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Spectral Studies on the Interaction of Dichloromethane with Hemoglobin

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SUMMARY

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In spectrophotometric studies of hemoglobin solutions dichloromethane caused a decrease in the affinity and the cooperativity of oxygen binding. The maximum effects observed were at a dichloromethane partial pressure of 50 torr with no significant effects below 25 torr. At 50 torr dichloromethane oxygen affinity was decreased 50% and Hill n values, used as a measure of cooperativity, decreased from 2.95 to 2.07. Dichloromethane affected the binding of carbon monoxide to hemoglobin in solution as described by changes in Haldane "M" values, a measure of the relative affinities of carbon monoxide and oxygen. Carbon monoxide binding was decreased by dichloromethane partial pressures as low as 0.03 torr. Maximum effects, involving a change in M from 257 to 143, occurred at 5 torr dichloromethane, a concentration that did not affect oxygen binding to hemoglobin. Changes in the proton NMR spectrum for hemoglobin solutions in the presence of dichloromethane were compared to the results of the oxygen and carbon monoxide binding studies and to information on the binding sites for dichloromethane determined by X-ray crystallography. The contact shifted resonance attributed to the β-heme was shifted approximately 1–2 ppm downfield in the presence of 100 torr dichloromethane. The upfield ring-current shifted resonance at +6.54 ppm, which has contributions from both the α- and β-E11 valine, was split into two peaks. Changes were also observed in the aromatic and methyl regions of the spectrum, suggestive of conformational changes in the protein with dichloromethane.

INTRODUCTION

Dichloromethane (methylene chloride, CH_2Cl_2) has been widely used as an industrial solvent and as a constituent of paint and varnish removers, cleaning fluids and aerosols. The most common toxicity associated with dichloromethane exposure has been elevated carboxyhemoglobin levels (1–3) and descriptions of the mechanisms underlying this effect center on the metabolic conversion of dichloromethane to carbon monoxide by the cytochrome P-450 mixed function oxidase system (2, 4–6). However in an *in vitro* study of carbon monoxide binding to purified hemoglobin, Settle (7) observed changes in carbon monoxide binding in the presence of dichloromethane, effects not due to its metabolic conversion. Thus a systematic study of the effects of this compound on oxygen and carbon monoxide binding was necessary in order to understand better the possible mechanisms underlying the toxicities associated with exposure to this compound.

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An understanding of the effects of dichloromethane on ligand binding to hemoglobin was also of interest with the *in vitro* observations that this molecule is effective in preventing and reversing hemoglobin sickling (8, 9). Most compounds introduced for the treatment of this disease are somewhat non-specific in their action (10) and may produce marked toxicities (11). The proposed mechanism for the anti-sickling effect of dichloromethane is more specific, involving the interaction of the molecule at specific sites in the hemoglobin tetramer to inhibit and reverse aggregation (8, 9).

The present study concerns the determination of oxygen and carbon monoxide binding to hemoglobin in solution as influenced by dichloromethane. The ligand-binding data are considered with respect to both changes in proton NMR spectrum in the presence of dichloromethane and binding sites reported from X-ray crystallographic analysis of hemoglobin (9).

MATERIALS AND METHODS

The following chemicals were used in this study: dichloromethane, analytical grade, Aldrich Chemicals; deuterium oxide, 99.8 and 100%, BioRad; Tris (hydroxymethyl) aminomethane base and the hydrochloride salt (Trisma), Sigma Chemical Company. All gases were obtained from Matheson Gas Products. The oxygen was both Research Purity and Ultra High Purity; the carbon monoxide, nitrogen and helium were all Ultra High Purity.

Hemoglobin was purified from outdated blood supplied by the Moffitt Hospital Blood Bank, using a method similar to that of Finch *et al.* (12). The erythrocytes were washed three times with 1.2% NaCl and twice with 1.5% NaCl prior to lysing.

Oxygen binding studies. Hemoglobin samples were deoxygenated by evacuation using a Cenco Hyvac 7 vacuum pump. Absorption spectra were obtained using a Perkin-Elmer 356 dual beam spectrophotometer equipped with a controlled temperature chamber and a Hitachi 156 recorder. The spectra were read from 450 to 600 nm. In examining the effects of dichloromethane on oxygen binding to hemoglobin, a modification of the technique described by Rossi-Fanelli and Antonini (13) was used. Hemoglobin solutions were diluted in 22 mM tris sulfate (pH 7.0) to give samples approximately 6×10^{-5} M in heme. Samples were equilibrated with oxygen at 0–2° under light to remove the residual carbon monoxide (monitored by examination of the first derivative of the absorbance spectrum) and then evacuated at 0–2° with gentle swirling. The deoxy sample was then brought to 30° and exposed to dichloromethane vapor at the desired partial pressure. After obtaining the oxygen dissociation curve, the absorbance for 100% oxyhemoglobin was determined graphically (14). The data were then applied to a Hill plot based on the equation

$$\log \frac{Y}{1 - Y} = \log K + n \log (O_2)$$

where Y is the fraction of hemoglobin in the oxy-form. The P_{50} values (partial pressure of oxygen required for 50% saturation) and the Hill n values at 50% saturation (cooperativity of oxygen binding) were calculated using a simple computer program.

Carbon monoxide binding studies. In describing the effects of dichloromethane on carbon monoxide binding at 30° the procedures of Benesch *et al.* (15) were modified to permit constant equilibration with dichloromethane. In these studies the relative affinities of carbon monoxide and oxygen were taken as an index of changes in carbon monoxide binding. The absorption spectra for samples of oxy- and carboxyhemoglobin were observed from 370 to 450 nm using a Perkin-Elmer 200 UV-Vis, double beam spectrophotometer connected to a Perkin-Elmer model 56 recorder. The hemoglobin sample (2×10^{-6} M in 22 mM tris sulfate at pH 7.0) was oxygenated as described previously then equilibrated with an oxygen or oxygen/dichloromethane mixture, as shown in Fig. 1. To regulate the concentration of dichloromethane-oxygen mixture bubbling through the sample, a series of flowmeters were used. In all cases the dichloromethane was controlled by a Matheson 610A flowmeter, whereas the oxygen was regulated by either a Matheson 610A flowmeter (0.3–100 ml air/m) or a Gilmont No. 11 flowmeter (10–300 ml air/m) de-

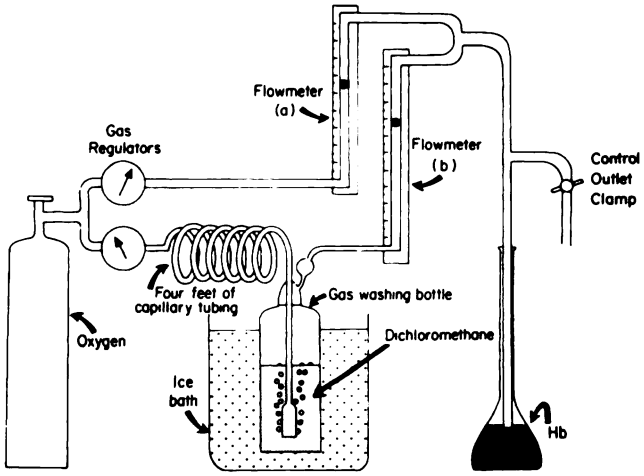


FIG. 1. *Gas equilibration system*

Relative concentrations of dichloromethane and oxygen were controlled by two flowmeters (a and b) in parallel. The dichloromethane liquid and hemoglobin solutions were maintained at constant temperature. The control outlet permitted constant rate of gas flow through the hemoglobin solution.

pending on the desired dilution factor. This system required a 2-3 hr equilibration period before the dichloromethane partial pressure in the final outlet remained constant, as measured by gas chromatography. To ensure that the hemoglobin sample was exposed to a constant partial pressure of dichloromethane throughout the experiment, the gas flow was continuously monitored. Dichloromethane partial pressures were determined using a Beckman GC-2A chromatograph equipped with a 6-foot molecular seive 13X column and a thermal conductivity detector. The current was set at 200 milliamperes, temperature at 160°, with a helium flow of 80 ml per minute. Under these conditions dichloromethane had a retention time of approximately 70 sec.

Concentrations of oxy- and carboxyhemoglobin were measured and the data used to calculate Haldane's M, a measure of the relative affinity of the two ligands, which can be expressed as

$$M = \frac{(\mathrm{HbCO})\ (\mathrm{O_2})}{(\mathrm{HbO_2})\ (\mathrm{CO})}$$

From a plot of $\log \frac{(\mathrm{HbCO})}{(\mathrm{HbO_2})}$ versus $\log \frac{(\mathrm{CO})}{(\mathrm{O_2})}$ the partition coefficient may be determined:

$$\log M = -\log \frac{(\mathrm{CO})}{(\mathrm{O_2})} \text{ where } \log \frac{(\mathrm{HbCO})}{(\mathrm{HbO_2})} = 0.$$

NMR studies. The Amicon Model 12 stirred cell equipped with a PM 30 membrane was used to prepare the hemoglobin sample in deuterium oxide. The Bruker HXS-360 spectrometer in the Stanford University Magnetic Resonance Laboratory was used to generate spectra by both correlation spectroscopy (16) and Fourier transform NMR with solvent peak suppression (17). The hemoglobin sample was deoxygenated in a 5 mm Wilmad glass-stoppered tube, and the probe temperature maintained at 23°. Contact-shifted resonances were examined using correlation spectroscopy to eliminate the HOD peak which leads to dynamic range problems with the computer (18). The spectra were analyzed after 3911 scans using a sweep width of 6000 Hz and sweep time of 0.8 sec. Spectra were also obtained using FT-NMR at 360 MHz. A sweep width of 16,000 Hz was used with an acquisition time of 0.253 sec. The spectra were analyzed after 8192 scans. The upfield contact shifted resonances were observed at +5.5 to +8.5 ppm from the HOD peak. The effects of dichloromethane on aromatic and aliphatic pro-

ton resonances of hemoglobin A were also observed at −4.8 to −0.8 ppm and −0.3 to +4 ppm, respectively.

RESULTS

Oxygen binding studies. The partial pressures of dichloromethane used in this study ranged from 5 to 100 torr, which included the pressures effective in preventing (25 torr) and reversing (50 torr) hemoglobin S aggregation (8). In describing the effects of dichloromethane on oxygen binding, the fractional saturation was calculated for each addition of oxygen as shown by the standard sigmoid-shaped oxygen dissociation curves in Fig. 2. After exposure to dichloromethane there was a dose-related shift of the curves to the right indicating a decrease in oxygen affinity.

The P_{50} and n were calculated using a Hill plot of the data (Table 1). The results showed an increase in P_{50}, corresponding to a decrease in oxygen affinity, with increasing pressures of dichloromethane. This effect was significant with pressures greater than 25 torr, with the maximal effect at 40 torr. The values at 50 and 100 torr were lower than for 40 torr which may be explained by the changes in cooperativity observed at these concentrations. There was a significant difference in cooperativity between the control value and that observed with 40, 50, and 100 torr dichloromethane, with no significant difference between 50 and 100 torr levels.

To determine the reversibility of this effect on oxygen binding, the oxygen dissociation curve was obtained as described previously in the presence of 40 torr dichloromethane. After removal of the dichloromethane by evacuation the oxygen dissociation curve was again observed for the same sample and found to be not significantly different from that obtained for the control samples. Gel electrophoresis of the

TABLE 1

Dichloromethane effects on the affinity and cooperativity of oxygen binding to hemoglobin in solution

Oxygen dissociation curves for hemoglobin solutions (6×10^{-5} M heme) in 22 mM Tris sulfate buffer at pH 7.0 and 30°, were derived from spectrophotometric measurements as described in MATERIALS AND METHODS. Values for P_{50} (partial pressure of oxygen at 50% saturation) and n (cooperativity) were calculated from a Hill plot of the data. Each value is the mean ± standard error of the mean.

Dichloromethane pressure	Number of experiments	Log P_{50}	P_{50}	Hill n
(torr)				
0	6	0.94 ± 0.04	8.71	2.95 ± 0.04
25	4	1.00 ± 0.05	10.00	2.80 ± 0.05
30	4	1.08 ± 0.05[a]	12.02	2.82 ± 0.05
40	3	1.16 ± 0.04[a]	14.45	2.40 ± 0.04[a]
50	4	1.11 ± 0.03[a]	12.88	2.07 ± 0.03[a]
100	3	1.09 ± 0.03[a]	12.30	2.05 ± 0.03[a]

[a] $p < 0.01$ versus control (no dichloromethane).

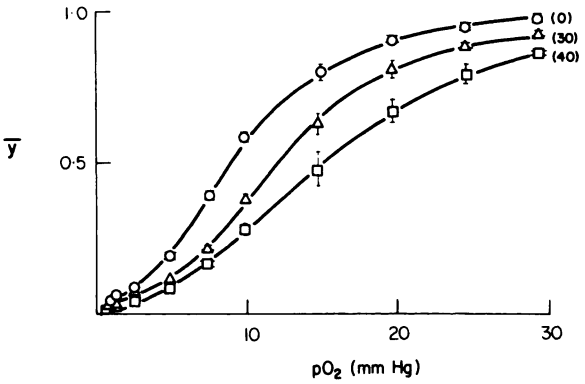


FIG. 2. *Oxygen dissociation curves for hemoglobin in the presence of dichloromethane*

Spectrophotometric measurements of oxygen binding to hemoglobin (6×10^{-5} M in 22 mM Tris sulfate at pH 7.0 and 30°) were carried out as described in MATERIALS AND METHODS. Each point represents the mean of 4–6 experiments with vertical bars as standard error of the mean. Numbers in parentheses are dichloromethane partial pressures in torr.

hemoglobin samples before and after exposure to dichloromethane showed no evidence for dimerization.

Carbon monoxide binding studies. In these studies a wide range of dichloromethane pressures were used to include the partial pressures relevant to the anti-sickling effect and oxygen binding effects, as well as lower partial pressures which may have toxicological relevance. Figure 3 shows some of the data in a graphical form derived from Haldane's equation. A dose-dependent shift of the curves to the right indicates a decrease in M value representing a change in the relative affinities of carbon monoxide and oxygen. As shown in Table 2 measurable effects on M value occurred at dichloromethane partial pressures as low as 0.03 torr with maximal effects at 5 torr. In the presence of dichloromethane all values of M were significantly different ($p < 0.05$) from the control value. Using the Studentized Range Test at $p < 0.05$, M values at 2.5 and 5 torr were shown to be significantly lower than the value at 0.3 torr dichloromethane and lower than the values at partial pressures of 10 torr and above. Although a change in affinity for either ligand could be reflected by a change in M, it seems reasonable to conclude that the effects at 5 torr and less were brought about by a decrease in carbon monoxide affinity since no change in oxygen binding was observed at these lower pressures of dichloromethane. The effects on M value did not follow a linear relationship with dichloromethane partial pressure. This is to be anticipated in view of the multiplicity of potential binding sites for dichloromethane (see DISCUSSION) and the direct effects of the compound on oxygen binding at higher concentrations. When the effects of dichloromethane on oxygen affinity were considered in the region of 25 to 50 torr and the necessary corrections made in Haldane's equation, it was found that the carbon mon-

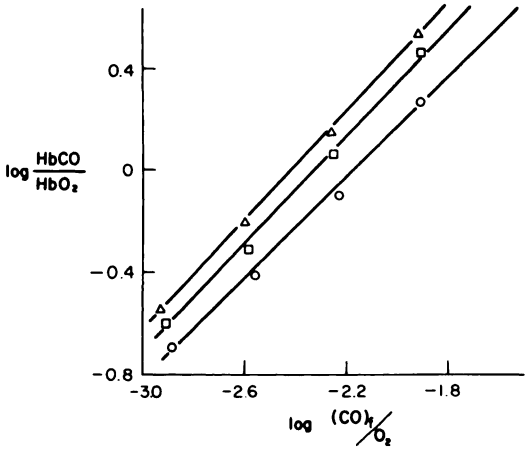


FIG. 3. *Relative oxygen and carbon monoxide binding to hemoglobin in solution*

Absorption spectra for oxy- and carboxyhemoglobin solutions (2×10^{-6} M in Tris sulfate at pH 7.0 and 30°) were determined following equilibration with dichloromethane as described in MATERIALS AND METHODS. Data are plotted in a form of Haldane's equation. △——△, control; □——□, 0.3 torr dichloromethane; ○——○, 5 torr dichloromethane.

TABLE 2

Dichloromethane effects on the relative binding of carbon monoxide and oxygen to hemoglobin in solution

Absorption spectra for oxy- and carboxyhemoglobin solutions (2×10^{-6} M heme) in 22 mM Tris sulfate at pH 7.0 and 30° were determined under conditions of equilibration with dichloromethane as described in MATERIALS AND METHODS. The relative concentrations of the two forms of hemoglobin at various partial pressures of oxygen and carbon monoxide were calculated. The data were used to determine the partition coefficient M, a measure of the relative affinity of carbon monoxide and oxygen, based on Haldane's equation. Values are means ± standard error at the mean.

Dichloromethane concentration	Number of experiments	M value
(torr)		
0	9	257 ± 3
0.03	8	193 ± 5
0.3	6	197 ± 8
2.5	6	169 ± 2
5	6	143 ± 10
10	6	211 ± 8
15	6	183 ± 6
25	7	205 ± 8
35	6	188 ± 12
50	4	173 ± 2

In the presence of dichloromethane all values of M were significantly different from control ($p < 0.05$). Using the Studentized Range Test M values at 2.5 and 5 torr were significantly lower ($p < 0.05$) than the value at 0.3 torr dichloromethane and lower than the values at partial pressures of 10 torr and above.

oxide binding at these partial pressures did not differ significantly from that for 5 torr.

NMR studies. The region of the spectrum containing the hyperfine shifted proton resonances is shown in Fig. 4. The peaks observed at approximately −12.5 and −18.4 ppm from HOD have been assigned to the heme methyls of the α- and β-subunits, respectively (19–21). In the presence of dichloromethane the contact shifted resonance at −18.4 ppm was shifted downfield to produce a large resonance at −19.2 ppm with a broad shoulder extending to −22 ppm indicating some interaction with, or rearrangement of the region around, the β-heme. The resonance at −12.5 ppm was not greatly affected, although the peak appeared somewhat broadened. The resonance at −14.5 ppm appeared to shift downfield to approximately −16 ppm in the presence of dichloromethane. Contributions to this resonance have not been formally assigned but have been suggested to involve changes in the β-heme on ligand binding (20).

Other regions of the spectrum were examined using FT-NMR. The upfield ring-current shifted proton resonances were of interest in that they are sensitive to changes in the tertiary structure around the heme pockets (22–24). The region of the spectrum containing these resonances is shown in Fig. 5. In the presence of dichloromethane the peak at +6.54 ppm, which has contributions from both the α- and β-E11 valines, was split into two peaks at +6.48 and 6.65 ppm.

The effects of dichloromethane on the entire spectrum, exclusive of the contact shifted resonances, are shown in Fig. 6. The appearance of a peak at −0.83 ppm with dichloromethane suggests a shift of approximately 1 ppm downfield for one of the peaks in the region of −0.3 to +0.1 ppm. Although the position of this peak prior to dichloromethane treatment could not be

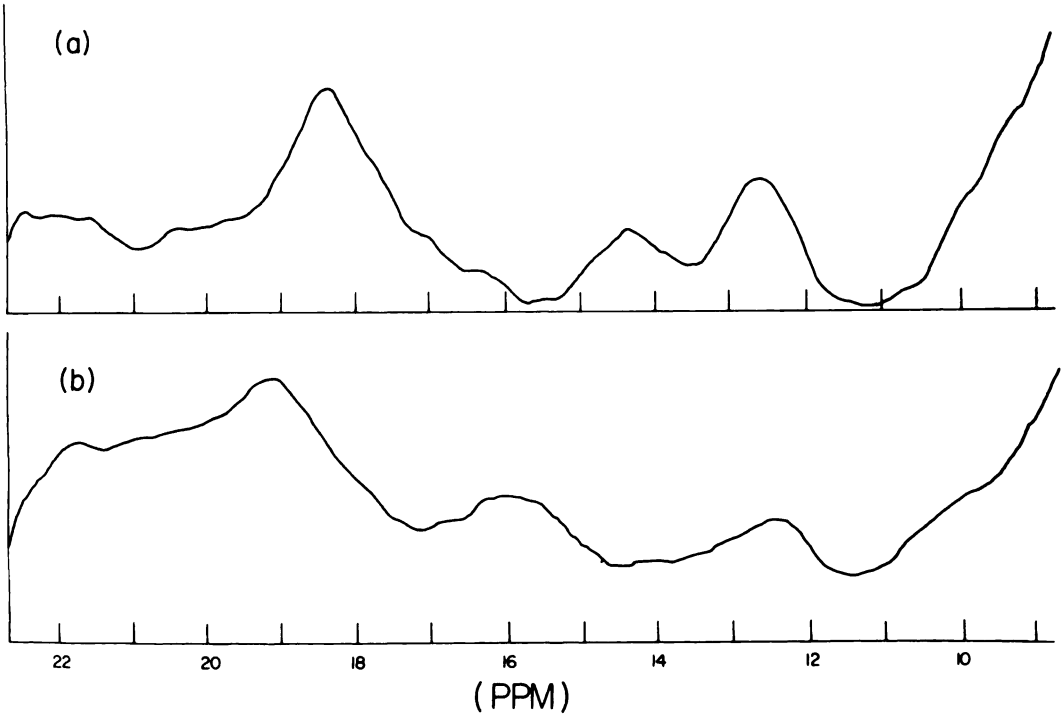


FIG. 4. *The 360 MHz hyperfine shifted proton resonances for deoxyhemoglobin A in 0.1 M phosphate in D_2O at pD 7.0 and 36°*

The units are given in ppm from HOD. a) deoxyhemoglobin control; b) deoxyhemoglobin in presence of 100 torr dichloromethane.

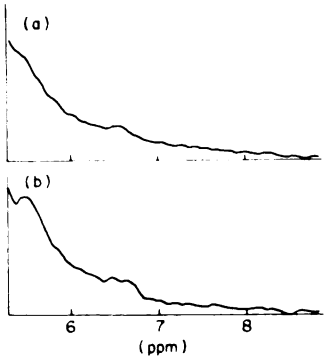


FIG. 5. *The 360 MHz ring-current shifted proton resonance for deoxyhemoglobin A in 0.1 M phosphate in D_2O at pD 7.0 and 36°*

The units are given in ppm from HOD. a) deoxyhemoglobin control; b) deoxyhemoglobin in presence of 100 torr dichloromethane.

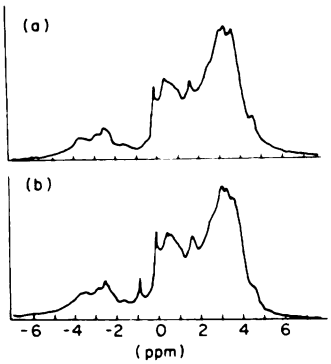


FIG. 6. *The 360 MHz proton NMR spectrum for deoxyhemoglobin A in 0.1 M phosphate in D_2O at pD 7.0 and 36°*

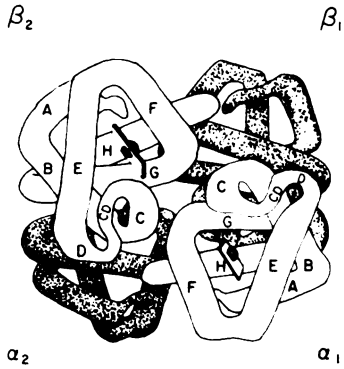
The units are given in ppm from HOD. a) deoxyhemoglobin control; b) deoxyhemoglobin in presence of 100 torr dichloromethane.

established, the resonances in this region are primarily attributed to protons of α-carbon methyls (25). Changes in the shape of the envelope from +3.05 to +3.65 suggests shifts in various peaks which may be due to conformational changes in the protein; however analysis of these peaks was complicated by the number of overlapping peaks and the poor resolution in this region.

DISCUSSION

These oxygen and carbon monoxide binding studies using purified hemoglobin solutions show that dichloromethane causes a decrease in affinity of the heme for the two ligands and decreases the cooperativity of oxygen binding. In recent studies using whole blood solutions carbon monoxide binding to hemoglobin was not changed in the presence of low partial pressures of dichloromethane (26, 27). It is possible that in whole cell preparations the high solubility of dichloromethane in lipids and its affinity for erythrocyte membranes (28) may have resulted in a marked decrease in its effective concentration at binding sites in the hemoglobin molecule. However, low partial pressures of dichloromethane caused a partial reversal of the carbon monoxide-induced shift of the oxygen dissociation curve (27). Such an effect is predicted by the present results in that low concentrations of dichloromethane should decrease the effect of carbon monoxide rather than decreasing affinity of oxygen for the heme directly. These direct effects of dichloromethane on ligand binding to the hemoglobin occur in addition to the established toxic actions that result from metabolic conversion of the compound to carbon monoxide (1-3). The physiological implications of a dichloromethane-induced decrease in oxygen affinity are not clear. However such an effect could result in increased oxygen delivery to tissues which may offset the toxic effects of carboxyhemoglobin formation to some degree.

The possible mechanisms involved include but are not limited to 1) a direct interaction of dichloromethane with the heme to alter heme-ligand interactions, 2) interaction of dichloromethane at the subunit interface, thereby changing ligand affinity and cooperativity by interfering with heme-heme interactions, and 3) binding of dichloromethane at any one of several sites within the tetramer which would cause a change in conformation and alter the binding characteristics by favoring the unliganded state. Potential binding sites for dichloromethane are shown in Fig. 7 which is a summary of data obtained by Schoenborn (9) from X-ray crystallographic analysis of hemoglobin in the presence of relatively high partial pressures of dichloromethane (350 torr). In considering the relationships between binding sites and effects on ligand affinity it must be kept in



Dichloromethane Binding Sites to Hemoglobin

SITE	CHAIN	HELIX
1	α_1 , α_2	A,G,E,H
2	β_1 , β_2	G,H,E
3	$\alpha_1\beta_2$,$\alpha_2\beta_1$ contact	H,F (β) C (α)
4	β_2,(β_1)	CD,E Heme pyrrole

FIG. 7. *Binding sites for dichloromethane in deoxyhemoglobin*

The data in the table are taken from Schoenborn (9) based on X-ray crystallographic analysis of deoxyhemoglobin in the presence of dichloromethane (350 torr). For clarity only the helices of the α_1 and β_2 chains are lettered. The designated helices of a particular chain are given to mark the boundaries of a potential binding site. For example, site 2 on the β_2 chain is located in a region of the subunit bounded by helices G, H and E.

mind that the present studies concern hemoglobin in solution at lower dichloromethane partial pressures and that no direct data are available on the relative affinities of the individual dichloromethane binding sites. From the X-ray data, binding sites 1 and 2 appear to be very similar and occur in both the α and β subunits. Evidence for such dichloromethane interactions at the heme in the NMR analysis is provided by the upfield ring-current shifted resonance spectrum where the single coalesced peak for the α- and β-E11 valines at +6.54 ppm was split into two peaks in the presence of dichloromethane. This effect could be caused by a direct interaction between the dichloromethane molecule and residues in these regions of the hemoglobin subunits. Dichloromethane binding sites 1 and 2 involve both the α-E15 leucine and the β-E15 phenylalanine (9) and these two residues are in close proximity to the E11 valines of the α and β subunit, respectively. An interaction of dichloromethane with such sites could represent the mechanisms underlying the observed changes in ligand binding. However changes in this region of the NMR spectrum for hemoglobin can also be caused by less specific agents including inorganic salts (24).

A third binding site for dichloromethane, apparent from crystallographic studies, is located at the subunit interfaces and appears to involve the β-H22 tyrosine and the β-H23 histidine. Although the present NMR data include evidence for changes in spectrum involving contributions from aromatic residues, no specific assignments could be made of direct relevance to the possible binding of dichloromethane to site 3. However if such binding occurs it could result in interference with the heme-heme interactions necessary for cooperativity of oxygen binding. The fourth binding site for dichloromethane appears to be located in the region of the β-heme and is observed in the β_2-chain only (9). The interacting residues at this site are the β-heme pyrrole, the β-E6 histidine, and the β-CD1 phenylalanine, all of which could contribute to the changes observed in the NMR spectrum. The observation that this site is present only in the β-subunit is especially meaningful when considered with the effects on the contact shifted resonances. The resonance at -12.5 ppm which has been assigned to the α-heme methyl protons was not appreciably affected, whereas the peak at -18.4 ppm associated with the β-heme was shifted downfield, suggesting some change in the structure around the β-heme induced by dichloromethane which might be expected to alter the affinity of ligand binding. If this binding site at the heme in the β subunit has high affinity for dichloromethane a possible explanation for why carbon monoxide binding is affected at much lower concentrations of dichloromethane than

those altering oxygen binding may be provided. Perutz (29) has suggested and Gray and Gibson (30) have demonstrated that oxygen binds preferentially to the α-subunit, whereas carbon monoxide binds preferentially to the β-subunit (19). It seems reasonable to expect that if dichloromethane binds only in the region of the β-heme, changes in affinity for carbon monoxide would be more readily apparent since binding to the first subunit proceeds more slowly than to the second and third subunits.

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