Action of Chloroform and Its Chlorinated Analogs on Hemoproteins*

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ABSTRACT: Chloroform and its chlorinated analogs, dichloromethane and carbon tetrachloride, when present in aqueous buffer systems, produce a spectral perturbation of ferrihemoglobin characterized visually by a "reddening" of the solution. In addition, sedimentation velocity of ferrihemoglobin decreases from 4.4 to 3.0 S in the presence of the chloromethanes. Molecular weight determination by gel filtration indicated dissociation of the ferrihemoglobin with interaction of chloroform during chromatography; however, sedimentation equilibrium studies showed that the molecular weight was unchanged under the conditions used for sedimentation velocity determination. Measurement of the diffusion coefficient showed that an increase in frictional ratio in the presence of chloroform was responsible for the lowered sedimentation rate. Although both spectral and shape perturbations of the ferrihemoglobin were enhanced by increased ionic strength and were readily reversible with removal of the chloromethane, they involved at least two different sites in the hemoglobin molecule. The spectral changes were complete within 2 min at any ionic strength and evidently represent an easily accessible site for chloromethanes near the heme group; on the other hand, conversion to the slowly sedimenting species at low ionic strength required several days. Myoglobin was inert to chloroform showing no detectable change in spectrum, sedimentation velocity, or optical rotation.

The importance of hydrophobic interaction in the stabilization of structure of proteins has become well established in the last decade. Elucidation of the structures of myoglobin and hemoglobin by X-ray crystallography has provided major evidence for hydrophobic stabilization of these molecules (Kendrew, 1962; Perutz *et al.*, 1964; Dickerson, 1964). In addition to internal hydrophobic interaction, it has been shown that these hemoproteins will bind hydrophobic molecules of diverse nature. Bonding has been reported for the short-chain hydrocarbon series of propane, butane, and pentane (Wishnia and Pinder, 1966; Taylor *et al.*, 1966) as well as for cyclopropane and xenon (Schoenborn, 1965, 1967; Schoenborn *et al.*, 1965). Bromothymol blue has been found to have its strongest affinity for hemoglobin at acid pH where it is largely nonionized (Antonini *et al.*, 1963). Kiehs *et al.* (1966) using equilibrium dialysis studied the interaction of 17 aromatic anilines and phenols with hemoglobin and found the amount of binding directly related to the degree of hydrophobic character of the organic compound; however, no adsorption to sperm-whale myoglobin was detected. A number of aromatic compounds apparently bind at two sites in myoglobin and alter its structural stability (Cann, 1969).

The interaction of chloroform with hemoglobin was noted as early as 1906 by Moore and Roaf (1906). They described a reddening of an aqueous solution of hemoglobin when it was partially saturated with chloroform. The reddening was found to be reversed upon evaporation of chloroform from the solution. They concluded that chloroform forms an unstable complex or aggregate with hemoglobin.

The current investigation attempted to characterize the physical changes occurring with the interaction of chloroform and hemoglobin and to view this in relation to effects of two other chloromethanes of greater and lesser hydrophobicity, carbon tetrachloride and dichloromethane, respectively. Furthermore, the apparent inertia of myoglobin to chloroform is noted.

Experimental Section

Bovine, equine, and human ferrihemoglobins and equine heart and sperm-whale ferrimyoglobins were obtained from Calbiochem. Rabbit oxyhemoglobin was prepared from fresh rabbit blood. To ensure complete oxidation of the proteins, three drops of 2% potassium ferricyanide solution were added to 25 ml of a 5% protein solution and the protein was then eluted from G-25 Sephadex to remove the oxidant. Reduced hemoglobin was formed by the addition of sodium dithionite. Oxyhemoglobin was formed by oxygenating after reduction, followed by passage over a mixed-bed resin (Bio-Rad AG 501-X8; Brown and Mebine, 1969) to remove residual dithionite. Protein concentration was determined by measurement of the optical density at the Soret maximum of the ferri state. Values for the extinction coefficient were obtained by dry weight measurement for equine ferrihemoglobin ($E^{1\%}_{406}$ 86) and from values in the literature for bovine ferrihemoglobin ($E^{1\%}_{405}$ 90) (Holden, 1936), equine ferrimyoglobin ($E^{1\%}_{407}$ 81) (Theorell and Ehrenberg, 1951), and sperm-whale ferrimyoglobin ($E^{1\%}_{407}$ 90) (Samejima and Yang, 1964).

Chloroform and carbon tetrachloride were obtained from Merck. Dichloromethane was obtained from Allied Chemical Co. Chloroform labeled with ^{14}C was a product of New England Nuclear Corp.

Buffers were equilibrated with chloromethanes in stoppered flasks placed in an ice bath in the cold room, using a magnetic stirrer operating at a medium rate for a minimum of 48 hr. The saturated buffers were then allowed to stand undisturbed for a minimum of 2 hr to permit dispersed solvent spherules

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TABLE I: Chloroform Concentration in Buffers of Varying Ionic Strength.

Buffer	pH	Conductivity 0° (mmhos)	$CHCl_3$ Concn at 100% Satn			
			0°		23°	
			mg/ml	M	mg/ml	M
Distilled H_2O	6.2	0.005	8.2	0.069	6.9	0.058
0.01 M P[a]	6.9	0.6	7.7	0.065	6.9	0.058
0.01 M P + 0.1 M NaCl	6.7	5.8	7.4	0.062	7.0	0.059
0.01 M P + 0.2 M NaCl	6.8	10.5	7.2	0.060	5.3	0.044
0.01 M P + 0.4 M NaCl	6.5	18.5	7.1	0.059	4.6	0.038
0.05 M P + 0.1 M NaCl	7.0	7.5	7.4	0.062	6.8	0.057
0.01 M P + 1.0 M NaCl	6.8	45.0	5.6	0.047	4.8	0.040

[a] P = sodium phosphate.

to coalesce and settle to the solvent layer. The aqueous buffer layers were then decanted and diluted to the desired level of solvent saturation. Chloroform concentration was determined using [^{14}C]HCl_3 for saturation of buffers at 0 and 23°; values are given in Table I. Concentrations for dichloromethane and carbon tetrachloride at saturation at 20° in water were obtained from the Merck Index: CH_2Cl_2, 26 mg/ml or 0.31 M; CCl_4, 0.8 mg/ml or 0.005 M.

Hemoprotein samples were dialyzed in 0.25-in. diameter dialysis tubing *vs.* the buffer at the desired level of chloromethane saturation in stoppered flasks with stirring at 4°. For most of the experiments, two-thirds total saturation with $CHCl_3$ was chosen as a desirable level. At 100% saturation, precipitation of hemoglobin invariably occurred. At three-fourths saturation the hemoglobin was stable at the temperature of saturation; however use of the two-thirds level ensured that any rise in temperature would not result in loss of protein. At the two-thirds level of saturation at various ionic strengths, the spectral changes were complete.

Column chromatography was performed using Sephadex G-75 and DEAE-Sephadex A-50 obtained from Pharmacia. Carboxymethylcellulose was a product of Bio-Rad. All columns were equilibrated a minimum of 24 hr with or without two-thirds saturation with chloroform. Hemoprotein samples (0.5–1.0%) were dialyzed 48 hr against the eluent buffer. Column dimensions were 90 × 1.7 cm for Sephadex G-75 and A-50 and 2.2 × 20 cm for CM-cellulose. Flow rate was maintained at 10–15 ml/hr and monitored at 520 mμ, the isobestic point for equine ferrimyoglobin and equine ferrihemoglobin ± $CHCl_3$. Blue Dextran (Pharmacia) was monitored at 625 mμ.

Spectra were recorded in a Model 11 or 15 Cary recording spectrophotometer at room temperature. Optical rotatory measurements were performed at 22° with a Cary Model 60 recording spectropolarimeter. Both spectral and optical rotatory measurements were made with hemoproteins that had been dialyzed in 0.01 M sodium phosphate–0.2 M sodium chloride, pH 6.8 buffer, unless noted otherwise.

Free-boundary electrophoresis was performed in a Perkin-Elmer Model 38-A Tiselius apparatus using a 2-ml rectangular Tiselius cell. Measurements of electrophoretic mobility were made in 0.05 M sodium phosphate buffer–0.1 M sodium chloride, at 2° with a current of 16 mA. Conductivity measurements were made using a Radiometer conductivity meter, type CDM 2C. The column and ampholytes (pH range 3–10) for isoelectric focusing were obtained from LKB.

Sedimentation velocity experiments were performed at or near 4° in a Spinco Model E analytical ultracentrifuge at 59,780 rpm in 0.01 M sodium phosphate–0.2 M sodium chloride (pH 6.8) unless otherwise indicated. Low-speed sedimentation equilibrium was performed and molecular weights determined according to the procedure of Richards *et al.* (1968). Diffusion coefficient measurements were made in the ultracentrifuge according to the procedure of Mommaerts and Aldrich (1958). Low-speed sedimentation equilibrium and diffusion coefficient measurements were performed using interference optics as adapted for use with red light (Bucher *et al.*, 1970).

Results

Spectra. Initial evidence of the interaction of chloroform and hemoglobin arose from the observation of an immediate reddening of a solution of equine ferrihemoglobin from its usual brown color on addition of $CHCl_3$-saturated buffer. Spectra were obtained for hemoglobin under varied conditions of pH, ionic strength, and chloromethane saturation. The effect of chloroform on several species and states of hemoglobin was investigated.

The characteristic spectrum found for equine ferrihemoglobin in the presence of two-thirds saturation with chloroform is shown in Figure 1. A decrease in absorption at 500 and 630 mμ is noted, accompanied by an increase in absorption at 530 mμ. A slight shift of the 280-mμ band is observed. A band appears at 360 mμ and the Soret band shifts from 405–406 to 411 mμ, accompanied by an approximate 20% loss of intensity. Ferrihemoglobin from other species (bovine, human, and rabbit) underwent spectral perturbations similar to equine throughout the range measured, 220–700 mμ. Dichloromethane and carbon tetrachloride produced similar spectral changes in equine ferrihemoglobin at two-thirds saturation although treatment with neither solvent resulted in changes so intense as those with $CHCl_3$.

The extent of the spectral shift was directly related to ionic strength at one-third saturation (Figure 1). At two-thirds saturation the spectrum at each of the ionic strengths was identical. Variation of pH between 6.0 and 8.0 produced no spectral changes in addition to those occurring at two-thirds saturation in a buffer of 0.01 M sodium phosphate–0.4 M NaCl (pH 7.0).

Oxy- and reduced hemoglobin showed no spectral changes after dialysis *vs.* two-thirds chloroform-saturated buffer as compared to untreated hemoglobin in these states. Sperm-whale

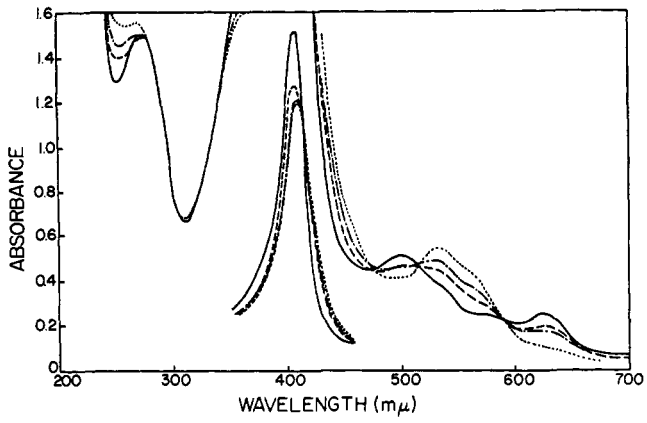

FIGURE 1: Absorption spectra of equine ferrihemoglobin at two-thirds saturation with $CHCl_3$–0.1 M phosphate–0.2 M NaCl (· · · · · ·) and at one-third saturation of chloroform at different ionic strengths. Concentration of protein, 0.89 mg/ml, except 0.18 mg/ml on the Soret region (around 400 mμ); insert: (—) no $CHCl_3$, 0.01 M phosphate–0.1 M NaCl; (- - -) $CHCl_3$–0.01 M phosphate; (— - — - —) $CHCl_3$–0.01 M phosphate–0.1 M NaCl; (superimposed on · · · · · ·) $CHCl_3$–0.01 M phosphate–1.0 M NaCl. All buffers pH 6.8.

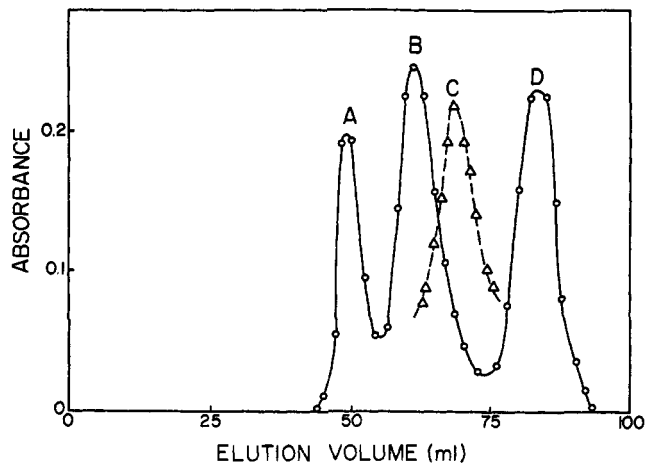

FIGURE 2: Chromatographic profile on G-75 Sephadex of the indicated substances (1.0 ml of each applied) after dialysis for 48 hr against buffer (0.05 M phosphate, pH 7.0) containing no chloroform, er against similar buffer two-thirds saturated with chloroform; absorbance read at 520 mμ for hemoglobin and myoglobin and at 625 mμ for Blue Dextran: A, Blue Dextran (1 mg/ml), presence *or* absence of chloroform; B, equine ferrihemoglobin (5 mg/ml), no chloroform; C, equine ferrihemoglobin (5 mg/ml), chloroform present; D, equine ferrimyoglobin (5 mg/ml), presence *or* absence of chloroform.

and equine heart ferrimyoglobin did not undergo any spectral perturbation with any degree of chloroform saturation.

All spectral changes of ferrihemoglobin were found to be complete within two minutes of dilution at room temperature using buffers with varied pH, ionic strength, and solvent saturation. Reversion to the original spectrum took place with the removal of chloroform from the buffer by evaporation, dialysis, or 50-fold dilution with solvent-free buffer.

Perturbed spectra were found to be identical when either distilled chloroform or that stabilized by 0.75% ethanol was used to saturate the buffers. Thus the possibility that these changes are attributable to traces of phosgene in the buffer is apparently precluded.

Column Chromatography. Equine ferrihemoglobin and ferrimyoglobin were chromatographed on Sephadex G-75 to determine the effect of the presence of chloroform in the media on size and/or shape of the macromolecules. The pattern of elution of a mixture of hemoglobin, myoglobin, and Blue Dextran is shown in Figure 2. The results are tabulated in Table II. The positions of the peaks were invariant with length of dialysis against buffers containing chloroform (minimum 48 hr, maximum 7 days). Blue Dextran and myoglobin were eluted at the same position whether or not chloroform was present. Molecular weights were obtained by interpolation from Andrews' calibration curve for globular proteins (Andrews, 1964). The low value for hemoglobin in the absence of chloroform is in agreement with the value found by Andrews for bovine hemoglobin.

Elutions of ferrihemoglobin were also performed from DEAE-Sephadex equilibrated with 0.05 M sodium phosphate buffer (pH 8.0) in the presence and absence of two-thirds saturation with chloroform. Under gradient elution to pH 6.0 or 0.5 M NaCl, the hemoglobin eluted as a single band.

Several forms of gradient elution were attempted on carboxymethylcellulose initially equilibrated with 0.01 M sodium phosphate (pH 6.0). These included pH gradients to pH 8.0 and salt gradients to 0.4 M NaCl. At two-thirds saturation, hemoglobin eluted as a single band. However, if the ferrihemoglobin was diluted with buffer at two-thirds saturation immediately prior to application to the column, rather than being subjected to 48-hr dialysis, and if elution was conducted

TABLE II: Parameters Determined by Sephadex Chromatography.[a]

Hemoprotein	+ or $-CHCl_3$	V_e/V_0	Mol Wt
Mb	+	1.70	17,200
Mb	−	1.70	17,200
Hb	+	1.24	41,700
Hb	−	1.38	29,600

[a] See legend for Figure 4 for experimental details.

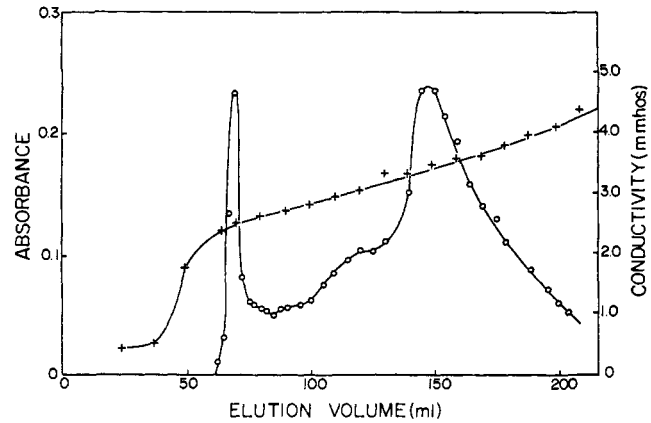

FIGURE 3: Chromatographic profile of carboxymethylcellulose column. One milliliter of a 10-mg/ml solution of equine ferrihemoglobin was applied to column immediately after dilution with two-thirds-saturated chloroform buffer; elution was with a shallow NaCl gradient: (○) absorbance of hemoglobin at 520 mμ; (+) conductivity, mmhos.

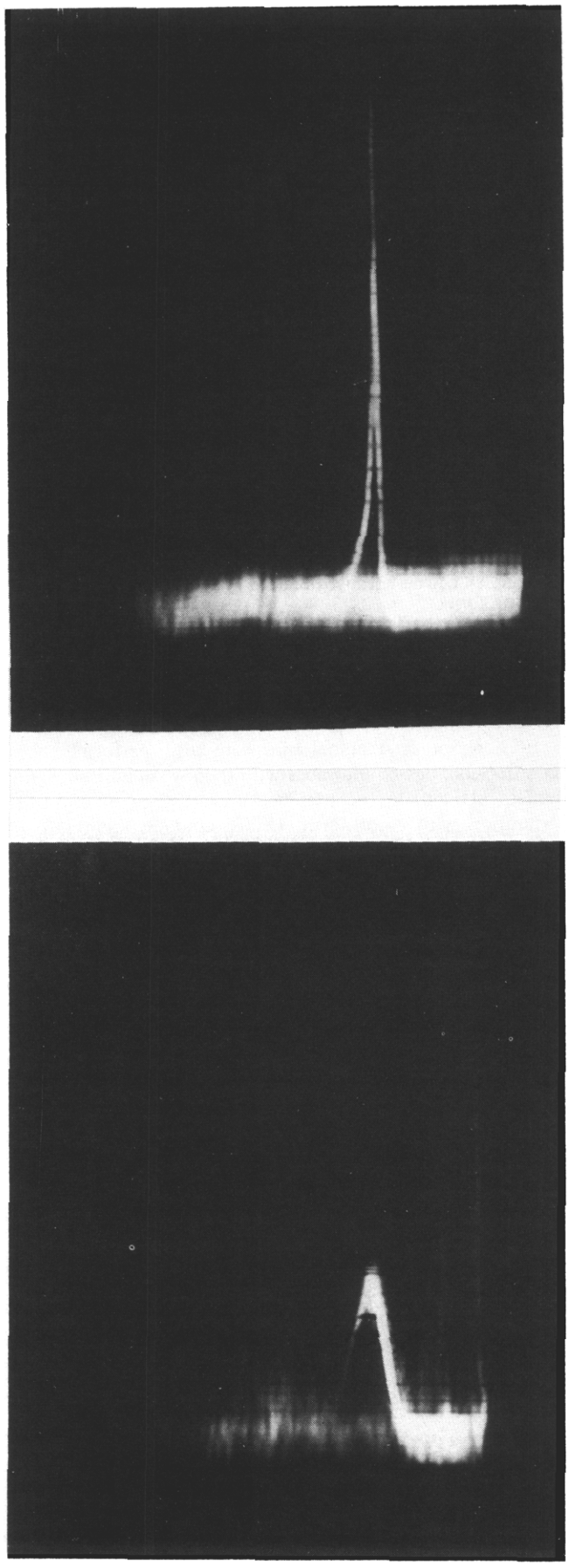

FIGURE 4: Schlieren patterns observed during free-boundary electrophoresis of equine ferrihemoglobin in the presence (top) and absence (bottom) of chloroform.

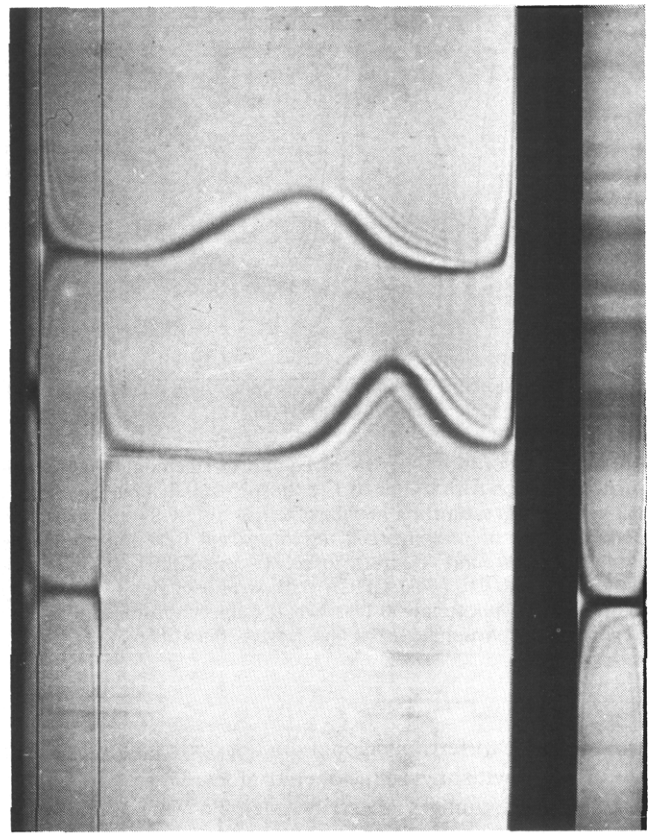

FIGURE 5: Sedimentation pattern of equine ferrihemoglobin 7.1, mg/ml, that had been dialyzed 24 hr *vs.* 0.01 M phosphate buffer (pH 6.8), containing 0.2 M NaCl and either no chloroform, or two-thirds saturated with chloroform. Picture taken after 202 min at 59,780 rpm; rotor at 3°, bar angle 60°. Upper pattern, chloroform present; slow component 3.1 S; fast component 4.1 S. Lower pattern, no chloroform; peak 4.3 S.

with a shallow salt gradient, the brown met form was rapidly eluted as a sharp band whereas the bright red chloroform perturbed hemoglobin was retarded (Figure 3). The brown met form was also converted to the red form before final elution to the collector.

Isoelectric Point Determination. The isoelectric point was found to be pH 6.8 for equine ferrihemoglobin in the presence or absence of $CHCl_3$ as determined by free-boundary electrophoresis. No difference in mobility was observed between the two forms over the pH range from 6.0 to 8.0 nor with dialysis against buffer containing chloroform for periods varying from 24 hr to 7 days. Under both conditions ascending and descending boundaries were enantiographic. However, a characteristic of hemoglobin in the presence of chloroform was the hypersharpness of the boundaries as seen in Figure 4. The chloroformated samples also appeared more heterogeneous near the isoelectric point.

The isoelectric point was also determined for equine ferrihemoglobin by isoelectric focusing. The isoelectric points were 7.27 ± 0.05 for unperturbed hemoglobin and 7.36 ± 0.04 for hemoglobin with chloroform. These results indicate only minor difference in the isoelectric points under the two conditions.

Optical Rotatory Dispersion. Ultraviolet rotatory dispersion was measured from 260 to 220 mμ and the specific rotation, [α], determined at the base of the trough for equine hemoglobin and myoglobin. A slight increase in $[m']_{min}$ was noted for ferrihemoglobin as well as for ferrimyoglobin in the presence of chloroform. In the case of oxyhemoglobin a shift in

TABLE III: Effect of Chloroform on Sedimentation Rates of Hemoglobins in Different Forms and from Different Species.[a]

Species	Form	Sedimentation Rate (S)	
		$+CHCl_3$	$-CHCl_3$
Equine	Ferri	3.1	4.3
	Reduced	2.8,[b] 5.4[c]	4.3
	Oxy	3.0,[b] 4.7[c]	4.6
Rabbit	Ferri	3.0	4.3
	Oxy	4.8	4.6

[a] Conditions similar to those given in legend for Figure 5. [b] Slow component. [c] Fast component.

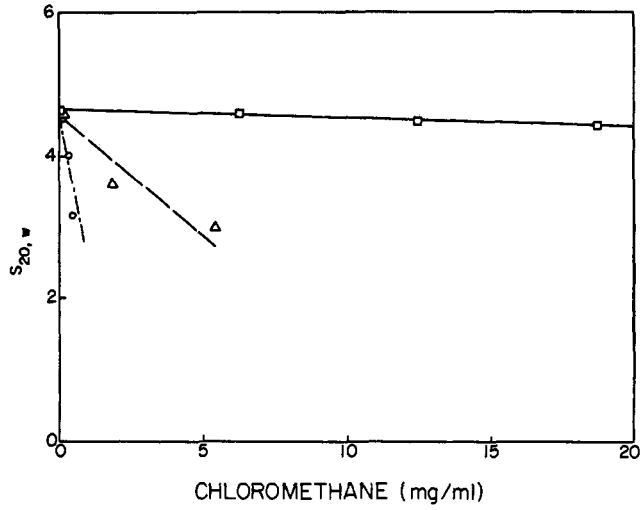

FIGURE 6: Variation of sedimentation rate of equine ferrihemoglobin with concentration of chloromethanes Protein concentration 11.9 mg/ml; dialyzed 72 hr *vs.* 0.01 M sodium phosphate buffer (pH 6.8), containing 0.2 M NaCl. (□) CH_2Cl_2, (△) $CHCl_3$, and (○) CCl_4.

the base of the trough from 232 to 232.5 was noted. For the reduced form, two additional rotatory bands appeared with chloroform present, a shoulder at 252 mμ and a second at 243 mμ. Furthermore, the $[m']_{min}$ increased from −7200 to −6300.

With addition of dichloromethane $[m']_{min}$ was increased for ferrihemoglobin from −7200 to −5200 accompanied by a shift in the trough minimum from 232.5 to 229 mμ. Carbon tetrachloride also apparently caused some diminution of the trough, $[m']_{min}$ changing from −7200 to −6500, with a slight shift in waevlength from 232.5 to 233 mμ.

Sedimentation Velocity. Chloroform produced a significant effect on the sedimentation rate and pattern of hemoglobin with no effect on that of myoglobin. Equine ferrihemoglobin showed a characteristic skewing of the boundary after 24-hr dialysis *vs.* buffer two-thirds saturated with chloroform (Figure 5). The rapidly moving peak had a value of 4.1 S; the shoulder peak, resolved before sedimentation was concluded, has a value of 3.1 S. In the absence of chloroform, ferrihemoglobin had a sedimentation rate of 4.3 S. After removal of the chloroform by evaporation, the hemoglobin sedimented with a single boundary of 4.2 S. With increased length of dialysis, the ferrihemoglobin was completely converted to the slowly sedimenting form in the presence of chloroform. After 7-days dialysis, all of the hemoglobin sedimented at 3.0 S. Sperm-whale myoglobin had the same sedimentation coefficient, 1.9 S, in the presence or absence of chloroform.

The influence of the chloromethanes on the sedimentation of equine ferrihemoglobin at various levels of saturation is shown in Figure 6. In terms of formation of slow component, equine hemoglobin is extremely sensitive to carbon tetrachloride and almost inert to dichloromethane.

The action of chloroform on the sedimentation of hemoglobin in the reduced and oxy states and on oxy- and ferrihemoglobin of two species was also examined. In the case of reduced equine hemoglobin, the fast component in the presence of chloroform sedimented at 5.4 S, significantly higher than the unperturbed reduced hemoglobin. The results are summarized in Table III.

The variation of sedimentation rate with protein concentration was examined in the presence and absence of chloroform from 3.3 to 16.5 mg per ml of protein concentration. Only slight variation for either form of hemoglobin occurred over this range.

Salt concentration plays an important role in determining the rate and amount of formation of slow component. At low ionic strength, formation of the slow component requires up to 5 days of dialysis. At high ionic strength, the slow component is formed by dilution. In addition, a rapidly sedimenting form was also seen with dilution to one-third chloroform saturation in the presence of 0.01 M NaCl or no NaCl. Values of 5.1 and 5.0 S were obtained, both significantly higher than for the controls (4.5 and 4.4 S). Results are shown in Table IV.

Little effect of pH was noted on sedimentation performed in buffers at pH 6.0, 7.0, and 8.0. There appeared to be a slight increase in the area of the slow component at pH 6.0, with the area remaining constant at pH 7.0 and 8.0.

Low-Speed Sedimentation Equilibrium. From the results of G-75 Sephadex chromatography and the finding of a decreased sedimentation rate in the presence of chloroform, it was anticipated that a lowered molecular weight would be found by sedimentation equilibrium. However, on direct measurement of molecular weight using the same concentration of protein used for sedimentation velocity measurements, the hemoglobin was found to be undissociated under perturbation conditions.

Results are presented in Table V for equine ferrihemoglobin in the presence and absence of two-thirds saturation with chloroform. Under both conditions molecular weights tended to decrease with decrease in protein concentration. Equine ferrihemoglobin was dialyzed 24 hr against 0.01 M sodium phosphate buffer (pH 6.5), containing 0.4 M NaCl; this resulted in complete conversion to the 3.3S species of hemoglobin in the presence of two-thirds chloroform saturation. Although the relatively high sodium chloride concentration may be causing some dissociation, elevation of NaCl to 2.0 M resulted in a weight-average molecular weight of 61,000 at a protein concentration of 6.4 mg/ml.

$M_{w,app}$, apparent weight-average molecular weight, and $M_{Z,app}$, apparent Z-average molecular weight, values are those determined from white-light data. Corresponding computations from conservation of mass gave values in close agreement, within 0.5% of the white-light values.

High-Speed Sedimentation Equilibrium. To provide verification of results from the low-speed sedimentation equilibrium studies, one series of experiments was performed using the high-speed Yphantis technique of molecular weight determination. A four-cell rotor permitted simultaneous equilibrium of equine ferrihemoglobin at three levels of chloro-

TABLE IV: Effect of Ionic Strength and Length of Dialysis on Sedimentation Rate of Equine Ferrihemoglobin in the Presence and Absence of Chloroform.

Buffer[a]	No Dialysis (Diluted to $CHCl_3$ Satn)			Length of Dialysis at $CHCl_3$ Satn					
				6 hr		24 hr		120 hr	
	0	1/3	2/3	1/3	2/3	1/3	2/3	1/3	2/3
0.01 M phosphate	4.5	5.1	4.4	4.3	4.5	4.5	4.4 3.5[b]	4.0 3.2[b]	3.6
0.01 M phosphate, plus 0.1 M NaCl	4.4	5.0	3.9			4.0	3.7[c] 4.0[d]	3.6[c] 4.3[d]	3.3
0.01 M phosphate, plus 1.0 M NaCl	4.0	3.4	3.3						

[a] All pH 6.8. [b] $s_{20,w}$ of slow component. [c] $s_{20,w}$ of average (initial rate). [d] $s_{20,w}$ of fast component.

form concentration for three levels of protein concentration.

As was found with the low-speed technique, the molecular weight of equine ferrihemoglobin was not affected by the addition of chloroform to the buffer. Nearly all cells showed upward curvature in plots of log c *vs.* r^2 near the cell bottom, indicating aggregation of the protein.

Diffusion Measurements. Results of diffusion coefficient measurements are presented in Table VI. The diffusion coefficient for hemoglobin in the absence of chloroform had a linear relationship to protein concentration. However in the presence of two-thirds chloroform saturation, the diffusion coefficient at one concentration showed no obvious relationship to that at another; this probably reflects the heterogeneity of the hemoglobin species.

At the higher protein concentrations in the presence of chloroform the diffusion coefficients were significantly lowered. These lowered diffusion coefficients represent increased frictional ratios, f/f_0, of 1.25 to 1.4 compared to 1.16 for equine ferrihemoglobin at 7.5 mg/ml. Lamm and Polson (1936) found diffusion coefficients of 6.90×10^{-7} cm²/sec at a concentration of 8.2 mg/ml and 7.34×10^{-7} cm²/sec at 4 mg/ml using a stationary diffusion apparatus.

Discussion

The most obvious effect of the chloromethanes is on the spectrum of ferrihemoglobin. The three solvents each produce a reddening although the extent of the effect varies with the solvent. Such an intense change in the spectrum must necessarily be attributed to a change in the electronic structure of the heme group (Pauling and Coryell, 1936) and would be accompanied by a change in its magnetic properties.

Reddening of ferrihemoglobin after addition of a perturbant or denaturant is frequently used as an indication of hemochromogen formation. Such compounds on reduction show a reversal in height of the main bands in the green portion of the spectrum with extreme sharpness of a band at 550–560 mμ (Lemberg and Legge, 1949). However, reduced hemoglobin in the presence of chloroform shows no such reversal but has the same spectrum as in the absence of chloroform.

To produce such an intense effect on the electronic configuration of the heme, it would seem that one of the binding sites of the chloromethanes must be in close proximity to the heme (assuming the existence of more than one binding site). Furthermore from the rapidity of conversion of the spectra, no further change in spectra being seen after 2 min, one would assume that the site involved is readily accessible. It is known that the heme rests in a highly hydrophobic pocket for both hemoglobin and myoglobin (Dickerson, 1964) and would provide a hospitable environment for molecules of a similar nature, such as the chloromethanes. From the extent of Soret

TABLE V: Molecular Weight of Equine Ferrihemoglobin in the Presence and Absence of Chloroform as Determined by Low-Speed Sedimentation Equilibrium in the Ultracentrifuge.

Level of $CHCl_3$ Satn	Hb Concn (mg/ml)	Hr at Equilibrium Speed	$M_{w,app}$	$M_{z,app}$
0	1.0	15	53,000	56,000
0	2.6	15	55,000	69,000
0	4.9	13	66,000	72,000
0	8.9	12	62,000	73,000
2/3	1.1	12	52,000	64,000
2/3	1.8	15	61,000	66,000
2/3	4.5	13	63,000	77,000
2/3	7.4	12	59,000	65,000

TABLE VI: Diffusion Coefficients of Equine Ferrihemoglobin in the Presence and Absence of Chloroform.[a]

Level of $CHCl_3$ Satn	Hb Concn (mg/ml)	$D_{20,w}$ (cm²/sec × 10^7)	f/f_0
0	0.89	7.5	1.1
0	5.4	7.2	1.1
0	8.9	6.9	1.2
2/3	2.1	7.6	1.0
2/3	3.8	5.6	1.4
2/3	6.9	6.3	1.3
2/3	7.4	6.0	1.3

[a] Hemoglobin was dialyzed against 0.01 M phosphate buffer (pH 6.5) containing 0.4 M NaCl and chloroform as indicated for 24 hr. Measurements were performed in the analytical ultracentrifuge at 4°, 4,059 rpm.

shift occurring with chloromethanes, chloroform is causing the greatest effect on the environment of the heme group and carbon tetrachloride the slightest, dichloromethane being intermediate. Since the environment of the heme is apparently very similar for both hemoglobin and myoglobin, the lack of any spectral change for myoglobin in the presence of the chlorinated solvents is particularly striking.

The second aspect of the solvent–protein interaction is the dramatic effect on the shape of the hemoglobin molecule. The large increase in frictional coefficient is reflected in a lowered sedimentation and diffusion rate in the presence of chloroform while the molecular weight remains constant.

Although the shape and spectral changes are both mediated by these organic solvents, the two effects are apparently independent and distinct and probably involve very different areas of the molecule. Whereas the spectral changes are complete within 2 min with the rate independent of the buffer system, the shape changes occur over periods of hours or days depending on the ionic strength involved. Also, although carbon tetrachloride produced the least perturbation of spectra, it was the most effective agent in altering the shape as reflected in sedimentation behavior. Perhaps the latter effect is dependent on a binding site(s) which is more hydrophobic than that near the heme.

The spectral and shape changes are both intensified by an increase in ionic strength. However, it appears that the chloromethanes will produce a shape change at any ionic strength if hemoglobin is exposed for a sufficient length of time, whereas the spectral change is complete within 2 min and no further changes occur with increased time of exposure.

Originally we thought that chloroform was acting as a dissociating agent for hemoglobin. Basing deductions solely on the sedimentation behavior and the increased retardation by gel filtration in the presence of chloroform, it appeared that hemoglobin was being split into α–β subunits. The sedimentation velocity of the chloroform-perturbed hemoglobin, 3.2 S, corresponds to that expected for symmetrically dissociated hemoglobin (Rossi-Fanelli *et al.*, 1964) and the partition coefficient on G-75 Sephadex would represent a molecule with a molecular weight of 29,500. Several observations prevented acceptance of the phenomenon of a lowered sedimentation rate as being due to dissociation. A primary observation was in the free boundary electrophoresis experiments where the most characteristic aspect of those solutions containing chloroform was the hypersharpness of the boundaries. Since the ultracentrifugation patterns showed more heterogeneity in the presence of chloromethanes under the conditions of electrophoresis than in their absence, the hypersharpness could not have been a result of increased homogeneity.

The elution from Sephadex had been performed at a low concentration of hemoglobin (0.5 mg/ml during elution). Since hemoglobin behaves as though it were a smaller molecule on this column material (Andrews, 1964), it seemed imperative to perform a direct measurement of the molecular weight of hemoglobin in the presence and absence of chloroform at the concentrations used for the sedimentation velocity experiments. From such measurements it was evident that hemoglobin in the presence of chloroform was remaining undissociated at least at the concentrations used for the sedimentation velocity experiments; the decreased sedimentation rate must be a result of an increase in assymetry.

The use of a second procedure for molecular weight determination, the Yphantis technique, gave results that showed little difference in molecular weight between hemoglobin with chloroform and that in its absence.

Values for diffusion coefficients determined in separate experiments suggested an increase in assymetry of the hemoglobin molecule in the presence of chloroform. Heterogeneity of the preparations was reflected in diverse values for the diffusion coefficient in the presence of chloroform.

Although reports of lowered sedimentation coefficients for hemoglobin are frequently attributed to dissociation, some investigators have found hemoglobin to become assymetric, resulting in a lowered sedimentation rate. Kurihara and Shibata (1960) found a large increase in assymetry for equine hemoglobin as determined from the diffusion and sedimentation coefficients. They found an increase in the frictional ratio from 1.3 at pH 7.0 to 1.6 at pH 11.6, as determined from a sedimentation rate of 4.2 S at pH 7.0 and 3.5 S at pH 11.6 and diffusion coefficients of 6.0×10^{-7} and 5.0×10^{-7} cm^2 per sec, respectively. Gottlieb *et al.* (1967) found a decrease in sedimentation rate at pH 5.7 with no decrease in molecular weight. Gutter *et al.* (1956) found no molecular weight decrease for human hemoglobin in 4 M urea but found f/f_0 ratio increased from 1.3 to 1.8.

The assymetric species produced evidently is not the same for each of the solvents. Dichloromethane produced only slight change in sedimentation velocity; however, the optical rotation was definitely perturbed indicating some strong conformational change. With chloroform, only slight change occurred as measured by optical rotatory dispersion, whereas carbon tetrachloride showed an intermediate change in conformation.

With Sephadex chromatography we are introducing another factor which normally promotes dissociation of ferrihemoglobin (Andrews, 1964) and causes thorough dissociation of the chloroform-perturbed ferrihemoglobin. Merrett (1966) found different degrees of dissociation of hemoglobin depending on the state of the iron with reduced hemoglobin being the most difficult to dissociate. Morris (1964) in observations of the behavior of hemoglobin on thin-layer chromatography (using Sephadex as the bed media) found that hemoglobin behaved as though it had a molecular weight of 33,000. He noted that similar observations had been made in his own laboratory with polyacrylamide gel and by others using agar (Andrews, 1962) and G-100 Sephadex (Whitaker, 1963).

The absence of any detectable effect on myoglobin by any of the measurements used in this study suggests that the difference in behavior could be attributed to the subunit composition of hemoglobin, *i.e.*, the provision of hydrophobic interfaces for sequestering of chloromethanes. Kiehs *et al.* (1966) found no binding of phenols by myoglobin although the compounds were readily bound by hemoglobin. These authors suggested this as evidence that the compounds were held on the subunit interfaces rather than within the protein folds.

The chloromethanes are relatively soluble but primarily hydrophobic agents. Their interaction with hemoglobin, but not myoglobin, may provide information about action of other hydrophobic compounds. Use of the three chloromethanes gives a spectrum of hydrophobicity with relatively constant radii of the interacting compounds. Saturation is reproducible and easily maintained at a desired level with a minimum of precautions, permitting the use of a wide range of techniques to gauge changes in protein structure.

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Specificity and Mechanism of Clostripain Catalysis*

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ABSTRACT: The mechanistic basis for the unique specificity of bacterial protease clostripain for arginyl residues in preference to lysyl substrates was studied by the steady-state kinetic method using synthetic substrates and inhibitors. The binding affinity of alkylguanidine, as determined by K_i was two to three orders of magnitude higher than alkylamines. Only a small difference was observed in k_{cat} for an identically substituted pair of arginine and lysine substrates. Therefore, it was proposed that the specific action on arginine substances was due to a specifically elevated affinity for the alkylguanidine structure of its side chain. Contrary to trypsin, no catalysis was observed with a neutral, nonspecific glycine substrate, nor was the inductive activation of catalysis by alkylguanidines or amines demonstrable. Again differing from trypsin, esters of acylhomoarginine and -norarginine were not hydrolyzed. On the other hand, *p*-nitrophenyl *p*-guanidinobenzoate, an inhibitor of trypsin, was hydrolyzed by clostripain. The amide and ester of identically substituted arginines were found to have identical k_{cat} values indicating the deacylation of the acyl-enzyme intermediate is the rate-limiting step. The pH profiles of k_{cat} and k_{cat}/K_m revealed the presence of catalytically functional groups with pK's of 6.7 and 8.2. The energy of activation of the clostripain catalysis was found to be considerably lower than the values for trypsin and papain.

Clostripain (EC 3.4.4.20) isolated from the culture filtrate of *Clostridium histolyticum* is a sulfhydryl protease (Kocholaty and Krejci, 1948). Unlike other sulfhydryl enzymes from plants and animals this enzyme has a very narrow substrate specificity. Ogle and Tytell (1953) have shown that, of all synthetic substrates studied, only esters and amides of arginine and lysine are hydrolyzed. Amides and peptides of neutral amino acids were not attacked. Using chromatographically purified enzyme, Labouesse and Gros (1960) were able to show that clostripain has a rigid substrate specificity analogous to that of trypsin (EC 3.4.4.4). However, in contrast to trypsin, clostripain hydrolyzed arginyl substrates much more readily than identically substituted lysyl substrates. Using a number of peptides and proteins of known sequence, Mitchell and Harrington (1968) have demonstrated that clostripain can be used for the selective cleavage of the arginyl peptide bond without significant lysyl peptide-bond hydrolysis. Mitchell (1968) has also reported that the

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