Hemin Intercalated in Micellar Cetyltrimethylammonium Bromide and Triton X-100. A Kinetic, Spectral, and Equilibrium Study with Cyanide†

Jon Simplicio* and Kathryn Schwenzer

ABSTRACT: An investigation of hemin in the spectral region from 330 to 650 nm in the presence of micelles of cetyltrimethylammonium bromide ($CetMe_3NBr$) and Triton X-100 (TX) in aqueous solution has revealed that above the critical micellar concentration, hemin, normally dimeric, is both solubilized and monodispersed between a pH of 2.9 and 13.5. The intercalated hemin has associated with it an acid–base equilibrium between a red diaquo form and a green monoaquomonohydroxy form. The molar absorptivity spectra and pK_a values have been characterized. Above a pH of 8.5 the intercalated hemin reacts with cyanide to form a red dicyanohemin complex. The presence of isosbestic points suggests the existence of primarily two species, although an unstable N-bound isomer has been spectrally and kinetically characterized. The equilibrium constants have been evaluated over a wide range of pH, cyanide, and hemin concentration in 0.1 M tetramethylammonium bromide (Me_4NBr) for ionic strength. Differences in the equilibrium constants of the dicyanohemin complexes in neutral TX ($K = 1.83 \times 10^2$ M^{-1}) and cationic $CetMe_3NBr$ ($K = 5.91 \times 10^4$ M^{-1}) micelles presumably reside in both the charge differences at the micelle–water interface and possible orientation differences of the hemin in the respective micelles. The kinetics between intercalated hemin and cyanide have been carried out over a temperature range of 15–25° and a pH range from 9.0 to 13.5. Stopped-flow studies reveal a three-step process; the formation of a steady-state monocyanide–hemin complex in the rate determining step rapidly followed by the formation of a dicyanohemin N-bound isomer and the latter's subsequent intramolecular linkage isomerization to a more stable C-bound isomer. The rate constants for most of these processes have been evaluated. Decomposition of both N-bound and C-bound isomers can be affected by high base and the unimolecular rates have been evaluated. Rate constants have been combined to yield a kinetically determined overall equilibrium constant which agrees with those determined by spectral titration. Comparisons of these to metmyoglobin are discussed.

The iron complexes of porphyrins are known to form the active site of a variety of vitally important enzyme systems such as catalases, peroxidases, and hemoglobins (Falk, 1964).

The immediate biological environment imposed upon the metalloporphyrin can produce rather unique chemical reactivity properties compared to the free metalloporphyrin. It has been suggested that heme or hemin reactivity is greatly influenced by either the protic character of its immediate environment (Caughey *et al.*, 1966) or the hydrophobic environment (Wang, 1958) imposed by the native protein. A fruitful approach toward understanding the relative importance of these parameters, since a protein imposes both on the metalloporphyrin (Muirhead *et al.*, 1967), has been to remove the heme from the protein and study its reactivity in a variety of protic and aprotic solvents of varying hydrophobic character (Caughey *et al.*, 1966; Wang, 1958). The enzymatic activity of hemin dissociated from its native protein has been investigated (Brown and Jones, 1968) and is found to possess catalase activity somewhat lower than while bound to the protein. Such studies are complicated by the limited solubility of the metalloporphyrin, its insolubility under acidic conditions, and the monomer–dimer equilibrium established under basic conditions (Brown *et al.*, 1970). The use of sulfonated porphyrin derivatives (Fleischer *et al.*, 1971) has been used to solubilize the metalloporphyrin, although the monomer–dimer equilibrium still exists in basic solution. Previous workers have shown that heme is highly solubilized (Phillips, 1963; Porra and Jones, 1963) in the presence of detergents such as sodium lauryl sulfate, $CetMe_3NBr$,[1] and Emasol 4130. A recent study on the interactions between sodium lauryl sulfate and hemin (Simplicio, 1972a,b) has revealed that hemin is monodispersed and intercalated into the micelle of sodium lauryl sulfate. The intercalated hemin has been observed to form an equilibrium system with cyanide, histidine, and imidazole (Cornell, A., and Simplicio, J., unpublished data).

The use of micellar systems to simulate the electrostatic and hydrophobic interactions in biological systems has stimulated reasearch in micelle-catalyzed reactions (Fendler and Fendler, 1970). The rate effects on reactions in micellar systems can be attributed to electrostatic and hydrophobic interactions between the substrate and the surfactant aggregate. Using simple electrostatic considerations (Hartley, 1934), one expects cationic micelles to enhance the rate of reaction of nucleophiles with substrates, anionic micelles to retard the rate, and nonionic micelles to have little or no effect on the rate. The incorporation of Cu(II) into protoporphyrin IX when the porphyrin is intercalated in the anionic micelle

† From the Department of Chemistry, University of Miami, Coral Gables, Florida 33124. *Received October 30, 1972.* Supported by a grant from the National Institutes of Health (NIH 101R HE-13111-01A1).

[1] The intercalated hemin monomer H_2O–M–OH (charges omitted) will be denoted as M(OH)·$CetMe_3NBr$ or M(OH)·TX. The corresponding dicyanohemin complexes will be denoted as $M(CN)_2$·$CetMe_3NBr$, $M(NC)_2$·$CetMe_3NBr$, $M(CN)_2$·TX, or $M(NC)_2$·TX noting the possibilities of both N-bound and C-bound isomers. Evidence suggests that only one cyanide is N bound. Abbreviations used are: $CetMe_3NBr$, cetyltrimethylammonium bromide; TX, Triton X-100; Me_4NBr, tetramethylammonium bromide.

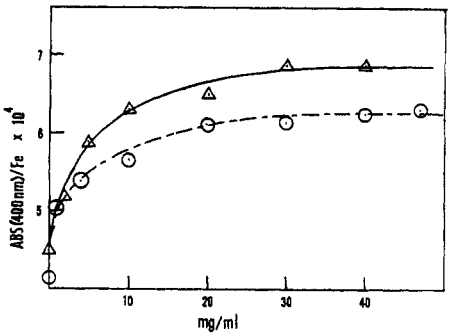

FIGURE 1: Spectral changes accompanying the addition of $CetMe_3NBr$ (△) and Triton X-100 (○) to solutions of constant hemin concentration at pH 9.50, 25°.

sodium lauryl sulfate compared to the cationic micelle $CetMe_3NBr$ (Lowe and Phillips, 1961) has borne this out. A kinetic and spectral study (Simplicio, 1972a,b) has revealed that the rate of formation of a dicyanohemin complex from cyanide and hemin intercalated in micelles of sodium lauryl sulfate is enhanced when the concentration of Me_4NBr is increased. This cationic counterion presumably helps neutralize the negative charge of the micelle in the Stern layer and makes the penetration of cyanide anion occur more easily. The present study was undertaken to characterize the kinetic, spectral, and equilibrium parameters associated with cationic $CetMe_3NBr$ and neutral TX micelles as compared to anionic sodium lauryl sulfate. The contrasting micellar systems provide unique and simple models for hemoprotein reactivity that can be compared to the reactivity of metmyoglobin with cyanide (VerPloeg and Alberty, 1968; VerPloeg *et al.*, 1971).

Experimental Section

Materials and Methods. All materials used were reagent grade and were used without further purification. The $CetMe_3NBr$ and TX were obtained from Sigma Chemical Co. and the Me_4NBr was obtained from Eastman Chemicals. The isolation of hemin from cow's blood was essentially that of Labbe (Labbe and Nishida, 1957). The preparation and standardization of hemin solutions and stock potassium cyanide solutions are as previously described (Simplicio, 1972a). To adjust the pH with either concentrated nitric acid or potassium hydroxide it was necessary to briefly keep the GK 2301B combination glass electrode (Radiometer) in a micellar solution just prior to standardizing it with the appropriate Beckman prepared buffers. This prevented some slight drift in the pH reading when measuring pH in micellar solutions (presumably due to coating of the electrode by the micellar material). Except where noted all solutions contained 0.1 M Me_4NBr for the maintenance of constant ionic strength and either 4% $CetMe_3NBr$ or 3% TX. Ultraviolet (uv) and visible spectra were obtained with a Cary 17 recording spectrophotometer.

Continuous Flow Apparatus. Spectra of the transient (N-bound) isomer were obtained by a continuous flow apparatus. This is a Sage Instruments Syringe Pump Model 355. Two separate and well-thermostated 50-ml syringes at 10° were filled respectively with potassium cyanide (~0.01 M at pH 9.5) solution and hemin. The solutions were driven out and mixed together in a Hamilton T valve made of Kel-F. The solutions continued through a flow cell (1.0-cm path length) seated in the Cary 17 where spectra were obtained at a convenient speed. The time between mixing and observation was about 2.5 sec. This ensured that the (C-bound) stable isomer was present to the extent of less than 5%.

Kinetic Measurements. Kinetic measurements too fast for the Cary 17 (about 10 sec half-life) were measured with the stopped-flow portion of a stopped-flow–temperature jump apparatus previously described (Erman and Hammes, 1966).

Decomposition of the unstable N-bound intermediate by high base concentrations was effected in the stopped flow by generating the intermediate from separate hemin and cyanide solutions which were rapidly drawn into and simultaneously mixed in one of the two syringes situated on the stopped-flow apparatus. The unstable intermediate generated in this way was then rapidly mixed with base and its decomposition rate observed at an appropriate wavelength. The total time from generation of the intermediate to its decomposition was roughly 5–10 sec. Only one unimolecular rate is observed. If the mixing of the intermediate with base was delayed, the unimolecular decomposition was observed to be biphasic, a fast rate corresponding to the N-bound decomposing and a slower rate clearly identified with the decomposition of the more stable C-bound isomer.

All reactions were driven to completion to maintain pseudo-first-order conditions. Such conditions were determined by spectral studies. Reactions were followed in the visible region either at 400 nm (the peak of M(OH)·micelle) or at 430 nm (the peak of $M(CN)_2$·micelle). A least-squares analysis was applied to all data and usually a minimum of three kinetic runs were carried out to obtain an average half-life with an estimated error of ±8%.

Results

Monomer Formation. At 25° a series of spectra was obtained by the addition of either $CetMe_3NBr$ or TX to a solution of known hemin at constant pH 9.5. No spectral changes were observed until the critical micellar concentration (cmc) of the detergent had been reached. Spectra were taken over the range from 330 to 450 nm and a plot of absorbance at 400 nm/Fe_{total} against $CetMe_3NBr$ or TX (milligrams/milliliter) yields the two curves shown in Figure 1. The leveling off point is taken as the molar extinction spectra for the new intercalated hemin species. The limiting spectra for these new complexes are shown in Figures 2a,b and 3a,b. As a matter of practice all spectral and equilibrium studies of the intercalated species were run in 4% $CetMe_3NBr$ or 3% TX. This ensured the exclusive presence of the monodispersed systems.

Acid–Base Equilibrium. The addition of nitric acid to basic solutions of the green M(OH)·micelle results in a change to a reddish material below a pH of about 5. The existence of isosbestic points suggests the following equilibrium

$$M(H_2O)_2\cdot\text{micelle} \overset{K_a}{\longleftrightarrow} M(OH)\cdot\text{micelle} + H^+ \qquad (1)$$

A representative plot at constant wavelength for the M(OH)·$CetMe_3NBr$ is shown in Figure 4 from which the associated pK_a may be obtained. At 25° the pK_a values in micelles of $CetMe_3NBr$, TX, and sodium lauryl sulfate are, respectively, 6.10 ± 0.10, 4.70 ± 0.10, and 5.50 ± 0.10. Within experimental error all three $M(H_2O)_2$·micelle complexes have λ_{max} 393 nm with a molar extinction coefficient of 0.90×10^5 M^{-1} cm^{-1} at pH 3.3 (on the basis of limiting spectra). Measurements involving the M(OH)·micelle–cyanide equilib-

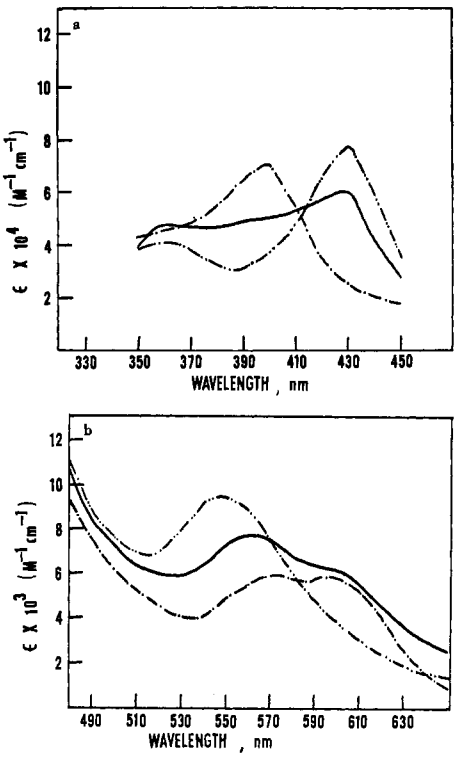

FIGURE 2: Spectra of M(OH)·$CetMe_3NBr$ (—·—), $M(CN)_2$·$CetMe_3NBr$ (—··—), and the unstable intermediate $M(NC)_2$·$CetMe_3NBr$ (—); pH 9.50, 25°.

rium were made above pH 9.0; thus equilibrium 1 made no contribution to this study.

Spectra of $M(CN)_2$·Micelle. Under basic conditions the green solutions of M(OH)·micelle undergo a color change to red upon the addition of potassium cyanide. The spectra of the final dicyanohemin complexes were obtained by maintaining a solution at constant hemin concentration, constant pH 9.5, and adding stock potassium cyanide until no further spectral changes occurred. The spectra for the $CetMe_3NBr$ and TX are shown in Figures 2a,b and 3a,b. A dicyanohemin complex may also be generated from a hemin solution that contains no micellar material and is shown in Figure 3a.

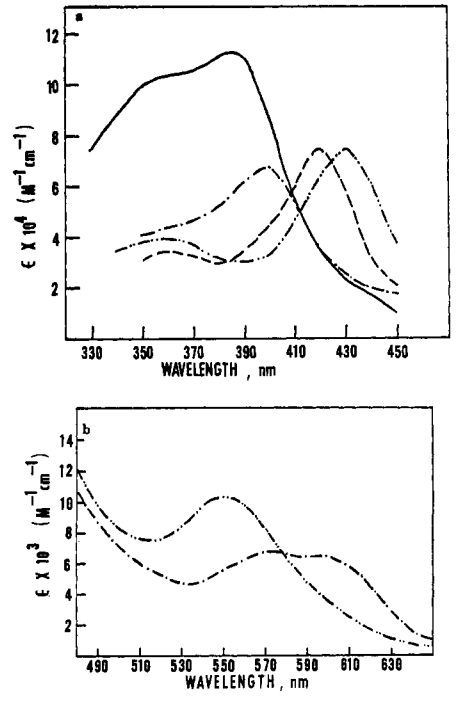

FIGURE 3: Spectra of hemin dimer (—), M(OH)·TX (—·—), $M(CN)_2$·TX (—··—), and $M(CN)_2$ (— —); pH 9.50, 25°.

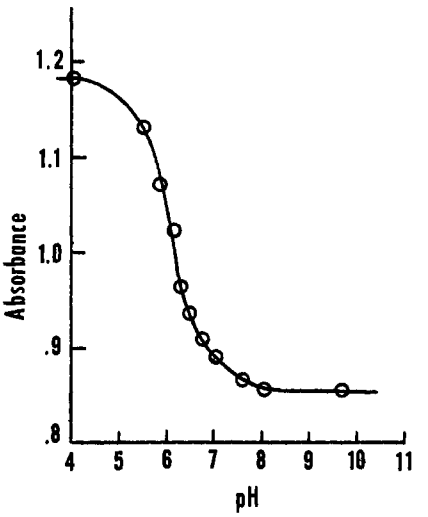

FIGURE 4: Absorbance change at 393 nm, [hemin] = 1.3×10^{-6} M, in 4% $CetMe_3NBr$ as a function of pH; T = 25°.

Equilibrium Studies. The addition of cyanide to M(OH)·micelle solutions at constant hemin concentration yields spectra that exhibit isosbestic points (Figure 5) providing that the spectra are taken 10–15 min after the addition of an

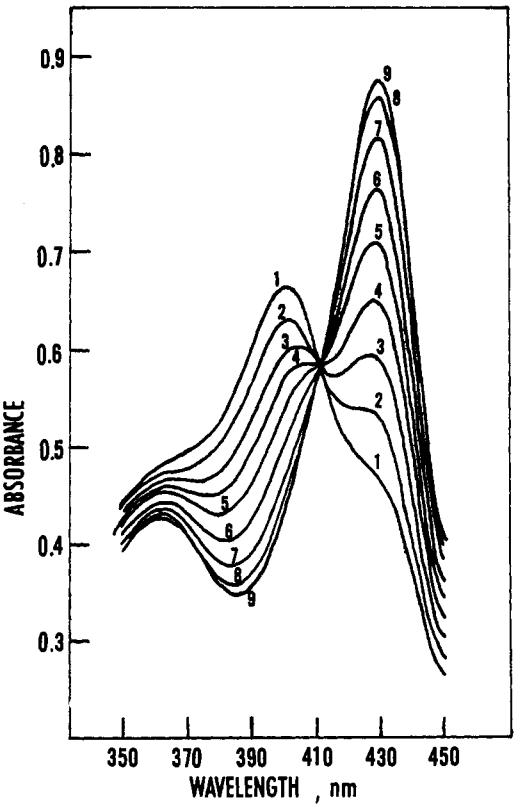

FIGURE 5: Family of curves showing isosbestic point in the equilibrium between $M(CN)_2$·TX and M(OH)·TX. For conditions see Table I.

TABLE I: Equilibrium Parameters for M(OH)·Micelle + CN^- at 25° and 0.1 M Me_4NBr.[a]

Fe_{tot} × 10^{-6}	CN_{free}^- × 10^{-3}	Abs (430 nm)	pOH	K_{eq} × 10^4 (M^{-1})	
		M(OH)·$CetMe_3NBr$ + $2CN^-$			
8.76	5.48	0.510	3.76	5.23	
	5.67	0.480	3.55	6.54	
	5.75	0.575	3.43	7.32	
13.1	5.55	0.723	3.70	4.96	
	5.69	0.680	3.53	5.72	
	5.78	0.615	3.38	5.70	
	5.82	0.586	3.28	5.98	
				K_{av} = 5.91	
		M(OH)·TX + $2CN^-$			
				K_{eq} × 10^2 (M^{-1})	Curve
13.2	1.85	0.465	2.64	2.11	1
	1.84	0.526	2.92	1.74	2
	1.84	0.590	3.07	1.86	3
	1.83	0.646	3.18	2.03	4
	1.81	0.705	3.35	1.99	5
	1.78	0.760	3.53	1.92	6
	1.72	0.815	3.79	1.67	7
	1.57	0.858	4.14	1.32	8
	1.18	0.875	4.62		9
				K_{av} = 1.83	

[a] K_{eq} = 14.4 M^{-1} for M(OH)·sodium lauryl sulfate + $2CN^-$ (Simplicio, 1972b).

aliquot of cyanide. Table I presents the data of the equilibrium solutions. Consistent with these changes is the following overall equilibrium (eq 2). Combining eq 4 and 5 one obtains the equilibrium constants listed in Table I.

$$M(OH)\cdot micelle + 2CN^- \overset{K_{eq}}{\longleftrightarrow} M(CN)_2\cdot micelle + OH^- \quad (2)$$

$$K_{eq} = \frac{[M(CN)_2\cdot micelle][OH^-]}{[M(OH)\cdot micelle][CN^-]^2} \quad (3)$$

$$\text{absorbance}_\lambda = \epsilon_\lambda^{M(OH)\cdot micelle}[M(OH)\cdot micelle] + \epsilon_\lambda^{M(CN)_2\cdot micelle}[M(CN)_2\cdot micelle] \quad (4)$$

$$Fe_{tot} = M(OH)\cdot micelle + M(CN)_2\cdot micelle \quad (1\text{-cm path length}) \quad (5)$$

Spectra of $M\cdot(NC)_2\cdot CetMe_3NBr$. Kinetic evidence indicated the existence of an intermediate in the formation of the $M(CN)_2\cdot CetMe_3NBr$ and $M(CN)_2\cdot TX$ complexes. This was also supported by the fact that isosbestic points could only be obtained after ~10 min of "aging" the solutions after cyanide addition. Spectra taken within a few seconds of mixing cyanide and M(OH)·$CetMe_3NBr$ (pH 9.5) on the continuous flow apparatus are shown in Figures 2a and b (dark line). Under these reaction conditions no M(OH)·$CetMe_3NBr$ is present and little isomerization to the $M(CN)_2\cdot CetMe_3NBr$ has occurred.

TABLE II: Summary of Formation Rate Data.

pH	$10^3k_{-1}/k_1k_2$ (M^2 sec)	$10^5/k_1$ (M sec)
	25°, 4% $CetMe_3NBr$, 0.1 M Me_4NBr	
9.50	3.20	7.08
9.80	2.87	8.03
10.00	4.65	8.32
10.20	3.70	12.9
10.50	3.33	10.5
	k_1(av) = 1.12 × 10^4 M^{-1} sec^{-1} ± 0.2	
	k_{-1}/k_1k_2(av) = 3.55 × 10^{-3} M^2 sec ± 0.5	
	25°, 3% TX-100, 0.1 M Me_4NBr	
pH	k_{-1}/k_1k_2 (M sec)	$10^3/k_1$ (M sec)
9.50	*a*	1.70
10.00	1.57	2.34
10.50	1.56	*b*
	k_1(av) = 5.1 × 10^2 M^{-1} sec^{-1} ± 0.6	
	k_{-1}/k_1k_2(av) = 1.57 M^2 sec	

[a] Slope is too sensitive to error limit. [b] Intercept is too sensitive to steep slope; therefore, it is unreliable.

Formation of $M(NC)_2\cdot Micelle$. The data for the kinetics of binding of cyanide over the concentration range 0.32 mM to 0.12 M for M(OH)·$CetMe_3NBr$ and 10 mM to 0.12 M for M(OH)·TX are summarized in Table II and partly displayed in Figure 6.

Two different mechanisms equally consistent with kinetic and equilibrium data are

Mechanism I

$$M(OH)\cdot micelle + CN^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} M(OH)(NC)\cdot micelle + H_2O \quad (6a)$$

$$M(OH)(NC)\cdot micelle + HCN \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} M(NC)_2\cdot micelle + H_2O \quad (6b)$$

$$M(NC)_2\cdot micelle \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} M(CN)_2\cdot micelle \quad (6c)$$

$$K = \frac{k_1k_2k_3}{k_{-1}k_{-2}k_{-3}} \quad (6d)$$

Mechanism II

$$M(OH)\cdot micelle + CN^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} M(H_2O)(NC)\cdot micelle + OH^- \quad (7a)$$

$$M(H_2O)(NC)\cdot micelle + CN^- \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} M(NC)_2\cdot micelle + H_2O \quad (7b)$$

$$M(NC)_2\cdot micelle \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} M(CN)_2\cdot micelle \quad (6c)$$

$$K' = K(K_w/K_a) \quad (7c)$$

Process 6c is an intramolecular linkage isomerization process that may involve either one or two unstable N-bound cyanides rearranging to the C-bound stable isomer. The first two steps

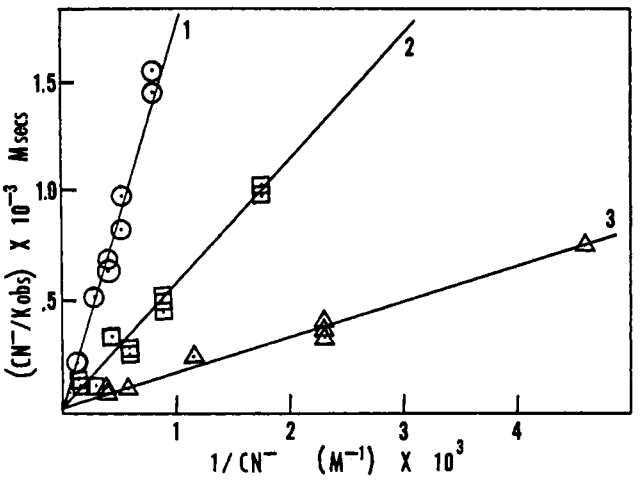

FIGURE 6: Plots for eq 3 or 4 from stopped-flow data at 25°, 0.1 M Me_4NBr and 4% $CetMe_3NBr$; curve 1 at pH 10.50; curve 2 at pH 10.00; curve 3 at pH 9.50.

are well separated from the isomerization and may be treated separately from eq 6c. With the reactions driven to completion (*i.e.*, with the exclusive formation of the N-bound isomer) and a steady state on the monocyanide intermediate species, the following two kinetically indistinguishable rate laws are obtained: for Mechanism I in which H_2O is displaced first by cyanide

$$\frac{[CN^-]}{k_{obsd}} = \frac{k_{-1}[OH^-]}{k_1k_2[CN^-]}\left[\frac{K_a}{K_w}\right] + \frac{1}{k_1} \qquad (8)$$

and for Mechanism II in which OH^- is displaced first from its axial position to the porphyrin ring

$$\frac{[CN^-]}{k_{obsd}} = \frac{k_{-1}[OH]}{k_1k_2[CN^-]} + \frac{1}{k_1} \qquad (9)$$

K_a is the acid dissociation constant for HCN and K_w is the dissociation constant for water. A plot of $[CN^-]/k_{obsd}$ against $1/[CN^-]$ will give a straight line (Figure 6), at constant pH, with an intercept of $1/k_1$ and slopes, respectively, of $(k_{-1}/k_1k_2)[K_a/K_w]$ and k_{-1}/k_1k_2.

Isomerization Reaction. A slow reaction occurs after the initial formation of a dicyanohemin complex. The half-life is independent of the hemin, cyanide concentrations, and pH, provided irreversible kinetics are employed. This is consistent with an intramolecular isomerization process envisioned in eq 6c. Data for this process, *i.e.*, k_3, are found in Table III. The C-bound isomer is present, at equilibrium, to at least 96%. Its rate of formation is essentially first order and may be treated as irreversible.

Decomposition of the Stable C-bound Isomer. Mechanism I or II indicates that the equilibrium may be shifted to the left by the addition of base. Solutions containing $M(CN)_2$· micelle for these studies were aged for approximately 20 min to ensure that the C-bound isomer predominated in solution. The addition of concentrated base caused the breakdown of the dicyano product with the subsequent formation of the original M(OH)·micelle (except at $[OH^-] > 0.1$ M where spectra indicate some differences, presumably due to $M(OH)_2$· micelle). The decomposition is independent of base from 0.05 to 0.6 M KOH or cyanide and the unimolecular process is associated with k_{-3}. These data are summarized in Table III.

TABLE III: Summarized Data at 25°.

	$CetMe_3NBr$ Micelles	TX-100 Micelles
k_{-2} (sec^{-1})	0.43	8.15×10^{-2}
	2.04×10^{-2} (15°)	
k_3 (sec^{-1})	0.152	5.67×10^{-2}
	2.5×10^{-2} (15°)	
k_{-3} (sec^{-1})	8.45×10^{-4}	2.05×10^{-3}
	2.0×10^{-4} (15°)	

Decomposition of the N-Bound Unstable Isomer. The unstable intermediate was rapidly generated at pH ~9.5 and subsequently decomposed by base (~0.16–0.35 M KOH) before any significant isomerization occurred. The rate of decomposition is independent of base concentration and is different from the stable C-bound isomer, providing kinetic evidence for its existence. Table III summarizes these data. The kinetic data may be combined to give an equilibrium constant (eq 6d) for both micelle systems shown in Table IV.

Discussion

Studies of hemin monomers (Brown *et al.*, 1970) indicate large spectral differences from dimerized hemin. Investigations of the spectral properties of hemoproteins (Brill and Sandburg, 1968), where the prosthetic group is hemin in the protein environment of micrococcus catalase, reveal spectral profiles clearly associated with those of monomers of hemin. The present work has demonstrated that micelles of $CetMe_3NBr$ and TX are also capable of forming monodispersed solutions of hemin *via* intercalation into the hydrophobic interiors of micelles. This interaction apparently overcomes the monomer–monomer stacking interactions causing the dimers to dissociate.

The spectra of M(OH)·micelle for sodium lauryl sulfate (Simplicio, 1972b), $CetMe_3NBr$, and TX are different from one another with regard to both position of maximum wavelength absorption and molar extinction coefficients. This suggests that the orientation or location of the porphyrin is somewhat different in each case and is supported by the different structures (Reiss-Husson and Luzzati, 1964) of the sodium lauryl sulfate and $CetMe_3NBr$ micelles. The TX micelle presents an ambiguity since hemin interaction may occur with either the hydrocarbon chain or polyethoxy chain. Previous workers (Brown *et al.*, 1970) have found that the pK_a associated with the monomer of deuteroferriheme is 7.10. This is substantially higher than that found for any of the three micelle systems here and suggests that a hydro-

TABLE IV: Equilibrium Constants at 25°.[a]

	M(OH)· $CetMe_3NBr$	M(OH)·TX
K(kinetic) (M^{-1})	8.85×10^4	2.16×10^2
K(spectral) (M^{-1})	5.91×10^4	1.72×10^2

[a] The kinetically determined equilibrium constants have an estimated error of ±20%.

phobic environment increases the acidity of one of the axial water protons. The trend in pK_a values does not parallel what might be expected on the basis of simple first-order considerations of micellar charge, although the charge density and type of charge in the Stern layer no doubt makes some contribution as reflected in the cyanide–hemin equilibrium constants. The corresponding pK_a for ferrimyoglobin (Breslow and Gurd, 1962) is 8.9. This large value may reflect the influence of a *trans*-imidazole residue present in the native protein (Kendrew *et al.*, 1960).

The interaction of M(OH)·micelle with cyanide involves two cyanides. While the existence of isosbestic points supports two major species in the overall equilibrium, kinetic measurements reveal a third species, presumably an unstable N-bound isomer. The spectrum of this intermediate and its minute presence at equilibrium make it understandable that isosbestic points will occur and why eq 5 and 6 are adequate for use in calculating the overall equilibrium constants. The stability constants for the cyanide complexes for the three detergent systems, unlike the pK_a values, reveal a thermodynamic stability that is consistent with charge differences at the micelle–water interface, this order being cationic $CetMe_3NBr$ > neutral TX > anionic sodium lauryl sulfate. The spectrum of $M(CN)_2$·micelle parallels those changes associated with the addition of cyanide to catalases (Brill and Sandberg, 1968).

The uptake of cyanide by metmyoglobin has been extensively studied (VerPloeg *et al.*, 1971; Awad and Badra, 1967; George and Hanania, 1955). Such studies have often led to ambiguities as to the attacking nucleophile, *i.e.*, CN^- or HCN. This has resulted from rapid acid–base equilibrium associated with the native globular protein since the transition state involving CN^- and the protonated form of metmyoglobin is identical with that generated from HCN and an unprotonated metmyoglobin. The present studies extend and confirm, for neutral and cationic micelles, what has been found for hemin in anionic sodium lauryl sulfate, that the attacking nucleophile is most likely CN^-. The initial uptake of CN^- in the TX and $CetMe_3NBr$ micelles is very close to those found for metmyoglobin (VerPloeg *et al.*, 1971).

The intermediate dicyano product for $CetMe_3NBr$ and TX appears to undergo a linkage isomerization to the C-bound stable product. Such isomerizations are common for Cr(III) (Espenson and Bushey, 1971) where a $CrNC^{2+} \rightleftharpoons CrCN^{2+}$ occurs by a Cr–N cleavage, followed by a rotation to a transition state that is likely stabilized by π bonding between the d orbitals of appropriate symmetry on the metal and the π orbitals on CN^-. It has been suggested (Awad and Badra, 1967) that a linkage isomerization occurs with metmyoglobin if the attacking agent is HCN, hence PFe + HCN $\rightleftharpoons$ PFeNC + H^+ and PFeNC $\rightarrow$ PFeCN, whereas PFe + CN^- $\rightarrow$ PFeCN, directly. In Mechanism I the first step requires that CN^- displace H_2O and since OH^- is a better nucleophile this seems more reasonable than CN^- first displacing OH^- as in Mechanism II. For the isomerization reaction to occur the hemin must be attacked by HCN. Only Mechanism I is consistent and if it operates, then the isomerization is a linkage change of only one cyanide. While linkage isomerization is observed in metmyoglobin, M(OH)·$CetMe_3NBr$, and M(OH)·TX, none has been found for M(OH)·sodium lauryl sulfate. This may be a reflection of a large k_3 preventing the N-bound isomer from building up. The $K_3(k_3/k_{-3})$ for the $CetMe_3NBr$ and TX are 136 and 27.7, respectively.

Equations 6 and 7 are further substantiated by combining individually determined rate constants to give an overall equilibrium constant in excellent agreement with that determined spectrally.

Surface charge of metmyoglobin is suggested (George and Hanania, 1955) to play a role in its reactivity with cyanide. The k_1 rate constants for the sodium lauryl sulfate, TX, and $CetMe_3NBr$ micelle systems are, respectively, 3.35×10^3, 5.1×10^2, and 11.2×10^3 M^{-1} sec^{-1}. On the basis of simple electrostatics one would envision the rates in the order $CetMe_3NBr$ > TX > sodium lauryl sulfate, but this is not borne out. A general trend has been noted for the incorporation of Cu(II) into porphyrins that are themselves intercalated in micelles of sodium lauryl sulfate and $CetMe_3NBr$ (Lowe and Phillips, 1961) with the sodium lauryl sulfate micelle catalyzing the metal incorporation nearly 20,000 times faster than the cationic $CetMe_3NBr$. The dissociation of $M(CN)_2$·micelle is of the expected order $CetMe_3NBr$ < TX < sodium lauryl sulfate on the basis of charge considerations.

These results appear to indicate that charge differences and possible differences in orientation of the intercalated hemin play major roles in determining kinetic and thermodynamic properties of these systems. These results also suggest their importance in hemoproteins.

The study of intercalated metalloporphyrins in micelles of various charge types has been little explored. This work suggests that these systems may be used as models for the more complex hemoproteins. Currently in progress are studies on the catalase activity of intercalated hemin and the stability of the molecular oxygen–Fe(II)–porphyrin adducts in micelles.

References

Awad, E. A., and Badra, R. G. (1967), *Biochemistry 6*, 1978.

Breslow, E., and Gurd, F. R. N. (1962), *J. Biol. Chem. 237*, 371.

Brill, A. S., and Sandberg, G. E. (1968), *Biophys. J. 8*, 669.

Brown, S. B., Dean, T. C., and Jones, P. (1970), *Biochem. J. 117*, 733.

Brown, S. B., and Jones, P. (1968), *Trans. Faraday Soc. 64*, 999.

Caughey, W. S., Fijimoto, W. Y., and Johnson, B. P. (1966), *Biochemistry 5*, 3830.

Erman, J. E., and Hammes, G. G. (1966), *Rev. Sci. Instrum. 37*, 746.

Espenson, J. G., and Bushey, W. R. (1971), *Inorg. Chem. 10*, 2457.

Falk, J. E. (1964), Porphyrins and Metalloporphyrins, New York, N. Y., Elsevier.

Fendler, E. J., and Fendler, J. H. (1970), *Advan. Phys. Org. Chem. 8*, 271.

Fleischer, E. B., Palmer, J. M., Srivastava, T. S., and Chatterjee, A. (1971), *J. Amer. Chem. Soc. 93*, 3162.

George, P., and Hanania, G. I. H. (1955), *Discuss. Faraday Soc.* 216.

Hartley, G. S. (1934), *Trans. Faraday Soc. 30*, 444.

Kendrew, J. C., Dickerson, R. E., Strandberg, B. E., Hart, R. G., Davies, D. R., Phillips, D. C., and Shore, V. C. (1960), *Nature (London) 185*, 422.

Labbe, R. F., and Nishida, G. (1957), *Biocheim. Biophys. Acta 26*, 437.

Lowe, M. B., and Phillips, J. N. (1961), *Nature (London) 190*, 262.

Muirhead, G., Cox, J. M., Mazzarella, L., and Perutz, M. F. (1967), *J. Mol. Biol. 28*, 117.

Phillips, J. N. (1963), *Compr. Biochem. 9*, 34.

Porra, R. J., and Jones, O. T. G. (1963), *Biochem. J. 87*, 186.
Reiss-Husson, F., and Luzzati, V. (1964), *J. Phys. Chem. 68*, 3504.
Simplicio, J. (1972a), *Biochemistry 11*, 2525.
Simplicio, J. (1972b), *Biochemistry 11*, 2529.
VerPloeg, D. A., and Alberty, R. A. (1968), *J. Biol. Chem. 243*, 435.
VerPloeg, D. A., Cordes, E. H., and Gurd, F. R. N. (1971), *J. Biol. Chem. 246*, 2525.
Wang, J. G. (1958), *J. Amer. Chem. Soc. 80*, 3168.

Dilatometry of Biological Membranes†

Donald L. Melchior*‡ and Harold J. Morowitz

ABSTRACT: Use of a precision dilatometer with a small sample size has allowed characterization of the thermal volume changes of erythrocyte ghosts, *Mycoplasma laidlawii* membranes, and vesicles made from dipalmitoyl-L-α-lecithin associated with cytochrome *c* in dilute aqueous suspensions. Erythrocyte ghosts were found to have a mass coefficient of expansion, ϵ_T, which increases with temperature, going from 0.005 ml per gram (ghost) per degree at 16° to 0.014 ml per gram (ghost) per degree at 40°. These large values of ϵ_T are comparable to those of lecithin vesicles. Reproducible details also appear in the plot of ϵ_T *vs.* temperature for erythrocyte ghosts. *M. laidlawii* membranes, submitted to treatment which either denatures (heat) or removes (Pronase) the bulk of the membrane protein, have an average ϵ_T of about 0.004–0.005 ml per gram (membrane) per degree, with little temperature dependence. The ϵ_T of native *M. laidlawii* membranes exhibits a large increase (0.003 ml per gram (membrane) per degree) in the 32–50° temperature range. This hump is destroyed by treatment which destroys or denatures protein. The second feature of the ϵ_T *vs.* temperature plots of *M. laidlawii* membranes is a sharp dip in ϵ_T at 22°. This very striking phenomenon occurs over a 5° range. In fresh membrane preparations this dip extends to negative values. It is destroyed in a systematic manner by processes which denature membrane protein. Thin-walled vesicle preparations of pure synthetic phospholipids or solutions of cytochrome *c* display no dips in their ϵ_T *vs.* temperature plots. If, however, cytochrome *c* is bound to these vesicles, a sharp dip in the ϵ_T of this aggregate occurs at 22°. Calorimetric findings together with the dilatometric results indicate a highly cooperative effect. The likelihood of this being a water-related effect is diminished by membrane suspensions in D_2O having 22° dips indistinguishable from those of membrane preparations in H_2O. The 22° dip appears to be a phenomenon involving protein, present in lipid bilayer systems acting as a class.

The development of a precision dilatometer requiring small samples (Rothman *et al.*, 1972) provides us with the opportunity of using coefficients of thermal volume change to characterize biological membranes. We have previously applied this technique to study phase transitions in phospholipid vesicles (Melchior and Morowitz, 1972) and in the present study we extend the technique to the plasma membranes of *Mycoplasma laidlawii* and human erythrocytes as well as lecithin vesicles associated with cytochrome *c*.

Our findings indicate dilatometry to be a potentially useful tool in the investigation of biological substances. Each of the systems studied was found to have a characteristic volume *vs.* temperature curve. Several distinctive features were found which should aid in increasing knowledge about biological membranes. The most notable of these bears on protein associated with lipid bilayers.

Materials and Methods

Preparation of Mycoplasma laidlawii Membranes. M. laidlawii B were grown on a medium consisting of 18 g of Tryptose (Fisher, Fair Lawn, N. J.), 8 g of Yeast Extract (Fisher), 4 g of Tris, 5 g of NaCl, and 900 ml of water. After autoclaving, 100 ml of 10% sterile glucose solution and 10 ml of sterile Bacto-PPLO serum fraction (Difco, Detroit, Mich.) were added. For cell growth 1 l. of medium was inoculated with 10 ml of a 24-hr culture and incubated at 37°. The cells were harvested at late log phase and lysed in deionized water, and membranes were prepared by the method of Engelman *et al.* (1967).

Preparation of Red Blood Cell Ghosts. Human red blood cell membranes, "ghosts," were prepared by the method of Hoffman and Ryan (Parker and Hoffman, 1964). Fresh red blood cells were hemolyzed in 10 vol of cold 1×10^{-4} M EDTA at pH 7.4. The resulting ghosts were washed until hemoglobin free with a solution containing 0.0153 M sodium chloride, 0.0017 M Tris, and 1×10^{-4} M EDTA (pH 7.4).

Cytochrome c. Cytochrome *c* (horse heart, Miles-Seravac, Maidenhead, Berks., England) was dissolved in deionized water and the pH was adjusted to 8.0.

Preparation of the DPL Vesicle–Cytochrome c Complex. Synthetic DPL[1] (Calbiochem, Los Angeles, Calif.) was formed into thin-walled vesicles with diameters of 0.2–5 μ by the method of Reeves and Dowben (Melchior and Morowitz, 1972). The vesicles were brought to a concentration of about 0.2%, the pH was adjusted to 8.0, and cytochrome *c*

† From the Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520. *Received December 14, 1972.* This work was supported by the National Institutes of Health.

‡ Part of this material was submitted in partial fulfillment of the Ph.D. requirements of Yale University.

[1] Abbreviation used is: DPL, dipalmitoyl-L-α-lecithin.