MOLECULAR PHARMACOLOGY, 16, 1046-1058

Characterization of the Interaction of the Aromatic Hydrocarbons Benzene and Toluene with Human Hemoglobin[1]

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(Received April 23, 1979)
(Accepted July 18, 1979)

SUMMARY

NOVAK, RAYMOND F., MARK DERSHWITZ AND FRANCES C. NOVAK. Characterization of the interaction of the aromatic hydrocarbons benzene and toluene with human hemoglobin. *Mol Pharmacol.* **16:** 1046-1058 (1979).

The longitudinal relaxation rates, T_1^{-1}, and transverse relaxation rates, T_2^{-1}, were determined for the protons of benzene and the methyl and phenyl protons of toluene in aqueous media in the presence of varying concentrations of human ferrihemoglobin. The T_1^{-1} relaxation rate of the protons of 18.8 mM benzene and the methyl and phenyl protons of 8.2 mM toluene increased from 0.065 sec^{-1}, 0.083 sec^{-1} and 0.14 sec^{-1} in aqueous medium in the absence of hemeprotein to 2.5 sec^{-1}, 5.6 sec^{-1}, and 5.3 sec^{-1}, respectively, at 140 μM human ferrihemoglobin. Similar increases were also observed in the transverse relaxation rates with values of T_2^{-1} for the protons of benzene and the methyl and phenyl protons of toluene increasing from 1.1 sec^{-1}, 2.7 sec^{-1} and 2.3 sec^{-1} in the absence of hemeprotein to 3.2 sec^{-1}, 6.7 sec^{-1}, and 6.3 sec^{-1} in the presence of 140 μM ferrihemoglobin. Formation of cyanoferrihemoglobin *in situ*, which changed the paramagnetic spin state of the heme iron atom from $S = 5/2$ to $S = 1/2$, decreased the T_1^{-1} values for the protons of benzene and the methyl and phenyl protons of toluene. For example, the methyl and phenyl proton T_1 relaxation rates of toluene decreased from 5.6 sec^{-1} and 5.3 sec^{-1} in the presence of 140 μM ferrihemoglobin to 2.0 sec^{-1} and 2.1 sec^{-1}, respectively, in the presence of 140 μM cyanoferrihemoglobin. In contrast, formation of fluoroferrihemoglobin *in situ*, which enhanced the paramagnetic effect, increased the T_1 relaxation rates for the protons of benzene and the methyl and phenyl protons of toluene in comparison with an equal concentration of ferrihemoglobin. For example, T_1^{-1} for the methyl and phenyl protons of toluene increased dramatically from 3.8 sec^{-1} and 3.6 sec^{-1} at 91 μM ferrihemoglobin to 7.7 sec^{-1} and 13.0 sec^{-1} in the presence of 91 μM fluoroferrihemoglobin. The *in situ* formation of fluoroferrihemoglobin produced dramatic changes in the relative relaxation rates of the phenyl and methyl protons of toluene with methyl > phenyl. In all cases carbonmonoxyferrohemoglobin, the diamagnetic form of the hemeprotein, was used to determine the paramagnetic contributions ($T_{1_p}^{-1}$, $T_{2_p}^{-1}$) to the observed T_1 and T_2 relaxation rate increases. T_1^{-1} for the protons of benzene and the methyl and phenyl protons of toluene decreased from 2.4 sec^{-1}, 5.6 sec^{-1} and 5.3 sec^{-1} in the presence of 140 μM human ferrihemoglobin to 0.64 sec^{-1}, 1.4 sec^{-1}, and 1.4 sec^{-1}, respectively, in the presence of 140 μM carbonmonoxyferrohemoglobin. The relaxation rates of the protons of benzene and the methyl and phenyl protons of toluene in the presence of cyanoferrihemoglobin were significantly greater than those observed in the presence of an identical concentration of carbonmonoxyferrohemoglobin, suggesting that these aromatic ligands are not displaced

[1] This work was supported in part by Grant C77-14 from the Chicago and Illinois Heart Association to RFN and National Institutes of Health Training Grant GM07263 to the Department of Pharmacology.

[2] National Institutes of Health Predoctoral Fellow.

0026-895X/79/061046-13$02.00/0

by cyanide but continue to interact with cyanoferrihemoglobin in proximity to the paramagnetic center. T_2^{-1} changes for the protons of benzene and the methyl and phenyl protons of toluene in the presence of cyano- or fluoroferrihemoglobin or carbonmonoxyferrohemoglobin agreed qualitatively with those observed in the longitudinal relaxation rate, T_1^{-1}. Variable temperature experiments confirmed that exchange between free ligand and the hemeprotein-complexed ligand occurred in the fast exchange region of the variable temperature profile. Although both paramagnetic and diamagnetic components contribute to the observed enhancement of relaxation rate the paramagnetic contribution is approximately an order of magnitude greater. Thus these results support an interaction between benzene or toluene and human hemoglobin and show that this interaction occurs at or in proximity to the heme iron atom.

INTRODUCTION

Certain drugs, drug metabolites and environmental contaminants, such as the aromatic hydrocarbons, have long been associated with the induction of non-immune hemolytic anemia or methemoglobinemia. In particular, episodes of non-immune hemolytic anemia have been observed in industrial workers exposed to the organic solvents benzene and toluene (1–3). Postulated mechanisms in the pathogenesis of such anemia include either hemoglobin-catalyzed oxidative modification of certain agents to reactive metabolites capable of effecting irreversible damage to the erythrocyte membrane or drug-enhanced hemoglobin-catalyzed formation of H_2O_2 or some activated oxygen species (4, 5). In this regard, evidence has recently been presented suggesting that hemoglobin may indeed occupy a functional role as an oxygenase enzyme in addition to its well-established role as an oxygen carrier hemeprotein (4, 6–9). For example, hemoglobin has been found to convert aniline to para-aminophenol in a reconstituted enzyme system consisting of hemoglobin, cytochrome P = 450 reductase, NADPH and molecular oxygen (6, 7), as well as in intact erythrocytes (8, 9). Hemoglobin has also been implicated in the demethylation of benzphetamine (7) and in the o-demethylation of ortho methylated metabolites of the catecholamines (10). Additional substrates of hemoglobin-catalyzed oxidation include acetylphenylhydrazine (4) and membrane lipids (11). Furthermore, several of these substrates, aniline, various aniline derivatives and acetylphenylhydrazine, have been associated with the induction of such blood dyscrasias as methemoglobinemia (12) and nonimmune hemolytic anemia (2, 4) suggesting that hemoglobin-mediated bioactivation might well be a plausible mechanism in the pathogenesis of non-immune hemolytic anemia or methemoglobinemia. It has also been demonstrated by Castro *et al.* (13, 14) that certain organic hydrocarbons and inorganic reducing agents are capable of causing the conversion of oxyhemoglobin to methemoglobin in erythrocytes, lysed cells and isolated human oxyhemoglobin. These data have been interpreted as demonstrating that the oxy complex possesses some degree of radical character (13). Although many drugs and agents have been implicated in such blood dyscrasias as methemoglobinemia and non-immune hemolytic anemia (1–4, 12), and simple organic hydrocarbons have been shown to convert oxyhemoglobin to methemoglobin (13, 14), little information is available regarding the degree or specificity with which these drugs or agents may interact with human hemoglobin or the molecular level requirements or restrictions for such an interaction to occur.

NMR relaxation time measurements represent a sensitive method for investigation of molecular interactions involving paramagnetic centers and may be effectively employed in the characterization of such interactions ultimately providing information on the nature of the interaction (i.e., direct coordination), accessibility to the binding site, distances of approach to the paramagnetic center and orientation of the molecule in complex.

We have previously reported the use of ^1H Fourier transform nuclear magnetic resonance relaxation rate measurements in the

study of aniline interactions with human ferrihemoglobin (15) and 2,6-dimethylaniline interactions with myoglobin, human hemoglobin and mammalian cytochrome P = 450 (16). It was suggested from results obtained in these studies that aniline and 2,6-dimethylaniline may not directly coordinate with the heme iron atom and may be capable of continued interaction with human hemoglobin in the vicinity of the heme in the presence of directly-coordinating ligands such as cyanide or fluoride. In addition, the pronounced increase in relaxation rate of the aromatic protons of aniline and 2,6-dimethylaniline in the presence of hemeprotein suggested that attractive forces other than those directly associated with the positively charged iron atom may be involved in the complexation of these molecules to hemoglobin and that a nitrogenous basic site on the molecule and/or direct coordination with the heme-iron center may not be absolute requirements for "ligand-ferrihemoglobin" interactions. Thus, the ability of these aromatic hydrocarbons to induce non-immune hemolytic anemia, coupled with our previous observations that suggest the presence of additional attractive forces in ligand binding to hemoglobin, prompted this characterization of the interaction of benzene and toluene with human hemoglobin. Some evidence implicating benzene in complexation with human ferrihemoglobin has been presented (17).

EXPERIMENTAL PROCEDURES

Materials. A stock solution of ferrihemoglobulin was prepared by dissolving hemoglobin (human, type IV, twice recrystallized, Sigma) in 0.02 M KP_i, pH_{obs} 7.5 in ^2H_2O (99.8%, Aldrich) until saturation. Excess potassium ferricyanide was added to convert the hemoglobin to the fully oxidized form, Hb^{3+}, and the solution was passed over two Sephadex G-25 columns previously equilibrated with 0.02 M KP_i buffer pH_{obs} 7.5 in ^2H_2O to achieve deuteration and to remove ferri- and ferrocyanide ions. A stock solution of carbonmonoxyferrohemoglobin was prepared by bubbling a saturated solution of hemoglobin in phosphate buffer pH_{obs} 7.5 with carbon monoxide (Matheson) for 10 minutes and then adding an excess of sodium dithionite (Fisher) to convert the hemeprotein to the reduced carbon monoxide form. The solution was then passed over two Sephadex G-25 columns previously equilibrated with phosphate buffer to achieve removal of the dithionite and exchange labile protons on the protein molecule for deuterium. The solutions of hemoglobin and benzene or toluene used for experimental measurements were prepared by adding an aliquot of the appropriate hemoglobin stock solution to a saturated solution of the aromatic hydrocarbon in ^2H_2O. The stock solutions of benzene in ^2H_2O were prepared by pipetting 5 μl of benzene into 1.5 ml of ^2H_2O, 0.02 M in KP_i, pH_{obs} 7.5. The toluene stock solution was prepared by pipetting 5 μl of toluene into 2.0 ml of ^2H_2O and KP_i buffer was added to yield a solution 0.02 M in KP_i, pH_{obs} 7.5. The final concentration of benzene or toluene in the aqueous stock solution was determined according to the following procedure: To 300 μl of the benzene (or toluene) stock solution was added 100 μl of 9.30 mM ethylene glycol solution in ^2H_2O. A ^1H Fourier Transform NMR spectrum of this solution was recorded and the phenyl peak of the aromatic hydrocarbon and methylene signal of the glycol integrated. The ratio of the integrated signal intensities of the aromatic moities to that of the glycol methylene signal multiplied by the known concentration of the ethylene glycol and the respective proton ratios (i.e., the benzene/ethylene glycol ratio is 6/4) gave the final concentration of the aromatic compound in aqueous medium. The results of 8 separate determinations using this approach yielded benzene and toluene stock solutions of 25.1 mM and 10.9 mM, respectively. Using this approach, standard deviations of ≤2% and ≤4% were determined for variations in the benzene and toluene concentrations, respectively. All data reported herein represent a minimum of 5 experiments per data point except in cases of T_2^{-1} studies where the relatively smaller changes in signal linewidth required a larger number of individual measurements for each data point. The cyanoferrihemoglobin and fluoroferrihemoglobin deriva-

tives were prepared directly in the NMR tube by adding microliter quantities of 1.0 M KCN, pH_{obs} 7.5, or NaF in ^2H_2O. The 1.0 M KCN stock solution was previously adjusted to pH_{obs} 7.5 using KH_2PO_4. A Teflon vortex plug was inserted directly into the NMR tube just above the surface of the solution in order to minimize evaporation of the aromatic hydrocarbons at ambient magnet temperature (29°).

Methods. ^1H NMR spectra were recorded using a Varian CFT-20 spectrometer operating at 80 MHz in the Fourier transform mode utilizing a single sideband crystal filter for enhancement of signal-to-noise. Spectra were recorded at ambient magnet temperature, 29°, except for the variable temperature experiments in which the probe temperature was varied over the range 5°–30°. Temperatures were measured directly with a thermometer inserted into the probe in place of an NMR tube. ^1H homonuclear solvent suppression of the residual HDO signal was employed throughout to enhance signal-to-noise and minimize solvent signal interference.

The ^1H longitudinal relaxation rates, T_1^{-1}, of benzene and toluene in an aqueous medium were determined using the standard 180°-τ-90° inversion-recovery sequence. The 90° pulse width was 20 μsec and a full pulse delay of 5 times the largest T_1 value for each experiment was employed throughout the T_1 relaxation rate determinations. The ^1H transverse relaxation rates, T_2^{-1}, were estimated by measuring the full linewidth of the peak at half-maximal intensity and using the linewidth multiplied by π as an approximation for T_2^{-1}.

The experimentally-determined relaxation rate, T_1^{-1}, of the protons of benzene or the methyl and phenyl protons of toluene in a solution of human ferrihemoglobin represents the sum of several separate contributions. These include the paramagnetic effect which results from interaction of the agent with the high spin (S = 5/2, 85%)[3] heme iron atom, the diamagnetic apoprotein contribution which results from agents interacting with the apoprotein, and the contributions of buffer (KP_i) and dissolved oxygen in the sample. It thus becomes necessary to separate those contributions that may result from aromatic hydrocarbon-protein interactions from those aromatic hydrocarbon-Hb^{3+} complexations involving the paramagnetic center. One mechanism for accomplishing this involves conversion of the hemeprotein to a diamagnetic form such as carbonmonoxyferrohemoglobin. Thus the paramagnetic contribution to the observed relaxation rate may be expressed as:

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

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

The paramagnetic and diamagnetic contributions should vary linearly with protein concentration while the contribution of buffer and dissolved oxygen should be independent of protein concentration in the absence of selectively enhanced O_2 solubility by the protein. Hence, use of the diamagnetic form of the hemeprotein also permits the selective removal of additional contributions, such as buffer and dissolved O_2, to the enhanced relaxation rate.

RESULTS

The ^1H NMR spectrum of benzene consists of a singlet at δ = 7.43 ppm while the ^1H NMR spectrum of toluene consists of a singlet for the phenyl protons at δ = 8.3 ppm and a singlet for the methyl protons at δ = 2.0 ppm as referenced to DSS in ^2H_2O. A typical series of partially relaxed ^1H Fourier transform NMR spectra, from which relaxation rate values were calculated for the phenyl and methyl protons of toluene, is shown in Fig. 1. A plot of the natural log of $M_\infty - M_\tau$ as obtained for the phenyl and methyl protons of toluene in aqueous media is shown in Fig. 2. Here M_τ is defined as the value of the amplitude, either positive or negative, for any of the signals at the various τ (delay time) values as indicated in the figure, and M_∞ refers to the positive, fully relaxed NMR spectrum that can be achieved when τ is equal to or

[3] The percentage of high-spin ferrihemoglobin existing under ambient conditions was determined by comparison of the positions of the hemoglobin Soret absorption band with that of the fluoro (S = 5/2, 100%) and cyano (S = 1/2, 100%) derivatives using UV-visible spectroscopy.

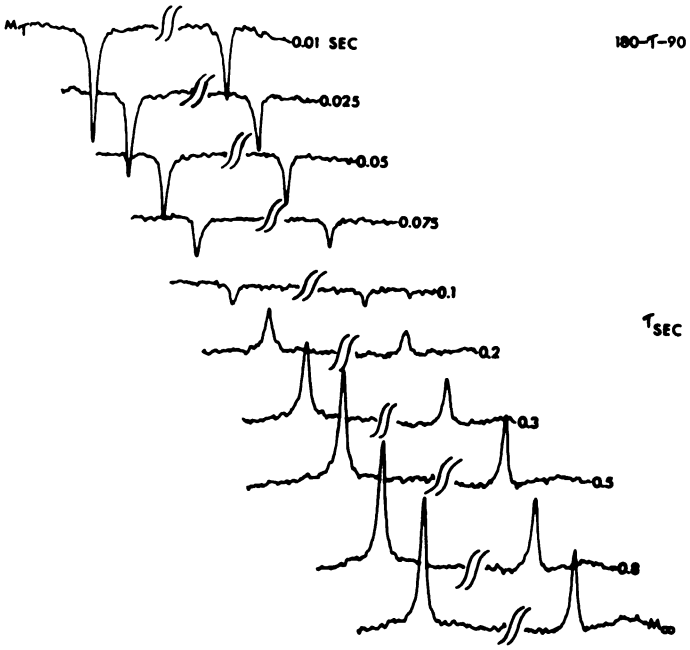

FIG. 1. *A typical 180-τ-90 inversion-recovery sequence obtained for 8.2 mM toluene in ^2H_2O that was 0.02 M in phosphate buffer, pH_{obs} 7.5 and 140 μM in ferrihemoglobin*

The spectra were recorded using 25 acquisitions over a sweep width of 650 Hz (8.13 ppm) utilizing 8192 data points. The instrument was internally locked on the ^2H signal of the ^2H_2O solvent. Toluene evaporation from the ^2H_2O solution at magnet temperature (29°) was minimized through the use of Teflon vortex plugs that were inserted into the NMR tube and situated just above the surface of the liquid. In the figure the values for the delay time τ are given adjacent to each spectrum and the value of the positive or negative signals constitutes the amplitudes for M_τ values. M_∞ is the maximum signal amplitude obtained for a fully relaxed spectrum where $\tau \geq 5$ times T_1.

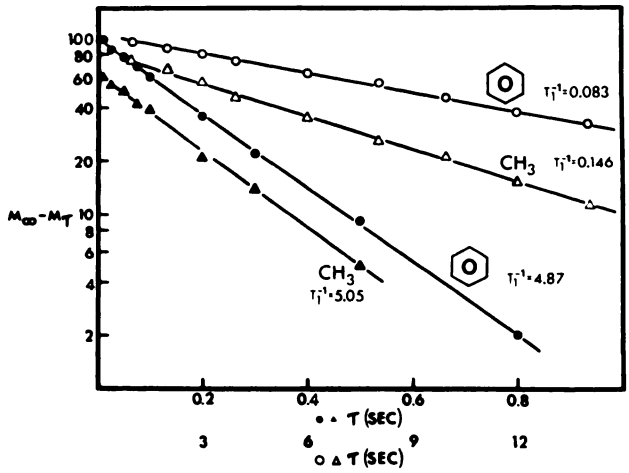

FIG. 2. *A plot of the natural log of $M_\infty - M_\tau$ for the phenyl (○) and methyl (△) protons of toluene in aqueous media, 0.02 M KP$_i$ pH_{obs} 7.5 (open symbols) and for the phenyl (●) and methyl (▲) protons of toluene in the presence of 140 μM ferrihemoglobin (closed symbols)*

The slopes of the lines yield the relaxation rates, T_1^{-1}, for the methyl and phenyl protons of toluene.

greater than 5 times the largest value of T_1. The slopes of the lines yield the relaxation rates, T_1^{-1}, for the phenyl and methyl protons of toluene. The second set of data in Fig. 2 shows the effects of the addition of 140 μM ferrihemoglobin to the toluene solution. It can be seen from the slopes of the lines and their respective τ values that a very marked increase in the relaxation rate of the phenyl and methyl protons of toluene occurs upon addition of human ferrihemoglobin. In all cases the paramagnetic contribution to the relaxation rate change, $T_{1_p}^{-1}$, was determined by subtracting from the relaxation rate change observed for the aromatic hydrocarbons in the presence of ferrihemoglobin, cyanoferrihemoglobin and fluoroferrihemoglobin the relaxation rate for an equivalent concentration of carbonmonoxyferrous hemoglobin (see METHODS).

The increase in relaxation rate, $T_{1_p}^{-1}$, of the benzene protons in aqueous media with increasing concentration of ferrihemoglobin is provided in Fig. 3. This figure also shows the resulting effects of the presence of other ligands such as cyanide or fluoride upon the relative relaxation rate values.

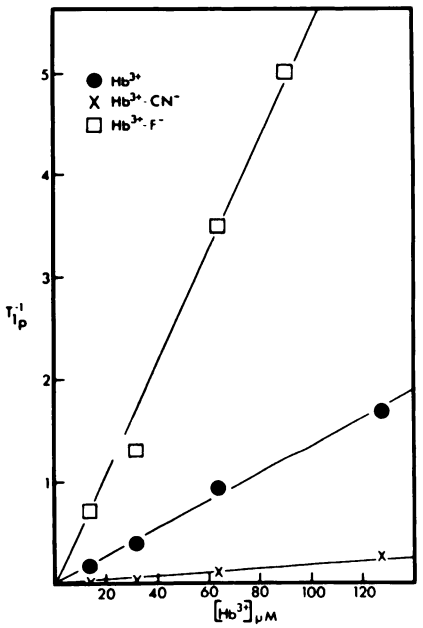

FIG. 3. *The paramagnetic contribution to the observed relaxation rate $T_{1_p}^{-1}$ for benzene in the presence of ferrihemoglobin, cyanoferrihemoglobin and fluoroferrihemoglobin*

The addition of cyanide ion to ferrihemoglobin changes the spin-state of the iron atom in the ferrihemeprotein from high spin (S = 5/2, 85%)[4] to low spin (S = 1/2, 100%). Based on this change of spin state, or upon the ability of cyanide to displace the ligand, a decrease in relaxation rate of the benzene protons or the phenyl and methyl protons of toluene in comparison with that observed for these aromatic hydrocarbons in the presence of an equivalent concentration of ferrihemoglobin would be expected. This was observed for both benzene and toluene and is shown for benzene in Fig. 3 and for toluene in Fig. 4. Conversely, fluoride ion is known to enhance the paramagnetic effect through conversion of the hemeprotein to the 100% high spin form[4] with a concomitant increase in the electron spin relaxation time (18). Such an increase in the paramagnetic effect should thus produce a further enhancement in relaxation rate in comparison with that measured at an identical concentration of ferrihemoglobin under ambient conditions. Such an effect was observed for benzene (Fig. 3) and toluene (Fig. 4). In order to take into consideration the relative effects of the diamagnetic apoprotein contribution to the observed change in relaxation rate, it becomes necessary to convert the hemeprotein to the reduced carbonmonoxyferrous form which is completely diamagnetic. Thus formation of carbonmonoxyferrohemoglobin should therefore result in a substantial decrease in relaxation rate in comparison with that obtained using an equivalent concentration of ferrihemoglobin. This decrease in relaxation rate was indeed observed both for benzene and toluene.

The $T_{1_p}^{-1}$ values for the phenyl and methyl protons of toluene were equivalent, to within experimental error, in the presence of ferrihemoglobin; similar results were also obtained for the phenyl and methyl protons of toluene in the presence of cyanoferrihemoglobin. A small but significant paramagnetic relaxation rate value remains as-

[4] The complete conversion of ferrihemoglobin from high spin (S = 5/2, 85%) to low spin (S = 1/2, 100%) by cyanide and to high spin (S = 5/2, 100%) by fluoride under the experimental conditions employed was established using UV-visible spectroscopy.

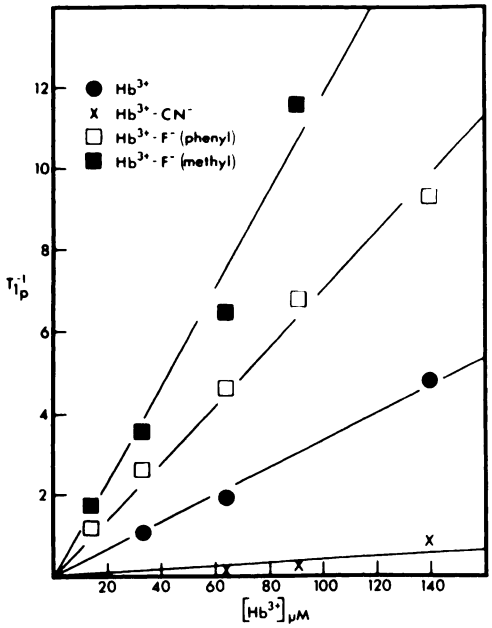

FIG. 4. *The paramagnetic contribution to the relaxation rate, $T_{1_p}^{-1}$, for the phenyl and methyl protons of toluene*

The paramagnetic relaxation rates $T_{1_p}^{-1}$ were observed to be equal for both the phenyl and methyl protons to within experimental error for ferrihemoglobin and cyanoferrihemoglobin. In the case of fluoroferrihemoglobin, however, where the paramagnetic effect is substantially enhanced by fluoride, a marked difference in relaxation rates of the phenyl and methyl protons is observed with the relative changes being methyl (■) > phenyl (□).

cribable to the protons of benzene and the phenyl and methyl protons of toluene in the presence of cyanoferrihemoglobin (Figs. 3 and 4). These results suggest that benzene or toluene may continue to interact with cyanoferrihemoglobin and that addition of cyanide ion does not appear to result in the complete displacement of benzene or toluene from ferrihemoglobin.

Toluene was chosen as the second aromatic hydrocarbon for investigation because, in addition to its possessing some degree of water solubility, it also provided the opportunity for monitoring individual changes in relaxation rates for the phenyl and methyl protons in the presence of hemeprotein. Thus, toluene represented a potential source of information on the orientation of the molecule in complex with hemoglobin via measurement of differential changes in phenyl and methyl relaxation rates. Although ferrihemoglobin and cyanoferrihemoglobin produced virtually identical $T_{1_p}^{-1}$ values for the phenyl and methyl protons of toluene, the *in situ* formation of fluoroferrihemoglobin produced a marked difference in $T_{1_p}^{-1}$ values of the phenyl and methyl protons (Fig. 4). In this regard, the methyl protons exhibited a substantially greater relaxation rate than the phenyl protons in the presence of all concentrations of fluoroferrihemoglobin employed in these experiments.

In addition to the observation of T_1^{-1} relaxation rate changes of the aromatic hydrocarbons with increasing ferrihemoglobin concentration, appreciable changes in signal linewidth, T_2^{-1}, were also observed. Figures 5a and 5b show the relative effects of ferrihemoglobin upon the signal linewidths at half-maximal intensity of the protons of benzene and the phenyl and methyl protons of toluene in aqueous media. For comparative purposes, the data are shown at a constant concentration of 140 μM hemoglobin. The conversion of ferrihemoglobin to cyano- and fluoro-derivatives produces a narrowing and broadening of the benzene and toluene proton signals, respectively, in agreement with the expected decrease and increase in paramagnetism of the hemeprotein. For example, the benzene signal linewidth at half-maximal intensity broadened from 3.9 Hz in the presence of 140 μM ferrihemoglobin to 4.4 Hz in the presence of 140 μM fluoroferrihemoglobin and decreased from 3.9 Hz to 2.9 Hz in the presence of 140 μM cyanoferrihemoglobin. Similar results were observed for the phenyl and methyl proton signals of toluene (Fig. 5b). Again, in order to assess the paramagnetic effect, carbonmonoxyferrohemoglobin was used as a diamagnetic standard to take into consideration apoprotein contributions to the observed linewidth changes. The signal linewidth in the presence of 140 μM carbonmonoxyferrohemoglobin is 2.1 Hz, approximately a factor of 2 greater than that observed for benzene in aqueous medium in the absence of ferrihemoglobin. Figure 5b shows that the difference between the carbonmonoxyferrohemoglobin

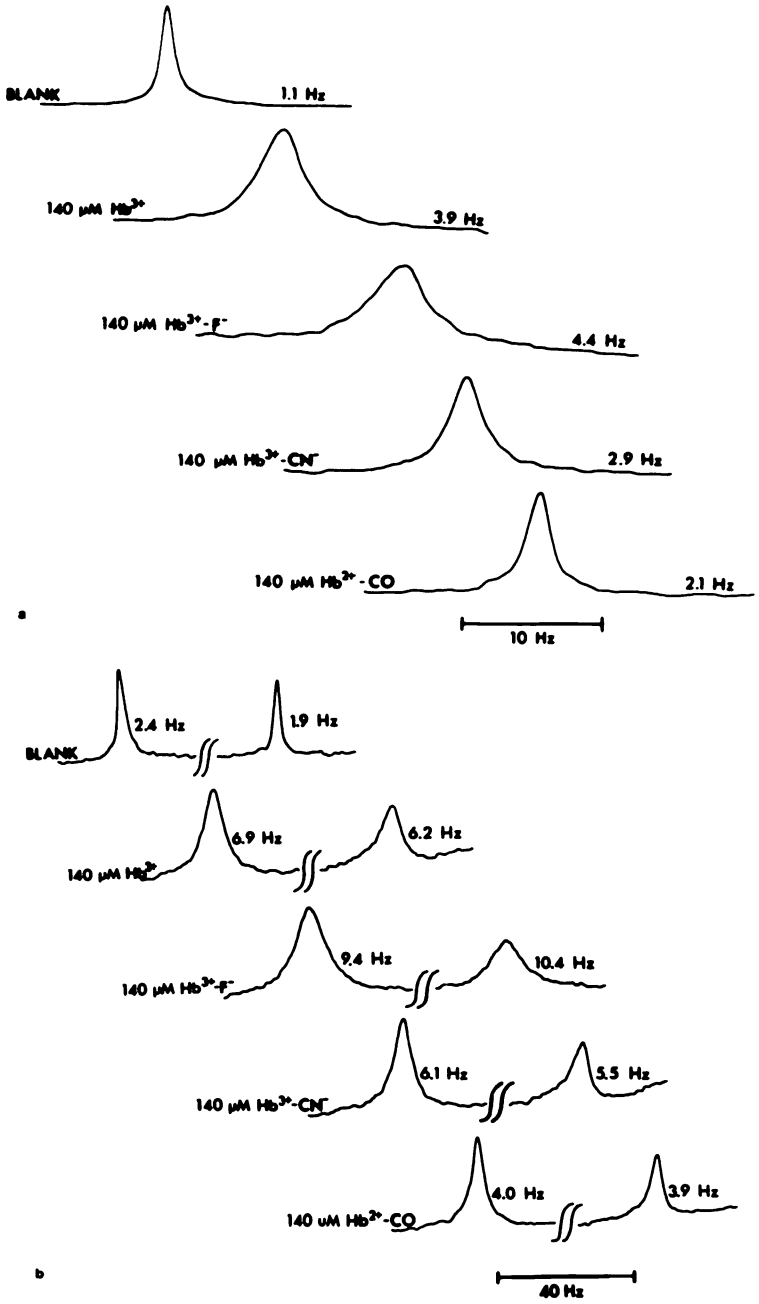

FIG. 5. *Figures 5a and 5b show the linewidths of the benzene proton signal (5a) and the phenyl and methyl proton signals (5b) of toluene in aqueous medium and the relative effects of 140 μM ferrihemoglobin, 140 μM cyanoferrihemoglobin, 140 μM fluoroferrihemoglobin and 140 μM carbonmonoxyferrohemoglobin upon these linewidths*

and toluene blank in aqueous media is approximately 1.6 Hz for the phenyl protons and 2.0 Hz for the methyl protons. Figure 6 shows the paramagnetic contribution to the transverse relaxation rate $T_{2_p}^{-1}$ as observed from the signal linewidth at half-maximal intensity. $T_{2_p}^{-1}$ was determined by subtracting out the protein contribution to

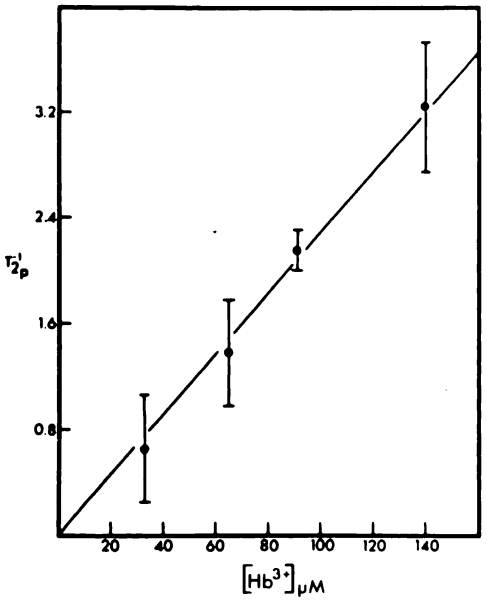

FIG. 6. *The paramagnetic contribution, $T_{2_p}^{-1}$, to the observed change in transverse relaxation rate for the protons of benzene*

Here the paramagnetic contribution was determined by subtracting out the protein contribution to the linewidth using the diamagnetic carbonmonoxyferrohemoglobin.

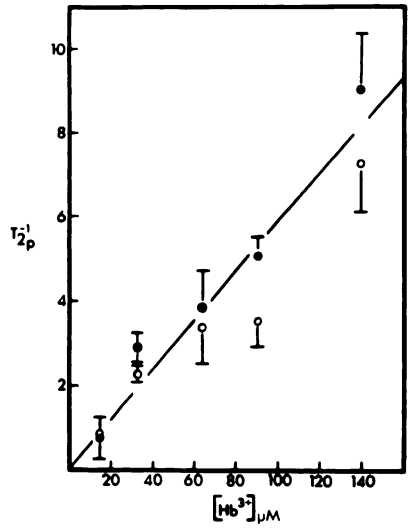

FIG. 7. *The increase in the paramagnetic contribution to the relaxation rate of the phenyl* (○) *and methyl* (●)*protons of toluene with increasing concentrations of ferrihemoglobin*

the linewidth using the diamagnetic carbonmonoxyferrohemoglobin. Figure 7 shows the increase in the paramagnetic contribution to the transverse relaxation rate of the phenyl and methyl protons of toluene with increasing concentration of ferrihemoglobin. Both the phenyl and methyl protons exhibit equivalent relaxation rates to within experimental error in the presence of increasing concentrations of ferrihemoglobin.

Arrhenius variable temperature profiles for changes in paramagnetic relaxation rates, $T_{1_p}^{-1}$ and $T_{2_p}^{-1}$, with temperature for the protons of benzene and the phenyl protons of toluene are shown in Figs. 8 a and b. The observed enhancement of the paramagnetic relaxation rates $T_{1_p}^{-1}$, $T_{2_p}^{-1}$ with decreasing temperature for the interaction of benzene and toluene with ferrihemoglobin suggest that these aromatic hydrocarbons are in the rapid exchange region of the NMR time scale (19, 20). Calculation of activation energies, E_{act} from the slope of the lines in Figs. 8a and b gave average values of 2.2 and 3.9 kcal/mole for benzene and toluene, respectively. These activation energy values lie within the range of 1–5 kcal/mole and may be associated with the temperature dependence of τ_s, the electron-spin correlation time (19, 20). Typical activation energies for ligand exchange processes, which have longer residence times of species in complex, are on the order of 10–15 kcal/mole (19).

In summary, the data presented here support the conclusion that benzene and toluene interact with human ferrihemoglobin in proximity to the paramagnetic center. Furthermore, additional experiments utilizing cyanide, fluoride, and carbonmonoxyferrohemoglobin suggest that benzene and toluene continue to interact with human hemoglobin in the presence of such directly coordinating ligands as cyanide and fluoride. A differential change in relaxation rate for the phenyl and methyl protons of toluene was observed only in the presence of fluoroferrihemoglobin.

DISCUSSION

The addition of human ferrihemoglobin to aqueous solutions of benzene or toluene produces a marked increase in the T_1^{-1} and T_2^{-1} relaxation rates of the protons of ben-

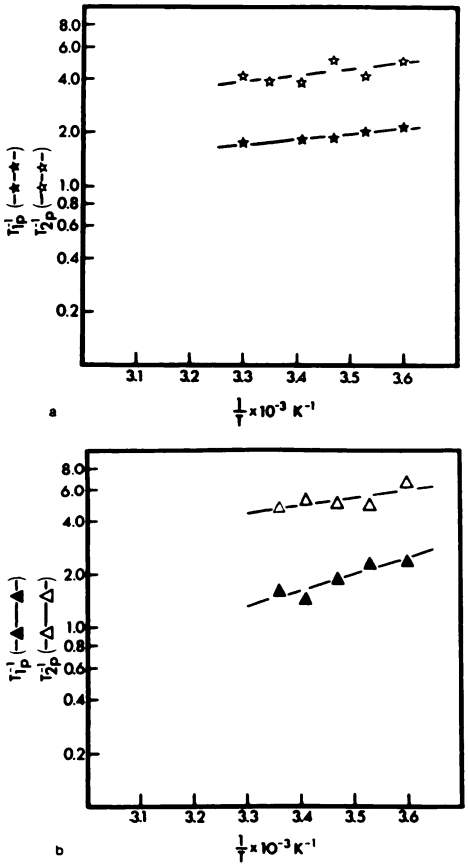

FIG. 8. *Variable temperature profiles of the change in the paramagnetic contribution to the paramagnetic relaxation rate, $T_{1_p}^{-1}$, $T_{2_p}^{-1}$, with temperature for the protons of benzene (8a) and the phenyl protons of toluene (8b)*

The results suggest that exchange between free benzene or toluene and that in complex with ferrihemoglobin occurs in the rapid exchange region of the NMR time scale.

zene and the phenyl and methyl protons of toluene. Experiments in which the oxidation state and/or spin state of the heme iron atom were modified demonstrated that the changes in the T_1^{-1} and T_2^{-1} relaxation rates of the proton moieties of the aromatic hydrocarbons, upon addition of ferrihemoglobin, result primarily from the interaction of the aromatic hydrocarbons directly with, or in proximity to, the paramagnetic center of hemoglobin. As can be observed from data presented in Fig. 9, although a significant contribution by the apoprotein of the human ferrihemoglobin to the relaxation time changes exists, the $T_{1_d}^{-1}$ values are substantially less than those of the paramagnetic contribution at a given hemoglobin concentration. The diamagnetic apoprotein contributions to the observed T_1 relaxation rate changes of the protons of the aromatic hydrocarbons may be determined nevertheless through the use of carbonmonoxyferrohemoglobin and comparison of the T_1^{-1} values in the presence of this diamagnetic derivative with those of ferrihemoglobin in various liganded or oxidation states (i.e., oxyferrohemoglobin). Since this experimental approach enables one to assess the protein contributions to the relaxation rate changes, it may be possible to characterize diamagnetic substrate-ferrohemeprotein complexes and determine whether such protein binding is significantly altered upon changes in conformation that accompany changes in oxidation state and/or the presence of ligands. For example, the study of diamagnetic complexes involving oxyferrohemoglobin may provide insight with regard to the sequence of events in reactions such as those occurring in hemeprotein-catalyzed oxidative modifications, in particular

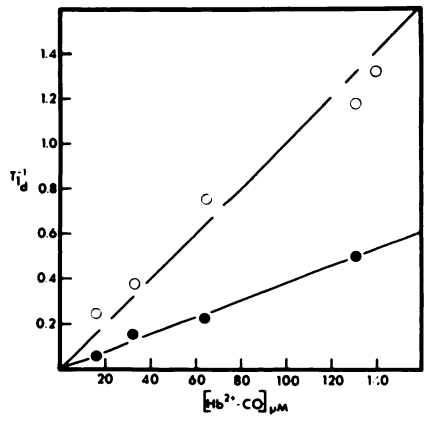

FIG. 9. *The increase in the diamagnetic apoprotein contribution to the relaxation rate of the protons of benzene* (●) *and the phenyl protons of toluene* (○)

The diamagnetic contribution to the relaxation rate, $T_{1_d}^{-1}$, was determined by subtracting the relaxation rates for the protons of benzene and the phenyl protons of toluene observed in the absence of hemeprotein from the relaxation rates in the presence of carbonmonoxyferrohemoglobin. The phenyl and methyl protons of toluene gave equivalent values for $T_{1_d}^{-1}$ to within experimental error.

those involving hemoglobin or cytochrome P-450.

A small but significant difference in relaxation rates of the proton moieties of the aromatic hydrocarbons exists between identical concentrations of cyanoferrihemoglobin and carbonmonoxyferrohemoglobin. These results suggest that direct coordination between the aromatic hydrocarbon and heme iron atom may not occur and that the aromatic hydrocarbon may not be completely displaced by cyanide but rather may continue to interact with cyanoferrihemoglobin. Such continued interaction of aromatic hydrocarbon with the heme iron atom in the presence of a small ligand is clearly demonstrable in the case of fluoroferrihemoglobin where a significant enhancement of relaxation rate is observed. Similar observations have been reported for 2,6 dimethylaniline and aniline-ferrihemoglobin interactions (15, 21). In particular, a comparative study was conducted on aniline and imidazole-hemoglobin interactions. Imidazole directly coordinates to the heme iron atom and was shown to yield virtually identical relaxation rate values in the presence of cyanoferrihemoglobin and carbonmonoxyferrohemoglobin, while fluoroferrihemoglobin produced no significant enhancement in the relaxation rates of the imidazole protons. The T_1^{-1} relaxation rates of the aniline phenyl protons, however, were significantly different in the presence of identical concentrations of cyanoferrihemoglobin and carbonmonoxyferrohemoglobin. Thus it appears that an interaction between certain "ligands" and cyanoferrihemoglobin may well occur.

The differential relaxation rates of the methyl and phenyl protons of toluene observed in the presence of fluoroferrihemoglobin may reflect a change in the molecular orientation of this aromatic hydrocarbon in complex with fluoroferrihemoglobin. This effect may occur when the average position of the methyl group is closer to the paramagnetic center than that of the phenyl group; alternatively, additional relaxation mechanisms may be present that give rise to enhanced methyl relaxation rate changes.

Both benzene and toluene appear to complex ferrihemoglobin equieffectively. When the ratio of the concentration of hemoglobin to benzene or hemoglobin to toluene is plotted versus the paramagnetic contribution to the relaxation rate ($T_{1_p}^{-1}$), an equivalent line is observed for the protons of benzene and the methyl and phenyl protons of toluene (Fig. 10). Thus these results would tend to indicate that the association constant for complex formation between benzene and human ferrihemoglobin or toluene and human ferrihemoglobin is similar for both aromatic hydrocarbons.

A wide variety of drugs including the antimicrobial nitrofurantoin, the antimalarial primaquine, the sulfonamides, and menadione induce non-immune hemolytic anemia in susceptible patient populations that exhibit a deficiency in the enzyme glucose-6-phosphate dehydrogenase (1-3). While the mechanisms that have been proposed in the pathogenesis of such hemolytic anemia include hemoglobin-catalyzed oxidative modification of the drug (possibly to reactive or toxic metabolites) and formation of activated oxygen (possibly facilitated by the presence of drug), very little is known concerning the ability of such drugs to interact with human hemoglobin in proximity to the O_2 binding site (i.e., the affinity of such drugs for human hemoglobin, or the

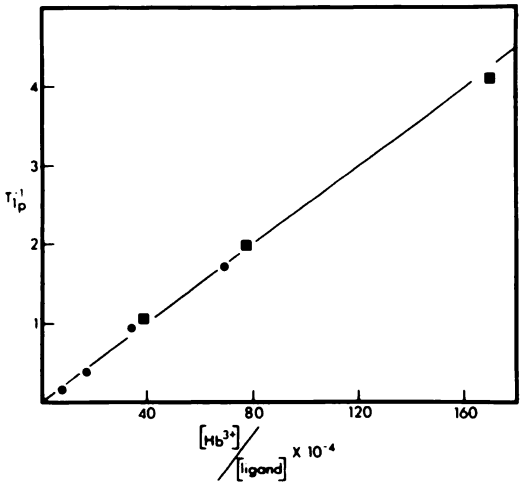

FIG. 10. *The increase in the paramagnetic contribution to the relaxation rate of the protons of benzene* (●) *and the phenyl protons of toluene* (■) *as a function of the ratio of the concentration of ferrihemoglobin to the concentration of the organic ligand*

structural or electronic requirements for such interactions to occur). Indeed, it has been shown that aniline, which is oxidized to para-aminophenol by hemoglobin both in a reconstituted enzyme system (7) as well as in intact red cells (8, 9), binds human ferrihemoglobin cooperatively with a Hill coefficient of 2.2 (22). Recently the influence of various ring substituents on the binding of nitrosobenzene to ferrohemoglobin has been investigated utilizing UV-visible spectroscopy (23). Results obtained from these studies have demonstrated that many of these nitrosobenzene derivatives bind cooperatively, and that large neutral para substituents, which cannot directly affect the formation of an Fe-NO bond, inhibited binding although not to the same degree as ortho substituents (23). It was concluded from these data that the affinity of substituted nitrosobenzenes for ferrohemoglobin depended in part on the interaction of the hydrophobic part of the ligand molecule with the heme crevice (perhaps causing distortion of the crevice), in addition to the electrostatic interaction occurring between the nitroso group and the heme iron atom.

Other studies have focused on the ability of certain agents to oxidize human oxyferrohemoglobin to ferrihemoglobin. It has been shown that oxidation of human oxyferrohemoglobin by organic agents such as nitrosoarenes (23) and cyclohexadiene and certain alkyl halides proceeds with recrystallized protein, lysed cells, and intact human erythrocytes (13, 14). Wallace and Caughey (24), for example, have demonstrated that reaction of a series of phenols with oxyferrohemoglobin produced methemoglobin. Thus the pathogenesis of nonimmune hemolytic anemia or methemoglobinemia may arise from a series of mechanisms active within the erythrocyte including bioactivation of the drugs or agents to a reactive metabolite, a drug-induced increase in hemoglobin-catalyzed generation of hydrogen peroxide (24, 25), or superoxide anion (4, 5, 26). While hydrogen peroxide and superoxide anion may play a role in such hemolysis and drug-induced methemoglobinemia, recent evidence (8, 9) also lends credence to the concept of reactive intermediate formation when certain drugs or agents interact with oxyhemoglobin. Little molecular-level information, however, is available on the nature of drug-hemoglobin complexation, distance of the drug to the paramagnetic center, orientation of the drug in complex with hemoglobin, or the role of the geometrical orientation in drug modification or hemoglobin oxidation. Information is also lacking as to stuctural limitations or requirements for such drug/agent-hemoglobin interactions to occur. Thus we have undertaken an investigation using ^{1}H Fourier Transform NMR relaxation time technique to characterize the interaction of certain aromatic hydrocarbons with human hemoglobin. The results presented herein suggest that these aromatic hydrocarbons do not directly coordinate with the heme iron atom but rather that benzene and toluene bind in proximity to the paramagnetic center, continue to interact with cyano- and fluoroferrihemoglobin, have rapid accessibility to the binding site and experience substantial relaxation rate contributions from the diamagnetic apoprotein. These results also suggest that a change in environment and/or geometry may occur in the presence of fluoride anion as evidenced by the differential change in relaxation rates of the phenyl and methyl protons of toluene. Such an approach as illustrated here may provide specific information regarding the molecular mechanisms of substrate-hemeprotein association and the overall role of this complexation in the oxidative modification of substrates and/or pathogenesis of non-immune hemolytic anemia or methemoglobinemia.

ACKNOWLEDGMENT

The authors wish to express their gratitude to Drs. P. H. Stern and K. P. Vatsis for critical review of this manuscript.

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