemoprotein com-

[7] Schenkman *et al.* have concluded that type II substrates displace CO from carbonmonoxy-$P450^{2+}$-CO (6) and have interpreted these data as indicative of direct coordination of type II substrates with the heme iron atom. We have reported that displacement of CO and resultant autoxidation of hemoglobin or myoglobin can also occur when substrates such as aniline are added to a solution containing the reduced carbonmonoxyhemoglobins in an air atmosphere (12), but we suggested that this need not be reflective of direct interaction of the substrate with the iron atom. For example, 2,3-diphosphoglycerate when added to oxyhemoglobin displaces O_2 even though it does not coordinate directly with the iron atom (35). 2,3-Diphosphoglycerate does not bind to heme iron atoms, but its binding lowers the affinity of Hb for oxygen and concomitantly accelerates autoxidation (36). We confirmed, for the present case, that the addition of xylidine to a cuvette containing reduced carbonmonoxymyoglobin resulted in spectral changes reflective of the displacement of CO and concomitant autoxidation of the hemoprotein. However, when such an experiment was repeated in reverse order under CO (i.e., reduction of the hemoprotein in the presence of substrate under an atmosphere of CO) and an excess of dithionite and a positive pressure of CO was maintained, no autoxidation was observed. This was verified for both Hb and cytochrome P450, using ultraviolet difference spectroscopy. Cytochrome P450 determinations made in the presence and absence of substrate gave the same absorbance at 450 nm to within 10%, and no decrease in the absorbance was observed within approximately 10 min. Thus the conditions were established under which the $P450^{2+}$-CO form could be maintained in the presence of substrate. This was important in establishing a diamagnetic control, which allowed values of T_{1_p} (relaxation due to the paramagnetic heme iron) to be determined.

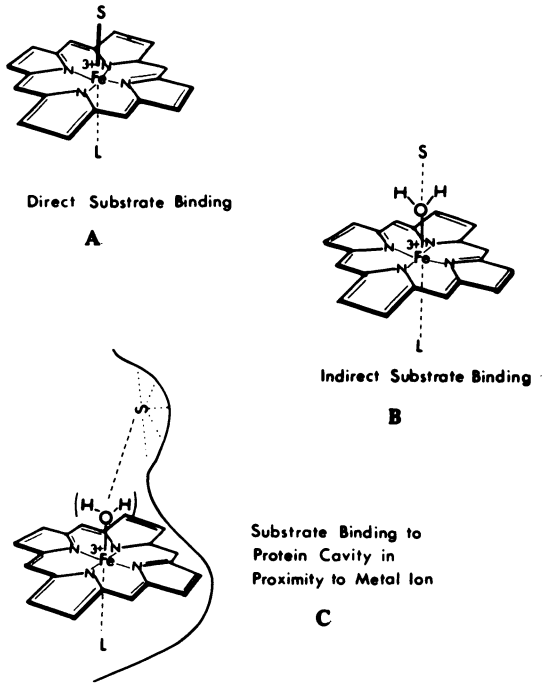


FIG. 7. *Proposed models for substrate-hemoprotein interactions and calculated distances between methyl and phenyl protons of xylidine and heme iron atom in xylidine-cytochrome P450 and xylidine-hemoglobin complexes*

The Mb^{3+}-H_2O proton distance was calculated to be 2.5 Å ($\tau_c = 3 \times 10^{-11}$), using the reduced carbonyl derivative of the hemoprotein as the diamagnetic standard. This value of proton-to-iron distance is in agreement with previously published values (7, 20).

Xylidine-P450 distances			
Xylidine	τ_c	r(Fe−H)	
		$S = 5/2$	$S = 1/2$
	sec	*Å*	*Å*
Methyl	10^{-10}	5.55	3.68
Phenyl	10^{-10}	5.09	3.38
Methyl	10^{-11}	3.81	2.53
Phenyl	10^{-11}	3.51	2.32

Xylidine-Hb^{3+} distances[a]		
Xylidine	τ_c[b]	r(Fe−H)
	sec	*Å*
Methyl	10^{-10}	9.07
Phenyl	10^{-10}	8.43

[a] K_D estimated as 0.1 M (see below).

[b] τ_c estimated as 10^{-10} sec from aquomethemoglobin (24, 25).

plexes (e.g., substrate-P450-O_2) via this technique and thus gain insight with regard to the sequence of events in P450-catalyzed reactions.

The difference in T_1 values between the cyano derivatives of Hb and Mb and the blank (minus hemoprotein) was significantly greater than that obtained from the corresponding difference between the carbonmonoxyferrous forms and the blank. This suggests that xylidine remains in complex with Mb and Hb even in the presence of cyanide. The decrease in $T_{1_{obs}}$ upon addition of fluoride anion was consistent with this interpretation. Hence the results of both these experiments (Tables 1

and 2) suggest that the substrate continues to interact during ligation of the iron atom of Mb and Hb with CN^- or F^-. This finding indicates that the technique employed may be used to detect the simultaneous interaction of the substrate with the paramagnetic center in the presence of other ligands.

Estimation of substrate-heme iron atom distances. For the cytochrome P450–xylidine complex, average proton-to-iron distances were calculated using Eq. 2a and b. These distances were calculated with the assumption that the dipolar interaction is the primary relaxation mechanism[8] and that the correlation time τ_c for the xylidine-hemoprotein complex lies in the range $10^{-11} < \tau_c < 10^{-10}$.

Analysis of EPR spectra taken of solubilized liver microsomal cytochrome P450 prepared from phenobarbital-induced rats indicated that only a small fraction of the total heme iron of this hemoprotein is in the high-spin state ($S = 5/2$) (37). Addition of the substrate benzphetamine or benzpyrene caused an increase in the amplitude of the $g = 8$ EPR signal, which was interpreted as indicating an increase in high-spin P450 (37, 38). These EPR experiments, however, were performed in the temperature range −269° to −196° and did not provide information concerning the effect of temperature on spin equilibrium. Similar EPR experiments performed at −267° showed that substrate-free, camphor-induced *P. putida* $P450^{3+}$ was approximately 100% low-spin while the camphor-bound enzyme was approximately 80% high-spin over the temperature range −173° to −23° (9). On the other hand, bathochromic shifts in the Soret band of hemoproteins (as caused by xylidine) have been interpreted to reflect an increase in the low-spin form of the hemoprotein (14). Therefore, in the absence of direct measurements (i.e., magnetic susceptibility) of the spin state of cytochrome P450 under ambient conditions and in the presence of xylidine, it was not possible to assess the spin state of the hemoprotein. Hence the calculation of proton-to-iron distances were made for both the high-spin ($S = 5/2$) and low-spin ($S = 1/2$) states.

The calculation of distances is based on the assumption of rapid chemical exchange between the free and complexed species in solution; i.e., $\tau_M \ll T_{1_M}$. The observed enhancement of the relaxation rate $(1/\alpha_M)$ $(\Delta(1/T_1))$ with decreasing temperature for the interaction of xylidine with Mb^{3+} and $P450^{3+}$ was indicative of the condition of rapid exchange (29, 33); i.e., $\tau_M \ll T_{1_M}$. The high ratio of xylidine to cytochrome P450 concentrations and the low K_D value for the complex permit the assumption that the mole fraction of substrate in complex will be essentially independent of temperature for this small range (5–40°), thereby allowing determination of E_{act}. (The same may not be true for the xylidine-Mb complex, which may have a K_D as large as 0.1 M.) Calculation of E_{act} from the slope of the line of Fig. 5B yielded approximately 2.7 kcal/mole for the xylidine exchange process. This energy of activation is probably reflective of the temperature dependence of τ_s, which is expected to be in the range of 1–3 kcal/mole (29). Typical activation energies for ligand exchange processes which are dominated by τ_M range from 10 to 15 kcal/mole (28). The theoretical relationship between T_1 and temperature for the limiting regions of rapid exchange, chemical control, and slow exchange is given in Fig. 5C (29, 33). The entire temperature profile as given in Fig. 5C allows determination of τ_M, the residence time, and the activation parameters $\Delta H‡$ and $\Delta S‡$ for the two-site exchange problem (29). The activation energy measured supports the conclusion that T_{1_M} is greater than τ_M, but

[8] The assumption was made for these ^1H NMR studies that the dipolar interaction is the primary relaxation mechanism. It is possible that other relaxation mechanisms may be present for the phenyl protons of xylidine. For example, anisotropic rotation of the complexed xylidine molecule or π-π interactions between xylidine and aromatic amino acids (or the porphyrin ring) may also contribute to the observed changes in T_1 relaxation. In contrast, ^{13}C relaxation times are governed primarily by the dipolar relaxation mechanism, and studies employing ^{13}C NMR relaxation time changes may provide verification of substrate–iron atom distances.

the limited range of temperatures over which these experiments could be conducted[9] precluded an accurate determination of τ_M, which would be required to establish unequivocally the condition $\tau_M \ll T_{1_M}$. However, it is unlikely that the system is in the region of slow exchange, since the stoichiometry (i.e., [substrate] to [hemoprotein] ratio) employed in this study would preclude the observation of any marked changes in T_1. For example, at [substrate] to [hemoprotein] ratios greater than 100:1 (Fig. 3A and B) for Mb and Hb or greater than 5000:1 (Fig. 3C) for cytochrome P450, less than 1.0% of the substrate would be fixed in complex and would not be expected to contribute to any alteration in the T_1 of the bulk of the substrate, which would be free in solution. In fact, at these ratios, marked changes in T_1 were observed, which is consistent with rapid exchange of free and bound substrate. Hence, the conclusion of rapid exchange for xylidine-myoglobin and xylidine-P450 appears valid in view of the results obtained from the variable-temperature studies.

The T_{1_μ} values (Table 3), in conjunction with the dissociation constant for the xylidine-P450 complex ($K_D = 4.1 \times 10^{-4}$ M) and estimates of the correlation time for the complex, allowed calculation of distances between the heme iron atom and specific portions of the substrate molecule. Since the exact spin state of cytochrome P450 at 35° in the presence of xylidine and the precise value of τ_c for the complex are not known, a range of distances was calculated by inserting values for both high- ($S = 5/2$) and low- ($S = 1/2$) spin forms of P450 and correlation times of 10^{-10} sec and 10^{-11} sec into Eq. 2a and b. The distance estimates range from 2.44 to 5.35 Å for the methyl protons and from 2.30 to 5.00 Å for the phenyl protons of the xylidine molecule. The lower extreme in distances (about 2.5 Å) for $\tau_c = 10^{-11}$ sec and $S = 1/2$ could indicate direct coordination of the amino group of xylidine to the heme iron atom, but the upper extreme (about 5 Å), for $\tau_c = 10^{-10}$ sec and $S = 5/2$, suggests that the xylidine molecules may exist either as an outer sphere complex or bound to a protein cavity, as shown in Fig. 7b,c. Previously, ultraviolet difference spectra have been employed to classify substrates for P450 as type I or type II according to the spectral change that they elicited. Type I substrates are thought to bind in the vicinity of the heme (i.e., protein-binding cavity, Fig. 7c), while type II substrates are thought to coordinate directly (Fig. 7a) to the heme iron atom (6). Xylidine elicits a typical type II spectral change (bathochromic shift in the Soret band), but the larger distances estimated from the NMR data suggest that it may not coordinate directly to the heme iron atom. Clearly, in the case of the xylidine-Mb and xylidine-Hb complexes, the cumulative evidence suggests a binding site separate from the heme site. As more precise information on the spin state and correlation time for the xylidine-P450 complex becomes available, the average orientation of the bound substrate with respect to the heme iron atom can be more clearly defined.

Unlike ultraviolet spectroscopy, which does not provide information on substrate orientation, the NMR approach illustrated here can provide specific information in this regard. For example, a more extensive survey of substrates may reveal a systematic correlation between the substrate orientation in complex with the hemoprotein (in the ferric or ferrous oxidation state), relative to the O_2-binding heme site, and the molecular site of hydroxylation of the substrate. In this manner the use of NMR relaxation time measurements could provide basic information on the molecular mechanism of catalysis by this hemoprotein.

[9] The variable-temperature experiments could be conducted only over the limited temperature range of 5–40°, since D_2O freezes at about 4° and the enzyme is denatured above 40°.

ACKNOWLEDGMENTS

The authors thank Drs. T. J. Swift and A. F. Boyne for critical review of the manuscript.

REFERENCES

1. Estabrook, R. W., Gillette, J. R. & Liebman, K. C., eds. (1972) *Second International Sympo-*

sium on Microsomes and Drug Oxidations, Williams & Wilkins, Baltimore.
2. Gunsalus, I. C., Pederson, T. C. & Sligar, S. G. (1975) *Annu. Rev. Biochem.*, **44**, 377–407.
3. Imai, Y. & Sato, R. (1966) *Biochem. Biophys. Res. Commun.*, **22**, 620–626.
4. Mannering, G. J. (1971) in *Drugs and Cell Regulation*, E. Mihich, ed., Academic Press pp. 197–225, New York.
5. Orrenius, S., Wilson, B. J., vonBahr, C. & Schenkman, J. B. (1972) *Biochem. Soc. Symp.*, **34**, 55–77.
6. Schenkman, J. B., Remmer, H. & Estabrook, R. W. (1967) *Mol. Pharmacol.*, **3**, 113–123.
7. Hershberg, R. D. & Chance, B. (1975) *Biochemistry*, **14**, 3885–3891.
8. Vuk-Pavlovic, S. & Benko, B. (1975) *Biochem. Biophys. Res. Commun.*, **66**, 1154–1159.
9. Griffin, B. W. & Peterson, J. A. (1975) *J. Biol. Chem.*, **250**, 6445–6451.
10. Ruckpaul, K., Maricic, S., Janig, G. R., Benko, B., Vuk-Pavlovic, S. & Rein, H. (1976) *Croat. Chem. Acta*, **48**, 69–86.
11. Mieyal, J. J., Ackerman, R., Blumer, J. & Freeman, L. S. (1976) *J. Biol. Chem.*, **251**, 3436–3441.
12. Mieyal, J. J. & Blumer, J. L. (1976) *J. Biol. Chem.*, **251**, 3442–3446.
13. Van Kampen, E. J. & Zijlstra, W. G. (1961) *Clin. Chim. Acta*, **6**, 538–544.
14. Antonini, E. & Brunori, M. (1971) *Hemoglobin and Myoglobin in Their Reactions with Ligands*, North-Holland Publishing Co., Amsterdam.
15. Lu, A. Y. H., Junk, R. W. & Coon, M. J. (1969) *J. Biol. Chem.*, **244**, 3714–3721.
16. Autor, A. P., Kaschnitz, R. M., Heidema, J. K. & Coon, M. J. (1973) *Mol. Pharmacol.*, **9**, 93–104.
17. Omura, T. & Sato, R. (1967) *Methods Enzymol.*, **10**, 556–561.
18. Schenkman, J. B., Cha, Y. N., Moldeus, P. & Cinti, D. L. (1973) *Drug Metab. Disp.*, **1**, 516–522.
19. Lowry, O. H., Roberts, N. R. & Kapphahn, J. I. (1957) *J. Biol. Chem.*, **224**, 1047–1064.
20. Kendrew, J. C., Dickerson, R. E., Strandberg, B. E., Hart, R. G., Davies, D. R., Philips, D. C. & Shore, V. C. (1960) *Nature*, **185**, 422–427.
21. Solomon, I. (1955) *J. Chem. Phys.*, **99**, 559–565.
22. Bloembergen, N. (1957) *J. Chem. Phys.*, **27**, 572–573.
23. Swift, T. J. & Connick, R. E. (1962) *J. Chem. Phys.*, **37**, 307–320.
24. Asakura, T. (1973) *Ann. N. Y. Acad. Sci.*, **222**, 68–85.
25. Gupta, R. K. & Koenig, S. H. (1971) *Biochem. Biophys. Res. Commun.*, **45**, 1134–1143.
26. Gupta, R. K. & Mildvan, A. S. (1975) *J. Biol. Chem.*, **250**, 246–253.
27. Asakura, T., Leigh, J. S., Jr., Drott, H. R., Yonetani, T. & Chance, B. (1971) *Proc. Natl. Acad. Sci. U. S. A.*, **68**, 861–865.
28. Koenig, S. H. & Schillinger, W. E. (1969) *J. Biol. Chem.*, **244**, 6520–6526.
29. Luz, Z. & Meiboom, S. (1964) *J. Chem. Phys.*, **40**, 2686–2692.
30. Pople, J. A., Schneider, N. G. & Bernstein, H. J. (1959) *High Resolution Nuclear Magnetic Resonance*, pp. 233–296, McGraw-Hill, New York.
31. Sharrock, M., Debrunner, P. G., Schulz, C., Lipscomb, D., Marshall, V. & Gunsalus, I. C. (1976) *Biochim. Biophys. Acta*, **420**, 8–26.
32. Champion, P. M., Lipscomb, J. D., Munck, E., Debrunner, P. & Gunsalus, I. C. (1975) *Biochemistry*, **14**, 4151–4158.
33. Swift, T. J. (1974) in *NMR of Paramagnetic Molecules* (LaMar, G. N., Holm, R. & Horrocks, W. D., eds.), pp 53–83, Academic Press, New York.
34. Hofstee, B. H. J. (1952) *Science*, **116**, 329–333.
35. Kilmartin, J. V. & Rossi-Bernardi, L. (1973) *Physiol. Rev.*, **53**, 836–890.
36. Mansouri, A. & Winterhalter, K. H. (1974) *Biochemistry*, **13**, 3311–3314.
37. Stern, J. O., Peisach, J., Blumberg, W. E., Lu, A.Y.H. & Levin, W. (1973) *Arch. Biochem. Biophys.*, **156**, 404–443.
38. Nebert, D. W., Robinson, J. R. & Kon, H. (1973) *J. Biol. Chem.*, **248**, 7637–7647.